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UNRESTRICTED

THE SIGNIFICANCE OF ANTIBODY PRODUCTION IN  
HUMAN RENAL TRANSPLANTATION

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A thesis submitted in partial fulfilment of the  
requirements of the Open University for  
the degree of Doctor of Philosophy

October 1997

**Sponsoring Establishment:**  
**Guy's and St Thomas's NHS Trust**  
**London**

in collaboration with  
The Oxford Transplant Centre

Author no. P9270318  
Date of award: 28<sup>th</sup> January 1998

## ABSTRACT

Screening for HLA specific antibodies is an important part of laboratory testing for transplantation. The conventional technique for screening is complement dependent lymphocytotoxicity. The development and increasing use of the sensitive flow cytometric crossmatch technique has meant that this conventional screening method is often less sensitive than the final crossmatch. The aim of this study was the development of a flow cytometric screening technique which would be as sensitive as the flow cytometric crossmatch and the investigation of the newly developed ELISA screening method PRA-STAT. These methods were used to investigate antibody production in patients with failed transplants.

Flow cytometric analysis of antibody binding to pooled cells was found to be a reliable and sensitive method for the detection of HLA class I and class II specific antibodies. PRA-STAT also detects both class I and class II specific antibodies. Both flow cytometric and PRA-STAT screening methods were shown to be more sensitive than conventional cytotoxic screening.

Screening of patients with failed transplants by these sensitive methods showed that the majority of patients produce both HLA class I and class II donor specific antibodies following failure of a primary transplant. Flow cytometric and PRA-STAT screening detected antibody production earlier than cytotoxic screening in some patients. HLA matching was shown to be related to both graft survival and to the levels of antibody produced following graft failure with poorly matched grafts resulting in higher levels of sensitisation than well matched grafts. Patients with detectable post graft failure antibodies had a lower chance of receiving a second transplant and had significantly worse regrant survival than patients with no antibody. The results of the study suggest that HLA class II specific antibodies and repeat class II mismatches may be detrimental in regrafts and this requires further study.

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## Abbreviations

AHG	Anti-human globulin
CDC	Complement dependent lymphocytotoxicity
CFT	Complement fixation test diluent
CLL	Chronic lymphocytic leukaemia
DTT	Dithiothreitol
EBV	Epstein Barr virus
ELISA	Enzyme linked immunosorbant assay
FCXM	Flow cytometric crossmatch
FITC	Fluorescein isothiocyanate
PBL	Peripheral blood lymphocyte
PE	R-Phycoerythrin
PR	Panel reactivity
PRA	Panel reactive antibody

## Acknowledgements

There are many people who have helped me during the course of this study but there are 2 people in particular without whom I could not have completed this thesis. Dr Ken Welsh provided the original inspiration for the work and his guidance, help and encouragement have been greatly appreciated. I am also very grateful to Dr Bob Vaughan whose unstinting support and advice has been so important.

The serological typing and crossmatching of recipients and donors and cytotoxic screening was performed as part of the Guy's Tissue Typing Laboratory's routine work and I am very grateful to all the members of staff, past and present, involved in this work, in particular I wish to thank Mieke van Dam and Gareth Page who have, each in turn, overseen the day to day running of the laboratory during the time covered in this study. Thanks are also due to Alasdair Heads who has helped to maintain the EBV cell lines and prepare the cell pools, Elli Kondeatis for teaching me the techniques for DNA typing and all the staff involved in making up the stock solutions for DNA work, and Paul Norman for help with the use of statistical analysis programs.

I am grateful to Maggie Sutton and Anita Bayne from the Oxford Transplant Centre who played an important part in the development of CLL screening, preparing the CLL cells for pooling and performing the cytotoxic screening with CLL cells. I would also like to thank Professor Dudley Dumonde of the Immunology Department at UMDS for his support and Alain Segers of SangStat Atlantique.

Finally, to go back to the beginning, I would like to thank Geoff Koffman for introducing me to Tissue Typing at Guy's in the first place, which is how this all got started.

**CHAPTER 1**  
**INTRODUCTION**

## 1.1 History of Renal Transplantation

The first seriously reported experiments attempting renal transplantation were performed in the early years of the 20th century by Emerich Ullmann who reported the successful autotransplant of a dog kidney from the normal position into the neck where it did produce some urine in 1902. A number of workers continued to investigate transplantation in animals and in a small number of human kidney xenografts were performed but such grafts never functioned for any longer than one hour (Hamilton 1994). The surgical techniques necessary for performing transplants were established during this period but interest in clinical transplantation faded.

During the 1920's, 30's and 40's studies into the immunological nature of graft rejection were carried out in tumours and skin grafts and the work of Gorer, Snell and Medawar laid the foundations upon which modern transplantation was built (Brent & Sells 1989). Eventually the first partially successful renal transplant was performed by Hufnagel et al in 1946 when a cadaveric kidney was transplanted to the arm of a patient with septicaemia and anuria (reported in Moore 1964). The kidney functioned for a short time but was rejected within 3 days, however the patient's own kidneys had recovered function by that time and the patient survived (Brent & Sells 1989).

During the early 1950's a number of workers carried out isolated human renal transplants which generally failed to function for any length of time although there was some degree of survival of the transplanted kidney, thought to be due to the immunosuppressed nature of the uraemic patient (Hamilton 1994). Finally in 1954 the first truly successful renal transplant was performed by Murray and others in Boston, U.S.A. when they transplanted a kidney from an identical twin into the patient



(Merrill et al 1956). This graft functioned for several years. A number of transplants between identical twins followed.

Renal allografts from non-identical donors were attempted at this time but generally failed to function for any length of time. Attempts at immunosuppression were made using total body irradiation but this resulted in only a few isolated cases of successful transplantation (Hamilton 1994). It was the discovery by Calne in 1960 of the immunosuppressive effect of 6-mercaptopurine, allowing successful transplantation in dogs, which introduced the era of effective immunosuppression for transplantation. A derivative of 6-mercaptopurine, azathioprine was used by Calne and Murray in dogs and was found to be a better immunosuppressive agent (Calne et al 1962). Murray et al (1963) used azathioprine for human recipients resulting in the first long term survival of non-identical renal allografts. Renal transplantation as a treatment for renal failure began to be increasingly used with azathioprine and steroids being used for immunosuppression. The developments in tissue typing which were made during the 1960's (covered in section 1.7) helped to increase the success of clinical transplantation.

During the 1970's the improvement in graft survival rates did not rise as quickly as had been hoped. A major advance however occurred towards the end of the decade with the discovery of the fungal metabolite Cyclosporin A (Borel et al 1976). Calne performed the first trials of Cyclosporin in human renal allografts and demonstrated improved graft survival compared with the conventional immunosuppression at that time (Calne et al 1978). The introduction of Cyclosporin throughout transplant centres followed during the early 1980's resulting in a marked

increase in graft survival and renal transplantation has become recognised as the treatment of choice for renal failure.

## 1.2 Introduction to Antibodies

The term isoantibody was first used by Bordet in 1898 (Grabar 1987) who observed agglutination of red blood cells by serum from rabbits which had earlier been injected with red blood cells. The discovery of factors in the serum of guinea pigs sensitised by injection of lymph node preparations from rabbits and rats which caused agglutination and lysis of lymphocytes was made by Metchnikoff in 1899 (Brent and Sells 1989). The subsequent isolation of pure antibodies by Heidelberger and Kendall in 1936 (Grabar 1987) paved the way for subsequent studies which elucidated the nature of antibodies.

Antibodies are serum proteins, produced by plasma cells, which specifically bind the antigen which induced their production. In addition B cells carry surface bound immunoglobulin which acts as a receptor. The binding of antibody to the target antigen may bring about the removal of the target by a number of methods. Bound antibody may fix complement and lead directly to cell lysis or may trigger cell mediated responses by Fc receptor bearing cells. (Baldwin et al 1986, Whitley et al 1990).

The antibody molecule, or immunoglobulin, is composed of 2 identical heavy and 2 identical light chains. These are joined together by di-sulphide bonds, with a hinge region such that the antibody can be schematically represented as a Y shaped molecule. The heavy chain contributes to the 'stem and arms' of the molecule and the light chain to the 'arms'. The regions of the light and heavy chains at the tip of the

molecule are areas of considerable sequence variation and it is this variation which leads to the multiplicity of individual immunoglobulin molecules which each recognise different target antigens (Roitt 1988).

There are 5 classes of immunoglobulin in man, IgG, IgA, IgM, IgD and IgE, these classes are based on the structure of the heavy chain. IgG comprises the largest proportion of circulating antibody, around 80% of total immunoglobulin. IgA and IgM account for most of the rest of the circulating antibody with less than 1% of total immunoglobulin being IgD and IgE. IgG, IgA, IgD and IgE exist as single peptide units whereas IgM is a pentamer of the basic peptide unit (IgA is also found as a dimer). IgM and IgG are the immunoglobulin classes which cause complement fixation by the classical pathway, with IgM being the most effective complement fixing antibody. There are 4 subclasses of IgG immunoglobulins, IgG1, IgG2, IgG3 and IgG4. The effectiveness of the subclasses at fixing complement varies, with IgG1 and IgG 3 being the most efficient, IgG2 less so and IgG4 being non-complement fixing (Roitt et al 1985).

The ability of bound antibody to fix complement is dependent on both the antibody class and concentration and on the antigen density (Whitley et al 1990). A single pentameric IgM molecule needs to bind to a number of antigenic sites so that the conformation of the molecule alters such that the Fc portions of the molecule are exposed and can initiate complement fixation (Baldwin et al 1986). For IgG antibodies to fix complement efficiently binding of 2 molecules in close proximity is necessary (Whitley et al 1990). The phenomenon of antibody mediated damage to transplanted kidneys is covered in section 1.4.

### 1.3 Discovery of the MHC and Specific Antibodies in Transplantation

The production of antibodies directed against antigens of the major histocompatibility complex (MHC) following tissue transplantation was first described by Peter Gorer. The discovery of antigens forming part of the MHC was made by Gorer through his work on the transplantation of tumours in mice. He showed that erythrocytes from different strains of mice possessed different antigens as detected using sera from immunised rabbits. Transplants of tumours from mice possessing 'Antigen II' into mice lacking this antigen showed that the tumours regressed, leading to Gorer's conclusion that antigenic differences between strains could lead to resistance to the tumour (Gorer 1937). As part of this work the animals which proved resistant to the transplanted tumours were bled and their sera was shown to specifically agglutinate cells possessing antigen II. The discovery of antigen II and the 'iso-agglutinins' was the start of the unravelling of what was to become known as the MHC.

It was almost 20 years later that Dausset described serum alloantibodies reacting with human leukocytes (Dausset 1958). This began the investigation of the human leukocyte antigen system (HLA) and the development of tissue typing, which progressed rapidly over the next 10 years, by which time the importance of the HLA system in clinical kidney transplantation was becoming clear.

The association of pre-existing antibodies with hyperacute rejection of renal allografts was described by Kissmeyer-Nielsen et al in 1966. Two cases of patients whose transplanted kidney stopped working within 1 hour of engraftment were described. Both recipients were shown to have antibodies which reacted strongly with both leukocytes and kidney extracts from their respective donors. These antibodies



were present in serum collected prior to transplantation. Similar findings were made by Williams et al (1968). In seven cases of rejection (at least 6 of which could be described as hyperacute) antibodies reacting with donor cells were found in the pre-transplant sera of 6 patients. The remaining patient received an ABO incompatible graft whose rejection may be attributed to anti-blood group antibodies.

In these studies antibodies were detected by agglutination assays, although Williams (1968) had, in addition, employed a cytotoxicity technique developed by Terasaki, initially for use as a method for typing for HLA antigens. In addition to typing HLA antigens Terasaki was also using his cytotoxicity technique to look for antibodies in transplant recipients (Morris et al 1968). A large number of serum samples from over 40 transplant recipients were tested against a panel of 10-40 lymphocytes. A higher proportion of patients were shown to have cytotoxic antibodies following transplantation than pre-transplant, with 9 of 10 cases of graft loss leading to antibody production. This method of screening for HLA specific antibodies became the standard technique used by tissue typing laboratories and, with some refinements, is still the most common method of antibody detection in use today.

The critical importance of pre-existing antibodies to renal allografting was emphasised by Patel and Terasaki in 1969. In this study patients had been screened as previously described (Morris et al 1968). Immediate rejection was seen in 42.5% of recipients with pre-formed antibodies as compared to only 2.4% of those with no detectable antibodies. The patients with pre-formed antibodies had crossmatch tests carried out with donor lymphocytes and it was found that 80% of those with positive crossmatches lost their grafts (14.8% of negative crossmatch grafts failed). In



conclusion the authors stated that ‘The ethics of transplanting kidneys ..... across a known positive crossmatch result, can reasonably be expected to be questioned’. This was accepted by the transplant community and pre-transplant crossmatching became a routine requirement. Thus hyperacute rejections due to pre-formed antibodies were largely avoided. However elucidating the role of antibodies in transplantation and the difficulties associated with finding suitable grafts for patients with pre-formed antibodies are problems which have still to be fully solved.

#### 1.4 Antibody Mediated Rejection Mechanisms

The association of preformed antibodies with hyperacute rejection of renal allografts found by a number of studies suggested that the antibodies played a primary role in the mechanism of rejection. The role of antibodies produced following transplantation in subsequent rejection episodes (both acute and chronic) is less clear.

##### 1.4.1 Hyperacute Rejection

The mechanism of hyperacute rejection is the most clearly understood. Circulating antibodies which bind to the HLA (or blood group) antigens of the donor expressed on the endothelial cells of the allografted organ cause activation of the complement system which can lead to direct damage of the endothelial cells and to cell lysis. The histologic examination of hyperacutely rejected kidneys shows accumulation of granulocytes and platelets with coagulation leading to formation of microthrombi. The vessels become obstructed by thrombi leading to ischaemia and finally necrosis of the graft tissue (Porter, 1976).

The direct involvement of antibodies has been shown by immunohistochemical studies. Williams et al (1968) showed deposition of IgG in the capillaries of 4 hyperacutely rejected kidneys, 3 due to HLA specific antibodies and 1 case of blood group incompatibility. Hyperacute rejection has also been demonstrated when kidneys have been perfused with an antibody containing perfusate. Cross et al (1974) described 2 cases of hyperacute rejection of a pair of kidneys which were perfused with cryoprecipitated plasma. Subsequent screening of the plasma showed a specific antibody directed against an HLA antigen present on the perfused kidneys. The importance of the specificity of the antibody for the donor antigen was demonstrated by the fact that a second pair of kidneys, not bearing the relevant antigen, perfused by the same plasma were not rejected.

#### 1.4.2 Acute Rejection

Acute rejection is generally regarded as primarily mediated by the lymphocyte, particularly the T cells and most current immunosuppressive protocols are aimed at suppressing the T cell response to the allografted tissue. In cellular rejection lymphocytes, macrophages and granulocytes are seen infiltrating the graft tissue during the course of rejection (Tilney et al 1979, Tilney et al 1983). However, whilst the principle cause of graft destruction appears to be due to specific cell lysis by recipient cytotoxic lymphocytes along with a non-specific inflammatory response (Hayry, 1984, Chandler & Passaro 1993), there is also evidence of antibody production in cases of acute rejection. Whether the antibody is directly involved in graft destruction or is simply a consequence of the cellular immune response to the allogenic tissue is not clear.

Porter (1976) described 'acute humoral rejection' where early onset of graft failure was accompanied by the appearance of antibodies specific to donor HLA antigens, although in most cases this was accompanied by some degree of lymphocyte infiltration. The involvement of both cellular and humoral immune responses in renal allograft loss was shown by Tilney et al (1979). Viable lymphocytes recovered from rejected grafts were shown to specifically lyse donor cells, with the principle component of the infiltrate being T cells in 9 of the 10 cases studied. In addition to this evidence of donor specific cellular activity within the graft, donor specific antibodies were eluted from 4 of the kidneys and immunoglobulins were demonstrated in the vessel walls of 3 of the kidneys. In 1982 the same group demonstrated the presence of immunoglobulin secreting plasma cells amongst the infiltrating cells recovered from a number of failed renal allografts (Garovoy et al).

Thus whilst T cells may be regarded as the main cause of acute graft loss there is evidence of humoral responses taking place within the graft alongside the cell mediated events, emphasising that the immune response has many different effector mechanisms, more than one of which may be involved in the same process.

Whilst the production of antibodies may be secondary to the cellular immune response it is also possible that they may initiate cell mediated graft damage by the mechanism of antibody dependent cell-mediated cytotoxicity (ADCC). In ADCC antibody bound to antigen on the allogeneic cell surface may lead to cell lysis by Fc receptor positive cells such as natural killer cells or macrophages. Graft infiltrating cells have been shown to mediate cellular lysis of antibody coated cells by Strom et al (1977) and Tilney et al (1979) demonstrated that antibodies eluted from rejected grafts had ADCC activity to donor cells.

### 1.4.3 Chronic Rejection

Whilst the proportion of allografts lost during the early post transplant period has decreased over the years the attrition rate over the long term has not changed. Chronic rejection is a major cause of long term graft failure (death with function being the other principle cause of loss). Chronic rejection is a well defined process with specific histological and functional characteristics. The principle feature of chronic rejection in renal allografts is progressive thickening of vessel walls, leading eventually to obliteration of the lumen (Tullius and Tilney 1995, Hayry et al 1997).

The causes of chronic rejection are not clearly understood. It is probable that a number of different factors are involved, both alloantigen dependent and independent. One factor which several studies have found to be significantly associated with the development of chronic rejection is the occurrence of previous acute rejection episodes (Almond et al 1993, van Sasse et al 1995, Opelz et al 1997).

A hypothesis has been put forward suggesting that chronic rejection is the result of a continuous inflammatory process, which is the result of previous injury to the graft tissue (Halloran et al 1997). The association of early injury due to acute rejection with chronic graft failure supports this idea. The fact that allografts from living donors do not display the same tendency to develop chronic rejection as do cadaveric grafts, which may suffer injury due to the brain death of the donor or to cold ischaemia, also supports the theory that early damage is an important factor in the subsequent development of chronic rejection.

The role of antibodies in the chronic rejection process is not understood. There is evidence for the involvement of endothelial specific antibodies in chronic rejection of cardiac allografts (Crisp et al 1994). Whether antibodies specific for



endothelial antigens or HLA are a factor in chronic rejection of renal allograft is not well documented. There have been reports linking antibodies with chronic rejection in renal transplantation (Mohanakumar et al 1981, Davenport 1994) but there is little direct evidence of antibody involvement in histologically defined chronic rejection. Since acute rejection episodes are associated with the subsequent development of chronic rejection as discussed above it is possible that early antibody mediated graft damage occurring during an acute rejection could predispose to chronic rejection thus playing an indirect role in the development of rejection.

### 1.5 The HLA System & Specific Antibodies

The human leukocyte antigen (HLA) system is coded for on the short arm of chromosome 6. The system is highly polymorphic and there are 2 classes of HLA antigens termed class I and class II with each class having several different expressed loci. The class I antigens were the first to be described with antigens being defined on the basis of serologic reactions. Early reports used differing types of nomenclature for the antigens being detected but by 1975 a clear nomenclature for the system was established with the class I loci HLA-A, B and C being described and individual antigens of the locus being assigned a numerical identifier e.g. A1, A2. The class II antigens were initially detected by cellular methods and were known as HLA-D antigens, but with the advent of molecular methods of HLA typing three different loci were identified and named HLA-DR, DQ and DP in 1984 (reviewed in Bodmer 1997).

Some sera whose reactions were initially used to define a new HLA antigen were with time found not to be reactive with a single monomorphic antigen but with a number of different antigens which could be individually defined by reactions with

other sera. This led to the development of the categories of broad antigens and splits where for example the broad antigen originally defined as HLA-A9 was later split into HLA-A23 and HLA-A24 (Bodmer et al 1975), with some sera reacting with cells expressing either antigen but other sera being specific for only one of the 2 splits. The reactions of some sera with a number of different antigens also led to the recognition of cross reactive groups (CREGs) (Legrand & Dausset 1972 ,Schwartz et al 1979, Oldfather et al 1986). A CREG contains a number of different antigens, some of which may be splits of broad antigens, to which sera may be reactive although the stimulus for the response did not contain all of these antigens. The antigens comprising each CREG are determined on the basis of these serological reactions. CREGs are found where the different antigens have one or more structural areas (epitopes) in common, these are known as public epitopes, whereas the individual allele will possess unique, or private epitopes.

The reactions which different sera display are determined by the HLA antigens present in the individual making the antibody and the HLA type of the stimulus for antibody production. The HLA-antigen must be recognised as foreign by the responder for antibody to be produced, recognition being dependant on the structure of the antigen.

The HLA class I antigen consists of a polymorphic heavy chain encoded by the class I genes and a nonglycosylated polypeptide,  $\beta$ 2microglobulin. The heavy chain is divided into 3 domains,  $\alpha$ -1, $\alpha$ -2 and  $\alpha$ -3. The polymorphism is found within the  $\alpha$ -1 and  $\alpha$ -2 domains. The structure of the HLA class I antigen was first demonstrated by Bjorkman et al in 1987 by X-ray crystallography of the HLA-A2



antigen. The  $\alpha$ -1 and  $\alpha$ -2 domains each contribute to one of the 2 alpha helices and to the beta pleated sheet which form the sides and base of a peptide binding groove.

The class II molecule consists of 2 chains, the  $\alpha$  and  $\beta$  chains each of which has 2 extracellular domains. Following the elucidation of the class I structure it was generally believed that the  $\alpha$ -1 and  $\beta$ -1 domains combined to form a peptide binding groove like that found in class I molecules. This was finally shown to be the case, with some slight differences in structure, by Brown et al in 1993. The polymorphic residues of the HLA-DR molecules are mainly confined to the  $\beta$  chain, whereas for HLA-DQ molecules regions of polymorphism are found in both the  $\alpha$  and  $\beta$  chains.

Antibodies recognise one or more epitopes of the HLA antigen. An epitope may be defined by a single amino acid at a specific position. Epitopes may be found uniquely on a single allele or, more frequently, the same epitope is found on a number of different alleles. The relationship of HLA antibody reactions to antigen structure and epitopes was first shown by Fuller et al (1990a, 1990b) who devised epitope maps for the HLA-A2 and HLA-B7 CREGS, identifying the positions of the epitopes found on all the antigens present in the CREG and on sub-groups of antigens or individual antigens (public and private epitopes). The majority of the epitopes described were found in the alpha helices of the class I molecules, although some were located on exposed, connecting loops. Park et al (1990) analysed a large series of serum screening results together with class I sequence data to determine whether the antibody responses observed could be explained by epitopes defined at the amino acid level. The results clearly showed that the patterns of antibody reactivity fitted those predicted by the amino acid epitopes.

## 1.6 HLA Matching in Renal Transplantation

The importance of the degree of HLA match between donor and recipient is a subject which continues to generate opposing opinions. The relationship between HLA matching and graft outcome has been a continuing subject of investigation since the early years of HLA typing. In 1971 a number of studies by individual centres demonstrated that recipients receiving allografts with fewer mismatched antigens had superior graft survival when compared to those with more mismatches (e.g. Festenstein et al, Morris, van Rood et al). However in a large, retrospective study published in 1974 no significant association was found between HLA matching and graft outcome although there was a non-significant trend towards higher graft survival in better matched pairs (Opelz et al). At this time HLA typing was still in the early stages of development, relatively few of the antigens now known to exist had been identified. Therefore the matching data was necessarily incomplete. Following the identification of a number of DR antigens in 1977 (Bodmer et al) Ting and Morris (1978) retrospectively typed donors and recipients to assess DR matching and found that graft survival in patients matched for both DR antigens was superior to those matched for 1 antigen which in turn was better than those mismatched for both DR antigens, although with very small numbers of fully matched grafts this did not reach significance. This observation was followed by a study of HLA A, B and DR matching (Persijn et al 1978) which confirmed the stratified effect of DR matching on graft survival. The best survival was seen in patients with 0 or 1 DR mismatch and only 1 mismatch at the A or B loci indicating that both class I and class II matching had an effect although the DR matching was found to have the greater influence on graft survival. The difference in graft survival found in this study was striking with

87% of well matched grafts ( as defined above) functioning at 18 months as compared with 48% of those less well matched.

Following the introduction of the immunosuppressive agent Cyclosporin A in the early 1980's, with the accompanying improvement in graft survival rates, initial studies showed that HLA matching did not appear to have any influence on graft survival in Cyclosporin treated patients (Harris et al 1985). This was refuted by the results of the collaborative transplant study, which showed that whilst Cyclosporin A treated patients did have superior graft survival compared with those on other immunosuppressive regimes there was still an effect of HLA matching within the Cyclosporin treated group (Opelz et al 1985). The study emphasised the importance of DR matching, with an additive effect of matching for B as well as DR. In contrast Lundgren et al (1986) found no effect of HLA matching in Cyclosporin treated patients, although there were fewer rejection episodes in the well matched patients.

This pattern of conflicting studies has continued over the years. In general the large, multi-centre studies have continued to show that matching for HLA (DR and B especially) does have a beneficial effect on outcome (Opelz 1991, Terasaki 1991, Opelz et al 1993). Organ sharing schemes in Europe and the USA have used this premise as the basis for shipping kidneys between centres for well matched patients.

Recent studies have investigated HLA matching in live donor transplantation. Two large studies have shown that recipients of kidneys from unmatched living donors have better graft survival than recipients of cadaveric kidneys. Jones et al (1994) found no difference in survival rates between completely mismatched and fully HLA-A,B,DR matched grafts from living related donors. Terasaki et al (1995) showed that allografts from unrelated living donors, which were not HLA matched,



had a survival rate comparable with that of allografts from parental donors, which are matched for at least one haplotype. In contrast the most recent study by Opelz (1997) has found a significant effect of matching for live unrelated transplants. It has always been the case that kidneys from living donors have had superior survival compared with cadaveric donor organs, most probably because very little damage is sustained by the kidney taken from a live donor whereas organs from cadaveric donors may be at risk of damage both before the organs are removed and during the storage period before a suitable recipient is identified and the transplant operation can take place. It may be that damage to cadaveric organs leads to an increased risk of recognition and attack by the host immune system and that this may account for a tendency for better matched grafts to have a lower risk of rejection than those which are poorly matched. However, the data published by Opelz (1997) suggests that lack of cold ischaemic damage in live donor kidneys does not completely abrogate the influence of HLA matching in category of transplants.

## 1.7 Techniques for the Identification of HLA Specific Antibodies

### 1.7.1 Lymphocytotoxic Crossmatching

The crossmatch test which identified patients at risk of hyperacute rejection and has remained the principle test of donor/recipient compatibility for over 25 years is based on the lymphocytotoxic test developed by Terasaki & McClelland in 1964 for the detection of antigens on human lymphocytes. A number of modifications following the original publication of the method were made, including the development of the 60 well plastic Terasaki Tray and multi-channel dispensing syringes, resulted in a more reproducible test which was easy to perform (Mittal et al

1968). The basic test involves the incubation of recipient serum with donor cells, followed by the addition of third party complement (usually rabbit). Following a further period of incubation cell viability is determined by microscopy. Any cell death above the background level of the negative control indicated the presence of complement fixing antibodies in the patients serum and is regarded as a positive crossmatch. This test is known as the complement dependent lymphocytotoxic (CDC) crossmatch and has continued to be the recommended method for HLA typing by serology, crossmatching and antibody screening throughout the years since its introduction (Dick & Crichton 1972, Darke & Dyer 1993, Martin & Claas 1993).

A so called standard test does in theory exist and is the basic NIH CDC test described in the NIAID Manual of Tissue Typing Techniques. However there are many variations on this technique with different incubation times and temperatures and differences in cell/serum ratio occurring in different laboratories, the technique is still often referred to as the 'standard technique' despite the slight deviation from the published version. There are also widely recognised modifications of the technique which are frequently used in published studies. Increased incubation times have frequently been used to increase the sensitivity beyond that of the standard technique (Ting et al 1973). The AMOS modification includes additional washing steps to remove unbound serum which may contain factors which inhibit complement lysis and therefore may increase the sensitivity compared with the standard test (Amos 1969). The antiglobulin or AHG method employs heterologous complement-fixing antibodies which bind to human immunoglobulins of both complement and non-complement fixing classes. This allows the detection of non-complement fixing HLA specific antibodies as well as those which are complement fixing by a CDC assay

(Johnson et al 1972). The method is also more sensitive than the standard CDC crossmatch or CDC with extended incubation times (Cross et al 1977). The DTT modification of the CDC assay allows IgM antibodies to be distinguished from IgG antibodies. The addition of the reagent dithiothreitol to either serum or cells causes reduction of the di-sulphide bonds of the IgM molecules thus preventing complement fixation. IgG antibodies are not affected by the addition of DTT. Therefore a positive reaction which can be abolished by treatment with DTT can be attributed to IgM antibody (Chapman et al 1986, Rudy & Opelz 1987).

### 1.7.2 Flow Cytometric Crossmatching

The use of flow cytometry for crossmatching recipient serum with donor cells was first described by Garovoy in 1983. This was a single colour technique in which donor cells were incubated with recipient serum. Following the incubation step unbound serum was removed by washing steps and a second antibody, an anti-human immunoglobulin reagent which is conjugated to a fluorochrome was then added. Following a further incubation unbound secondary antibody was removed by washing and recipient antibody binding to the donor cells detected by single colour flow cytometry. Garovoy (1983) found the method to be more sensitive than CDC. In the early studies of the flow cytometric crossmatch (FCXM) unseparated lymphocytes were most commonly used, resulting in two separate populations of cells with different background fluorescence intensities due to the differences in immunoglobulin binding between T and B cells. It was therefore difficult to distinguish between T and B cell reactions in some tests. Some workers used separated T cells to overcome this problem (Thistlethwaite et al 1986).



The technique was further developed by Cook et al (1987) who introduced a second fluorochrome conjugated antibody so that immunoglobulin binding to T cells could be specifically determined. This was achieved by using a monoclonal antibody to CD3 conjugated to R Phycoerythrin (PE) in addition to an anti-human IgG monoclonal antibody conjugated to fluorescein isothiocyanate (FITC). Following this study the 2 colour FCXM has become the most popular method used. The same method can also be used to perform specific B cell FCXMs by the use of a CD19 or CD20 monoclonal antibody conjugated to PE. (Bray et al 1989). More recently the development of flow cytometers has led to detectors for 3 or 4 different wavelengths of fluorescent emissions and it has become possible to use 3 colour flow cytometry to specifically label both T and B cells as well as bound immunoglobulin in a single reaction tube (Robson and Martin 1996).

Analysis of the FCXM is carried out according to policies developed within individual laboratories and a positive FCXM may be defined by variable degrees of linear or logarithmic channel shift, relative fluorescence or molecular equivalents of bound antibody. The differences in analysis of results may lead to differences in definition of positivity (Harmer et al 1996). Similarly differences in methodological variables may also affect the result of the crossmatch (Shenton et al 1997).

### 1.7.3 Antibody Screening by Lymphocytotoxicity

It is of great importance that any pre-formed antibodies in patients awaiting transplantation, whether as a result of previous failed transplant, blood transfusion or pregnancy are identified prior to crossmatching with a potential donor. The interpretation of the crossmatch test without some knowledge of the patients

antibodies is problematic as not all positive crossmatches are necessarily a contra-indication to transplantation as will be discussed in section 1.8. Knowledge of the level of sensitisation to HLA antigens also provides a measure of the likelihood of finding a crossmatch negative donor for a specific patient which can indicate whether a patient may have a prolonged wait for a suitable kidney to become available. Screening of patient's serum for pre-formed antibodies has therefore become a major part of the transplant laboratories work.

The complement dependent lymphocytotoxic technique has been the standard test used for antibody screening throughout all transplant laboratories. The majority have performed the 'standard' CDC test using a panel of cells to determine the percentage of cells against which a positive reaction is detected giving the panel reactivity of the sample. Analysis of the positive and negative reactions against cells of known HLA phenotype is carried out to determine the antibody specificity. The DTT modification is frequently used to distinguish between IgG and IgM antibodies.

The accuracy of specificity determination is dependant upon the composition of the panel used. It is necessary for a sufficiently large number of HLA antigens to be represented in the panel. The antigens present should be controlled so that all those which might reasonably be expected to occur within the population which is being studied are represented. In many areas the patients requiring screening are from a number of different ethnic groups and the population from which potential donors may come will similarly comprise different ethnic groups. It can be the case that antigens occurring in ethnic minority groups are not always represented in the panels used due to the limitations in acquiring cells suitable for screening purposes. In addition cells expressing antigens which are present in only a small proportion of the

population are also difficult to obtain. The selection of a panel of cells expressing all the required HLA antigens is not in itself sufficient to ensure accurate specificity determination. It is necessary to attempt to construct the panel so that antigens of the different HLA loci are expressed on the cells in a variety of combinations and that those antigens which are commonly found in linkage disequilibrium do not dominate the pool in these combinations. If this does occur it becomes impossible to determine which of the antigens co-expressed on the cells is the target for the antibody detected.

The analysis of the CDC test is subjective, relying upon the individual's ability to distinguish a significant level of cell death. Due to time constraints it is not practical for those who are reading the screening tray to physically count the numbers of live and dead cells in each individual well, therefore an estimate of the percentage of dead cells is generally made. The ability of each individual to estimate percentages will vary. In addition the percentage of dead cells which is considered significant varies between different laboratories. Some workers consider any cell death which is detectable above the background to be significant whereas others have used a cut off of 50% cell death to distinguish a positive from a negative reaction. Such wide variation in practices will inevitably lead to differences in the results obtained.

The cells used in the panels may be freshly obtained, frozen or maintained in culture. Cells from freshly drawn blood of healthy individuals are likely to have the highest viability making it easier to detect low levels cell lysis. The use of fresh cells however makes the screening procedure a lengthy one. It is not possible in most laboratories to process the numbers of cells which would be considered necessary for a complete panel in a short period of time. Therefore sera are screened in batches with a large number of samples being collected and dispensed onto trays and then



screened with cells from only a few donors on each day. It can therefore take several weeks before the screening process is complete.

Frozen trays have been used to avoid the delays in obtaining a final result (Sinnott et al 1985). In these cases the panel cells are dispensed into the wells of the tray and the trays are then frozen. A single serum sample can then be screened against the complete panel in one day. This can be a very effective method of obtaining results rapidly but there may be some loss of cell viability due to the freezing process and it also fixes and limits the panel composition.

The use of fresh cells may enable the laboratory to have a random panel. Although a single panel composed of randomly obtained cells may be more limited than a selected panel where close attention has been given to the phenotypes of the cells used, the use of random panels over a period of time should ultimately lead to a greater number of cells of differing phenotypes being used. Thus, although screening of a single sample from an individual patient may not be sufficient to determine all possible antibody specificities, screening of serial samples over a period of time should provide a more complete analysis of the antibody specificities for that patient.

Using cells derived from peripheral blood provides a population of cells which consists principally of T cells, indeed many laboratories use procedures to purify the population so that it contains almost exclusively T cells. Therefore antibodies specific for HLA class II antigens are not detected by this method. Peripheral blood does not provide sufficient quantities of B cells for testing so it is necessary to find another source of cells if class II screening is to be undertaken. Cells from patients with chronic lymphocytic leukaemia (CLL cells) are the most commonly used source of B cells for screening (Martin & Class 1993). However their availability is not as

universal as cells from healthy volunteers and many laboratories are not able to routinely screen for antibodies to HLA class II. The lack of agreement about the clinical significance of class II specific antibodies which will be discussed in the following sections has also meant that many laboratories have not felt it necessary to screen for these antibodies.

Whereas crossmatching techniques have commonly been enhanced by laboratories to provide a level of sensitivity greater than that of the 'standard' test this has not generally been the case for screening. It has been suggested that, since the understanding of a patient's antibody status is important for the interpretation of crossmatching results, the screening technique should be at least as sensitive as the most sensitive crossmatch test used by the laboratory (Fuller 1991). Indeed Fuller comments that the use of a crossmatch technique with enhanced sensitivity implies a lack of confidence in the screening method used.

Since the introduction of the flow cytometric crossmatch it is the case that the CDC screening techniques used are likely to be significantly less sensitive than the final crossmatch technique. Indeed in 2 studies where flow cytometry was used to investigate sensitisation, rather than for direct donor recipient crossmatching, the method was shown to be more sensitive than CDC testing (Scornik et al 1984, Rodey et al 1987). However, despite these indications, a method suitable for screening large numbers of samples by flow cytometry has not been developed. One of the aims of the work contained in this study has been the development of such a screening technique.

#### 1.7.4 Screening by ELISA

Since the development of the screening technique to be described in Chapter 3 the enzyme linked immunosorption assay (ELISA) has been introduced as a method for the detection of HLA specific antibodies. Buelow et al (1995) described a method in which soluble HLA class I antigens were captured onto the bottom of the wells in a 96 well plate using a monoclonal antibody specific for a non-polymorphic region of class I. HLA antigens from 46 different EBV cell lines were used. Serum is incubated in the antigen coated wells and then removed by washing. A peroxidase conjugated anti-human IgG antibody is then added, incubated and unbound antibody removed by washing. A chromogenic substrate is then added and colour development occurs, the intensity of the colour being proportional to the amount of conjugate present in the well. The absorbance of each well is then read using an ELISA microplate reader and the amount of human IgG antibody binding to the HLA antigens is determined by comparing the OD of the test wells with those of control wells.

### 1.8 The Clinical Significance of the Crossmatch Test

#### 1.8.1 Lymphocytotoxic Crossmatching

Whilst a positive crossmatch is generally regarded as a contra-indication to transplantation it has been clear from the first that not all positive crossmatches are associated with immediate failure. In Patel and Terasaki's 1969 study 20% of grafts with positive crossmatches did not fail, but the risk associated with transplanting across a positive crossmatch meant that this rarely happened when crossmatching became an essential pre-transplant test.



However, over the years many studies have looked at the significance of the crossmatch test and tried to find ways of determining which positive crossmatches are predictive of transplant failure and which are not. The main areas of investigation have been immunoglobulin class, T and/or B cell reactivity, autoreactivity and timing of positive results in relation to time of transplantation. The significance of the sensitising event in relation to several of the above has also been examined.

In 1976 Cross et al first described the phenomenon of auto reactivity in crossmatching for transplantation. In a small series of patient's sera which gave positive crossmatches against donor cells were also found to be positive against the recipients own cells. The transplants were performed and there were no cases of acute graft loss. A series of reports appeared during the following year in which successful transplants in the face of positive crossmatches were described. Lobo et al and Ting and Morris (1977) found that patients with a positive B cell, but negative T cell crossmatch, did not reject grafts at a higher rate than those with negative T and B cell crossmatches. Park et al (1977) suggested that positive B cell crossmatches were due to auto antibodies as they found that the proportions of patients with B cell autoreactivity and those with reactivity to allogeneic B cells at 5°C were the same. Ting and Morris did not agree that all B cell reactivity was necessarily associated with autoantibodies and split their B cell positive crossmatches into those that were due to 'auto' or to 'allo' reactivity. No significant difference was found between the 2 groups in terms of graft outcome, and in fact patients with negative crossmatches had the poorest outcome of all in this series. Reekers et al (1977) confirmed Cross's observation that autoantibodies did not adversely affect graft outcome in 2 cases. In

contrast to these reports DeJelo & Williams (1977) described a case of hyperacute rejection in a patient with a positive B cell crossmatch.

As described above B cell antibodies were often, but not exclusively, associated with autoreactivity. Another factor associated with autoreactivity is immunoglobulin class. Lobo (1981) found that autoreactivity was due to IgM antibody in every case tested with no evidence of IgG autoantibodies being found in patients on haemodialysis. The associations of these three separate characteristics has often led to the assumption of autoreactivity where B cell and/or IgM reactivity is detected and a corresponding belief that these antibodies are not deleterious to graft survival.

However, closer investigation shows that the above conclusion is too simplistic. Some studies have clearly demonstrated that the IgM antibodies identified in the crossmatch test are not only reactive with the donor cells but also with the recipients' own cells, i.e. the antibody is an IgM autoantibody. This can be further confirmed by absorption of the serum with the autologous cells, leading to loss of IgM activity against donor (and other 3rd party cells). It is possible that some IgM antibodies which are not HLA specific are also not autoreactive. It is not clear how common this type of IgM antibody may be as not all studies have exhaustively tested all serum samples for both auto and 3rd party reactivity. The demonstration that antibody reactivity with no apparent HLA specificity is of the IgM class often leads to the assumption of autoreactivity without proof positive being sought. This has resulted in a situation where the majority of IgM antibodies are considered non-deleterious to renal transplants but the policy of whether to transplant in the presence of an IgM antibody varying between centres. Some centres consider all IgM

antibodies to be harmless, some require proof that the antibody causing the positive reaction is indeed autoreactive and some will accept that IgM antibodies with no evidence of HLA specificity are not a contra-indication to transplantation. IgM antibodies which are HLA specific are also found in some patients but there is again no clear consensus on whether these antibodies are deleterious to allografts.

There has continued to be disagreement on the clinical significance of B cell positive, T cell negative crossmatches which are not caused by auto-reactive IgM antibody. Noreen et al (1987) found that a B cell positive crossmatch was associated with lower graft survival than the negative crossmatch group for a group of primary transplant recipients, although when these were split into groups receiving cadaveric or living donor grafts and those on different immunosuppressive protocols this association was no longer significant. Russ et al (1987) found an association between positive B cell crossmatches and rejection, the antibodies causing the positive crossmatch were further analysed by screening and those which were shown to have specificity for class II antigens were found to have the strongest association with rejection. Other studies have found no association between a positive B cell crossmatch and graft failure, indeed Hourmant et al (1990) suggested that a positive B cell crossmatch was associated with better outcome in regrant patients, although the antibody class was not determined in this study.

The importance of determining the antibody specificity causing positive B cell crossmatches, as was done in the study by Russ et al (1987) has been shown in a number of subsequent studies. Sumitran-Karuppan et al (1990) used blocking antibodies to determine HLA specificity of donor reactive B cell antibodies. This study showed that B cell reactivity due to HLA class I specific antibody was



associated with poorer graft outcome than that due to class II specificity, the authors did however note that whilst the majority of the class I antibodies were IgG most of the class II antibodies were IgM. Scornik et al (1992) reported 3 cases of hyperacute rejection associated with positive B cell crossmatches due to HLA class II specific antibody. The proposal that the class II antibody was causative of the rejection in these cases is strengthened by the elution of class II specific antibody from the rejected kidney. Ten Hoor et al (1993) also showed that IgG antibodies reactive with donor B cells which were specific for HLA class II were associated with poor graft survival whereas B cell reactive IgG antibodies which were not specific for HLA or IgM antibodies were not.

There is general agreement that a positive T cell crossmatch with 'current' serum, i.e. a serum sample taken immediately prior to transplantation or the most recent sample obtained depending on the individual laboratories policy, is a contra-indication to transplantation. However the clinical significance of a positive T cell crossmatch with historic samples but a negative result with current serum is less clear. The significance of current negative, historic positive B cell crossmatches is yet more indistinct since there is no general agreement on the significance of current positive B cell crossmatches. Cardella et al (1982) first reported a number of successful renal transplants performed in the face of a current negative, historic positive T cell crossmatch in highly sensitised patients. There was no significant difference in actuarial graft survival between these patients and those for whom all the samples tested were negative. It is interesting to note that within the current negative, historic positive T cell crossmatch group there were 4 patients who had positive B cell



crossmatches with the current serum only 1 of which was still functioning at the time the report was published.

Taylor et al (1989) investigated many of the factors discussed above by characterising the antibodies causing historic positive, current negative crossmatches in terms of immunoglobulin class and HLA specificity, which was determined by using blocking antibodies specific for HLA class I or class II antigens. In common with most other studies IgM, non HLA specific antibodies were not associated with inferior graft survival. IgM antibodies with specificity for HLA class I had no association with poor graft outcome if found only in historic sera but historic positive T cell crossmatches due to HLA specific IgG antibodies were associated with poor graft survival, a finding duplicated by ten Hoor et al in 1993. Taylor et al (1989) found that historic positive, current negative B cell crossmatches showed a different picture to T cells with both IgG and IgM antibodies having no association with graft loss in primary grafts but with poor survival in regrafts. As the antibodies were shown to be specific for HLA this observation raises the question as to whether the sensitising event is a factor for consideration.

Sensitisation to HLA can occur due to transfusion, pregnancy or previous transplant. Sanfilippo et al (1982) showed that of these 3 possible sensitising events previous failed transplant had the greatest quantitative effect on antibody production as measured by the panel reactivity, with pregnancy producing an intermediate effect and transfusion the lowest rise in panel reactivity. When there are combinations of 2 or more of these sensitising events for one patient this can have an additive effect on antibody production, Scornik et al (1984) found that blood transfusions in patients who had had previous pregnancies or failed grafts produced a persistent rise in panel

reactive antibody more frequently than was the case in patients with no previous graft or pregnancy.

The effects of these different sensitising effects on subsequent graft survival was investigated by Sanfilippo et al (1982). The different effects of the combinations of sensitising effects were complex but it was shown that previously high antibody levels in transfused males, with failed grafts or transfused females with previous pregnancies were associated with poor outcome. High antibody levels resulting from transfusion alone were not associated with decreased survival compared with patients with no evidence of antibody production. These results indicate that sensitisation to previous failed grafts or pregnancy may be more harmful to subsequent grafts than transfusion induced antibodies. Aprile et al (1989) also found that primary graft recipients with high levels of peak (historic) panel reactive antibodies had similar survival to those with no antibody whereas regrant recipients with any significant antibody production fared less well than those with no antibody, supporting the idea that antibodies resulting from failed grafts are more deleterious to graft survival than those due to transfusion.

The sensitising effect of previous transplants was also shown by Barger et al (1988) and Senitzer et al (1988) when regrafts which repeated HLA mismatches encountered on a previous graft were shown to have poor survival. Welsh et al (1988) however found that repeat mismatches did not increase the risk of graft loss if it could be shown by previous, comprehensive screening that no antibody specific for the mismatched antigen had ever been produced. Gnant et al (1992) also found that regrafts with a repeat mismatch had similar graft survival to those with no repeat mismatches, despite historic positive, current negative crossmatch results. Poor graft

survival in regrafts with repeat DR mismatches was shown by Cecka and Terasaki (1994) in an analysis of transplant registry data. Whilst such studies have the advantage of large numbers of cases there are disadvantages in that the different centres have a variety of different screening and crossmatch policies and the authors acknowledge that in many cases DR specific antibodies would not have been tested for and advocate the use of B cell crossmatches which are currently not performed by many laboratories.

From the variety of different factors examined in many studies over the years it is clear that antibody specificity is a crucial factor in determining the significance of positive crossmatches, whether with historic or current sera. A comprehensive knowledge of each individual patient's antibodies to both HLA class I and class II antigens would provide the best basis for interpreting crossmatch results.

### 1.8.2 Flow Cytometric Crossmatching

The first study of flow cytometric crossmatching by Garovoy et al in 1983 showed the method to be more sensitive than conventional CDC crossmatches for the detection of antibody. This was also found to be the case by Scornik et al in 1984 when they investigated the relative ability of flow cytometry and CDC tests to detect antibody in patients prior to blood transfusion. It was found that flow cytometry detected antibody in a number of patients who were CDC negative and that these flow positive patients had a higher risk of developing persistent, CDC detectable, HLA specific antibodies following blood transfusion. The greater sensitivity of flow cytometry and the association of a positive flow crossmatch and graft rejection was also confirmed by Chapman et al (1985) and Thistlethwaite et al (1986) showed the



technique to be more sensitive than the AHG augmented CDC crossmatch as well as the standard method. Rodey et al (1987) demonstrated that extra reactivities detected by flow cytometry could be attributed to definable HLA specificities, supporting the possibility that positive flow cytometry crossmatches may be of clinical significance.

In 1987 Cook et al found a clear association between a positive flow crossmatch and graft failure in CDC crossmatch negative renal allograft recipients. This association was significant only in sensitised recipients (those with previous failed grafts or with panel reactive antibodies). However, within the sensitised group the positive flow crossmatch was predicative of 50% of all graft failures. Kerman et al (1990) also found that a positive FCXM correlated with rejection episodes and graft survival in sensitised recipients as did the AHG CDC crossmatch in this study. Other studies demonstrated associations between positive FCXMs and complications in first and regrafts (Lazda et al 1988, Talbot et al 1990), long term graft survival in recipients pre-treated with donor specific transfusions (Bou-Habib et al 1991), and improved graft survival when a negative FCXM was a pre-requisite for transplantation (Talbot et al 1992). In contrast other studies did not find a significant association between a positive FCXM and graft function (Horsburgh et al 1992, Evans et al 1992) although the latter study did show a non-significant trend towards poor graft survival in the small number of regrafts studied.

Small patient numbers in some studies may account for the lack of significant associations between FCXMs and outcome. In one of the largest studies of FCXM and primary kidney transplantation there was a significant association between a positive T cell FCXM and reduced graft survival at one year (Ogura et al 1993). The difference between the positive and negative FCXM groups was only 7 percentage



points, but with the large numbers was significant. It could be reasonably suggested that a graft survival at one year of 75% (compared with 82% in FCXM negative recipients) is within acceptable limits but the authors argue that as the supply of cadaveric organs is insufficient for the demand kidneys would be best utilised in the group of recipients likely to have the highest graft survival rate and therefore prevent 'wastage' of a significant number of organs.

All the above studies have focused on the T cell FCXM. B cell FCXMs are also performed by increasing numbers of laboratories. Martin et al (1993) make this point by describing a significant association between positive T and B cell FCXMs and graft failure. The demonstration of antibody binding to both T and B cells suggests the antibody detected is likely to be directed at the HLA class I antigens. A T cell positive FCXM where there is no antibody binding with B cells suggests that the antibody may not be HLA specific. B cell positive FCXMs may occur when the T cell FCXM is negative. Lazda (1994) found that a strongly positive B cell FCXM was significantly associated with poorer graft survival at one year compared with those where negative, or weakly positive, B cell FCXMs occurred. This association was found only in those patients receiving allografts mismatched for at least one HLA-DR antigen suggesting that the antibody detected may be specific for class II. The clinical significance of the 'strongly positive' B cell FCXM as compared to that defined 'weakly positive' emphasises the possible influence differences in methodology and analysis may have on determining the relevance of the positive FCXM.

Sumitran-Karuppan (1992a) found that the FCXM could be used to great advantage in determining the likely outcome of renal allografts where the specificity of the antibody could be clearly allocated. This group had previously found that some

patients with weakly positive B cell CDC crossmatches could be transplanted without any increased risk as compared to CDC negative patients (Sumitran-Karuppan 1990). The FCXM study demonstrated that in the cases where the weak B cell reactions could be attributed to HLA specific antibodies there was an increased risk of rejection episodes, these patients also had a positive FCXM. A follow-up study investigated patients with negative CDC crossmatches but positive FCXMs and again found that if the antibody was HLA specific there was an increased risk of rejection (Sumitran-Karrupan1992b). These studies emphasise the importance of understanding the antibody profile of the patient serum samples when interpreting FCXMs. A screening technique of similar sensitivity would help in the interpretation of FCXM results.

The relatively large proportion of patients with pre-formed antibodies detected by flow cytometric crossmatching who do not experience any rejection, let alone hyperacute rejection, recalls the earlier observation that not all cytotoxic positive crossmatches result in hyperacute rejection. Whilst in many cases these antibodies have been shown to be non-HLA specific there are still reported cases where the antibodies are HLA specific (e.g. Taylor et al 1987). It is clear that whilst HLA specific antibodies are often associated with reduced graft outcome there are circumstances when this is not the case. It is possible that other, recipient associated factors such as differences in the complement system and its regulatory mechanisms may affect the way in which antibodies participate in graft damaging processes.

### 1.9 Antibody Production Post Transplantation

The *de novo* production of antibodies in renal allograft recipients was described shortly after the connection between pre-transplant HLA antibodies and

graft rejection was found. Morris et al (1968) looked for cytotoxic antibodies to a panel of lymphocytes in pre and post transplant sera and found that whilst only 4 of 21 recipients had pre-formed antibodies at the time of transplant this proportion of antibody positive patients rose post transplant with 9 of 10 graft failures producing antibodies. They also noted that the titre of the antibodies tended to rise following removal of the rejected kidney. Altogether only 3 of the 17 cases where post transplant antibody production was observed continued to have good graft function at the time of publication. The results of this study clearly demonstrated that antibodies were produced following transplantation and suggested a possible link between such antibodies and poor graft prognosis. In a follow-up study the same group demonstrated that the antibodies produced by some patients post nephrectomy were directed against HLA specificities which were present on donor but not recipient cells (Morris et al 1969). This provided evidence that the antibody production was a result of sensitisation by the donor tissue and suggested the possibility that the antibodies may be involved in the rejection process.

In 1978 Soullou et al investigated the presence of antibodies in a group of 18 renal allograft recipients. They found a significant association between the development of antibodies to donor B lymphocytes and early graft loss. Five patients experienced failure within the 1st month post transplantation, all produced antibodies compared with 3 of 13 patients with grafts functioning for more than 1 month. In only 1 of the 5 cases of early graft loss were the antibodies detected prior to graft failure. Similarly Lepage et al (1978) detected antibodies to PBL and separated B cells in only 3 of 9 cases of irreversible acute rejection prior to graft loss, but following transplant nephrectomy 3 of 4 patients with samples available for testing developed detectable



antibodies. The authors suggested the possibility that antibodies may have been absorbed onto the allograft preventing their detection in the circulation until the graft was removed. In this group of patients acute rejection was classed as graft loss within 2 months of transplant. When 1 year graft survival was examined it was found that only 20% of transplants where B cell antibodies had been demonstrated continued to function compared with 68% of those with no detectable antibody. In these recipients the antibodies appeared prior to graft loss and it was suggested that anti-B cell antibodies may be prognostic of what was termed by the authors 'chronic' graft loss. The following year the significance of anti B cell antibodies was questioned by Ting and Morris (1979). They failed to find any association between B cell antibodies and graft loss, with reactivity to donor B cells present in 63% of cases failure and 61% of successful transplants.

A possible reason for the different results obtained in these studies is that the anti-B cell antibodies detected may have different specificities. Soullillou (1978) attributes the anti-B cell reactivity in the patients tested to specificity for the then recently described DRw antigens. The serum samples were tested both unabsorbed and following absorption with pooled platelets. In 2 of 5 cases there was reactivity with T cells which was abolished by absorption suggesting antibodies to HLA class I. No data is given concerning the effect of absorption on B cell reactivity and in the 2 cases with class I antibodies additional DRw reactivity was assumed as the strength of the cytotoxic reaction was greater against B cells than against T cells or unseparated lymphocytes. However, as class I antigen expression is higher on B cells than the respective T cells, it is possible that these reactions could be due to the class I reactivity. In the study by Lapage (1978) B cell antibodies were demonstrated in sera



which had been platelet absorbed. In 50% of cases where B cell antibodies were found PBL antibodies which were removable by absorption were also demonstrated suggesting that class I specific antibodies were present in these patients. Ting and Morris (1979) found that there was no evidence of class I antibody in 23 of 24 cases where reactivity to donor B cells was demonstrated. Antibodies were shown to be reactive with the recipient's own B cells in 36% of patients. This highlights again the importance of determining the specificity of the antibodies being detected in these studies as in those investigating the antibodies detected by pre-transplant crossmatching.

Comparison of the results of different studies on the post transplant production of antibodies detected using B cells can be difficult for the reasons stated above. However in studies which have concentrated on the role of antibodies which react with T cells it is possible to compare the results of studies where the specificity of the antibodies detected is to HLA class I.

In 1987 Martin et al studied post-transplant antibody production in primary transplant recipients. Testing of serial serum samples showed that in the majority of cases there was no detectable antibody either pre or post transplant. The de novo development of antibodies was observed in 63 recipients with a reduced graft survival at both 1 and 5 years when compared with those recipients with no detectable antibody. Overall 44/63 patients lost grafts and had persistent post transplant antibodies. In 17 cases where a transient antibody production was observed the grafts continued to function. Using an exhaustive screening policy it was possible to assign antibody specificity from the PRA results in 26 cases and in 23 of these the specificity corresponded to antigens present in the donor. In only 2 cases where donor specificity

was demonstrated did the grafts continue to function with 21 cases of rejection. In ten of the failures the antibodies were detected in sera taken prior to graft failure whilst in 11 cases the antibodies only became detectable following graft nephrectomy.

As in the previous studies by Morris (1968,1969), Martin et al found that the antibodies were not always detected prior to removal of the transplanted organ. Thus it is unclear whether the antibody is involved in the rejection process or is merely a consequence of that process. In 1988 Tang et al carried out regular testing following transplantation using stored donor cells to detect donor specific antibody production. In 86.8% of cases of acute rejection there was coincident antibody production whereas only 7.9% of samples taken when there was no on-going rejection were found to contain detectable antibody. In only 1 case was antibody detected prior to diagnosis of rejection whereas samples taken on the same day as the diagnosis were positive for antibody. This indicates that the antibody production in these patients occurred at the time of rejection but was not predictive of rejection.

A study by Halloran et al in 1992 showed a similar pattern of antibody production post transplant as seen by Martin and Tang. All the antibody positive patients experienced at least one rejection episode although antibody was detected prior to diagnosis of rejection in only 4 of 15 cases. The antibody was shown to persist in cases where rejection did not respond to treatment but reactivity disappeared following successful treatment. There were also rejection episodes in 41% of patients with no detectable antibody demonstrating that in this group of patients whilst antibody production was always associated with rejection, rejection was not always accompanied by antibody production. Whilst this study like the previous ones did not find antibodies predictive of rejection it did provide some evidence that the antibodies

were involved in the rejection process. Biopsies from antibody positive patients showed evidence of direct antibody mediated damage together with features of cell mediated damage suggesting a mixed rejection picture. In antibody negative patients only features of cellular rejection were seen. This suggests that the antibody is a participant in the rejection process although whether it initiates the rejection or simply contributes to a process already underway cannot be deduced.

Lobo et al (1995) found that the outcome of rejection episodes associated with donor specific antibody production was poor compared to that of rejection episodes which were not accompanied by antibody production. As in the other studies described the antibody was detected at the time of onset of rejection (17 cases) or following graft nephrectomy (2 cases). Fifteen of the 19 patients lost their grafts compared with 17 of 63 with no antibody production. It is suggested that antibody production at the time of rejection is predictive of 'poor-prognosis acute rejections'. Such information may be useful in determining the treatment of such rejections, although the cases described in this study failed to respond to aggressive treatment with OKT3.

The studies described above all used complement dependent lymphocytotoxicity testing with either donor cells, lymphocyte panels or both. Only a small number of investigations of post-transplant antibody production by flow cytometry have been published as detailed below.

In 1989 Scornik et al evaluated antibody production by carrying out flow cytometric crossmatches of post transplant serum against donor lymphocytes. Antibody was detected in 19 of 48 patients experiencing rejection. In 6 cases the antibody was of the IgM isotype only whereas IgG was present in 13. No patients



without rejection episodes were found to develop IgG antibodies but 2 of 22 had IgM production. There was a significant association between IgG antibody production and rejection. Once again the antibody was not detected until after the onset of rejection in half the cases, limiting any potential predictive value. Thus the use of the sensitive flow cytometric test in this study confirmed the findings of workers using CDC but did not confer any greater predictive value to the antibody testing.

In contrast, Al-Hussein et al (1994) found that post transplant antibody production was predictive of severe rejection when flow cytometric detection was used. Rejections which were classed as mild were not accompanied by antibody production. One confounding factor was that donor specific antibodies were also detected in cases of delayed graft function thus it would not be possible to predict a rejection in a patient experiencing delayed graft function.

Morris and Terasaki (1968) found that 9 of 10 cases of failed transplants resulted in the production of HLA antibodies. However since this early work very few subsequent studies have investigated the proportion of failed transplants which result in circulating HLA antibodies. In 1987 Sanfilippo investigated antibody production following graft failure in relation to HLA matching. This study only considered matching at HLA-A and B with no assessment of DR matching. The authors found that patients with 0 or 1 mismatched antigens had significantly lower panel reactive antibody levels post graft failure than those patients mismatched for 2 - 4 antigens. The better matched patients, with lower PRs were more likely to be retransplanted during the study period. In contrast Matas et al reported in 1990 that whilst patients with high PRs post graft failure had a lower probability of receiving a second transplant the PRs found post graft loss were not related to the number of mismatched



antigens at HLA-A,B,DR. The screening method used by Matas was a CDC technique with peripheral blood lymphocytes as the target which would be unlikely to reliably detect any HLA class II specific antibody production.

HLA class II antibody production has remained a largely unexplored area, partly perhaps because of the lack of consensus of the clinical significance of B cell positive crossmatches and therefore of class II specific antibodies. The comparative difficulty of screening for class II specific antibodies as compared with detection of class I specific antibody may also be partly responsible for the smaller proportion of studies which have addressed this subject.

#### 1.10 Aims of the Study

The aim of this study is the development of a flow cytometric screening technique to provide a method for antibody detection which is as sensitive as the final crossmatch test used in the laboratory. Following successful development of this technique a comparative study of the flowscreen with a recently developed ELISA method will be carried out to determine whether the two methods are complimentary and could be used to gain information about antibody panel reactivity and specificity which standard CDC screening does not provide. Finally a combination of the available techniques will be used to study antibody production in patients with failed transplants. Graft function and antibody production will be related to HLA matching. The impact of antibody production on future transplantation will be investigated.

**CHAPTER 2**  
**MATERIALS AND METHODS**

## 2.1 Reagents and Consumables

The following list details the main reagents and consumables, and the suppliers, used in the techniques described in the following chapter.

Acridine Orange (BDH 46005)

Agarose (Sigma A-9539)

Albumin bovine fraction V (BDH 44155)

Ammonium Chloride (Sigma A9434)

Antibiotic/Antimycotic solution (Life Technologies)

Boric Acid (BDH Analar 10058)

Bovine Haemoglobin (Sigma H2652)

Bovine thrombin 50u (Sigma)

BSHI Primer Kit

Calcium Gluconate 10% w/v (Evans Medical Ltd)

CaliBRITE Beads (Becton Dickinson UK Ltd)

Complement Fixation Test diluent (ICN Flow)

Coulter-Clenz (Coulter)

Cystine (Sigma)

Dimethyl Sulphoxide (Sigma)

di-Sodium ethylenediaminetetra-acetic acid (EDTA) (BDH) 5% solution

Ethidium Bromide (Sigma E8751)

FITC conjugated anti-human IgG F(ab)<sup>2</sup> (Dako Ltd F0315))

Foetal Calf Serum (Sigma)

Glass Balls 2.5-3.5mm (BDH)

Immuno-Check Beads (Coulter)

Isoton II (Coulter)

Lymphoprep (Nycomed UK Ltd)

Lyophilised Rabbit Complement (Quest Biomedical)

Magnesium chloride (Advanced Biotechnologies)

Park-Terasaki Medium (Life Technologies)

Phosphate Buffered Saline (PBS) tablets (ICN Flow 28-103-05)

PRA-STAT (SangStat Medical Corporation)

Proteinase K (Boehringer Mannheim 1092 766)

RPMI1640 with Glutamax I (Life Technologies)

Sodium Azide (Sigma S2002)

Sodium Dodecyl Sulfate (SDS) 20% w/v (Biorad 161-0418)

Sodium Hydrogen Carbonate (BDH Analar10247)

tri-Sodium Citrate (BDH) 3.8% solution

Terasaki trays (Greiner)

Thermostable DNA Polymerase (Advanced Biotechnologies AB-0192)

Tris-Hydrochloric Acid (Sigma T-1378)

Ultrapure dNTPs set (Pharmacia 27-2035-01)

10X PCR Buffer IV (Advanced Biotechnologies AB-0289)

0.2ml reaction tubes (Advanced Biotechnologies AB-0264)

1.7ml microcentrifuge tubes (Sigma T-3406)

6ml tubes (Falcon 2054)

15ml tubes (Nunc 355581)

250 cm<sup>3</sup> culture flasks (Nunc 147589)



## **2.2 Equipment**

The following list details the main pieces of equipment used during this study.

**FACScan (Becton Dickinson UK Ltd)**

**EPICS XL MCL (Coulter Electronics Ltd)**

**Dynatech MR5000 Plate Reader (Dyantech)**

**Perkin Elmer 9600 thermal cycler (Perkin Elmer)**

**Heraeus Centrifuge (Heraeus)**

**Microcentrifuge (Hettich)**

**Electrophoresis tank (Biorad)**

**Power supply (Biorad)**

**UV Transilluminator (Flowgen)**

**Camera (Polaroid)**

**Incubator (Gallenkamp)**

## 2.3 Lymphocytotoxicity Testing

### 2.3.1 Introduction

Complement dependent lymphocytotoxicity testing has been the standard method used for HLA typing, antibody screening and crossmatching since the start of tissue typing for clinical transplantation (Terasaki & McClelland 1964). The principle of the assay is that HLA specific antibodies bind to the relevant HLA antigens on the cell surface of lymphocytes during an incubation period. Addition of rabbit complement after this incubation will cause lysis of any cells with antibody bound to surface receptors. Cell death may be visualised by staining the cells using a vital dye and examining by microscopy. The standard lymphocytotoxicity assay will detect any complement fixing antibodies but will not detect non complement fixing antibodies. The technique will detect antibodies other than those which are HLA specific if they are bound to lymphocyte surface receptors.

All cell preparation and lymphocytotoxic testing was performed according to the laboratory's standard operating procedures as detailed below.

### 2.3.2 Cell Preparation

#### 2.3.2.1 Peripheral Blood T cells

T cells were obtained from 20ml of sodium citrate anticoagulated blood. The blood was defibrinated by incubation on an orbital mixer with 75µl of 50U thrombin, 2ml calcium gluconate and 20 glass beads for 10 mins. The defibrinated blood was layered onto 4ml Lymphoprep in 15ml centrifuge tubes and centrifuged for 15 minutes at 1000g. The cells were collected at the interface and transferred to a clean tube. Ten ml of Complement Fixation Test Diluent (CFT) was added to the tube and

centrifuged at 400g for 5 minutes. The supernatant was decanted and the cell pellet resuspended in 10 ml CFT. The cells were centrifuged at 400g for 5 minutes and the supernatant decanted. The cells were resuspended in CFT and counted using a Neubauer counting chamber. The cell count was adjusted by addition of CFT to a final count of  $1.5 - 2.0 \times 10^6/\text{ml}$ .

#### 2.3.2.2 Peripheral Blood B Cells

B cells were obtained from 10ml of EDTA or sodium citrate anticoagulated blood. The blood was centrifuged at 1000g for 5 minutes and the buffy coat collected and mixed with 10ml PBS/citrate at 4°C. Fifteen  $\mu\text{l}$  of CD19 Dynabeads were added and mixed with the cell suspension for 5 minutes on an orbital mixer. The tube was placed on a magnetic particle concentrator (MPC) and left for 5 minutes to allow the Dynabeads and attached cells to migrate to the side of the tube next to the magnet. The supernatant was aspirated and the tube then removed from the MPC. The beads were washed twice by addition of PBS/citrate for the first wash and CFT for the second. The beads were resuspended in a final volume of 75 $\mu\text{l}$  CFT.

#### 2.3.2.3 Splenic Lymphocytes

Cadaver donor lymphocytes were obtained from a portion of spleen. A portion of spleen was placed in a Petri dish and CFT or RPMI added. The spleen was lacerated using scissors and cells were released from the spleen by gentle squeezing. The resulting cell rich solution was layered onto lymphoprep and centrifuged for 10 minutes at 1000g. The lymphocytes were removed from the interface and washed twice by addition of CFT and centrifugation at 400g for 5 minutes. The lymphocytes

were resuspended in CFT, counted and the concentration adjusted to a final count of  $1.5-2.0 \times 10^6/\text{ml}$ . If separated B lymphocytes were required 2ml of the unseparated lymphocyte suspension was transferred to a clean tube and 10 $\mu\text{l}$  of CD19 Dynabeads added. The B cells were then isolated with the beads as described above.

### 2.3.3 HLA Typing

HLA typing of recipients was performed in the routine laboratory using lymphocytes separated from peripheral blood. The HLA class I type was obtained using T cells and the HLA class II type using B cells. Class I typing trays were made in the laboratory using a variety of well defined sera obtained from the United Kingdom Transplant Support Service Authority (UKTSSA), other Tissue Typing Laboratories within the UK and serum from local patients. Class II typing trays were commercially obtained or produced using the National sera supplied by UKTSSA. HLA typing of cadaveric donors was carried out using lymphocytes obtained from spleen.

Cells used for antibody screening were obtained from a variety of cell donors including samples received for HLA typing for disease studies and local volunteers. HLA class I typing was performed using peripheral blood T cells.

### 2.3.4 HLA Specific Antibody Screening

#### 2.3.4.1 Samples

Serum samples were obtained regularly from patients awaiting renal transplantation and were stored at  $-20^{\circ}\text{C}$ . Sera from patients known to have well defined, high panel reactive antibodies would be assigned to screening plates which



would be tested with 40 cells which were not from preselected cell donors but were randomly selected from samples received in the laboratory, termed random cells.

Undefined sera or sera from patients known to have low panel reactive antibodies would be assigned to a screening plate to be tested with 60 random cells.

Samples which required screening with DTT to identify IgM antibodies were set up on parallel plates one containing untreated sera and one containing sera treated with DTT.

#### 2.3.4.2 Screening plate preparation

Sera for screening were dispensed into reservoir plates. A volume in excess of that required for the number of plates to be poured was added to each well of the reservoir plate. High panel reactive sera was placed in alternate wells with negative control sera in intervening wells. Plates were poured using the Biotest Autosera-dot. Eight  $\mu\text{l}$  of paraffin oil was dispensed into each well of a 60 well Terasaki tray. One  $\mu\text{l}$  of serum was dispensed into each well. The position of each serum sample on the plates was recorded on the screening sheet. Screening plates were stored at  $-20^{\circ}\text{C}$ .

#### 2.3.4.3 Screening method

Sera were screened against a random panel of cells. One  $\mu\text{l}$  of cells was added to each well of the screening plate and incubated for 30 mins at  $22^{\circ}\text{C}$ . Five  $\mu\text{l}$  reconstituted, lyophilised rabbit complement was added to each well of the plate and incubated for 60 mins at  $4^{\circ}\text{C}$ . Cells were then stained with ethidium bromide/acridine orange/haemoglobin stain.

Stock solution of stain was prepared as follows and stored as 2ml aliquots at

**-30°C:**

**0.14g ethidium bromide**

**0.07g acridine orange**

**1ml 100% ethanol**

**100g bovine haemoglobin**

**16.65g Sodium EDTA**

**666ml PBS**

**Plates were read by eye using an Olympus IMT2 microscope. Any dead cells above background were recorded with the following scores:**

<b>up to 20% above background</b>	<b>1</b>
<b>21-40% above background</b>	<b>2</b>
<b>41-60% above background</b>	<b>4</b>
<b>61-80% above background</b>	<b>6</b>
<b>81-100% above background</b>	<b>8</b>

**Individual plates were removed from the freezer and thawed prior to use.**

**Plates were set up with all suitable cells received in the laboratory until fewer than 10-20 plates of the original 40 or 60 prepared remained to be used. The HLA types of the cells used so far were then checked and cells for the remaining plates were selected to include antigens which were missing from the panel. The percentage panel reactivity was determined by dividing the number of positive reactions by the total number of cells screened. Antibody specificity was determined by manual analysis of positive and negative reactions, together with information on the patient's HLA type and known sensitisation.**

#### 2.3.4.4 DTT Screening Method

Sera for screening with DTT were treated by addition of 20µl 0.1M DTT to 180µl serum. The plates were then poured as described above. Complement to be added to DTT treated sera was supplemented with 25µl 0.1M cystine per 1ml complement. All other procedures were as described in section 2.3.4.3

### 2.4 Cell Culture

#### 2.4.1 Cell Lines

Epstein Barr Virus transformed B lymphoblastoid cell lines were obtained from the European collection or UKTSSA as growing cultures. Frozen aliquots of all cell lines were stored in liquid Nitrogen (LN<sub>2</sub>) for future culture. All cell lines were grown individually as required.

#### 2.4.2 Cell Culture Method

Cells were maintained in RPMI 1640 containing 10% foetal calf serum and antibiotic/antimycotic solution. Cell lines obtained as growing cultures were spun for 5 mins at 70g. Frozen cell lines from stock were thawed by placing in a water bath at 37°C and transferring the thawed cell suspension to Park-Terasaki medium at 37°C, then spun for 5 mins at 400g. The supernatant was decanted. The cells were washed by addition of 5ml culture medium and centrifugation. The cells were resuspended in culture medium to a concentration of 1x10<sup>6</sup>/ml and transferred to a culture flask. Growing cultures were kept at 37°C in an atmosphere of 5% CO<sub>2</sub>. Flasks were checked daily for cell growth and medium added as required until sufficient cells were present.

### 2.4.3 Preparation of Cell Pools

Cell pools were made by combining cells from a number of different cell lines. A small volume of cell suspension was removed from each flask by pipette and transferred to individual tubes. The cells were counted using a Neubauer counting chamber and the concentration calculated. The concentration and volume of each cell line was entered into a computer programme to calculate the amount of each cell line required to make the pool such that an equal number of cells from each line would be present. The appropriate volumes of each cell line were then pooled. In initial studies different numbers of cell lines were used for individual pools as detailed in figures 3.5 and 3.6 (p117, 118). Subsequently a total of 10 different cell lines were included in an individual pool and 2 different pools were used for all Flowscreens.

The pooled cells were thoroughly mixed and then spun down for 5 mins at 400g. The cells were resuspended at a concentration of  $2 \times 10^7$ /ml in a solution containing 45% RPMI, 45% foetal calf serum and 10% dimethyl sulphoxide. One ml aliquots were placed in cryotubes and the tubes placed in a freezing rack in the neck of a LN<sub>2</sub> dewar. The tubes were left in the rack in the vapour phase of the LN<sub>2</sub> for 30 mins and then the rack was lowered so that the tubes were in the liquid. Cells were left in LN<sub>2</sub> for at least 2 hours before transfer to storage in a -80°C freezer.

### 2.4.4 Use of Frozen Cell Pools

Aliquots of frozen pooled cells were retrieved from storage and placed in a water bath at 37°C until thawed. The cell suspension was transferred to a 15ml tube containing 2ml Terasaki Park medium at 37°C and left to recover for 5 minutes. Pooled cells were then used in the antibody binding assay as described in section 2.5.4



## 2.5 Flow Cytometry

### 2.5.1 Introduction

Flow cytometry is a technique which allows measurement of a number of parameters for single cells using detection of light scatter and light emission. Cells in suspension are introduced into the flow cell of a flow cytometer via a pressurised fluidics system such that a narrow stream of sheath fluid surrounds the sample stream ensuring that cells pass through a laser beam one at a time.

When each cell passes through the laser beam the light is scattered in a way which is determined by the size and density of the cell. The scattered light is detected by forward scatter and side scatter detectors. Forward scatter is the light scattered at angles close to the axis of the laser beam and is proportional to the size of the cell. Side scatter is the light scattered around a 90° angle to the axis and is proportional to cell granularity.

Other cellular characteristics may be measured by the use of fluorochrome conjugated antibodies. Membrane bound structures may be detected by addition of a monoclonal antibody specific for the structure of interest. If fluorochrome is bound to the cell surface via an antibody the fluorochrome will emit light of the appropriate wavelength when it passes through the laser beam. The light may then be detected by photomultiplier tubes. A series of filters ensures that only light of the required wavelength reaches the detector. The bench top flow cytometers in current use commonly have detectors for 3 different emission wavelengths.

The various parameters measured for each cell as it passes through the laser beam are converted into electronic signals which are recorded and can be analysed by

the computer. Data which is stored as listmode data may be analysed by computers other than those which are part of the flow cytometer if appropriate software is used.

## 2.5.2 FACScan

### 2.5.2.1 Introduction

The FACScan is a bench top five-parameter flow cytometer. It is fitted with a 488nm air-cooled, argon, ion laser. Light scatter is detected by forward and side scatter detectors. Emissions are detected by 3 photomultipliers which detect fluorescein isothiocyanate (FITC) emissions via a 530nm band pass filter, phycoerythrin (PE) via a 585nm band pass filter and red fluorescence via a >650nm band pass filter.

### 2.5.2.2 FACScan Operation

At the start of each daily session the machine alignment and sensitivity was checked using CaliBRITE beads. The amplifier gains and PMT voltages were set and spectral overlap compensated using the AUTOCOMP software. The instrument settings were recorded and a sensitivity test run and the results recorded. Forward scatter and side scatter measurements were carried out using a linear scale and fluorescence measurements made on a 4 decade log scale. All flow cytometry performed in this study was single colour using FITC. FITC was detected by the FL1 detector. FL2 and FL3 detectors were switched off. FITC data was collected using the log scale with resolution set at 256 channels.

Cell populations were identified by the forward and side scatter profiles (Figure 2.1, p90). The forward scatter gain and side scatter voltage were adjusted as

necessary to display the population in the FSC/SSC dot plot. The cell population of interest was live gated so that data on that population only was stored. For each sample analysed 10,000 events were collected. Listmode data was stored on floppy disks.

### 2.5.2.3 Data Analysis

Data analysis was performed using the LYSYS II analysis software. FL1 histograms were drawn for each sample to be analysed. An analysis gate was set on the negative control sample using the histogram statistics menu. The analysis gate set on the negative control was stored and copied to the histogram plot of each test sample. Histogram statistics were displayed and the percentage of events falling within the analysis gate was recorded. Figure 2.2(p 91) shows examples of positive and negative histograms with analysis gates and statistics. The percentage reactivity of the samples is given by the percentage of gated events.

## 2.5.3 EPICS XL MCL

### 2.5.3.1 Introduction

The EPICS XL MCL is a bench top five-parameter flow cytometer. It is fitted with a 488nm air-cooled, argon, ion laser. Light scatter is detected by forward and side scatter detectors. Emissions are detected by 3 photomultipliers which detect fluorescein isothiocyanate (FITC) emissions via a 525nm band pass filter, phycoerythrin (PE) via a 575nm band pass filter and red fluorescence via a 620nm band pass filter. The MCL attachment takes a carousel which allows 32 tubes to be run in an automated fashion.

### 2.5.3.2 EPICS XL MCL Operation

At the start of each daily session the start up programme was run and the machine alignment and detectors checked using Immunocheck beads. If half peak CVs were greater than 2.0 the prime function was performed as required until the half peak CVs fell within the acceptable range as instructed by the manufacturers. For single colour analysis the FSC, SSC and FL1 detectors were selected. Linear amplification was used for FSC and SSC and a 4 decade log scale was used for FL1 measurements. The cell population was identified by forward and side scatter. A gate was set around the population and used to set the stop count so that 10,000 events falling within the gate were collected. (Figure 2.3 p92) Listmode data on all events was stored for initial analysis on the hard disk and following analysis was archived to optical disk in case subsequent analysis was necessary.

### 2.5.3.3 Analysis

The listmode data on all test samples in a run was selected and the negative control sample was replayed to give the FL1 histogram. The analysis gate was set using the negative control and stored. The listmode data on all samples was then replayed as a batch with the analysis gate being copied to all histograms. The data was then displayed using the multigraph gallery command and the histograms and histogram statistics printed. The percentage of events falling within the analysis gate was recorded. Figure 2.4 (p93) shows the data print out for a series of 8 samples analysed in this way.



## 2.5.4 Flow Cytometric Antibody Binding Assay

### 2.5.4.1 The Principle of the Assay

The principle of the flow cytometric binding assay is that antibodies present in human serum will bind only to the antigens for which they are specific when cells and serum are incubated together. Unbound antibody is removed at the end of the incubation period by diluting and decanting the serum using washing steps. An anti-human immunoglobulin specific polyclonal antibody conjugated to a fluorochrome is then added and will bind to any human immunoglobulin of the appropriate class which has bound to cell surface antigen during an incubation period. Unbound polyclonal antibody is removed at the end of the incubation by washing steps. The cells are then analysed by flow cytometry to detect bound fluorochrome on the cell surface.

### 2.5.4.2 Target cells

The cells to be used in flow cytometric techniques were all washed and resuspended in a solution of phosphate buffered saline containing 0.1% bovine serum albumin and 0.1% sodium azide (FACS diluent). The cells to be used in the assay would be transferred to a 15ml tube and the volume of liquid in which the cells were suspended was made up to 10ml by adding FACS diluent. The cells were spun in a centrifuge for 5 minutes at 400g. The supernatant was decanted and the cells resuspended in 10ml FACS diluent. The cells were spun in a centrifuge for 5 mins at 400g. The supernatant was decanted and cells were resuspended in FACS diluent at a final concentration of  $5 \times 10^6$ - $5 \times 10^7$ /ml.

#### 2.5.4.3 Serum

A negative control serum was included in every assay. The negative control was pooled normal human serum. In early assays where the flow cytometric screening technique was under development the negative control serum was chosen on the basis of cytotoxic screening results and previous satisfactory performance in flow cytometric crossmatches. When the flow cytometric screening technique became established negative control sera were selected on the basis of cytotoxicity and flow cytometry screening results.

A positive control serum was included in each run. The positive control was a pool consisting of equal volumes of serum selected from 5 highly sensitised patients (panel reactivity >80%). The sera were selected on the basis of cytotoxic screening results such that the pool would be expected to be positive with all HLA class I antigens.

All sera were stored at a temperature below -20°C and were thawed immediately prior to use by placing in a water bath at 37°C.

#### 2.5.4.4 Antibody Binding

All reactions were carried out in 6ml tubes (Falcon). The tubes were labelled and the required volume of serum added to the tube (volumes varied according to the assay and will be detailed in each section ). The required volume of cells was then added and the cells mixed with the serum by vortexing for 2 seconds. Caps were placed on the tubes and the cells incubated for 30 minutes at 22°C.

Unbound antibody was removed after the incubation by adding 2ml FACS diluent to each tube, mixing and centrifuging at 400g for 5 minutes. The wash step

was then repeated. The supernatant was decanted and the cells resuspended in the residual FACS diluent in the tube (a volume of 30-40µl).

Four µl of a polyclonal anti-human IgG antibody conjugated with FITC was added to each tube. The cells were mixed by vortexing and incubated for 30 minutes at 4°C.

Unbound polyclonal antibody was removed by 2 wash steps using 2ml of FACS diluent and centrifuging at 400g for 5 minutes. The cells were resuspended in a final volume of 500 µl FACS diluent and were analysed immediately.

The analysis gate was set on the negative control as illustrated in figures 2.2 and 2.4. A background binding of between 0 and 10% was allowed within the analysis gate. The percentage of positive cells falling in the analysis gate was recorded for each sample. Antibody binding of 10% or greater above the background binding was classed as positive.

## 2.6 Enzyme Linked Immunosorbent Assay

### 2.6.1 Introduction

All enzyme linked immunsorbent assays (ELISA) were carried out using the PRA-STAT kits according to the manufacturer's instructions. The principle of the test is illustrated in figure 2.5. Soluble HLA molecules are captured onto the bottom of wells using monoclonal antibodies and these are supplied in the form of 96 well plates. HLA molecules from different cell lines are used to coat individual wells. Serum is added to the individual wells and incubated to allow antibody binding to the antigens coating the well if the antibody in the serum is specific for the antigen/s present. Following this incubation the plates are washed to remove unbound antibody.

An anti-human IgG antibody conjugated to peroxidase is then added to each well. This antibody will bind to human IgG which is bound to the antigens coating the well but will not bind if no human antibody is present and will not bind to immunoglobulins of different classes. Following the incubation with the anti-human antibody the plates are washed to remove unbound antibody. A chromogenic substrate is then added to the wells and colour will develop during the incubation with an intensity which is proportional to the amount of bound conjugate. The reaction is then stopped and the absorbance of each well is measured using an ELISA reader.

### **2.6.2 Plates**

Each kit contained 6 sets of 4 plates and allows for the testing of 8 samples per set of plates. Each plate consisted of 8 rows (row A-H) and 12 columns. Columns 1-12 of the assay on plate A, 13-24 on plate B, 25-36 on plate C and 37-48 on plate D. On plate A column 1 contained a control preparation of soluble HLA antigen, and column 2 had no antigen bound to the well. All the remaining columns on the 4 plates contained soluble HLA derived from an individual cell line, 46 cell lines in total.

### **2.6.3 Reagents**

A positive reference sample containing HLA class I specific antibodies and a negative reference human serum with no HLA specific antibodies were provided.

The specimen/conjugate diluent of bovine serum albumin in a stabilised buffer was supplied as a x4 concentrate. This was diluted 1:4 in distilled water immediately prior to use.



The wash buffer was provided in powder form and dissolved according to the manufacturers instructions prior to use.

The conjugate was a goat anti-human IgG and was supplied in lyophilised form. The conjugate was reconstituted by adding 1.5ml deionised water. The conjugate was stored in solution at 4°C following reconstitution.

The substrate solution was made immediately prior to use by dissolving o-phenylenediamine tablets in the buffer provided and placing in the dark for several minutes until the tablets were fully dissolved.

The stop solution was 1N hydrochloric acid.

#### **2.6.4 Method**

Each serum sample to be tested was diluted 1:101 by adding 70µl of serum to 7ml specimen/conjugate diluent and mixing well. The positive and negative reference samples were diluted 1:101 by adding 10µl to 1ml of specimen/conjugate diluent. 100µl of the positive reference was added to the wells in rows A-F of column 1 and 100µl of negative reference was added to wells G and H of column 1. 100µl of test serum was added to each of columns 2-48 of a single row. The plates were incubated for 2 hours at room temperature.

Washing was carried out manually. The serum was removed by inverting the plates and flicking quickly to expel the contents of the wells. Each well was filled to the rim with wash buffer and the buffer then removed by repeating the flicking procedure. This step was repeated twice. Any excess wash solution remaining in the wells was removed by inverting the plates and draining onto absorbent paper.

The conjugate was prepared by diluting 100µl of the conjugate stock solution in 900µl of specimen/conjugate diluent. This was then further diluted according to the manufacturers instructions to a final dilution which was lot specific. 100µl of the conjugate solution was added to each well and the plates were incubated for 1 hour at room temperature. Following this incubation the plates were then washed three times as described above.

The substrate solution was then added. A timer, set for 15 minutes, was started immediately following the addition of 100µl of substrate solution to row A of plate A and 100µl was dispensed into each subsequent row of plates A to D at a steady rate. The plates were then placed in the dark. At the end of the 15 minute incubation 100µl of the stop solution was immediately dispensed into row A of plate A and then at a steady rate into the subsequent rows.

The plates were read within 15 minutes of the addition of the stop solution using a Dynatech MR5000 plate reader, under computer control using the SOFT-STAT software. The measuring wavelength was set at 492nm with a reference wavelength of 620nm and the absorbance (OD) of each well was recorded using SOFT-STAT.

### 2.6.5 Analysis

Analysis of results was carried out using SOFT-STAT software and by manual analysis of the results produced by SOFT-STAT.

The following calculations were performed by the SOFT-STAT software.

For a valid assay the positive references in rows A-F of column 1 should have an OD between 0.67 and 1.76. The mean of the 6 positive reference OD values was calculated. A  $\Delta$  value was calculated for each well on the plate as follows

$$\Delta = \text{OD test well} - \text{OD of no antigen well}$$

A cut off value was determined by SOFT-STAT such that:

$$\text{cut off value} = \text{mean of positive reference} \times 0.35$$

If the  $\Delta$  value for a well was greater than the cut off value that well was judged positive with the specimen tested. The positive/negative results for each of the 46 test wells were calculated for each sample. A panel reactivity was calculated as follows

$$\% \text{ PRA} = \frac{\text{no. positive results}}{46} \times 100$$

46

The operator could obtain results reports in formats where results were sorted according to OD,  $\Delta$  or well no. Sorting according to  $\Delta$  produced a report which listed the reactions of the sera with the individual wells in order of the strength of the reaction. Fig 2.6 (p95) shows the standard result report used in this study sorted according to the  $\Delta$  value. The cut off value is shown at the top of the report and all wells with a  $\Delta$  greater than this were reported as positive. The operator derived positive threshold is indicated by the line drawn below the result for well 38. It can be seen from the result that although wells 37,32,47 and 38 are classed negative by the analysis software they fall only just below the cut off. There is a larger gap in the  $\Delta$  value of well 38 and well 12 where the operator derived cut off has been assigned.

The antibody specificity was determined by Fisher's analysis. These specificities were confirmed or adjusted by the operator following analysis of the reactions with all the wells in conjunction with the HLA type of the patient from

which the sample was obtained and also with any HLA data relating to known sensitising events. In all instances the HLA specificities assigned as a final result were those confirmed by the operator. The operator derived cut off such as that shown in fig 2.6 (p95) may be confirmed by the specificity analysis. As shown in this example the specificity of the reactions classed negative by the analysis programme but positive by the operator indicates that these reactions are consistent with the A2 specificity determined by SOFT-STAT. All results were analysed in this way and the PR determined by SOFT-STAT was amended by the operator where necessary.

## 2.7 DNA Typing

### 2.7.1 Introduction

The HLA typing data used in this study is mostly obtained from patients' records and was performed by lymphocytotoxicity testing as described in section 2.3. However, a number of the recipients included in this study had not been HLA class II typed at the time they were transplanted and class II typing was not subsequently carried out unless the graft failed and the patient returned to the transplant waiting list. Therefore the typing data was incomplete and analysis of HLA matching could not be carried out. In order to minimise the number of cases with incomplete HLA typing the transplant clinic staff were requested to supply 10ml of EDTA anti-coagulated blood from listed transplanted patients when they were seen in the out patient clinic.

Lymphocytotoxic typing was not considered a practical approach for these patients because the dates of sample receipt were not known in advance. It was therefore decided that DNA would be extracted from the patient's blood and stored until typing could be performed.



All DNA extraction and typing was performed according to the laboratory's standard operating procedures which are based on the technique of Olerup and Zetterquist (1992).

### 2.7.2 Reagent Mixtures

Red Cell Lysis Buffer 2ml (4.2g sodium hydrogen carbonate in 50ml water)

2L distilled water

15.4g ammonium chloride

Nuclei Lysis Buffer 23.37g sodium chloride

10ml 1M Tris-hydrochloric acid

4ml 0.5M EDTA

distilled water to a final volume of 1L

Proteinase K 5ml SDS

400µl 0.5M EDTA

90ml distilled water

200mg proteinase K

5x TBE Buffer 109g Tris-hydrochloric acid

55.6g Boric acid

40ml EDTA

Distilled water to a final volume of 2L

(Dilute with distilled water for 0.5x working solution)

### Deoxynucleoside triphosphate (dNTP) Mix

20 $\mu$ l each of dATP, dCTP, dGTP, dTTP

920 $\mu$ l sterile water

Primer Mix 20 $\mu$ l left hand primer

20 $\mu$ l right hand primer

1 $\mu$ l control 5' primer

1 $\mu$ l control 3' primer

60 $\mu$ l double distilled water

Agarose Gel 10g Agarose

1L 0.5x TBE

50 $\mu$ l ethidium bromide (10mg/ml stock)

### 2.7.3 DNA Extraction

The EDTA anticoagulated blood was centrifuged at 550g for 10 minutes. The buffy coat was then transferred to a clean 15 ml tube, mixed with 9mls of red cell lysis buffer and incubated for 20 minutes at room temperature. The cells were centrifuged at 550g for 10 minutes and the red cell lysate removed leaving the white cell pellet in the tube. The white cell pellet was resuspended in 3mls nuclei lysis buffer and 200 $\mu$ l SDS was added and mixed well. One hundred  $\mu$ l Proteinase K solution was added and the sample incubated for 16 hours at 37°C or for 2 hours at 55°C.

One ml of 6M NaCl was added and mixed well and the sample was then centrifuged at 2700g for 30 minutes. The supernatant was removed, taking care to

avoid the pellet and transferred to a clean universal tube. Two volumes of 100% ethanol were added to the supernatant and mixed by gentle inversion of the capped tube. The precipitated DNA was removed from the solution, transferred to a microcentrifuge tube and dissolved in 100-300 µl distilled water. The DNA was then stored at 4°C.

#### 2.7.4 Class II Typing by PCR-SSP

Reaction tubes were placed in a retainer. Pre-prepared primer mixes were used. The primer mixes were mixed and spun in a microcentrifuge prior to use. Five µl of primer mix was dispensed into each reaction tube. The DNA enzyme mixture was then prepared as follows:

70µl double distilled water

5µl DNA

30µl 10x Buffer IV

30µl dNTP mix

13µl MgCl<sub>2</sub>

2µl Thermostable DNA polymerase

The DNA/enzyme mixture was mixed well and spun in the microcentrifuge. Five µl of the DNA/enzyme mixture were added to each of the reaction tubes containing the primer mixes taking care not to contaminate the mixture with any primers.

The reaction tubes were capped and placed in the thermal cycler and the cycling programme started and run as follows:

Heat at 95°C for 5 mins

30 cycles of:

94°C - 20 seconds

65°C - 50 seconds

72°C - 20 seconds

#### **2.7.4 Identification of Amplified Product by Gel Electrophoresis**

An agarose gel was poured in a gel tray and the combs positioned in the gel. The gel was then left to set. When ready for use the gel was submerged in an electrophoresis tank containing 0.5xTBE buffer and the combs carefully removed. The reaction tubes were removed from the thermal cycler and the caps removed. One  $\mu$ l of gel-loading buffer was added to each reaction. 10 $\mu$ l of 100 base-pair ladder was added to the first well and the middle well of each row of the gel. The reaction samples were then loaded into the wells taking care not to carry any sample over into a consecutive one. As soon as all the samples were loaded the lid was placed on the tank and electrophoresis started. The gel was left to run for 30 minutes, checking the progress of the samples through the gel to ensure no sample was lost from the edge of the gel. The gel was then removed from the tank and placed on a UV transilluminator. The UV source was turned on and a photograph of the gel was taken.

The HLA type was determined by identification of amplified product of the appropriate size.



## 2.8 Statistical Analysis

### 2.8.1 Antibody Detection

Statistical analysis of antibody detection was carried out in 2 different ways. Ability of the different tests to detect antibody was determined by comparison of positive or negative results for each sample tested. Comparison of different tests to detect the level of antibody production in positive samples was carried out by analysis of panel reactivities.

Positive and negative results were analysed using 2 way frequency tables which can be used to relate 2 categorical variables. Analysis of frequency tables is based on hypothesis testing where the null hypothesis is that the 2 different variables are unrelated. The observed frequencies are compared with what would be expected if this hypothesis were true (Altman 1991). Calculations are based on the totals of the rows and columns of the tables as shown below

	Test 1 positive	Test 1 negative	Total
Test 2 positive	w	x	w+x
Test 2 negative	y	z	y+z
Total	w+y	x+z	N

The Chi squared ( $\chi^2$ ) test is used to analyse these results using the formula:

$$\chi^2 = \frac{N(wz-xy)^2}{(w+x)(w+y)(x+z)(y+z)}$$

A numerical value for  $\chi^2$  and a corresponding p value are generated, the p value suggesting whether there is an association between the variables. The smaller the p

value the lower the probability that the null hypothesis (that the variables are unrelated) is true. The value of p which is generally considered to be statistically significant is 0.05 which would correspond to 5% of observations falling outside the range which would suggest an association between the variables, a value which may be expected to occur by chance. If fewer than 5% of values are outside the range i.e.  $p < 0.05$  the null hypothesis may be rejected.

If sample numbers are small the  $\chi^2$  test described above may introduce some bias into the calculation and a correction for this has been devised, this is known as the Yates' correction. In order to allow for correction where sample numbers are small it is advisable to perform the Yates' correction in all calculations as the effect of the correction will be very small if numbers are sufficiently large (Altman 1991).

Where one or more of the expected values in a frequency table is less than 5 the Yates correction is not appropriate and it is necessary to perform the Fisher's exact test, which is also based on the observed row and column totals of the 2 way table.

$\chi^2$  and Fisher's exact test were performed using the computer programme EpiInfo 6 version 6.04a developed by the Center's for disease control and prevention (CDC), U.S.A.

Analysis of panel reactivities determined for the same sample by different methods requires testing of paired samples. The analysis of such data may be carried out by either parametric or non-parametric methods. For parametric methods the assumption that the data is normally distributed is made whereas non-parametric methods do not make assumptions about distribution. In analysis of paired data it is the differences between observations for the same samples which are of interest rather than differences between samples. The t-distribution is the principle parametric

method for analysing such data. This method compares the means of the values for the samples giving a t value:

$$t = \frac{\text{sample mean} - \text{hypothesised mean}}{\text{standard error of sample mean}}$$

for a paired t test the hypothesised mean is 0 as the hypothesis is that the 2 observation are the same. A p value can be derived from the t value, a significant p value (<0.05) will therefore reject the null hypothesis suggesting that the observations for the paired samples are significantly different.

The parametric t test assumes that the distribution of the within sample differences is normal. If this assumption is not valid a non-parametric method should be used. The corresponding non-parametric method for analysis of paired samples is the Wilcoxon matched pairs signed rank sum test where the differences between paired observations are calculated and assigned a rank.

If the distribution of the data is normal it is most appropriate to use a parametric method for statistical analysis, however if it is not clear whether the distribution is normal both parametric and non-parametric methods may be used. If the results of the 2 analyses are similar it is probable that the assumption of normal distribution is correct however if the results differ the non-parametric method will be the appropriate measure of significance (Altman 1991). The t distribution and the Wilcoxon test have been used for the comparison of panel reactivities using the statistical computer programme SPSS for Windows version 6.0.

Correlation coefficients may be used to provide a measure of the degree of association between 2 variables, they do not give a measure of how closely the variables agree. A measurement of correlation is therefore not appropriate for testing

the agreement of results produced by 2 different methods (Altman 1991), it can however provide an indication of the ability to predict the value of one measurement from the known value of the other measurement. The Pearson correlation coefficients have been calculated in some of the comparisons of PR determination by different methods to indicate whether the results obtained using one method may be used to predict those obtained with another method.

### 2.8.2 Antibody Production and HLA Matching

The analysis of levels of panel reactivity for groups of patients with failed grafts with different degrees of HLA match has been carried out by an analysis of variance. In this type of analysis the null hypothesis is that the mean and variance of different populations (different matching grades) will be the same. With ordered groups, such as those containing patients with increasing numbers of mismatches it is most appropriate to use a method which determines whether there is a trend across the groups rather than differences between each of the individual groups. The Kruskal-Wallis test is a non-parametric method for analysis of variance which can be used to assess trends (Altman 1991). The Kruskal-Wallis test was performed using the computer programme SPSS for Windows version 6.0.

### 2.8.3 Transplant Survival and HLA Matching

Analysis of graft survival has been performed using life tables and Kaplan-Meier survival curves. Actuarial survival curves have been generated using life tables. The null hypothesis in survival analysis is that the survival of different groups will be the same. Comparison of survival between groups has been performed using



the log rank test for Kaplan-Meier curves, which uses observed and expected incidences of failure during separate time intervals. This method is used for comparing 2 different groups, it is also possible to carry out an analysis for trend across 2 or more groups. The computer programme SPSS for Windows version 6.0 has been used to perform these analyses for matching at HLA-DR and HLA-A,B,DR.

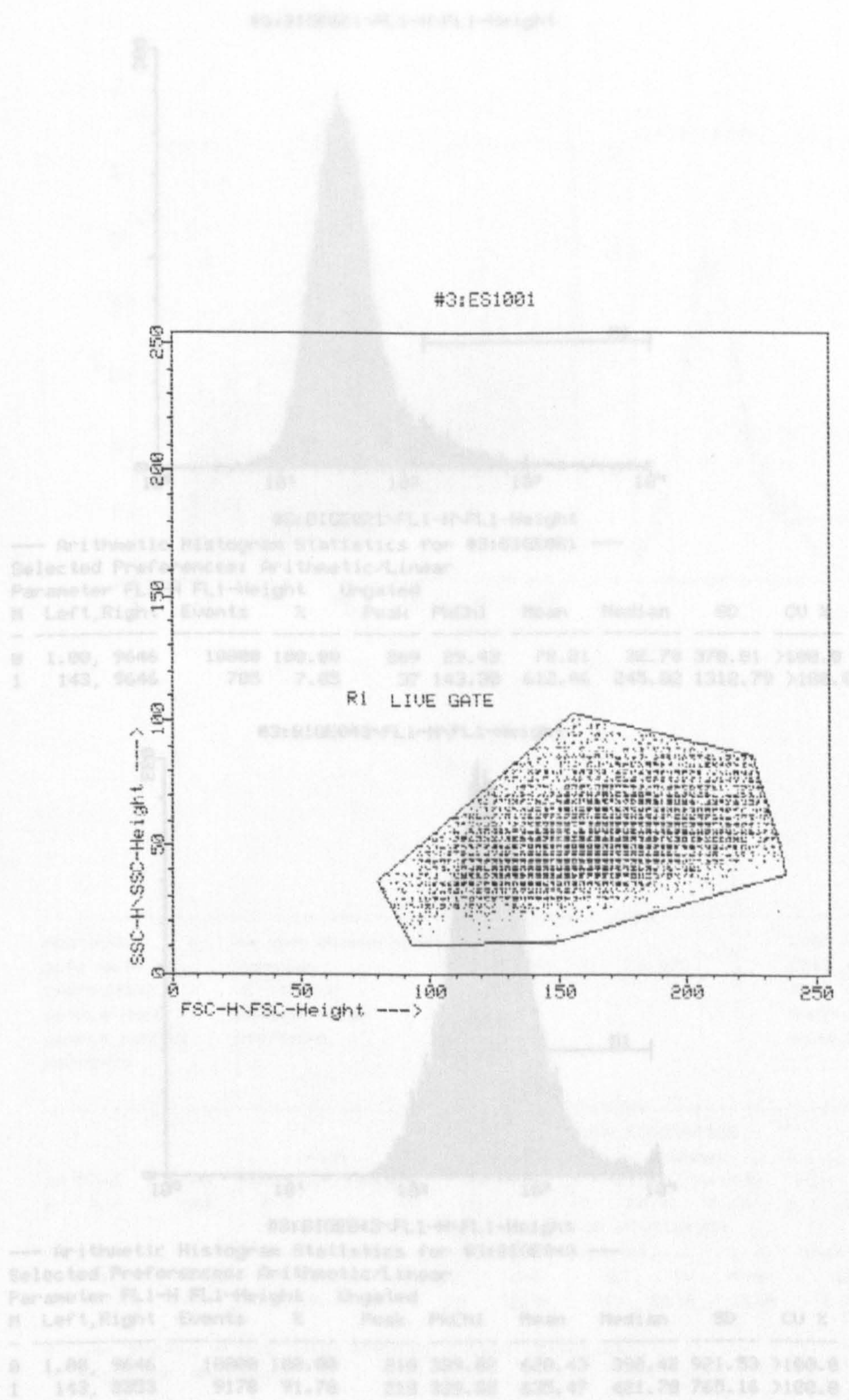


Figure 2.1 Gating of pooled EBV cells by FSC and SSC

Figure 2.2 Histograms of antibodies on the FACSscan on the FACSscan analysis gate and statistics generated by LYSYS II



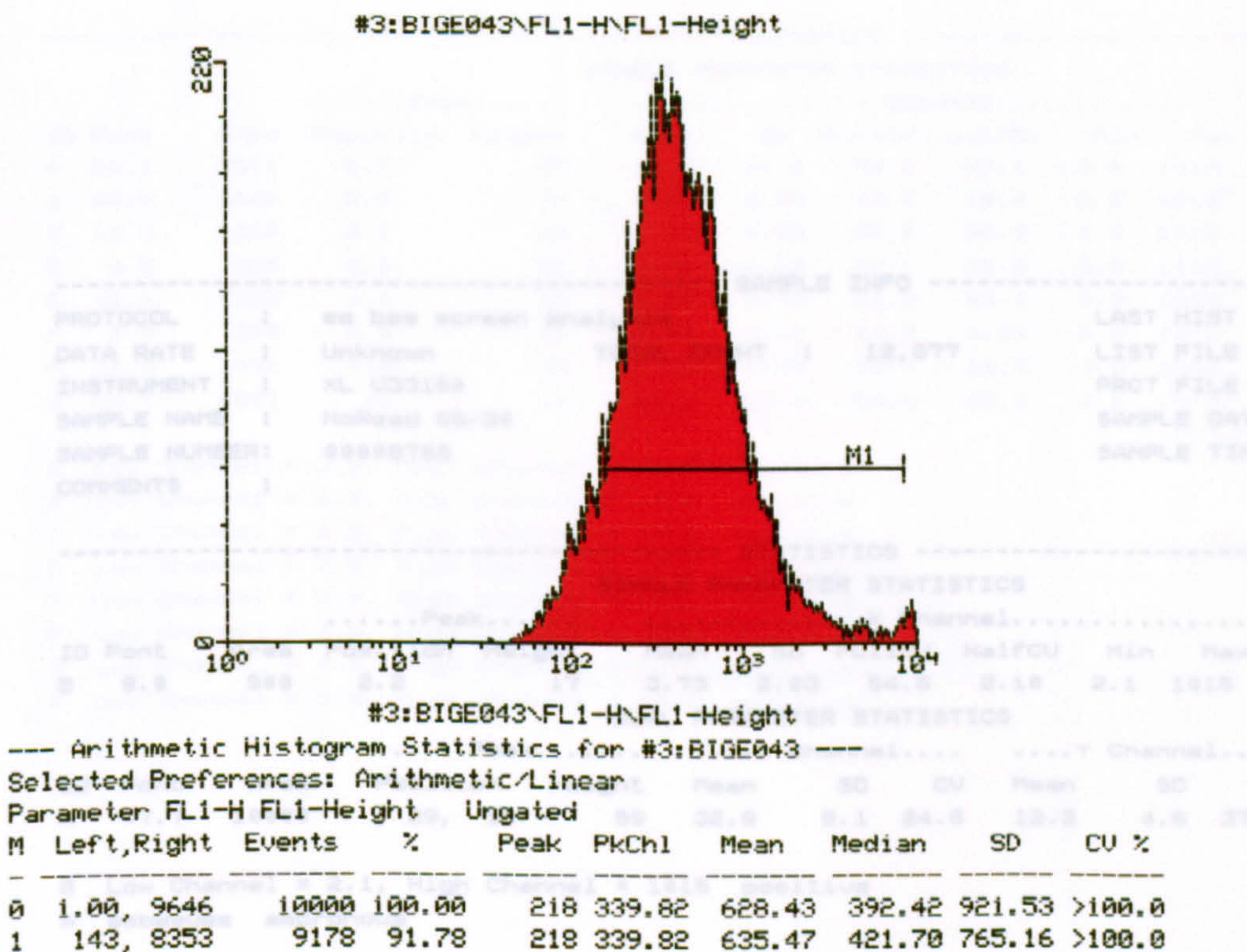
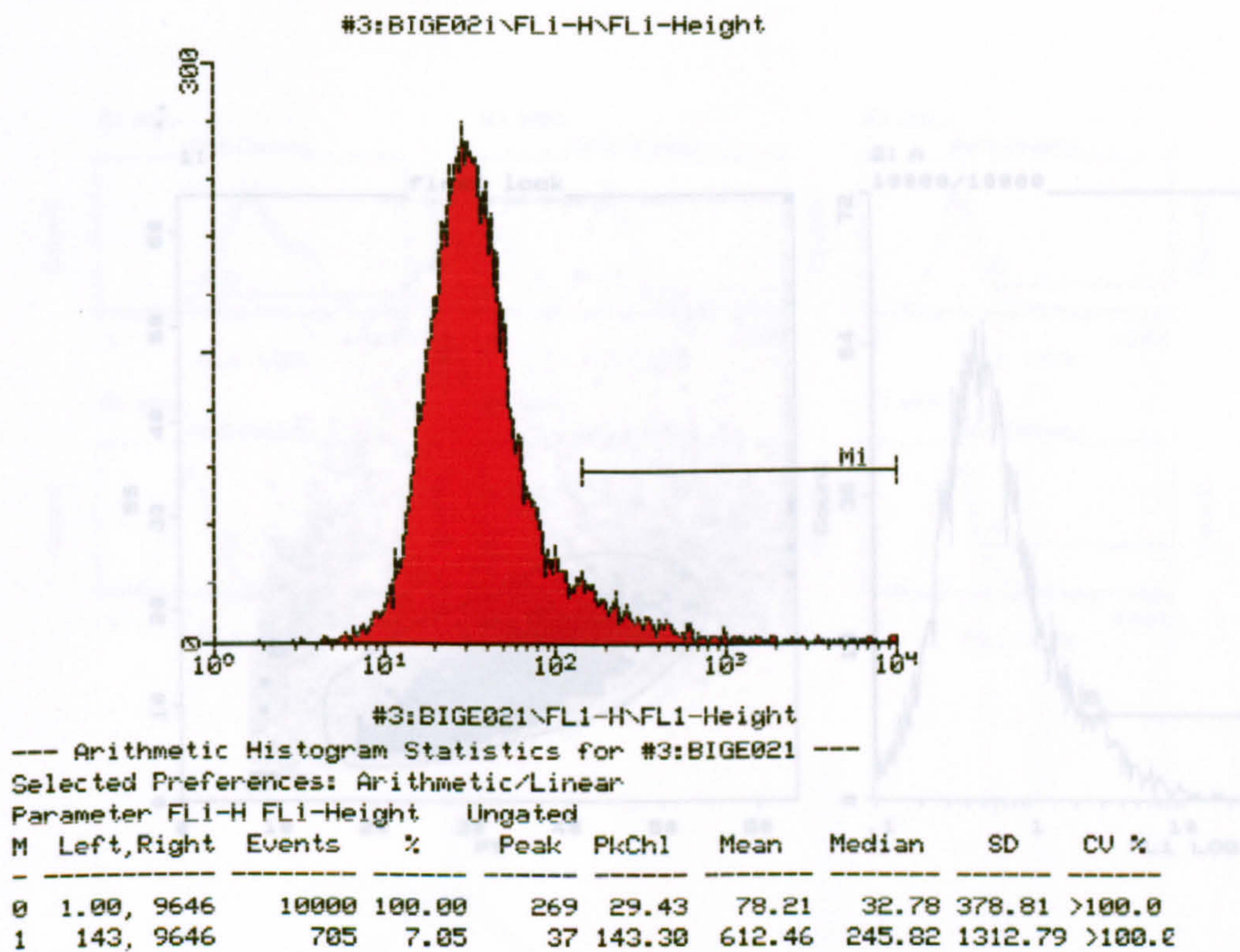


Figure 2.2 Histograms of antibody binding for negative and positive samples with analysis gate and statistics generated by LYSYS II



# GUYS HOSPITAL TISSUE TYPING

COULTER(R) EPICS(R) HISTOGRAM DISPLAY

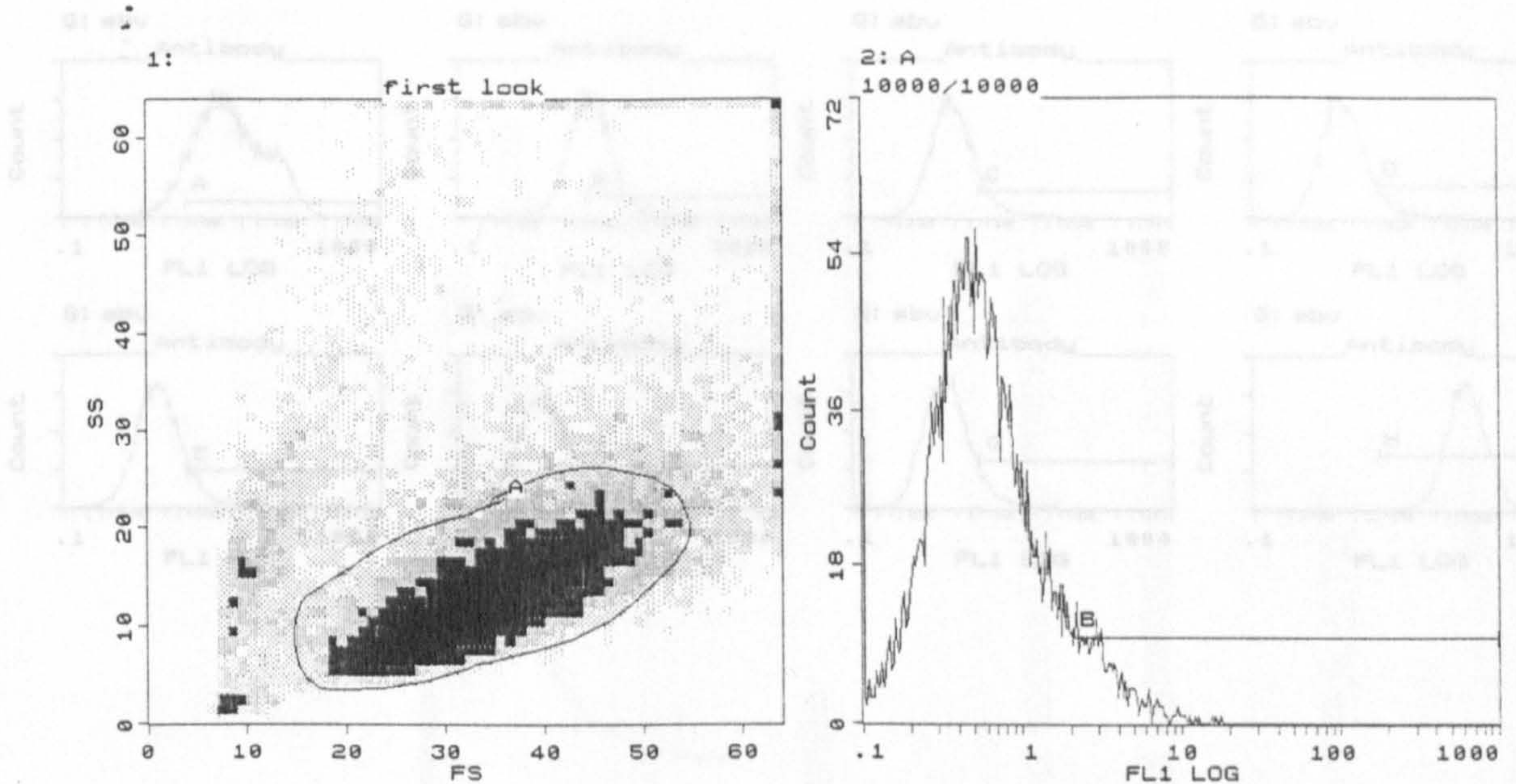
COULTER(R) EPICS(R) ANALYSIS HISTOGRAM

03May94 13:24:14

ee bee screen analysis

NoRead 69/30

#00008708



----- STATISTICS -----

SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Position	Height	Mean	SD	FullCU	HalfCV	Min	Max
A	26.1	8924	3.5	27	16.8	15.9	59.1	23.1	2.9	1015
B	24.4	6382	5.5	74	6.64	3.36	45.7	15.4	2.0	1015
C	13.0	1324	4.9	29	5.07	4.92	25.9	22.9	2.9	1015
D	3.5	305	2.9	27	3.29	3.29	11.9	11.9	2.9	1015

----- SAMPLE INFO -----

PROTOCOL : ee bee screen analysis  
 DATA RATE : Unknown  
 INSTRUMENT : XL V33150  
 SAMPLE NAME : NoRead 69/30  
 SAMPLE NUMBER: 00008708  
 COMMENTS :

LAST HIST : 00008708.H02  
 LIST FILE : 00008708.LMD  
 PROT FILE : EE000023.PRO  
 SAMPLE DATE: 03May94  
 SAMPLE TIME: 13:24:14

----- STATISTICS -----

SINGLE PARAMETER STATISTICS

ID	Pcnt	Area	Position	Height	Mean	SD	FullCU	HalfCV	Min	Max
B	9.0	900	2.2	17	3.73	2.03	54.5	2.10	2.1	1015

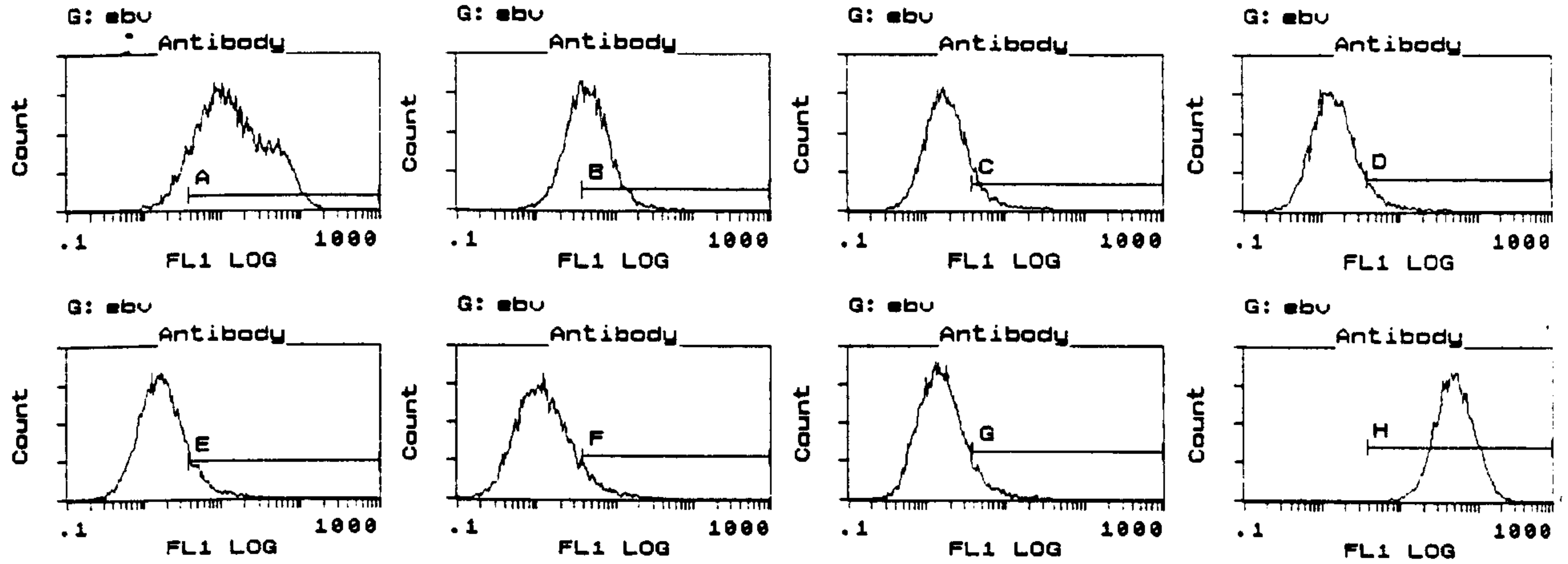
DUAL PARAMETER STATISTICS

ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	77.7	10000	29, 10	89	32.9	8.1	24.6	12.3	4.6	37.4

B Low Channel = 2.1, High Channel = 1015 positive  
 A eebeveee amorphous

Figure 2.3 Gating of pooled EBV cells by FS and SS, for and FL1 histogram on the EPICS-XL





----- STATISTICS -----  
 SINGLE PARAMETER STATISTICS

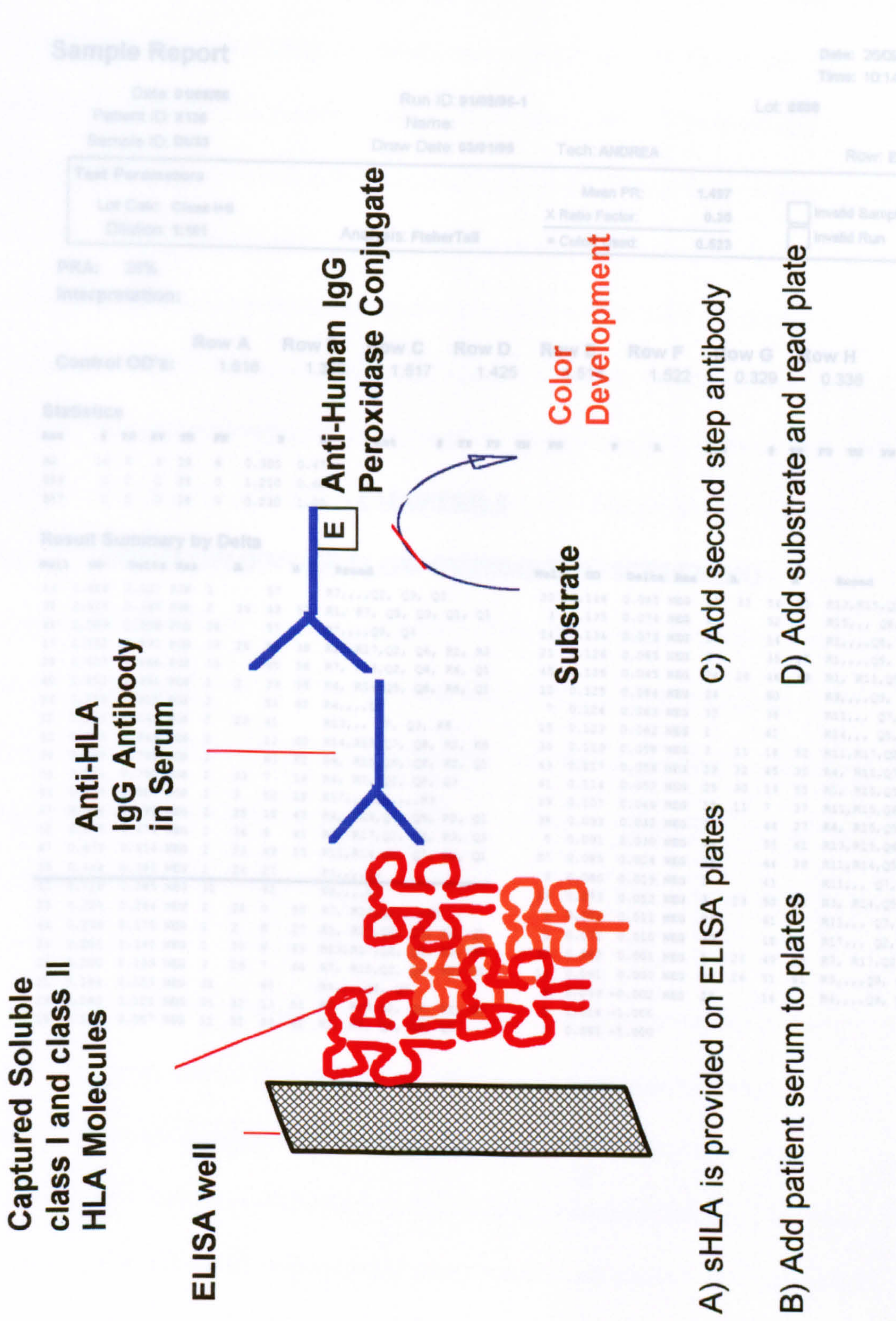
ID	Pcnt	Area	.....Peak.....		.....X Channel.....					
			Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	90.1	8920	8.6	47	16.8	15.5	92.1	23.1	3.9	1015
B	60.4	6022	5.6	74	6.94	3.38	48.7	16.4	3.9	1015
C	13.3	1320	4.0	29	6.87	4.52	65.8	68.8	3.9	1015
D	9.5	935	3.9	22	6.98	4.19	60.1	80.6	3.9	1015
E	12.4	1233	4.0	24	6.96	4.30	61.9	80.1	3.9	1015
F	9.6	955	3.9	21	7.42	5.10	68.7	4.09	3.9	1015
G	10.3	1031	4.0	19	7.08	4.49	63.4	10.6	3.9	1015
H	99.7	9901	46	76	50.2	29.8	59.4	26.6	3.9	1015

A	Low Channel = 3.9, High Channel = 1015	positive
B	Low Channel = 3.9, High Channel = 1015	positive
C	Low Channel = 3.9, High Channel = 1015	positive
D	Low Channel = 3.9, High Channel = 1015	positive
E	Low Channel = 3.9, High Channel = 1015	positive
F	Low Channel = 3.9, High Channel = 1015	positive
G	Low Channel = 3.9, High Channel = 1015	positive
H	Low Channel = 3.9, High Channel = 1015	positive

Figure 2.4 Histograms and statistics for antibody binding for 8 samples generated by EPICS-XL





A) sHLA is provided on ELISA plates

B) Add patient serum to plates

D) Add substrate and read plate

Figure 2.5 Principle of PRA-STAT

Sample Report

Run ID: 200804-1  
 Name: [Redacted]  
 Draw Date: 200804 Tech: ANDREA

Test Parameters	Units	Value
Anti-HLA IgG	U/ml	1.45
Anti-Human IgG	U/ml	0.55

Figure 2.6 Sample report generated by SOFT-STAT with cut-off point amended by operator



# Sample Report

Date: 26/09/97 Page: 1  
Time: 10:14:42

Date: 01/08/96 Run ID: 01/08/96-1 Lot: 8580  
Patient ID: X136 Name:  
Sample ID: DU33 Draw Date: 03/01/95 Tech: ANDREA Row: E

Test Parameters		Mean PR:	1.497	<input type="checkbox"/> Invalid Sample <input type="checkbox"/> Invalid Run
Lot Calc: Class I+II		X Ratio Factor:	0.35	
Dilution: 1:101	Analysis: FisherTail	= Cutoff Used:	0.523	

PRA: 26%

### Interpretation:

	Row A	Row B	Row C	Row D	Row E	Row F	Row G	Row H
Control OD's:	1.616	1.386	1.517	1.425	1.514	1.522	0.329	0.338

### Statistics

Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R
A2	14	8	4	28	6	0.300	0.47																
B58	2	2	2	28	0	1.210	0.68																
B57	2	2	0	28	0	0.230	1.00																

### Result Summary by Delta

Well	OD	Delta	Res	A	B	Broad	Well	OD	Delta	Res	A	B	Broad		
13	2.488	2.427	POS	1	57	R7,,,Q2, Q9, Q3	20	0.146	0.085	NEG	11	33	54	51	R13,R15,Q5, Q6, R2, R6
35	2.426	2.365	POS	2	26	49 57 R1, R7, Q5, Q9, Q1, Q3	3	0.135	0.074	NEG	1	52	R15,,, Q6, Q1, R2		
48	2.269	2.208	POS	24	57	R7,,,Q9, Q3	14	0.134	0.073	NEG	24	14	R1,,,Q5, Q1		
17	1.552	1.491	POS	33	28	58 38 R15,R17,Q2, Q6, R2, R3	21	0.126	0.065	NEG	3	35	27	R1,,,Q5, Q1	
28	1.527	1.466	POS	33	45	58 R7, R13,Q2, Q6, R6, Q1	45	0.126	0.065	NEG	24	28	44	49	R1, R11,Q5, Q7, Q1, Q3
40	1.452	1.391	POS	1	2	39 58 R4, R14,Q5, Q8, R6, Q1	10	0.125	0.064	NEG	24	60	R9,,,Q9, Q3		
23	1.376	1.315	POS	2	54	62 R4,,,Q4	7	0.124	0.063	NEG	32	38	R11,,, Q7, Q3		
22	1.203	1.142	POS	2	29	41 R13,,, Q7, Q3, R6	15	0.123	0.062	NEG	1	61	R14,,, Q5, Q1		
42	0.910	0.849	POS	2	13	60 R14,R15,Q7, Q8, R2, R6	30	0.119	0.058	NEG	3	11	18	52	R11,R17,Q2, Q7, R3, Q3
34	0.849	0.788	POS	2	63	52 R4, R15,Q6, Q8, R2, Q1	43	0.117	0.056	NEG	29	32	45	35	R4, R11,Q7, Q8, Q3
24	0.841	0.780	POS	2	33	7 14 R4, R7, Q2, Q8, Q3	41	0.114	0.053	NEG	25	30	13	55	R1, R15,Q5, Q6, R2, Q1
44	0.590	0.529	POS	2	3	50 18 R17,,, Q2,,,R3	39	0.107	0.046	NEG	25	11	7	37	R11,R15,Q6, Q7, R2, Q1
37	0.554	0.493	NEG	2	25	18 45 R4, R16,Q4, Q5, R2, Q1	36	0.093	0.032	NEG	24	44	27	R4, R15,Q5, Q7, R2, Q1,Q3	
32	0.535	0.474	NEG	2	34	8 41 R4, R17,Q2, Q8, R3, Q3	5	0.091	0.030	NEG	24	55	61	R13,R15,Q6, Q1, R2, R6	
47	0.475	0.414	NEG	2	23	49 55 R11,R14,Q5, Q7, R6, Q1	27	0.085	0.024	NEG	26	44	39	R11,R14,Q5, Q7, R6, Q1	
38	0.444	0.383	NEG	2	24	27 R4,,,Q4	9	0.080	0.019	NEG	1	41	R11,,, Q7, Q3		
12	0.326	0.265	NEG	31	62	R8,,,Q4	19	0.073	0.012	NEG	3	23	50	55	R3, R14,Q5, Q6, R6, Q1
25	0.325	0.264	NEG	2	28	8 50 R7, R17,Q2,,,R3	8	0.072	0.011	NEG	29	61	R11,,, Q7, Q3		
46	0.236	0.175	NEG	1	2	8 27 R1, R17,Q2, Q5, R3, Q1	4	0.071	0.010	NEG	30	18	R17,,, Q2,,,R3		
33	0.201	0.140	NEG	1	30	8 63 R13,R17,Q2, Q6, R3, R6	31	0.062	0.001	NEG	1	23	49	35	R7, R17,Q2, Q5, R3, Q1
26	0.200	0.139	NEG	3	29	7 44 R7, R15,Q2, Q6, R2, Q1	16	0.061	0.000	NEG	24	26	51	61	R9,,,Q9, Q3
11	0.184	0.123	NEG	31	60	R4,,,Q8, Q3	6	0.059	-0.002	NEG	23	14	R4,,,Q9, Q3		
29	0.182	0.121	NEG	31	32	13 51 R7, R15,Q2, Q6, R2, Q1	1	1.514	-1.000						
18	0.148	0.087	NEG	11	32	39 62 R1, R8, Q4, Q5, Q1	2	0.061	-1.000						

Figure 2.6 Sample report generated by SOFT-STAT with cut-off point amended by operator



## **CHAPTER 3**

### **DEVELOPMENT OF FLOW CYTOMETRIC SCREENING**



### **3.1 Introduction**

Flow cytometric crossmatching is a standard technique used for crossmatching all sensitised potential transplant recipients against donor cells in this centre. The technique used is based on that described by Cook et al (1987). This technique is known to be more sensitive than the standard crossmatch. One reason for developing a flow cytometric based screening technique was so that a screening method of similar sensitivity to the final crossmatch method was available. Cytotoxic screening is time consuming and it takes several weeks to get a result if random cell panels are used. Using flow cytometry for analysis of antibody binding allows data on many thousands of individual cells to be collected in a matter of minutes. The possibility of using pooled cells in a flow cytometric based technique therefore seemed feasible. Pooled cells cannot practically be used in cytotoxicity where the relatively small number of cells analysed in each well of a Terasaki plate would not guarantee adequate representation of each cell in the pool so that reactions with only a single component of the pool would be detected.

### **3.2 Methods**

Pooled platelets were obtained from the Tissue Typing Laboratory at the Oxford Transplant Centre. The pool contained platelets from approximately 150 donors. The pooled platelets were stored in suspension at 4°C until required.

CLL cell lines were obtained from the Oxford Transplant Centre. The cells were supplied as individual aliquots which were counted and pooled in volumes such that each cell was represented in equal numbers within the pool. The pooled cells

were washed twice by centrifuging at 400g for 5 minutes in FACS diluent. The pooled cells were resuspended in FACS diluent at a concentration of  $5 \times 10^6$ /ml.

EBV cell lines were grown and pooled as described in section 2.4.

The single colour flow cytometric antibody binding assay was performed as described in section 2.5.4. Analysis of the antibody binding was performed using the Becton Dickinson FACScan.

Cytotoxic screening against peripheral blood lymphocytes and CLL cells was performed as described in section 2.3.4.

### 3.3 Development of Platelet Screening

Platelets were selected as the initial target for developing a flow cytometric screening assay because they express HLA class I but not class II antigens and would therefore be an appropriate target for comparison with CDC screening against peripheral blood T cells. An initial series of four screening runs was carried out. A total of 38 test sera were screened in this first series (platelet series 1), 13 of which were repeat tested. The sera were selected on the basis of known antibody specificity as determined by CDC screening with a small number of known negative sera included.

A subsequent series (platelet series 2) was carried out using 60 test sera. The serum samples were not pre-selected but were 30 consecutive patient samples received in each of the Tissue Typing Laboratories at Guy's Hospital and the Oxford Transplant Centre.

### 3.3.1 Platelet Series 1

Twenty  $\mu\text{l}$  of serum was added to 30 $\mu\text{l}$  of pooled platelets to give a cell/serum ratio of 3:2 in the antibody binding assay, the same ratio used for the flow crossmatch.

Thirteen sera were rescreened using the same platelet pool as above and in addition sera were also tested at a dilution of 1:4 by adding 10 $\mu\text{l}$  of serum to 30 $\mu\text{l}$  platelets.

A significant correlation was found between cytotoxicity and platelet screening by flow cytometry for the sera tested ( $p=0.0078$ , Table 3.1, p107). There were however 9 samples with HLA specific antibodies detected by CDC screening which were negative by flow cytometry showing a false negative result in 31% of CDC positive cases. Analysis of the platelet screening results of sera known to contain HLA A2 specific antibody indicated that the pooled platelets did not produce consistent results. Table 3.2 (p108) shows the results of 8 of these sera. Serum 1 was clearly negative by platelet screening whilst the other 7 sera were positive although serum 4 showed antibody binding only just above background levels. Repeat testing of the sera at further dilutions demonstrated the inconsistencies of the technique. Serum 6 which showed the highest level of antibody binding at a dilution of 2:5 was negative at dilutions of 1:4 and 1:8 whereas serum 3 showed an increase in the level of antibody binding at 1:4 compared to 2:5.

As the flow cytometric analysis of pooled platelets did show a significant correlation with CDC screening a further series of sera were screened. However as there were inconsistencies in the platelet results it was decided to use CLL cells as an additional target and compare the results of CLL pools and platelet pools with cytotoxic screening against CLLs and PBLs respectively. The use of CLL pools



would have the advantage of providing a suitable target for the detection of HLA class II specific antibodies in addition to class I specific antibodies.

### **3.3.2 Platelet series 2**

Sixty sera were tested at dilutions of 1:4 and 1:8 against the platelet pool as described in 3.3.1. (The same samples were tested with CLL pools in CLL series 1).

#### **3.3.2.1 Results**

The platelet screening did not correlate sufficiently well with the results of CDC screening against T lymphocytes in this series of samples (Table 3.3, p109). There were 12 and 11 false negative results with platelets at 1:4 and 1:8 serum dilutions respectively, although at the 1:8 dilution there was a statistically significant correlation for detection. Figure 3.1 (p114) shows the comparison of panel reactivities detected by 2 methods with percentage antibody binding being used as a measure of PR for the flow screening. Platelet screening did not give a PR of greater than 40% in any of the samples tested whilst CDC screening showed 9 samples with PRs of over 40%. In view of the high number of false negative reactions and poor correlation for PR it was concluded that platelet screening was not a suitable method for the detection of HLA class I specific antibodies.

### **3.4 CLL Screening Development**

#### **3.4.1 CLL Series 1**

A total of 60 test sera were screened. Three groups of 20 sera each were screened with one of 3 CLL pools. Pools were made by combining equal volumes of

CLL cells at a concentration of  $5 \times 10^6$ /ml. Figure 3.2 (p115) details the CLL lines used. Pool 1 contained 10 CLLs, pool 2 contained 8 CLLs and pool 3 contained 11 CLLs.

Sera were tested at 2 dilutions. For a dilution of 1:4 10 $\mu$ l serum was dispensed into each tube and 30 $\mu$ l pooled CLL cells added. For a working dilution of 1:8 5 $\mu$ l of serum was dispensed into each tube and 35 $\mu$ l CLL cells added.

#### 3.4.1.1 Results

The correlation between flow cytometric screening of CLL pools and cytotoxic CLL screening results was highly significant at both the serum dilutions tested (Table 3.4, p110). At both dilutions there was only 1 sample which was positive by CDC screening which was not detected by flow cytometry. At 1:4 there were 3 samples which were flow positive, CDC negative whereas at 1:8 six samples were flow positive, CDC negative.

The panel reactivities of each sample determined by the two different methods were compared and the Pearson correlation coefficient calculated. These differed slightly at the 2 dilutions tested with overall coefficients of  $r=0.882$  for sera tested at 1:4 and  $r=0.916$  for sera tested at 1:8. The correlation coefficients for each individual pool are shown below:

Pool 1 (n=10) 1:4  $r = 0.954$  1:8  $r = 0.932$

Pool 2 (n=8) 1:4  $r = 0.893$  1:8  $r = 0.896$

Pool 3 (n=11) 1:4  $r = 0.921$  1:8  $r = 0.942$

The strong correlation between CDC and flow cytometric screening for estimation of panel reactivity with CLL cells was better than might have been

expected. The PR for the pools was based on antibody binding to 8, 10 or 11 cells whereas PR estimated by CDC screening was the result of testing against 30 cells. The smaller pools would not contain sufficient cells to cover all the HLA antigens represented in the CDC panel. The  $r$  values of between 0.893 and 0.954 may therefore indicate that the antibodies present in the serum samples tested were directed against the most commonly represented antigens in both the pools and the panel but that sera containing antibodies specific to a single or small number of less commonly represented antigens may not have been present in the randomly selected samples. In view of this possibility the serum samples to be tested in the next series of CLL screening were selected to contain a wide variety of panel reactivities and antibody specificities

### 3.4.2 CLL series 2

Thirty sera were selected based on previous CDC screening results to cover a range of panel reactivity levels and antibody specificities. The sera were tested against 2 pools of CLLs comprising a total of 18 CLL cells. Figure 3.3 (p116) gives the HLA types of the cells in each of the 2 pools. Sera were tested at a working dilution of 1:5. Ten  $\mu$ l of serum was dispensed into each tube and 40 $\mu$ l of pooled CLL cells added.

#### 3.4.2.1 Results

There was a significant correlation between flow screening of the CLL pools and the CDC screening against CLL cells (Table 3.5, p111). There were 14 samples which were positive by both methods, 9 of these were shown to contain HLA class I



specific antibodies by CDC screening of PBLs and CLLs and 5 contained class II specific antibodies only as was demonstrated by negative results with CDC screening of PBLs.

The use of 2 pools comprising 18 CLL cells in total did not produce a higher correlation found for panel reactivities, than those found with the smaller pools, with a correlation coefficient of 0.887 being found when the mean PR for the 2 pools was compared with the CDC PR. Figure 3.4 (p117) shows the PRs determined by flow and CDC screening for each sample. The pooled screening did detect antibodies with CDC panel reactivities of 10 and 13 % with pool PRs of 21 and 44 % respectively indicating that the pools contain sufficient cells to detect even low PR antibodies detected by CDC. There was a single sample which was CDC positive with a PR of 26% which was flow negative, this samples was negative with PBLs suggesting an HLA class II specific antibody was present. There was insufficient sera to test this sample with DTT so it is possible that the antibody detected by CDC screening was an IgM antibody. Four samples were found to be flow positive but were CDC negative, this may be due to the greater sensitivity of the flow technique or it may be possible that the antibodies detected were not HLA specific.

The overall results of the CLL screening series showed a significant correlation between CDC and flow cytometric screening of CLL cells with similar panel reactivities being determined by the 2 methods despite the smaller size of the panel used for flow screening. Both HLA class I and class II specific antibodies were detected by this method. The method was therefore adopted as a suitable technique for the rapid detection of HLA class I and class II specific antibodies.

Additional flow cytometric testing of pooled CLL cells was subsequently performed at the Oxford Transplant Centre. The results of screening serial dilutions of sera confirmed the greater sensitivity of the flow method compared with CDC screening (Sutton et al 1995).

### 3.5 EBV screening

CLL cells had been shown to be a suitable target for flow cytometric screening of pooled cells. However for the envisioned high volume, routine use of the screening method CLLs would not be available in sufficient quantities. It was therefore decided to assess the suitability of cell lines which could be maintained in culture to produce large quantities of cells as required. EBV transformed B lymphoblastoid cell lines were chosen as a potential target as they were well characterised with HLA class I and class II types confirmed by biochemical and/or DNA typing methods and were available from a number of sources. In order to validate the use of EBV cell lines for screening using the methods devised for CLL pools was performed.

#### 3.5.1 Validation of Pooled EBV Cell Screening

A first screening run using a pool of 5 EBV cells (Figure 3.5, p118) was carried out using 21 sera which were selected on the basis of antibody specificity determined by cytotoxic screening with 19 of 21 containing HLA specific antibody. The technique was identical to that used in the second series CLL screen with a serum cell ratio of 1:4 being used.

A second screening run using a pool of 10 EBV cells (Figure 3.6, p119). All the serum samples allocated to a plate for cytotoxic screening were screened by flow

cytometry immediately following pouring of the screening plate. The method used was as for the second series CLLs with a serum cell ratio of 1:4. Antibody binding was analysed by single colour flow cytometry using the FACScan. The results were determined and compared with the results of the cytotoxic screen when the screening plate was complete. Forty seven random cells were used for the cytotoxic screening.

### 3.5.2 EBV Screen 1

The results of the first EBV screen against a pool of 5 cells was as detailed below.

EBV Screen pos, antigen present in pool	9
EBV Screen pos, antigen not present	3
EBV screen neg, antigen not present	5
EBV Screen neg' antigen present	2
EBV Screen neg, cytotoxic neg	2

2 sera were EBV neg despite the antigen to which they had specific antibody being expressed by cells in the pool. Both were HLA A1 specific antibodies. Three other A1 antibodies were detected in this screen. Three sera were positive with the EBV pool although CDC screening results had shown specificities for HLA antigens not represented in the pool. This suggests that additional antibody specificities were detected. These could be HLA class II specific antibodies or additional class I specificities detected by the more sensitive flow cytometric technique. The correlation between the 2 methods for detection of antibody is significant ( $p=0.024$  table 3.6, p112) despite the small pool size and differences in target cells with respect to HLA antigen expression.



### 3.5.3 EBV Screen 2

The results of the second series EBV screening are shown in table 3.7a (p113). Eight serum samples were CDC positive but negative by flow screening with the EBV pool. Five of these samples were subsequently shown to be CDC positive due to the presence of IgM antibodies by DTT screening. Therefore the results were reanalysed with the 5 IgM samples excluded from the analysis (Table 3.7b, p113). Of the 3 remaining samples which were EBV negative 2 had specificity for HLA A2, although one of these was weakly positive with only 10 of 19 A2 expressing cells in the CDC panel. Two EBV cell lines in the pool were A2 positive and four other samples with antibodies specific for HLA A2 were detected by the EBV screening showing that the pool was able to detect A2 specific antibodies. The reason for the failure in 2 samples is not clear. The remaining 'false negative' sample gave weak reactions on CDC screening with no discernible specificity. No further samples were obtained from this patient for CDC screening and it was not possible to determine whether the reactivity shown by the CDC screen was due to HLA specific antibody. The correlation between the 2 methods was significant with a p value similar to that for the CLL screening indicating that EBV cell lines were an appropriate target for flow cytometric screening.

The results of the CLL and EBV pooled screening series indicated that flow cytometric analysis of antibody binding to pooled cells was a reliable method for the detection of IgG HLA specific antibodies. Flow cytometric screening of pooled EBV cell lines (Flowscreen) was therefore adopted as the screening method for detection of HLA class I and class II specific antibodies and was the principle method used in the study of antibody production in transplant patients

	Platelet Pos	Platelet Neg
Cytotoxic class I pos	20	9
Cytotoxic class I neg	1	8

$$\chi^2 = 7.11 \quad p=0.0078$$

**Table 3.1 Comparison of flow cytometric screening with pooled platelets and cytotoxic screening with T lymphocytes**



	2:5	1:4	1:8
1	5.74	NT	NT
2	38.09	25.8	36.07
3	31.05	40.32	22.16
4	18.67	8.17	3.06
5	26.66	NT	NT
6	43.98	15.77	14.03
7	20.88	15.65	10.60
8	36.04	NT	NT

	Platelet flow +	Platelet flow -
Cytotoxic PBL +	7	11
Cytotoxic PBL -	4	36

b.  
Fisher's exact p = 0.041

Table 3.2 Platelet PR for HLA A2 specific sera tested at 3 dilutions

Table 3.3 Comparison of flow screening of platelet pairs with cytotoxic screen of PBLs. Serum at dilutions of a) 1:4, b) 1:8



	Platelet flow +	Platelet flow -
Cytotoxic PBL +	6	12
Cytotoxic PBL -	8	34

a.

Fisher's exact p = 0.192

	Platelet flow +	Platelet flow -
Cytotoxic PBL +	7	11
Cytotoxic PBL -	6	36

b.

Fisher's exact p = 0.041

**Table 3.3 Comparison of flow screening of platelet pools with cytotoxic screen of PBLs. Serum at dilutions of a) 1:4, b) 1:8**

	Flow +	Flow -
Cyto +	20	1
Cyto -	3	36

a.

$$\chi^2 = 40.63 \quad p < 0.0001$$

Pearson correlation = 0.882

	Flow +	Flow -
Cyto +	20	1
Cyto -	6	33

b.

$$\chi^2 = 32.27 \quad p < 0.0001$$

Pearson correlation = 0.916

Table 3.4 Comparison of cytotoxic and flow screening methods for combined CLL pools at serum dilutions a) 1:4, b)1:8

	Flow +	Flow -
Cytotoxic +	14	1
Cytotoxic -	4	11

$$\chi^2 = 11.25 \quad p = 0.0012$$

Pearson Correlation Coefficient  $r = 0.887$

**Table 3.5 Comparison of flow screening of 30 sera with 2 pools of CLL, (mean of pools)**



	Flow pos	Flow neg
Cytotoxic +	9	2
Cytotoxic -	3	7

Fishers exact p = 0.024

Table 3.6 Comparison of PBL, CDC and Flow Cytometric screening of a pool of 5 EBV cell lines

	EBV Flow pos	EBV Flow neg
Cytotoxic pos	19	8
Cytotoxic neg	1	8

a.

Fisher's exact  $p = 0.0028$

	EBV Flow pos	EBV flow neg
Cytotoxic pos	19	3
Cytotoxic neg	1	8

b.

Fisher's exact  $p = 0.00017$

**Table 3.7 Comparison of flow screening of pooled EBV cell lines with cytotoxic screening a) including IgM containing sera b) excluding IgM containing sera**



A1,28  
A2  
A3,11  
A1,3  
A2  
A1,9  
A2,29  
A2,24  
A3,28  
A2  
A2,24  
A2,29  
A2,28  
A3,26  
A3,31  
A1,3  
A1,25  
A2,24  
A3,29  
A2,3

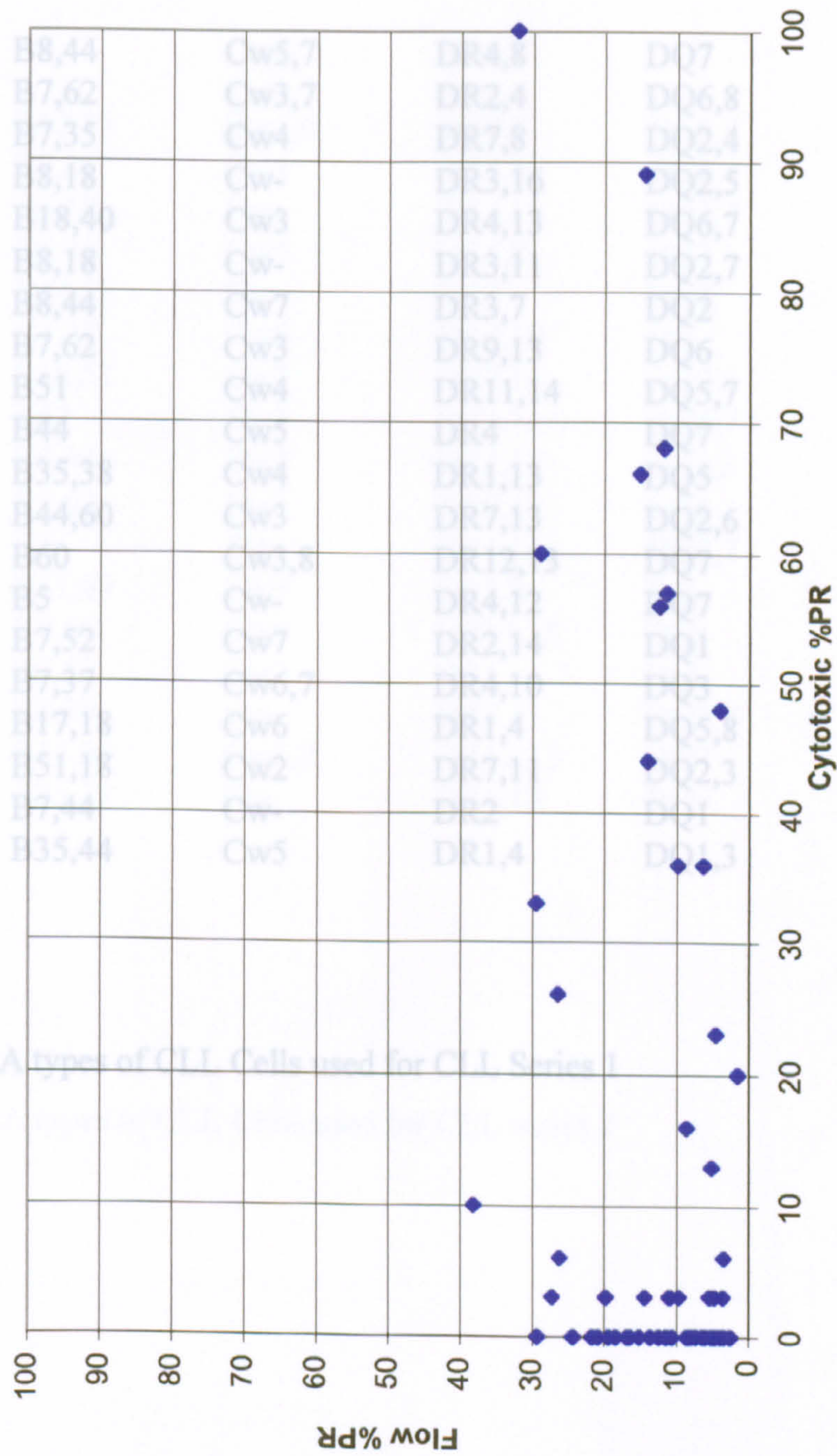


Figure 3.1 Percentage panel reactivity determined for individual samples by CDC screening of PBLs and flow cytometric screening of platelets



A1,28	B8,44	Cw5,7	DR4,8	DQ7
A2	B7,62	Cw3,7	DR2,4	DQ6,8
A3,11	B7,35	Cw4	DR7,8	DQ2,4
A1,3	B8,18	Cw-	DR3,16	DQ2,5
A2	B18,40	Cw3	DR4,13	DQ6,7
A1,9	B8,18	Cw-	DR3,11	DQ2,7
A2,29	B8,44	Cw7	DR3,7	DQ2
A2,24	B7,62	Cw3	DR9,13	DQ6
A3,28	B51	Cw4	DR11,14	DQ5,7
A2	B44	Cw5	DR4	DQ7
A2,24	B35,38	Cw4	DR1,13	DQ5
A2,29	B44,60	Cw3	DR7,13	DQ2,6
A2,28	B60	Cw3,8	DR12,13	DQ7
A3,26	B5	Cw-	DR4,12	DQ7
A3,31	B7,52	Cw7	DR2,14	DQ1
A1,3	B7,37	Cw6,7	DR4,10	DQ3
A1,25	B17,18	Cw6	DR1,4	DQ5,8
A2,24	B51,18	Cw2	DR7,11	DQ2,3
A3,29	B7,44	Cw-	DR2	DQ1
A2,3	B35,44	Cw5	DR1,4	DQ1,3

Figure 3.2 HLA types of CLL Cells used for CLL Series 1

A2	B13,15	Cw3,6	DR2,4	DQ6,8
A3,11	B7,35	Cw4	DR7,8	DQ2,4
A1,9	B8,18	Cw-	DR3,11	DQ2,7
A1,28	B8,44	Cw5	DR4,8	DQ7
A23,29	B44,13	Cw-	DR7	DQ2
A1,3	B7,37	Cw6	DR4,10	DQ-
A2,24	B18,51	Cw2	DR7,11	DQ2,3
A2,32	B62	Cw3	DR1,11	DQ5,7
A1,2	B8,44	Cw5	DR2,12	DQ1,3
A2,24	B7,62	Cw3	DR9,13	DQ6
A3,26	B5	Cw-	DR4,12	DQ3,7
A2	B51,62	Cw9	DR3,9	DQ-
A2,24	B7,55	Cw9	DR14,15	DQ1
A3,31	B7,52	Cw7	DR1	DQ-
A2	B8,39	Cw-	DR3,13	DQ2,6
A2,28	B13,60	Cw3,8	DR12,13	DQ7
A2,24	B35,38	Cw-	DR1,13	DQ5

Figure 3.3 HLA types of CLL Cells used for CLL series 2



A1,1  
 A30,68  
 A31,31  
 A29,29  
 A28,28

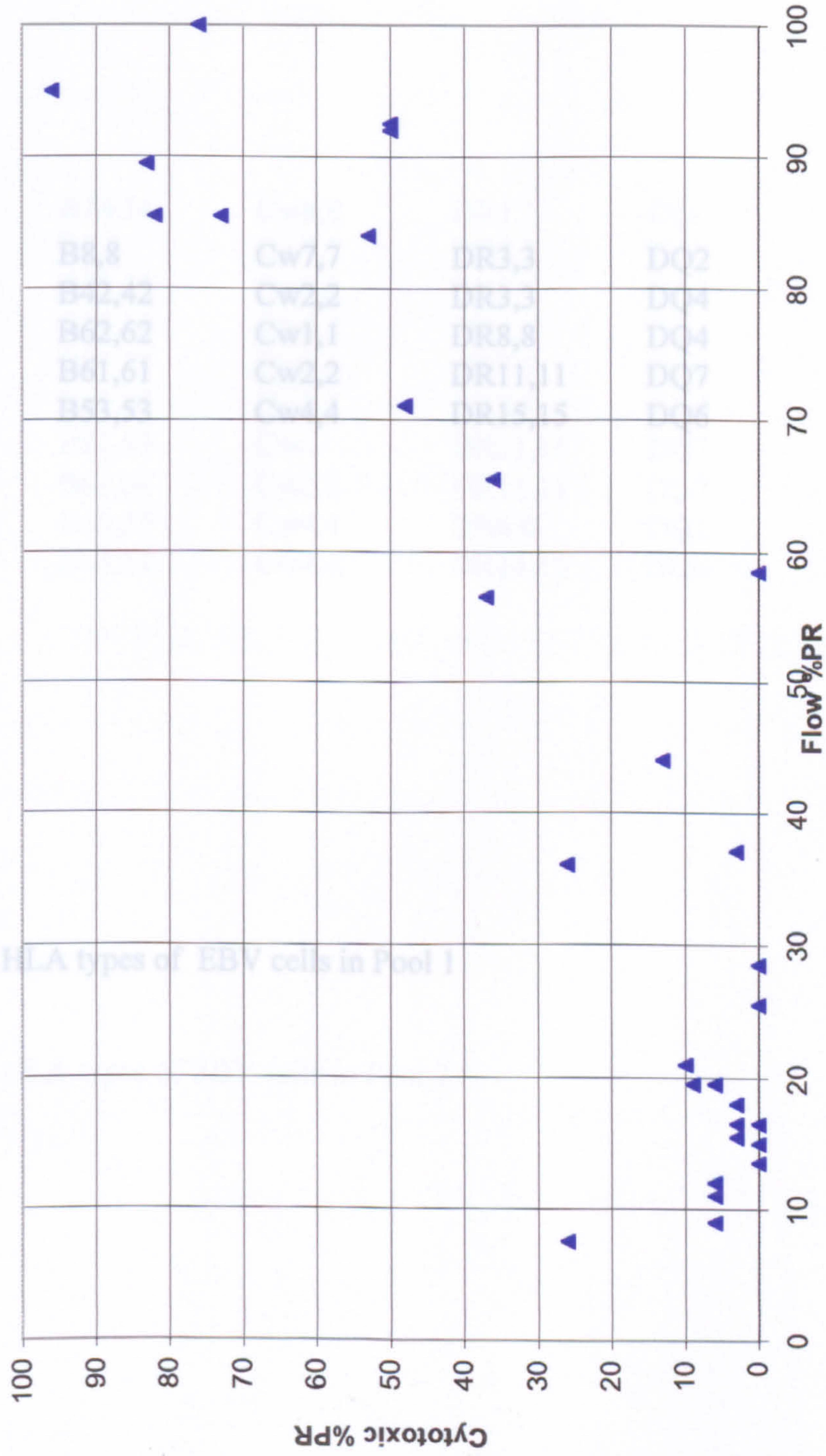


Figure 3.5 HLA types of EBV cells in Pool 1

Figure 3.4 Percentage panel reactivity determined for individual samples by CDC and flow cytometric screening of CLL cells



A1,1	B8,8	Cw7,7	DR3,3	DQ2
A30,68	B42,42	Cw2,2	DR3,3	DQ4
A31,31	B62,62	Cw1,1	DR8,8	DQ4
A29,29	B61,61	Cw2,2	DR11,11	DQ7
A28,28	B53,53	Cw4,4	DR15,15	DQ6

Figure 3.5 HLA types of EBV cells in Pool 1

A33,33	B14,14	Cw8,8	DR1,1	DQ-
A1,1	B8,8	Cw7,7	DR3,3	DQ2
A30,68	B42,42	Cw2,2	DR3,3	DQ4
A2,2	B44,44	Cw5,5	DR4,4	DQ3
A2,2	B57,57	Cw6,6	DR7,7	DQ2
A31,31	B62,62	Cw1,1	DR8,8	DQ4
A24,24	B51,63	Cw-,-	DR11,11	DQ7
A29,29	B61,61	Cw2,2	DR11,11	DQ7
A11,11	B35,35	Cw4,4	DR6,6	DQ1
A28,28	B53,53	Cw4,4	DR15,15	DQ6

Figure 3.6 HLA types of EBV cells in Pool 2

## **CHAPTER 4**

### **PRA-STAT SCREENING: COMPARISON WITH LYMPHOCYTOTOXICITY AND FLOW CYTOMETRY**



#### 4.1 Introduction

PRA-STAT is a commercially produced ELISA based method for the detection of HLA specific antibodies as described in section 2.6. It was marketed as a method which detected HLA class I specific antibodies only based on the premise that a class I specific monoclonal antibody was used to capture solubilised antigen and attach it to the bottom of the wells in 96 well plates. The method is designed to use an IgG specific conjugate and therefore only IgG HLA class I specific antibodies should be detected. It was therefore decided that the data obtained from PRA-STAT screening would be useful in addition to cytotoxic and flow cytometric screening techniques and help in the determination of antibody specificity. Cytotoxic screening detects both IgG and IgM antibodies and some non-HLA specific antibodies. Flowscreen detects IgG HLA class I and class II specific antibodies. If PRA-STAT could be used to conclusively detect IgG HLA class I specific antibodies comparison with Flowscreen results should indicate when Flowscreen was detecting IgG class II specific antibody in the absence of class I specific antibody.

#### 4.2 Samples

An initial assessment of PRA-STAT was carried out using sera from patients with well defined HLA class I specific antibodies detected by cytotoxicity along with some consistently negative sera and sera known to contain IgM antibodies. A total of 32 sera were tested.

For initial comparison of flow cytometric and PRA-STAT screening methods consecutive serum samples from 16 patients receiving renal allografts were analysed.

STAT results. Treating the sera with DTT prior to adding to the PRA-STAT plates did not alter the results with the PRs remaining exactly the same.

In four samples the same major specificity was found by both methods but extra class I specificities were shown by cytotoxic screening. Eleven samples showed detection of the same major specificity by both methods but higher panel reactivities were detected by PRA-STAT. In these cases the extra reactivity did not correspond with clearly defined class I specificities with the positive reaction, often making no apparent sense.

Although the results of this initial assessment were disappointing in that the specificity analysis did not appear to correspond with the results of the well established cytotoxic screening it was decided to continue with a comparative analysis of PRA-STAT and Flowscreen.

#### 4.3.2 Comparison of Flow Cytometric and PRA-STAT Screening Techniques.

The results of screening the sequential serum samples from 16 patients receiving renal transplants showed a significant correlation between the two screening methods ( $p < 0.001$ ). There were no examples of PRA-STAT positive, Flowscreen negative samples and only 6/121 were Flowscreen positive PRA-STAT negative (Table 4.3, p130). The panel reactivities for the 24 positive samples were similar with changes in panel reactivity being clearly shown by both methods. Figures 4.1 and 4.2 (p133 & 134) show an increase in panel reactivity following transplantation in 2 patients, these changes accompanied an irreversible rejection episodes in one of these patients. Figure 4.3 (p135) shows a patient with no antibody production being detected by either method. Only the samples from one of the 16 patients studied did



not produce similar results by both methods (Fig 4.4, p136). This patient was known to have a strong IgM autoantibody prior to transplantation. It is also possible that the antibody detected by FlowScreen in this patient was not HLA specific.

#### 4.3.3 Analysis of PRA-STAT Results for HLA class I and class II antibody specificity

The similarity of results of the PRA-STAT and FlowScreen against B cell lines suggested that the same antibodies were detected by both methods. FlowScreen detects both HLA class I and class II antibodies. After showing the results of the comparative study to SangStat representatives we were advised that the method was able to detect HLA class II specific antibodies and not class I only as previously stated. An updated list of panel HLA types was provided by SangStat giving the HLA A,B,DR and DQ types (Table 4.4, p131). This information was also provided as an update to the SOFT-STAT software. The results of genotyping the PRA-STAT cell lines by a PCR-SSP method at the Oxford Transplant Centre (Bunce et al 1995) were used for comparison, the HLA A,B,C, DRB1,3,4,5 and DQB1 types were given (table 4.5, p132).

The PRA-STAT results were reanalysed by the updated SOFTSTAT programme and by manual analysis using the genotyping data for HLA class I and class II specific antibodies. In the initial assessment of the PRA-STAT technique 11 samples gave extra reactions by PRA-STAT. In seven samples the extra reactions were shown to be due to HLA class II specific antibodies. In five of these seven cases the antibody was found to be specific for class II mismatched antigens on renal allografts (both HLA DR and DQ specific antibodies were detected), in the remaining



	EBV Flow pos	EBV Flow neg
Cytotoxic pos	19	8
Cytotoxic neg	1	8

a.

Fisher's exact  $p = 0.0028$

	EBV Flow pos	EBV flow neg
Cytotoxic pos	19	3
Cytotoxic neg	1	8

b.

Fisher's exact  $p = 0.00017$

**Table 3.7 Comparison of flow screening of pooled EBV cell lines with cytotoxic screening a) including IgM containing sera b) excluding IgM containing sera**

2 cases the class II types of the failed transplants were not known as the transplants were performed before class II typing was routinely available.

The results of the specificity analysis showed that 11 of the 24 positive sera in the comparative study had antibodies specific to HLA class I antigens only. Four samples had class II specific antibodies and 4 contained both class I & II specific antibodies. In five cases a panel reactivity of over 95% prevented accurate specificity analysis.

Figures 4.5 and 4.6 (p137 & 138) show the results analysing a sample using the class I panel or the class I + class II panel. The antibody specificity is demonstrated by the marking of antigens. The class I antibody specificity had been previously determined by cytotoxic testing to be A2. This corresponded to a mismatched antigen present on a failed renal allograft. It can be seen from the class I analysis that there are 19 extra positive reactions not due to the A2 specific antibody. However when the class I+ II panel is used it is clear that the extra reactions are due to the class II specific antibody. Only 3 of 33 positive reactions could not be accounted for by the A2, DR2, DQ1 specificity.

A comparison of the HLA class I types provided with the kits and the genotypes show a number of discrepancies in wells nos. 14,21,22,29,31,33 and 38 (tables 4.4 and 4.5, p131 & 132). In 2 cases these discrepancies show that an HLA B5 antigen detected by genotyping was not detected or was misassigned by the original phenotyping (wells 21 and 38). This is confirmed by analysis of samples from a patient who received a renal allograft mismatched for HLA B51 who was screened as part of the comparative study (Figure 4.7, p139). Of the 8 positive reactions 5 would be shown to contain HLA B5 antigens using the SOFT-STAT analysis whereas 7 of

the 8 were B5 positive when the genotyping results were used as is shown by the handwritten amendments to the results report. One B51 containing well was found to be negative but this had the highest delta value of all the negative results and was only just below the cutoff derived by SOFT-STAT.

This result strongly suggests that the genotypes be used as the definitive HLA type for the panel and the results of subsequent PRA-STAT assays have therefore all been manually checked using this data.

#### 4.3.4 Analysis of Sequential Serum Samples

PRA-STAT results had been shown to be consistently reproducible across individual plates and between plates within the same lot in the original study by Buelow et al (1995). In order to confirm this the ODs for each of the 46 test wells across the 4 plates were plotted for the sequential samples screened in the comparative study. Figure 4.8 shows the ODs for a patient where no antibody production was detected. The reactivity of each sample with the individual wells is clearly very similar for all 8 samples. Figure 4.9 (p141) shows the plot of ODs for 2 samples from the same patient tested on different plates and again the reproducibility of reactivity is clear.

OD plots were drawn for all 16 patients in the comparative study. In patients where no antibody production was demonstrated the plots were all similar to that shown in figure 4.8 (p140). In these patients the pre-transplant sample consistently showed the highest OD values with all the post-transplant samples mirroring the pre-transplant samples at slightly lower ODs. This corresponds to a slight decrease in the background binding seen with Flowscreen (figure 4.3, p135). When antibody is



detected the ODs rise above the pre-transplant level in both positive and negative wells as shown in figure 4.10 (p142).

Having detected the trend for ODs to fall below the pre-transplant level careful analysis of the ODs in patients where antibody production occurred indicated the possibility of using OD plots to provide early detection of antibody production.

Figure 4.11 (p143) shows the pre-transplant and 3 post-transplant samples from one patient. As in all cases the day 2 sample falls below the pre-transplant level.

However in sample 7B (the latter of 2 samples drawn several hours apart on day 7) several wells show a rise in OD above the baseline and there is a change in the overall pattern of reactivity. None of these reactions reached the positive cutoff value however and a PR of 0% was recorded. Figure 4.12 (pp144) shows the OD plot for these samples together with those of the subsequent sample. The difference in scale on the y axis recording the OD values should be noted. The OD values have risen significantly for all 46 wells with a panel reactivity of 98% on day 9, just 2 days after the first indication of a rise above the base level. Some of the strongest reactions in the day 9 samples are found in the wells where the small rise in OD was first detected and correspond to a specificity for a mismatched antigen

	CDC positive	CDC negative
PRA-STAT positive	26	0
PRA-STAT negative	2	6

Fisher's exact  $p < 0.0001$

**Table 4.1** Comparison of lymphocytotoxicity and PRA-STAT for the detection of HLA class I specific antibodies

	CDC	CDC + DTT	PRA-STAT	Flowscreen
Sample 1	66%	37% (A1)	24%	50%
Sample 2	64% (A1,36,23,24)		11%	
Sample 3	26% (A1,36)		7%	

**Table 4.2** Detection of IgG HLA A1 specific antibody in the presence of IgM autoantibody. Comparison of cytotoxic, PRA-STAT and Flowscreen methods.



	PRA-STAT Positive	PRA-STAT Negative
Flowscreen positive	24	6
Flowscreen negative	0	91

$\chi^2 = 85.85$        $p < 0.001$

**Table 4.3**      **Comparison of PRA-STAT and Flowscreen for the detection of HLA specific antibody in renal transplant recipients.**

well no.	A	A	B	B	DR	DR	DQ	DQ
3	1		52		15		6	
4	30		18		17		2	
5	24		55	61	15	13	6	
6	23		14		4		9	
7	32		38		11		7	
8	29		61		11		7	
9	1		41		11		7	
10	24		60		9		9	
11	31		60		4		8	
12	31		62		8		4	
13	1		57		7		2	9
14	24		14		1		5	
15	1		61		14		5	
16	24	26	61	51	9		9	
17	33	68	58	38	15	17	2	6
18	11	32	39	62	1	8	4	5
19	3	23	50	55	3	14	2	5
20	11	33	54	51	15	13	5	6
21	3		35	27	1		5	
22	2	29	41		13		7	
23	2		54	62	4		4	
24	2	33	7	14	4	7	2	8
25	2	68	8	50	17	7	2	
26	3	29	7	44	15	7	2	6
27	26		44	39	11	14	5	7
28	33		44	58	7	13	6	2
29	31	32	13	51	15	7	2	6
30	3	11	18	52	11	17	2	7
31	1	23	49	35	17	7	2	5
32	2	34	8	41	4	17	2	8
33	1	30	8	63	17	13	6	2
34	2		63	52	4	15	6	8
35	2	26	49	57	1	7	5	9
36	24		44	27	4	15	5	7
37	2	25	18	45	4	16	4	5
38	2	24	27		4		4	
39	25	11	7	37	11	15	6	7
40	1	2	39	58	4	14	5	8
41	25	30	13	55	1	15	5	6
42	2		13	60	15	14	5	6
43	29	32	45	35	4	11	7	8
44	2	3	50	18	3		2	
45	24	69	44	49	1	11	5	7
46	1	2	8	27	1	17	2	5
47	2	23	49	55	11	14	5	7
48	24		57		7		9	

Table 4.4 HLA-A,B,DR,DQ phenotypes of PRA-STAT panel

well no.	A	A	B	B	Bw	C	C	DRB1	DRB1	DRB3,4,5	DQB1	DQB1
3	1		52		4	12		15		51	601	
4	30		18		6	5		17		52	2	
5	24		55	61	6	3	9	15	13	51,52	6	
6	23		14		6	8		4		53	9	
7	32		38		4	12		11		52	7	
8	29		61		6	2		11		52	7	
9	1		41		6	17		11		52	7	
10	24		60		6	3		9		53	9	
11	31		60		6	3		4		53	8	
12	31		62		6	1	3	8			4	
13	1		57		4	6		7		53	2	9
14	24	33	14		6	2	8	1			5	
15	1		61		6	15		14		52	5	
16	24	26	61	51	4,6	8	14	9		53	9	
17	33	68	58	38	4	10	12	15	17	51,52	2	602
18	11	32	39	62	6	1	15	1	8		4	5
19	3	23	50	55	6	3	6	3	14	52	2	5
20	11	33	54	51	4,6	1	10	15	13	51,52	5	603
21	3		35	51	4,6	4	15	1			5	
22	2		41		6	17		13		52	7	
23	2		54	62	6	1	3	4		53	4	
24	2	33	7	14	6	8	15	4	7	53	2	8
25	2	68	8	50	6	6	7	17	7	52,53	2	
26	3	29	7	44	4,6	7	16	15	7	51,53	2	602
27	26		44	39	4,6	5	12	11	14	52	5	7
28	33		44	58	4	3	7	7	13	52,53	604-9	2
29	30	32	13	51	4	7	6	15	7	52,53	2	602
30	3	11	18	52	4,6	12	12	11	17	52	2	7
31	1	23	49	53	4	4	7	17	7	52,53	2	5
32	2	34	8	41	6	7	17	4	17	52,53	2	8
33	1	31	8	63	4,6	7		17	13	52	604-9	2
34	2		63	52	4	7	12	4	15	51,53	602	8
35	2	26	49	57	4	6	7	1	7		5	9
36	24		44	27	4	1	5	4	15	51,53	5	7
37	2	25	18	45	6	6	12	4	16	51,53	4	5
38	2	24	27	52	4	12	15	4		53	4	
39	25	11	7	37	4,6	6	7	11	15	51,52	602	7
40	1	2	39	58	4,6	10	12	4	14	52,53	5	8
41	25	30	13	55	4,6	6	3	1	15	51	5	601/2
42	2		13	60	4,6	3	6	15	14	51,52	5	602
43	29	32	45	35	4,6	4	6	4	11	52,53	7	8
44	2	3	50	18	6	5	6	3		52	2	
45	24	69	44	49	4	4	6	1	11	52	5	7
46	1	2	8	27	4,6	1	7	1	17	52	2	5
47	2	23	49	55	4,6	3	7	11	14	52	5	7
48	24		57		4	6		7			9	

Table 4.5 HLA class I class II genotypes of PRA-STAT panel



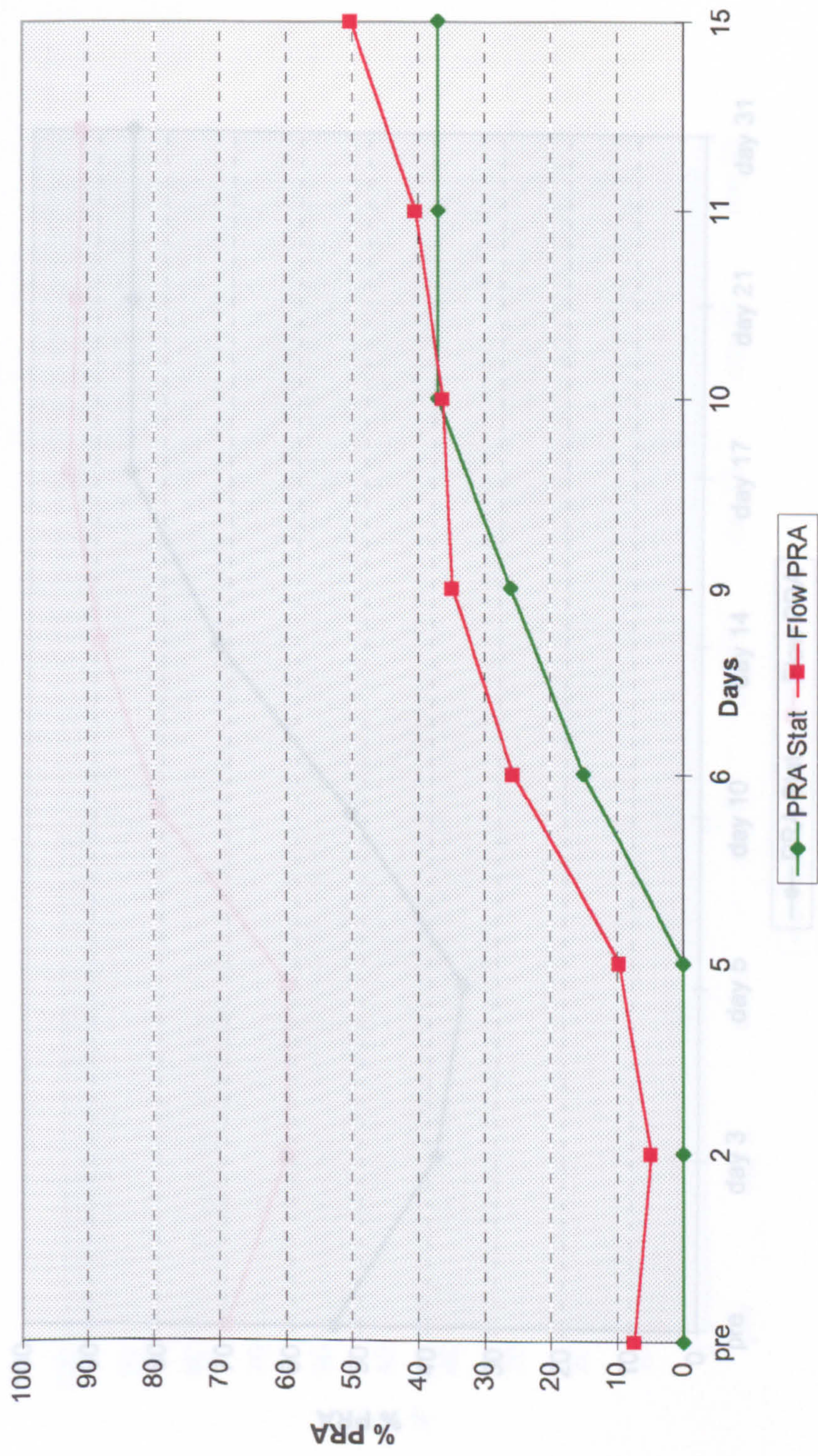


Figure 4.1 Percent PR determined by PRA-STAT and Flowscreen in serial samples showing antibody production post transplant



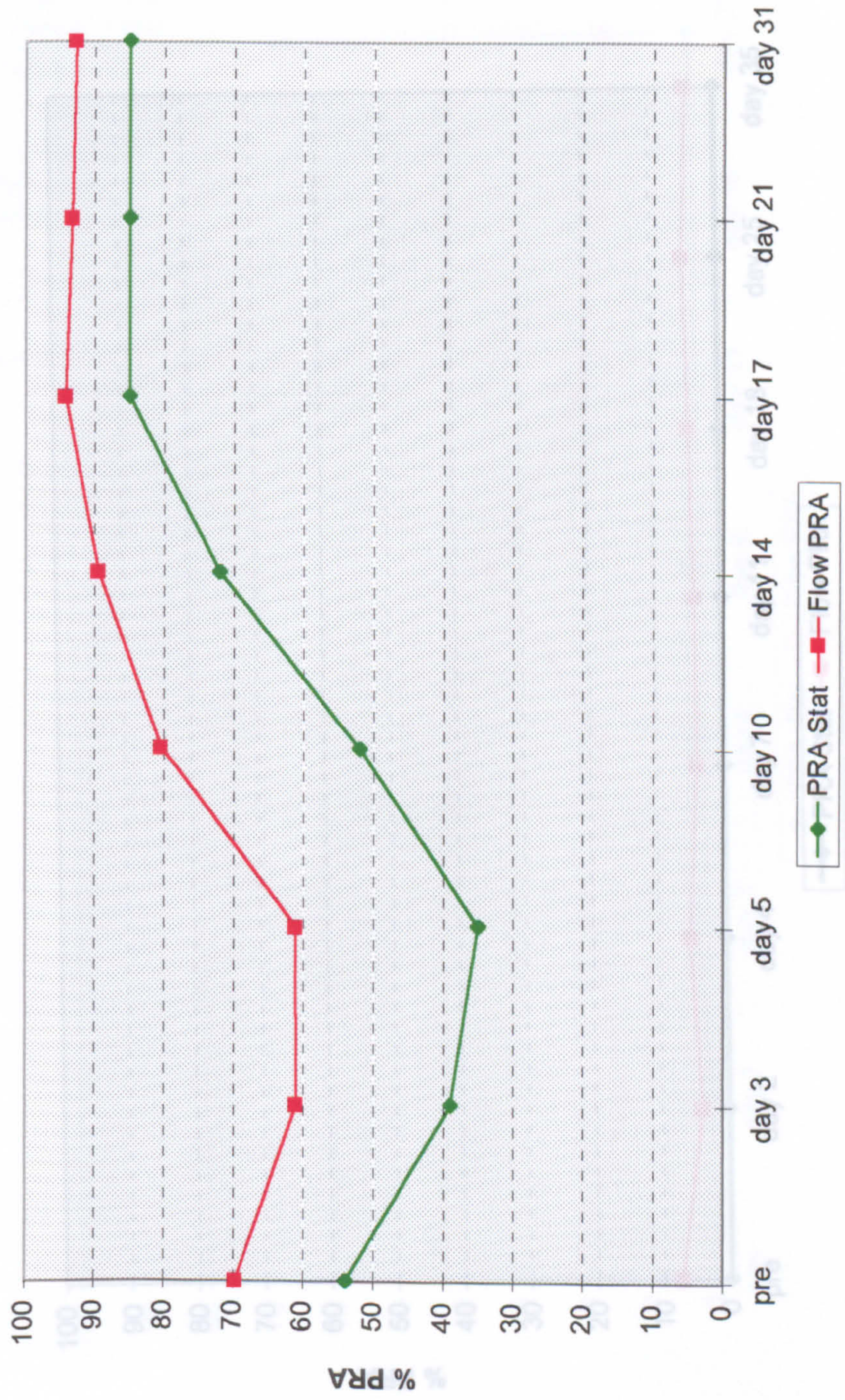


Figure 4.2 Percent PRA determined by PRA-STAT and Flowscreen in serial samples showing a rise post-transplant



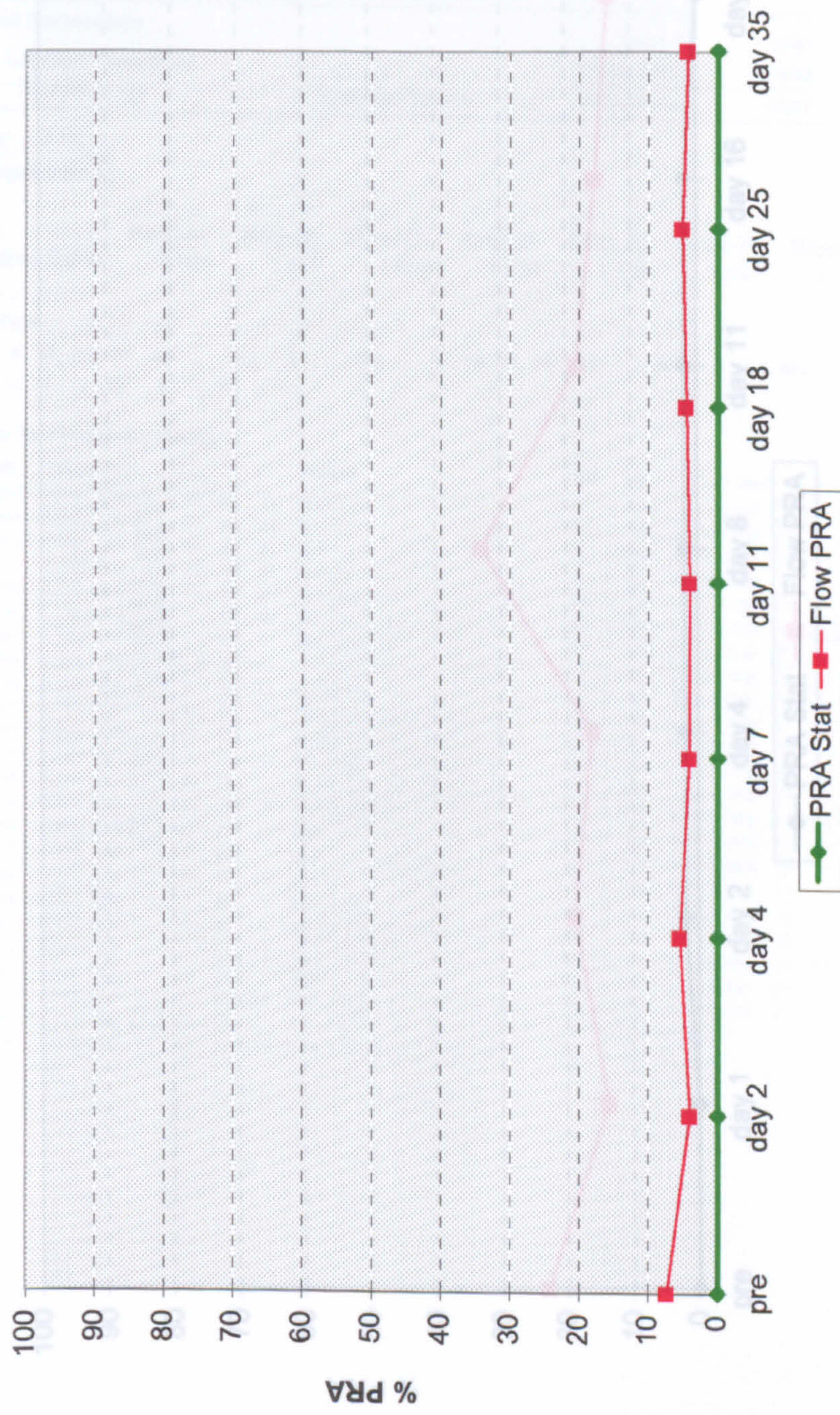


Figure 4.3 Percent PRA determined by PRA-STAT and Flowscreen in serial samples showing no post-transplant antibody production



### Sample Report

Date: 2/18/2016  
 Patient ID: X134  
 Sample ID: 003A  
 Run ID: 216788-1  
 Name:  
 Draw Date: 1/28/2016

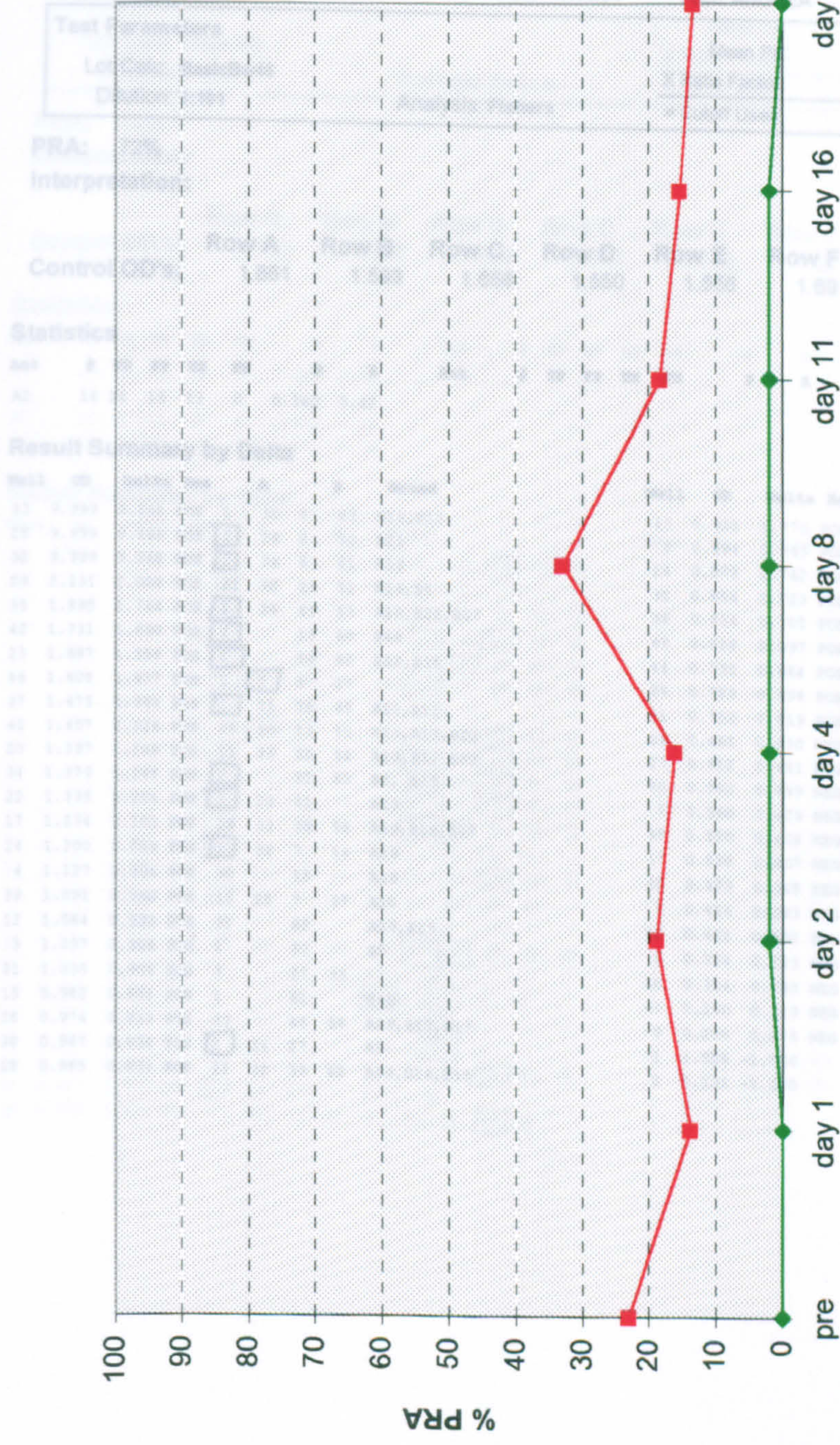


Figure 4.4 Percent PRA determined by PRA-STAT and Flowscreen in serial samples showing no post-transplant antibody production

Figure 4.5 SOFT-STAT report showing analysis using class I panel only



# Sample Report

Date: 26/09/97 Page: 1  
Time: 10:11:31

Date: 31/07/96 Run ID: 31/07/96-1 Lot: 8580  
Patient ID: X134 Name: Tech: ANDREA Row: E  
Sample ID: DE3A Draw Date: 17/06/94

Test Parameters		Mean PR:	1.651
Lot Calc: BasicBw46		X Ratio Factor:	0.35
Dilution: 1:101	Analysis: Fishers	= Cutoff Used:	0.577

Invalid Sample  
 Invalid Run

PRA: 72%

### Interpretation:

Control OD's:	Row A	Row B	Row C	Row D	Row E	Row F	Row G	Row H
	1.861	1.593	1.656	1.550	1.556	1.691	0.362	0.384

### Statistics

Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R
A2	14	14	19	13	0	0.340	0.42																

### Result Summary by Delta

Well	OD	Delta Res	A	B	Broad	Well	OD	Delta Res	A	B	Broad
33	9.999	9.868 POS	1	30	8 63 A19, B15	47	0.901	0.770 POS	23	49	55 A9, B21, B22
25	9.999	9.868 POS	3	28	8 50 B21	5	0.894	0.763 POS	24	55	61 A9, B22, B40
32	9.999	9.868 POS	4	34	8 41 A10	14	0.873	0.742 POS	24	14	A9
29	2.131	2.000 POS	31	32	13 51 A19, B5	31	0.854	0.723 POS	1	23	35 49 A9, B21
35	1.895	1.764 POS	2	26	49 57 A10, B21, B17	36	0.836	0.705 POS	24	27	44 A9, B12
42	1.731	1.600 POS	2	13	60 B40	40	0.828	0.697 POS	1	39	58 B16, B17
23	1.687	1.556 POS	2	54	62 B22, B15	44	0.795	0.664 POS	2	3	18 50 B21
46	1.608	1.477 POS	1	8	27	26	0.789	0.658 POS	3	29	7 44 A19, B12
37	1.475	1.344 POS	2	25	18 45 A10, B12	11	0.750	0.619 POS	31	60	A19, B40
41	1.457	1.326 POS	25	30	13 55 A10, A19, B22	19	0.661	0.530 NEG	3	23	50 55 A9, B21, B22
20	1.397	1.266 POS	11	33	51 54 A19, B5, B22	27	0.612	0.481 NEG	26	39	44 A10, B16, B12
34	1.379	1.248 POS	2	52	63 B5, B15	30	0.580	0.449 NEG	3	11	18 52 B5
22	1.335	1.204 POS	2	29	41 A19	7	0.560	0.429 NEG	32	38	A19, B16
17	1.234	1.103 POS	28	33	38 58 A19, B16, B17	45	0.555	0.424 NEG	24	28	44 49 A9, B12, B21
24	1.200	1.069 POS	2	33	7 14 A19	13	0.538	0.407 NEG	1	57	B17
4	1.127	0.996 POS	30	18	A19	48	0.479	0.348 NEG	24	57	A9, B17
39	1.091	0.960 POS	11	25	7 37 A10	6	0.414	0.293 NEG	23	14	A9
12	1.064	0.933 POS	31	62	A19, B15	10	0.411	0.280 NEG	24	60	A9, B40
3	1.037	0.906 POS	1	52	B5	8	0.354	0.223 NEG	29	61	A19, B40
21	1.035	0.904 POS	3	27	35	16	0.314	0.193 NEG	24	26	51 61 A9, A10, B5, B40
15	0.982	0.851 POS	1	61	B40	43	0.250	0.119 NEG	29	32	35 45 A19, B12
28	0.974	0.843 POS	33	45	58 A19, B12, B17	9	0.206	0.075 NEG	1	41	
38	0.967	0.836 POS	2	24	27 A9	1	1.556	-1.000			
18	0.965	0.834 POS	11	32	39 62 A19, B16, B15	2	0.131	-1.000			

Figure 4.5 SOFT-STAT report showing analysis using class I panel only



# Sample Report

Date: 26/09/97 Page: 1  
Time: 10:10:36

Date: 31/07/96 Run ID: 31/07/96-1 Lot: 8580  
Patient ID: X134 Name:  
Sample ID: DE3A Draw Date: 17/06/94 Tech: ANDREA Row: E

Test Parameters		Mean PR:	1.651
Lot Calc: Class I+II		X Ratio Factor:	0.35
Dilution: 1:101	Analysis: Fishers	= Cutoff Used:	0.577

Invalid Sample  
 Invalid Run

PRA: 72%

### Interpretation:

	Row A	Row B	Row C	Row D	Row E	Row F	Row G	Row H
Control OD's:	1.861	1.593	1.656	1.550	1.556	1.691	0.362	0.384

### Statistics

Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R
A2	14	14	19	13	0	0.340	0.42																
Q1	25	22	11	10	3	0.910	0.39																
R2	12	12	21	13	0	0.910	0.37																
R15	11	11	22	13	0	1.450	0.35																

### Result Summary by Delta

Well	OD	Delta	Res	A	B	Broad	Well	OD	Delta	Res	A	B	Broad
33	9.999	9.868	POS	1	30	8 63 R13, R17, Q2, Q6 R3, R6	47	0.901	0.770	POS	2	23	49 55 R11, R14, Q5, Q7, R6, Q1
25	9.999	9.868	POS	2	28	8 50 R7, R17, Q2, Q3	5	0.894	0.763	POS	24	55	61 R13, R15, Q6, Q1, R2, R6
32	9.999	9.868	POS	2	34	8 41 R4, R17, Q2, Q8, R3, Q3	14	0.873	0.742	POS	24	14	R1, Q8, Q1
29	2.131	2.000	POS	31	32	13 51 R7, R15, Q2, Q6, R2, Q1	31	0.854	0.723	POS	1	23	49 35 R7, R17, Q2, Q5, R3, Q1
35	1.895	1.764	POS	2	26	49 57 R1, R7, Q5, Q9, Q1, Q3	36	0.836	0.705	POS	24	44	27 R4, R15, Q5, Q7, R2, Q1, Q3
42	1.731	1.600	POS	2	13	60 R14, R15, Q7, Q8, R2, R6	40	0.828	0.697	POS	1	39	58 R4, R14, Q5, Q8, R6, Q1
23	1.687	1.556	POS	2	54	62 R4, Q4	44	0.795	0.664	POS	2	3	50 18 R17, Q2, R3
46	1.608	1.477	POS	1	2	8 27 R1, R17, Q2, Q5, R3, Q1	26	0.789	0.658	POS	3	29	7 44 R7, R15, Q2, Q6, R2, Q1
37	1.475	1.344	POS	2	25	18 45 R4, R16, Q4, Q5, R2, Q1	11	0.750	0.619	POS	31	60	R4, Q8, Q3
41	1.457	1.326	POS	25	30	13 55 R1, R17, Q5, Q6, R2, Q1	19	0.661	0.530	NEG	3	23	50 55 R3, R14, Q5, Q6, R6, Q1
20	1.397	1.266	POS	11	33	54 51 R13, R15, Q5, Q6, R2, R6	27	0.612	0.481	NEG	26	44	39 R11, R14, Q5, Q7, R6, Q1
34	1.379	1.248	POS	2	63	52 R4, R15, Q6, Q8, R2, Q1	30	0.580	0.449	NEG	3	11	18 52 R11, R17, Q2, Q7, R3, Q3
22	1.335	1.204	POS	2	29	41 R13, Q7, Q3, R6	7	0.560	0.429	NEG	32	38	R11, Q7, Q3
17	1.234	1.103	POS	33	28	58 38 R15, R17, Q2, Q6, R2, R3	45	0.555	0.424	NEG	24	28	44 49 R1, R11, Q5, Q7, Q1, Q3
24	1.200	1.069	POS	2	33	7 14 R4, R7, Q2, Q8, Q3	13	0.538	0.407	NEG	1	57	R7, Q2, Q9, Q3
4	1.127	0.996	POS	30	18	R17, Q2, R3	48	0.479	0.348	NEG	24	57	R7, Q9, Q3
39	1.091	0.960	POS	25	11	7 37 R11, R15, Q6, Q7, R2, Q1	6	0.414	0.283	NEG	23	14	R4, Q9, Q3
12	1.064	0.933	POS	31	62	R8, Q4	10	0.411	0.280	NEG	24	60	R9, Q9, Q3
3	1.037	0.906	POS	1	52	R15, Q6, Q1, R2	8	0.354	0.223	NEG	29	61	R11, Q7, Q3
21	1.035	0.904	POS	3	35	27 R1, Q5, Q1	16	0.314	0.183	NEG	24	26	51 61 R9, Q9, Q3
15	0.982	0.851	POS	1	61	R14, Q5, Q1	43	0.250	0.119	NEG	29	32	45 35 R4, R11, Q7, Q8, Q3
28	0.974	0.843	POS	33	45	58 R7, R13, Q2, Q6, R6, Q1	9	0.206	0.075	NEG	1	41	R11, Q7, Q3
38	0.967	0.836	POS	2	24	27 R4, Q4	1	1.556	-1.000				
18	0.965	0.834	POS	11	32	39 62 R1, R8, Q4, Q5, Q1	2	0.131	-1.000				

Figure 4.6 SOFT-STAT report showing analysis using class I + class II panel



# Sample Report

Date: 26/09/97 Page: 1  
Time: 10:17:22

Date: 24/10/96 Run ID: 24/10/96-1 Lot: Class I+II  
Patient ID: X147 Name: Row: G  
Sample ID: W143 Draw Date: 22/07/88 Tech: ANDREA

Test Parameters		Mean PR:	1.504
Lot Calc: Class I+II		X Ratio Factor:	0.35
Dilution: 1:101	Analysis: FisherTail	= Cutoff Used:	0.526

Invalid Sample  
 Invalid Run

PRA: 17%  
Interpretation:

	Row A	Row B	Row C	Row D	Row E	Row F	Row G	Row H
Control OD's:	1.669	1.519	1.552	1.337	1.400	1.544	0.420	0.439

## Statistics

Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R	Ant	#	TP	FP	TN	FN	P	R	
B52	3	3	5	38	0	0.370	0.58																	
B51	3	2	3	37	1	3.160	0.47																	

## Result Summary by Delta

Well	OD	Delta	Res	A	B	Broad	Well	OD	Delta	Res	A	B	Broad
29	1.325	1.203	POS	31	32	13 51 R7, R15, Q2, Q6, R2, Q1	18	0.258	0.136	NEG	11	32	39 62 R1, R8, Q4, Q5, Q1
20	1.089	0.967	POS	11	33	54 51 R13, R15, Q5, Q6, R2, R6	36	0.257	0.135	NEG	24	44	27 R4, R15, Q5, Q7, R2, Q1, Q3
34	1.074	0.952	POS	2	63	52 R4, R15, Q6, Q8, R2, Q1	26	0.250	0.128	NEG	3	29	7 44 R7, R15, Q2, Q6, R2, Q1
35	0.955	0.833	POS	2	26	49 57 R1, R7, Q5, Q9, Q1, Q3	4	0.250	0.128	NEG	30	18	R17, Q2, Q3, R3
38	0.788	0.666	POS	2	24	27 52 R4, Q4	15	0.246	0.124	NEG	1	61	R14, Q5, Q1
21	0.747	0.625	POS	3	35	51 R1, Q5, Q1	7	0.241	0.119	NEG	32	38	R11, Q7, Q3
3	0.666	0.544	POS	1	52	R15, Q6, Q1, R2	19	0.237	0.115	NEG	3	23	50 55 R3, R14, Q5, Q6, R6, Q1
30	0.663	0.541	POS	3	11	18 52 R11, R17, Q2, Q7, R3, Q3	37	0.231	0.109	NEG	2	25	18 45 R4, R16, Q4, Q5, R2, Q1
16	0.583	0.461	NEG	24	26	51 61 R9, Q9, Q3	14	0.225	0.103	NEG	24	14	R1, Q5, Q1
31	0.581	0.459	NEG	1	23	49 35 R7, R17, Q2, Q5, R3, Q1	24	0.221	0.099	NEG	2	33	7 14 R4, R7, Q2, Q8, Q3
47	0.544	0.422	NEG	2	23	49 55 R11, R14, Q5, Q7, R6, Q1	11	0.207	0.085	NEG	31	60	R4, Q8, Q3
45	0.498	0.376	NEG	24	28	44 49 R1, R11, Q5, Q7, Q1, Q3	10	0.191	0.069	NEG	24	60	R9, Q9, Q3
23	0.493	0.371	NEG	2	54	62 R4, Q4	9	0.190	0.068	NEG	1	41	R11, Q7, Q3
44	0.476	0.354	NEG	2	3	50 18 R17, Q2, Q3, R3	32	0.186	0.064	NEG	2	34	8 41 R4, R17, Q2, Q6, R3, Q3
33	0.386	0.264	NEG	1	30	8 63 R13, R17, Q2, Q6, R3, R6	39	0.186	0.064	NEG	25	11	7 37 R11, R15, Q6, Q7, R2, Q1
25	0.383	0.261	NEG	2	28	8 50 R7, R17, Q2, Q3, R3	46	0.183	0.061	NEG	1	2	8 27 R1, R17, Q2, Q5, R3, Q1
43	0.374	0.252	NEG	29	32	45 35 R4, R11, Q7, Q8, Q3	27	0.182	0.060	NEG	26	44	39 R11, R14, Q5, Q7, R6, Q1
12	0.334	0.212	NEG	31	62	R8, Q4	22	0.179	0.057	NEG	2	29	41 R13, Q7, Q3, R6
40	0.321	0.199	NEG	1	2	39 58 R4, R14, Q5, Q8, R6, Q1	41	0.177	0.055	NEG	25	30	13 55 R1, R15, Q5, Q6, R2, Q1
13	0.311	0.189	NEG	1	57	R7, Q2, Q9, Q3	8	0.154	0.032	NEG	29	61	R11, Q7, Q3
48	0.309	0.187	NEG	24	57	R7, Q9, Q3	6	0.152	0.030	NEG	23	14	R4, Q9, Q3
17	0.293	0.171	NEG	33	28	58 38 R15, R17, Q2, Q6, R2, R3	5	0.148	0.026	NEG	24	55	61 R13, R15, Q6, Q1, R2, R6
42	0.290	0.168	NEG	3	13	60 R14, R15, Q7, Q8, R2, R6	1	0.420	-1.000				
28	0.273	0.151	NEG	33	45	58 R7, R13, Q2, Q6, R6, Q1	2	0.122	-1.000				

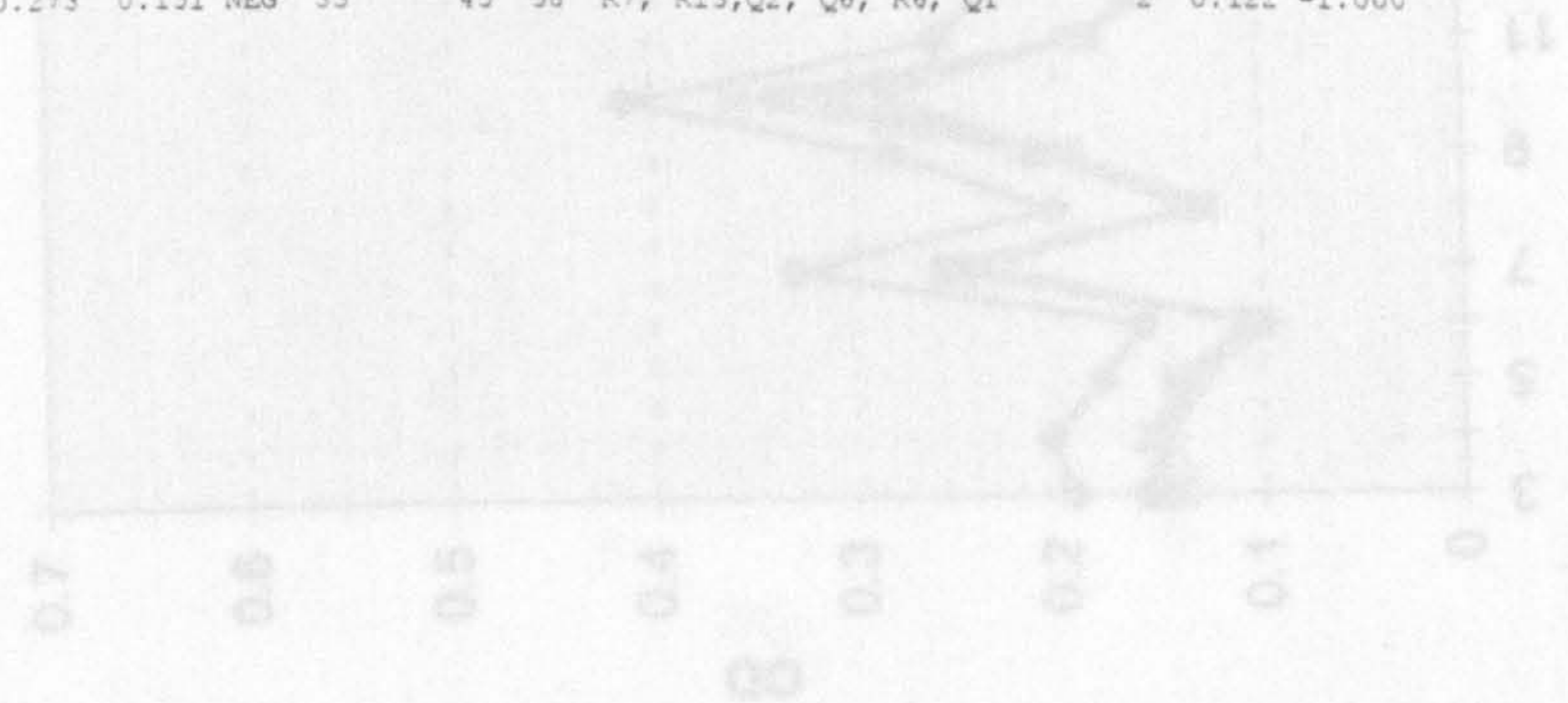


Figure 4.8 OD measurement

Figure 4.7 SOFT-STAT report with amended HLA types based on genotyping results



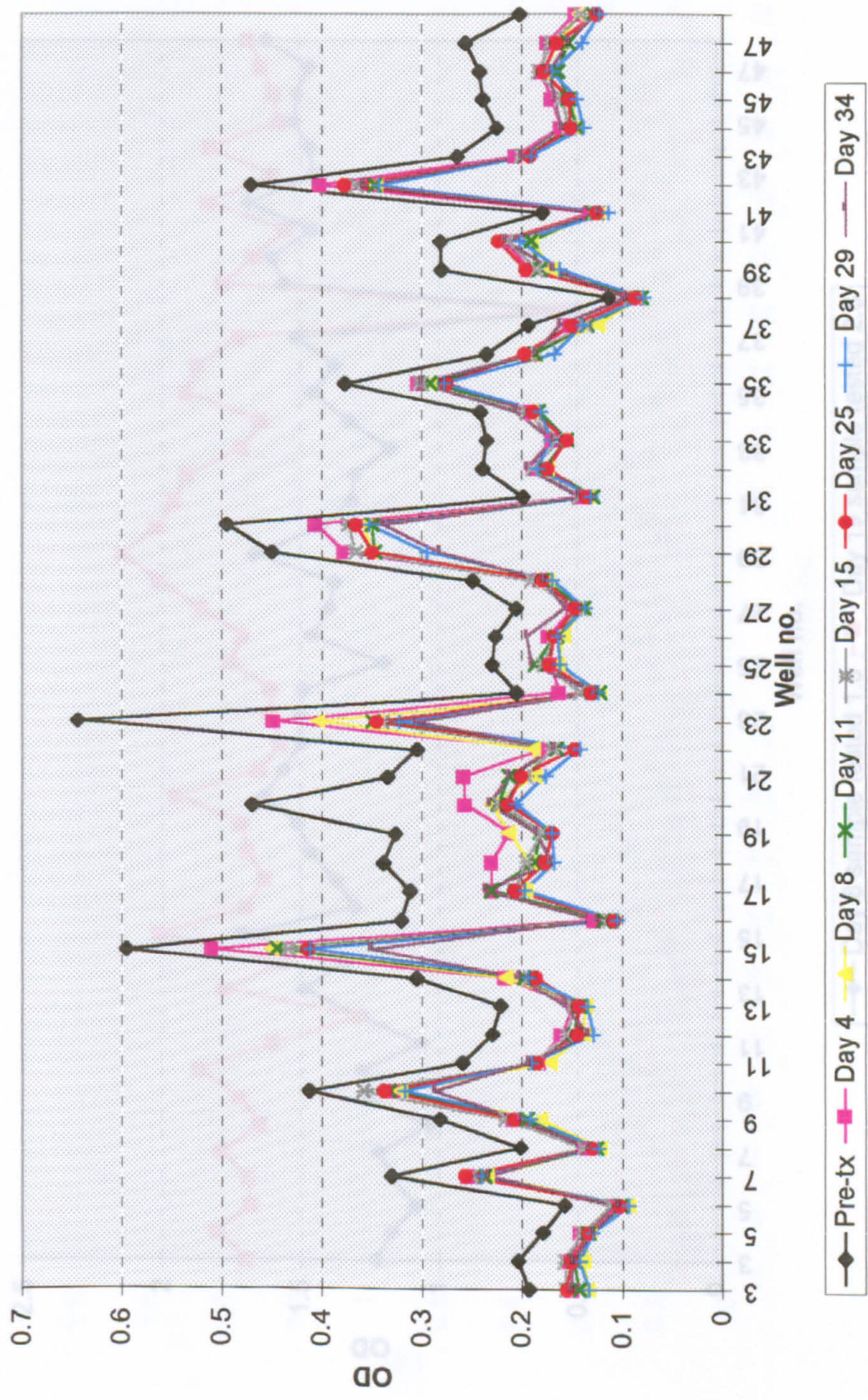


Figure 4.8 OD measurements for pre and post transplant samples in a patient with no rejection episodes



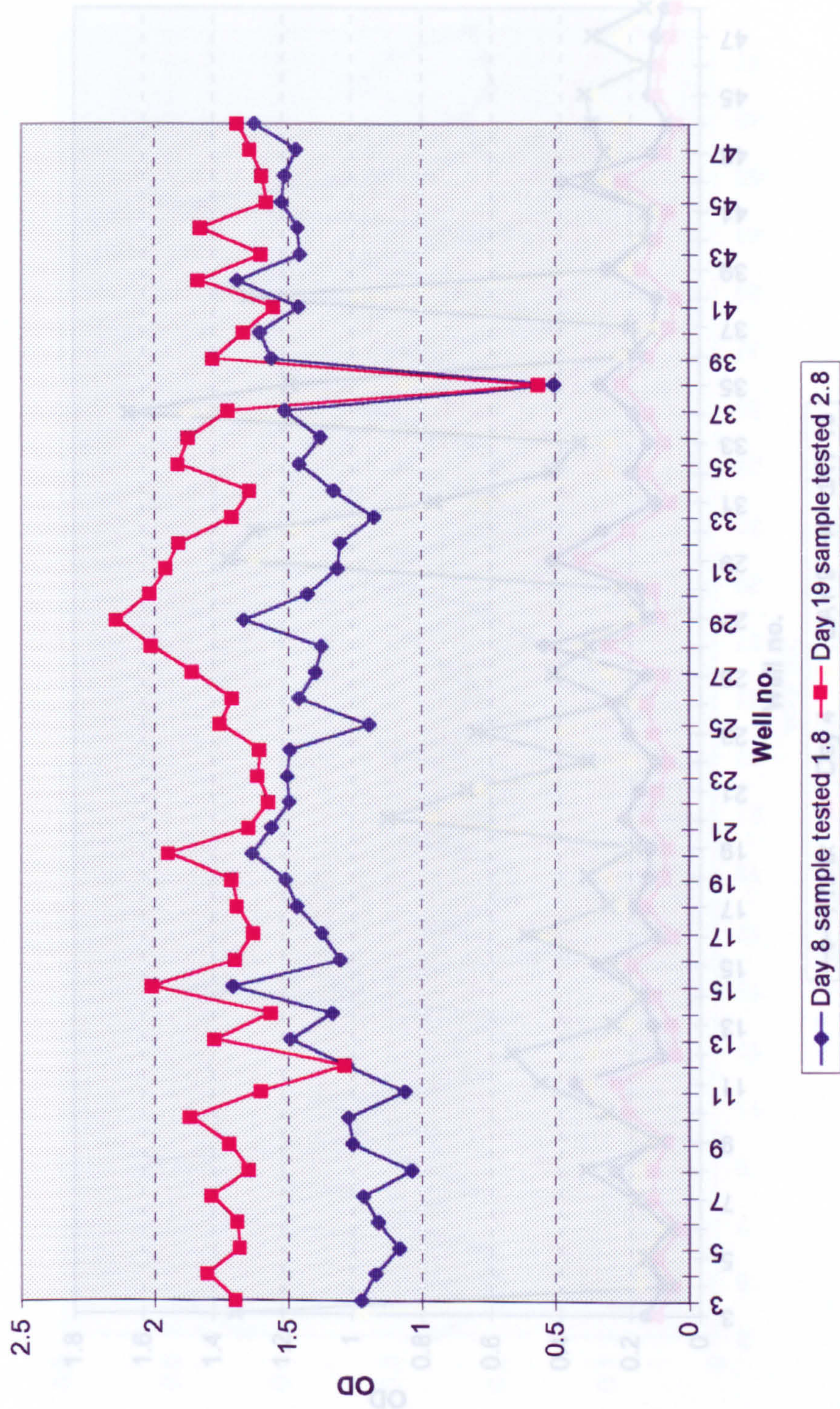


Figure 4.9 OD measurements for samples from the same patient tested on different days



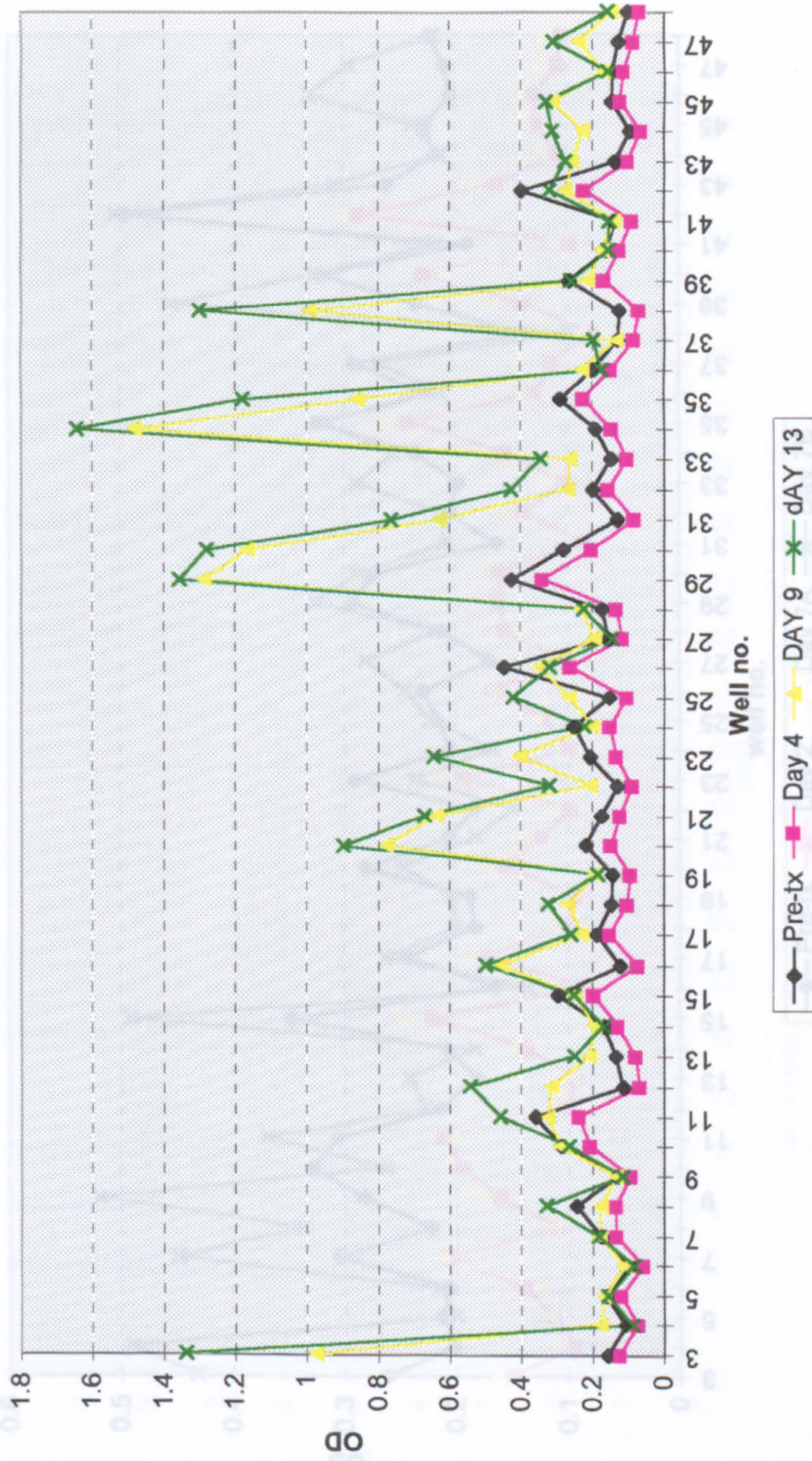


Figure 4.10 OD measurements showing a rise above pre-transplant levels in a patient with an ongoing rejection episode



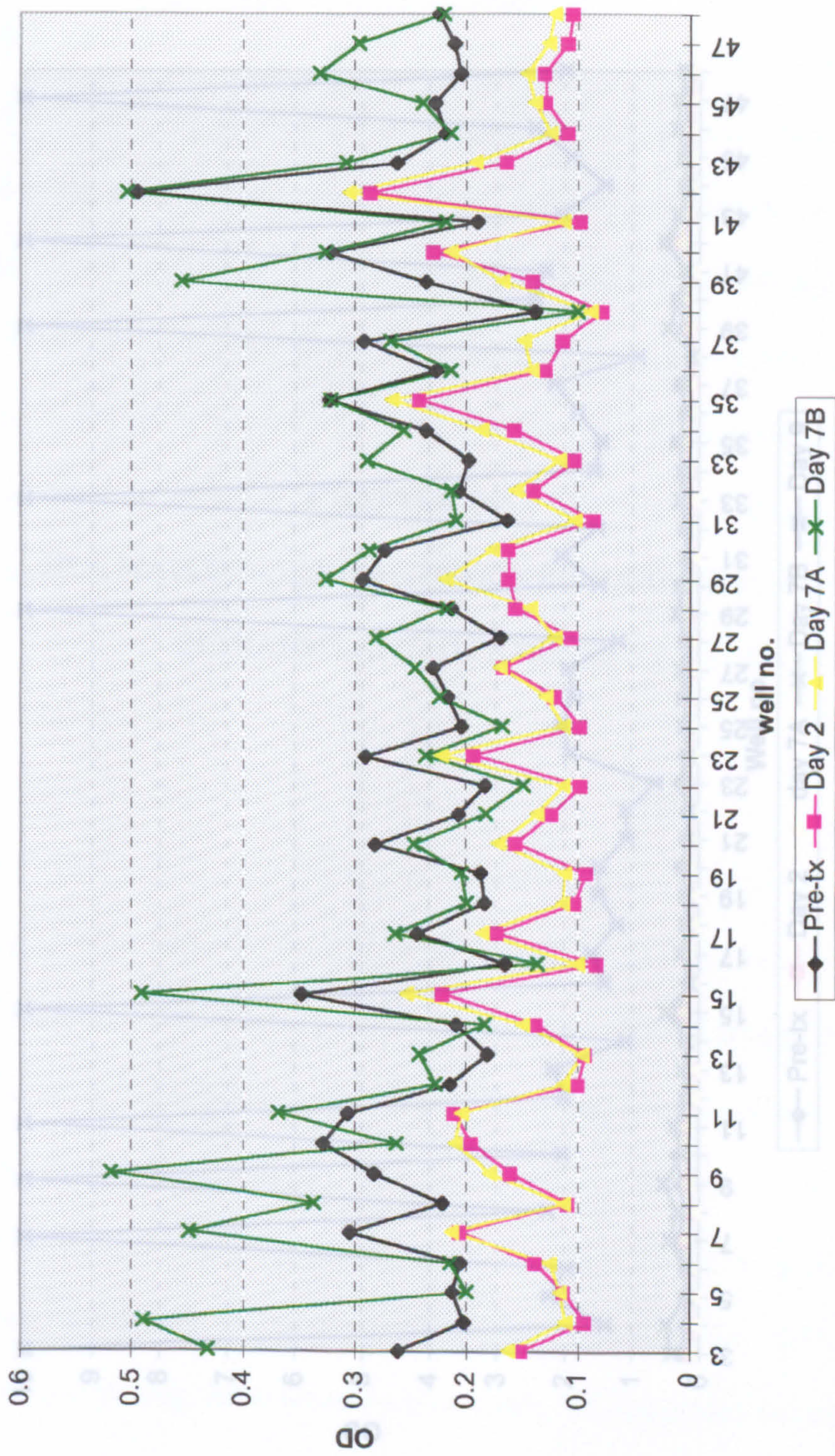


Figure 4.12 OD Measurements showing high antibody levels 2 days after the first, slight increase above pre

Figure 4.11 OD measurements showing a slight rise above pre-transplant levels at day 7B



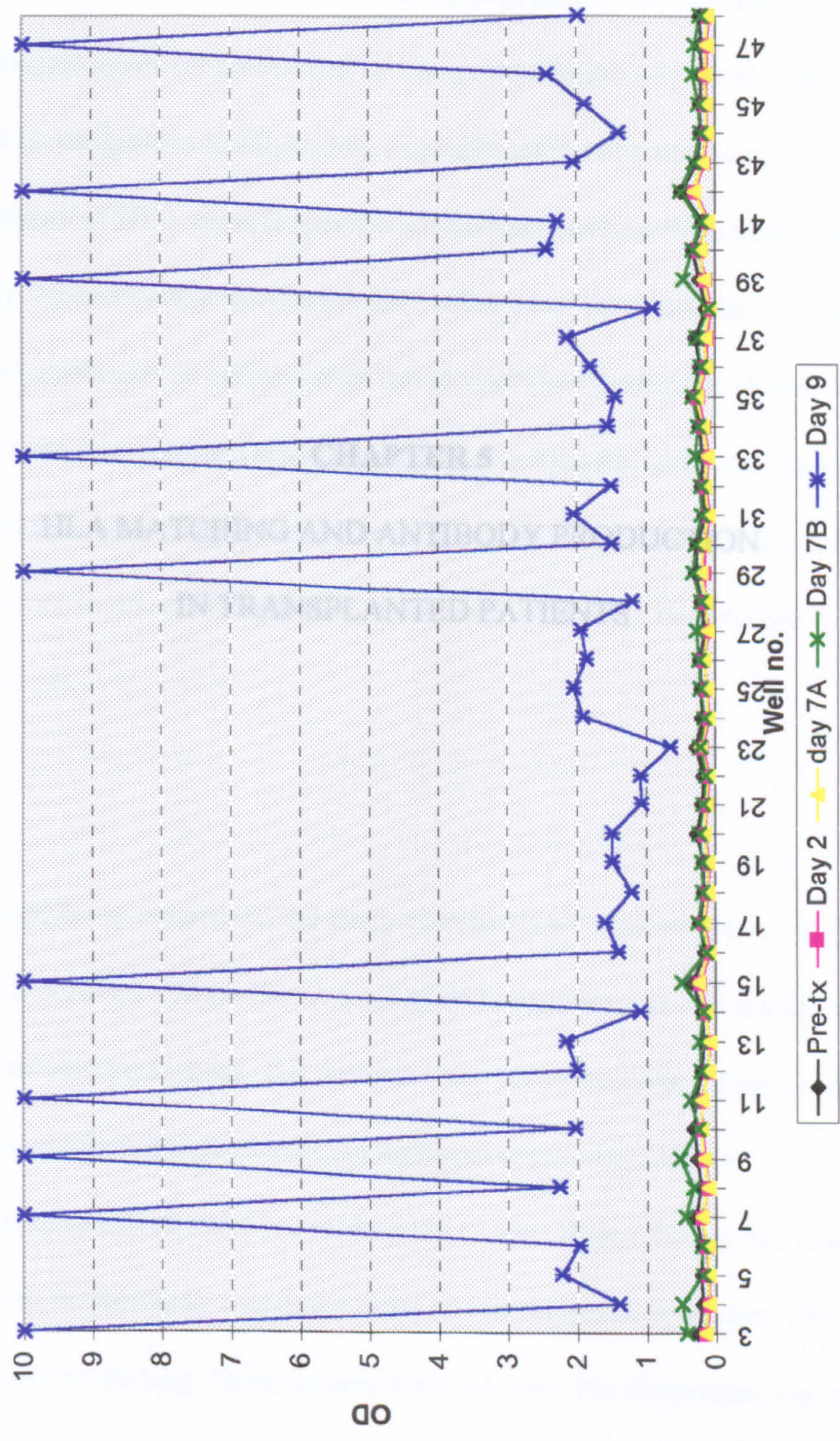


Figure 4.12 OD Measurements showing high antibody levels 2 days after the first, slight increase above pre-transplant levels



## **CHAPTER 5**

### **HLA MATCHING AND ANTIBODY PRODUCTION IN TRANSPLANTED PATIENTS**

## **5.1 Introduction**

Previous studies have investigated antibody production in transplanted patients, especially in relation to the occurrence of rejection episodes. Few studies have looked in detail at the occurrence of antibodies post transplant and the effect of HLA matching on antibody production in a large population of patients. Most studies have limited investigations to HLA class I specific antibodies and there is little data on the prevalence of HLA class II specific antibodies resulting from graft loss.

Using the techniques developed and evaluated in this study an investigation into antibody production in patients with failed grafts has been undertaken. The Flowscreen technique provides the most sensitive method for antibody detection. Previous studies have used cytotoxic techniques and it is possible that this may have resulted in failure to detect HLA specific antibodies which can be detected by more sensitive methods.

## **5.2 Patients**

The population chosen for the study comprised all adult recipients of primary renal allografts between 1984-1993. A total of 460 transplants in 177 female and 283 male recipients were performed. All patients were transplanted in a single unit and received Cyclosporin A and prednisolone immunosuppression. Other immunosuppressive agents varied according the current policy during this period. Patient outcome for the study was determined as current status in January 1995 and was recorded as functioning, failed or died with function. No distinction was made between immunological and non-immunological causes of graft failure.



## **5.3 Samples**

### **5.3.1 Post graft failure**

Cytotoxic screening was routinely performed on all samples collected following graft failure until such time as the patient was re-transplanted, died or was transferred. The results were analysed and samples were selected for further analysis on the basis of cytotoxic screening results. Patients with CDC panel reactivities of over 50% with clear HLA specificity in the first sample collected following graft failure were not considered to require further analysis. All other patients had samples screened by additional techniques. The first sample collected following the date of graft failure was chosen for analysis on all cases and subsequent samples were screened if a negative result was found with the first sample.

### **5.3.2 Functioning Transplants**

Samples were not routinely obtained from patients with functioning transplants. However during an 18 month period from January 1990-June 1991 post transplant samples were collected for another study. Samples were obtained at between 2 and 12 months post transplant. For this study a sample taken as close as possible to 6 months post transplant was screened to provide data on patients with stable function. No samples less than 2 months post transplant were screened.

## **5.4 Screening Techniques**

Flowscreening of EBV cell pools using the EPICS-XL was used as the principle technique for screening samples which required further analysis. Samples from patients with functioning grafts were screened by Flowscreen only. The majority

of samples from patients with failed grafts were screened by both Flowscreen and PRA-STAT.

### 5.5 Matching Data

Matching data was based on the recipient and donor types recorded in the patients records. Where no HLA class II type was recorded for a patient a sample was requested if the patient was still living. All samples received were class II typed by PCR-SSP as described in section 2.7 and the results were used to determine the matching for those patients. Where no patient samples were available for typing or in the few cases of incomplete donor types the transplants were excluded from matching analyses.

### 5.6 Results

#### 5.6.1 Functioning Grafts

Suitable post transplant samples were available from 46 patients with functioning grafts. The samples were collected between 52 and 386 days post transplant with a mean collection day of 140. The patients were transplanted between January 1990 and June 1991 and all still had functioning grafts in January 1995. Two of the forty six samples were found to be positive by Flowscreen. In both cases the reactivity was against only 1 of the 2 cell pools used with percentage reactivity against this pool of 44.7% and 19.05% respectively.



## **5.6.2 Failed grafts**

### **5.6.2.1 Screening Strategy**

Between January 1984 and December 1993 460 primary transplants were performed. One hundred and fourteen had failed by the census date in January 1995 (48 in female and 66 in male recipients). Of these 9 patients were excluded from the analysis because either no post graft failure samples had been obtained or because no serum remained to carry out further screening in addition to the initial CDC screen. A total of 105 patients were analysed for this study. In 31 cases the results of CDC screening did not indicate a need for further analysis, in the remaining 74 cases one or more samples were screened by additional methods. Overall 64 patients were screened by both Flowscreen and PRA-STAT, 9 by Flowscreen and 1 by PRA-STAT in addition to the CDC screening. Table 5.1 (p159) shows the screening methods used for samples split into 2 groups of those above and below 50% CDC PR. A small number of samples with CDC PR of >50% had further screening performed to confirm whether antibodies were IgG or IgM where no DTT testing had been carried out.

### **5.6.2.2 Comparison of Screening Methods for Antibody Detection**

Tables 5.2 -5.4 (p 160-162) show the correlation between the each of the 3 methods for the detection of antibody. There was a statistically significant correlation between all 3 methods for the detection of antibody. Thirteen samples were CDC positive, flowscreen negative (table 5.2, p160), all of these were IgM antibodies as confirmed by additional DTT or PRA-STAT screening. Nine of the ten CDC positive PRA-STAT negative samples were IgM. PRA-STAT detected HLA specific

antibodies in 4 patients where CDC screening did not detect any antibody formation. These 4 patients also had HLA specific antibody detected by Flowscreen. There was one patient for which Flowscreen detected HLA specific antibody production before either CDC or PRA-STAT as determined by testing sequential samples. In addition to this patient PRA-STAT failed to detect HLA specific antibodies in 2 patients who were CDC and Flowscreen positive. There were 3 patients where Flowscreen was positive but there was no evidence of HLA specific antibody production at any time following graft failure by CDC or PRA-STAT screening of sequential samples.

#### 5.6.2.3 Antibody Specificity and Panel Reactivity

Sixty three patients had samples tested by all three methods (table 5.1, p159). Twenty one were negative by all three methods and a further nine were shown to have only IgM antibodies. Of the remaining 33 patients there were 3 patients where Flowscreen demonstrated antibodies not detected by CDC or PRA-STAT. Thirty samples had detectable IgG HLA specific antibodies by at least 2 of the three methods used. All had CDC PRs of < 50%. The PRs detected by Flowscreen &/or PRA-STAT were >50% in 23 of these with only 8 patients where all 3 methods showed a PR of <50%. Figure 5.1 (p174) demonstrates the differences in PR determination by the 3 methods. Statistical analysis of the panel reactivities were carried out for all samples where either 2 of the 3 or all 3 methods were used. A total of 73 cases were tested by both CDC and Flowscreen, 63 by Flowscreen and PRA-STAT and 64 by CDC and PRA-STAT. Table 5.5 (p163) shows the mean PRs determined for all the samples tested for each comparison and the results of the t-distribution test and the Wilcoxon matched-pairs signed ranks test. The results of the parametric and non-



parametric analyses are very similar for each comparison indicating that the distribution of the within samples differences is normal and that the parametric analysis is therefore valid. The results show that there is no significant difference between the PRs determined by CDC and PRA-STAT ( $p=0.176$ ) whereas the Flowscreen PRs are significantly higher than those determined by either CDC or PRA-STAT ( $p<0.001$ ).

The results of the Flowscreen gives the percentage binding within the positive analysis gate. A background binding of up to 10% is taken to be an acceptable level with greater than 10% above the background being considered positive. Therefore samples which are effectively classed negative will still be recorded with a PR of between 0 and 19%, in practice the majority of negative samples have percentage binding of 5-15%. This could account for the significantly higher PRs detected by the Flowscreen method so an analysis of the results was carried out after subtracting 10% from the Flowscreen results. There was a difference between the parametric and non-parametric test results when this analysis was carried out indicating that the non-parametric test was the appropriate method. The results of the Wilcoxon test showed that there was still a significant difference between the results of the Flowscreen and CDC ( $p=0.002$ ) and PRA-STAT ( $p=0.006$ ) methods for detecting panel reactivity when the background binding was taken into account.

The panel reactivities of the antibodies produced in the positive patients screened by all 3 methods are illustrated in Figure 5.1 (p174). This graphical illustration of the results demonstrates the higher PRs produced by the Flowscreen technique, this is particularly evident in samples with CDC and PRA-STAT PRs of less than 50%. However there are a number of samples with high Flowscreen and

PRA-STAT but low CDC PRs. This apparent similarity in PRs determined by Flowscreen and PRA-STAT, despite the significant difference between the 2 methods which was shown by statistical analysis is confirmed by performing a correlation test for the 3 methods. Whilst correlation coefficients are not a suitable method for determining agreement between different methods of measuring the same factor they do give a measure of the likelihood of being able to predict the value of one variable from the known value of another variable. The correlation coefficient for Flowscreen and PRA-STAT is 0.86 compared with 0.64 for CDC and Flowscreen and 0.54 for CDC and PRA-STAT.

Analysis of specificity determination by PRA-STAT was compared with the class I specificities detected by CDC. In only 3 of the 30 patients were the CDC and PRA-STAT specificities concordant. In the majority of the patients PRA-STAT detected HLA class II specific antibodies in addition to the HLA class I antibodies detected by both CDC and PRA-STAT (26/30). In 8 of these patients PRA-STAT also detected antibodies to HLA class I antigens which were not shown by CDC screening, and one patient with no HLA class II specific antibody had additional class I specificities detected by PRA-STAT. Table 5.6 (p164) details the HLA specificities of the antibodies detected in the 9 patients with additional class I specificities. In 4 cases there was no evidence of any HLA specific antibody production by CDC screening and in another patient with a CDC PR of 42% there was no apparent specificity which could be determined by CDC testing. PRA-STAT failed to detect class I specificities found by CDC in 2 cases, patient 14 shown in table 5.6 (p164) had a CDC detectable B51 antibody. Another patient had an antibody specific for HLA B7



which was detected by CDC but not by PRA-STAT, antibody was detected by Flowscreen in this patient.

Although PRA-STAT detected additional specificities compared with CDC in the majority of patients there was no significant difference in the PRs determined by the 2 methods. This could be due to differences in the composition of the panels used for the screening. Table 5.7 (p165) shows the percentages of selected HLA antigens in the panels. Random panels were used for CDC screening, therefore a mean of five 40 cell panels and five 60 cell panels were taken as representative of the CDC panels used. HLA A1, A29, A30, A31 and A32 are found in approximately equal proportions of each panel whereas there is a slightly higher proportion of A23 and A24 in the PRA-STAT panel. However the other antigens surveyed appear at a higher proportion in the CDC panels. These results show that for some of the commonly found antigens which elicit antibody responses in the population studied the CDC PR would be expected to be higher than the PRA-STAT PR if exactly the same antibody specificities were detected by both methods. This could explain the fact that PRA-STAT PRs are not significantly higher than those determined by CDC despite the additional specificities detected.

The Flowscreen method produced PRs which were significantly higher than those given by CDC and PRA-STAT even taking into account the background binding present. Analysis of the actual and expected PRs determined by Flowscreen and PRA-STAT for a number of cases indicates that Flowscreen often gives higher percentage binding than would be expected from the antibody specificity. The expected PR is based on the frequency of the antigens in the individual pools and the expected mean percentage binding for the 2 pools. A range is given to allow for the background

binding of up to 10%. Table 5.8 (p166) shows the results of the analysis. This indicates that the PRA-STAT method, used to determine the antibody specificity gives a PR close to the expected result, with 1 or 2 false positive or negative results accounting for the slight difference. The Flowscreen however often produces PRs which are considerably higher than those which would be expected from the known specificity and pool composition. This could be accounted for if Flowscreen detects additional antibody specificities not found on PRA-STAT screening, although it is not possible to determine if this is the case using the methods available.

#### 5.6.2.4 HLA Specific Antibodies Resulting from Graft Failure

The results of the screening carried out showed that 21 of 105 patients at no time produced any detectable antibody following graft loss. Eighty four patients were shown to have detectable antibody. Twelve of these patients produced IgM antibody with HLA specificity being found in 5 cases. Four patients produced IgM antibody specific for mismatched donor antigen (A2 in 2 cases, B8 and DR4 in 1 case each), one patient produced an IgM antibody specific for A9, which was not present on the allograft. Three patients produced antibody which was detected by flow cytometry only, with antibody binding to 30, 35 and 36% of the cell pools respectively. The remaining 69 patients had HLA specific IgG antibody with specificity for mismatched donor antigens in 67 of the 69 cases. In forty of the 67 patients producing donor specific antibody additional non-donor specific antibodies were also demonstrated.

The impact of HLA-specific IgG antibody on future transplantation in these patients was examined. Table 5.9 (p167) shows the retransplant rates in the antibody positive and negative groups. Patients who never produce antibody were more likely



to receive a second graft, 58.3% compared with 34.8% of those patients with HLA specific IgG. The function of second transplants was also investigated with regards the antibody status of the patients. Forty five patients had received second grafts at the time of writing, table 5.10 (p168) shows graft outcome in the antibody positive and negative groups. The cumulative graft survival in patients with no evidence of antibody production is 80.9% which is significantly higher than the 41.7% of regrafts currently functioning in patients with antibody. The relationship between antibody specificity, and mismatching on the first and second grafts was investigated. Table 5.11 (p169) shows that there is no significant effect of repeating a previous mismatch on a second graft. There were 16 patients who received a second transplant with repeat mismatches. Ten patients received grafts with repeat class II mismatches, 3 of which also had repeat class I mismatches, 5 of these 10 grafts failed. One failed graft was shown to have produced antibody to the repeat mismatch, 1 was not tested for class II antibody and 3 had no detectable class II antibody. A further six patients had repeat class I mismatches, only 1 of which failed.

The policy regarding repeat mismatches during this time would ensure that a mismatch would not have occurred if CDC screening had demonstrated an antibody was produced to that mismatched antigen. The results of the PRA-STAT screening demonstrated antibodies specific for HLA antigens, not detected by CDC, which were present on subsequent grafts in 3 patients, all 3 grafts failed. Only one of these was due to a previous mismatched antigen (DQ1), one was a pregnancy induced antibody specific for A1 and the remaining antibody was specific for DQ3. Six of eleven grafts with no evidence of antibody specific for the mismatched antigens on the

second graft failed. In the remaining 10 patients the class II antibodies had not been determined or the donor class II type was not known.

Four of the five patients who produced IgM HLA specific antibodies received second grafts, one of which failed. None of these grafts were mismatched for the antigen to which the recipient was sensitised by the first graft, although 2 of the grafts did have repeat mismatches to which no antibody was detected following failure of the first graft (an A1 mismatch in the patient with B8 antibody and a DQ1 mismatch in the patient with DR4 antibody). Both grafts were functioning at the census date.

Figure 5.2 (p175) illustrates the overall graft survival in all second transplants performed between 1984 and 1993 together with that for first grafts. The survival in regrafts is no different to that of first grafts. This suggests that sensitisation due to a previous failed transplant does not significantly decrease graft survival and that the relatively small number of antibody positive patients who received both first and second grafts during the study period may not be representative of the overall experience for regrafts.

#### 5.6.2.5 HLA matching and Antibody Levels Following Graft Failure

The analysis of antibody levels and HLA matching was performed using the highest recorded PR following graft failure. In some cases the PR was found to continue to rise over several months after significant antibody production was first detected, and specificity analysis showed that these antibodies were related to graft loss. When patients were found to have a marked rise in antibody post-graft failure due to a subsequent blood transfusion the highest PR prior to the transfusion was used for the analysis. Table 5.12 (p170) shows the mean PR for the different DR mismatch



groups and table 5.13 (p171) for A,B,DR mismatch. There were no cases of 0 A,B,DR mismatched grafts in the failure group. The Kruskal-Wallis 1-way analysis of variance was used to determine whether there was a significant trend across the mismatched groups for antibody production. The results shown on tables 5.12 (p170) and 5.13 (p171) indicate that when DR matching alone is analysed the increase in PR with number of DR mismatches is not statistically significant, however when mismatching at A,B,DR is considered there is a significant trend for increasing antibody levels with increases in the number of mismatched antigens ( $p=0.0257$ ).

#### 5.6.2.6 HLA Matching and Graft Survival

The influence of HLA matching on graft survival was analysed for primary transplants performed between January 1984 and December 1993. There were 395 donor recipient pairs with complete matching data for HLA A,B,DR. Sixty five cases were excluded from the analysis because DR types were not known for one or both of the pair. The cases analysed contained 364 cadaveric transplants and 31 living related transplants. An overall analysis and separate analyses for cadaveric and living donor transplants were performed. All failures and all cases of death with function were classed as graft losses for the survival analysis.

##### 5.6.2.6.1 DR matching

Figures 5.3, 5.4 and 5.5 (p 175-177) show the actuarial survival for all grafts, cadaveric and living donor transplants respectively according to DR match. Table 5.14 (p172) shows the cumulative graft survivals for each of the groups. Although there is 0% graft survival in the living donor grafts mismatched for both DR antigens

this relates to only a single case. This is seen in figure 5.4(p176) as curtailment of the appropriate line at the time of this failure. The survival in the 0 and 1 DR mismatch groups are less than 2 percentage points different. There is an overall difference of over 20% in the cumulative survival of the cadaveric grafts. Log rank analysis of the Kaplan-Meier curves for trend show that for all 395 transplants there is a significant association between DR mismatch and graft failure ( $p = 0.0035$ ). Individual log rank tests show that there is no significant effect for matching in the living donor group and that the overall significance is a result of differences observed in the cadaveric group.

#### 5.6.2.6.2 A,B,DR matching

Figure 5.6, 5.7, and 5.8 (p179-181) show the actuarial survival curves for all grafts, cadaveric and living donor transplants respectively according to A,B,DR mismatch. There were no living donor grafts mismatched for more than 3 antigens. The overall cumulative survival for the living donor grafts is 87.1% compared with 63.46% for cadaveric grafts. Table 5.15 (p173) shows the cumulative graft survivals for each group according to total mismatches. Log rank analysis of the Kaplan-Meier curves for trend show that for all 395 transplants there is a significant association between A,B,DR mismatch and graft failure ( $p = 0.0006$ ). Individual log rank tests show that there is no significant effect for matching in the living donor group and that the overall significance is a result of the differences observed in the cadaveric group. In the no mismatch group there was a single graft loss due to the death of the patient, with a functioning graft at over 7.5 years post-transplant. The actuarial survival curve therefore shows graft survival at 100% until the time of this loss. There are no further observations in this group beyond this time point therefore the line is curtailed.



	CDC + Flowscreen + PRA-STAT	CDC + Flowscreen	CDC + PRA-STAT	CDC
CDC PR < 50%	60	7	0	0
CDC PR > 50%	3	3	1	31

**Table 5.1 Screening techniques used for determining antibody production in patients following transplantation**

	CDC +	CDC -
Flowscreen +	27	8
Flowscreen -	13	25

$$\chi^2 = 11.88 \quad p < 0.001$$

**Table 5.2 Comparison of Flowscreen and CDC screening for the detection of antibody**



	CDC +	CDC -
PRA-STAT +	26	4
PRA-STAT -	10	24

$$\chi^2 = 18.97 \quad p < 0.0001$$

**Table 5.3 Comparison of PRA-STAT and CDC screening for the detection of antibody**

	PRA-STAT +	PRA-STAT -
Flowscreen +	29	5
Flowscreen -	0	29

$$\chi^2 = 42.46 \quad p < 0.0001$$

**Table 5.4 Comparison of Flowscreen and PRA-STAT for the detection of antibody**



	mean % PR	t-test	Wilcoxon test
CDC vs Flowscreen	20.77 vs 37.05	t = -4.61 p < 0.001	z = -4.5 p < 0.001
PRA-STAT vs Flowscreen	24.63 vs 39.75	t = 7.24 p < 0.001	z = -5.61 p < 0.001
CDC vs PRA-STAT	20.09 vs 25.70	t = -1.37 p = 0.175	z = -1.35 p = 0.176

Table 5.5 Comparison of the percentage panel reactivities determined by different screening methods



Patient	Graft mismatched antigens	CDC PR	Specificity by CDC	PRA-STAT PR	Specificity by PRA-STAT
4	A2, B40, Cw3, DR4,7,DQ3	9%	none	87%	A2, Cw3, DR4,7 DQ3
13	A1,B49,61 Cw2 DR5,6	42%	none apparent	87%	A9, B15 B60,61 DQ1
16	A1,29 B8,51 Cw2 DR4,14	44%	B51,52	26%	B8,51,52
14	A2,B44,51 DR4,7	28%	B44,51	30%	A2,B44 DR4,7
18	A2 B18 DR3	0%		91%	A2,A19,B51, 52 DR3
30	A1,3 B7,8 Cw7 DR3	27%	A1 B8	96%	A1,3,19 B7,8 DR3 DQ3
68	A1,2 B7,37 DR4	7%	none	57%	A2 DR4 DQ3
78	A24 B27,39 Cw1 DR4	39%	A24	57%	A1 DR2,4
99	A11 B7 DR2,5	0%		91%	A11 B7 DR2,5 DQ1

Table 5.6 Panel reactivities and antibody specificities determined by CDC and

PRA-STAT screening in patients with additional class I specificities

detected by PRA-STAT



HLA Antigen	40 Cell CDC panel (mean of 5 panels)	60 cell CDC panel (mean of 5 panels)	PRA-STAT panel
A1	22.5	17.6	17.4
A2	56.5	50.0	30.0
A3	28.5	30.6	10.9
A23	3.5	2.3	8.7
A24	12.0	11.6	17.4
A29	6.0	5.0	6.5
A30	9.0	9.0	6.5
A31	3.0	9.0	6.5
A32	7.5	10.3	8.7
B7	13.5	16.3	6.5
B8	20.5	19.6	8.7
B35	19.5	16.3	4.3
B44	18.5	18.0	10.9
B60	13.0	9.6	6.5

Table 5.7 HLA antigen frequency of selected antigens in 40 and 60 cell CDC and PRA-STAT panels

Antibody specificity	Flowscreen		PRA-STAT	
	Actual %PR	Expected %PR	Actual %PR	Expected %PR
A24 DR7	50	38-48	30	33
A1 B12 DR2	83	52-62	57	52
B57 DR7	45	24-34	22	17
B51,52	75	14-24	17	22
B8,51,52	50	24-34	26	24
A23 B44 DR7	71	28-38	41	33
DR4 DQ3	71	43-53	54	57

**Table 5.8 Actual and expected percentage panel reactivities for Flowscreen and PRA-STAT screening of samples containing HLA specific antibody**



	IgG antibody positive	IgG antibody negative
Regraft	24	21
No regraft	45	15

$$\chi^2 = 4.44 \quad p=0.033$$

**Table 5.9 HLA-specific IgG antibody and regrafts in patients with failed primary transplants**

	antibody positive	antibody negative
working	10	17
failed	14	4

$$\chi^2 = 5.66 \quad p = 0.017$$

**Table 5.10 Second graft outcome in patients with or without HLA-specific IgG antibody following failure of a first transplant**



	repeat mm	no repeat mm
working	10	17
failed	6	12

$$\chi^2 = 0.004 \quad p = 0.95$$

**Table 5.11 Graft outcome in patients receiving second transplants with or without repeat mismatched antigens**

DR Mismatches	No. patients	Mean maximum PR	Standard deviation
0	5	26.60 %	42.69
1	54	50.98 %	34.51
2	36	61.58 %	35.74

Kruskal-Wallis 1-way ANOVA  $\chi^2 = 4.61$   $p = 0.0998$

(corrected for ties)

Table 5.12 HLA-DR mismatch and mean percentage panel reactivity following graft failure



A,B,DR mismatches	No. patients	Mean maximum PR	Standard deviation
1	2	0 %	0
2	6	31.33%	26.57
3	21	49.43 %	37.36
4	21	63.81%	34.11
5	33	51.36 %	34.79
6	12	70.17 %	34.72

Kruskal-Wallis 1-way ANOVA  $\chi^2 = 12.76$   $p = 0.0257$

(corrected for ties)

Table 5.13 HLA-A,B,DR mismatch and mean percentage panel reactivity following graft failure

DR mismatch	No. cases	Overall graft survival	Cadaveric graft survival	Live donor graft survival
0	68	77.94%	75.44%	90.91%
1	216	67.59%	65.48%	89.47%
2	111	53.15%	53.64%	0%

Table 5.14 Cumulative graft survival and HLA-DR matching



A,B,DR mismatch	No. cases	Overall graft survival (n=395)	Cadaveric graft survival (n=364)	Live donor graft survival (n=31)
0	13	92.31%	85.71%	100%
1	18	77.78%	78.57%	75.00%
2	51	82.35%	80.49%	90.00%
3	85	62.35%	59.46%	81.82%
4	103	66.99%	66.99%	-
5	101	56.44%	56.44%	-
6	24	45.83%	45.83%	-

Table 5.15 Cumulative graft survival and HLA-A,B,DR matching



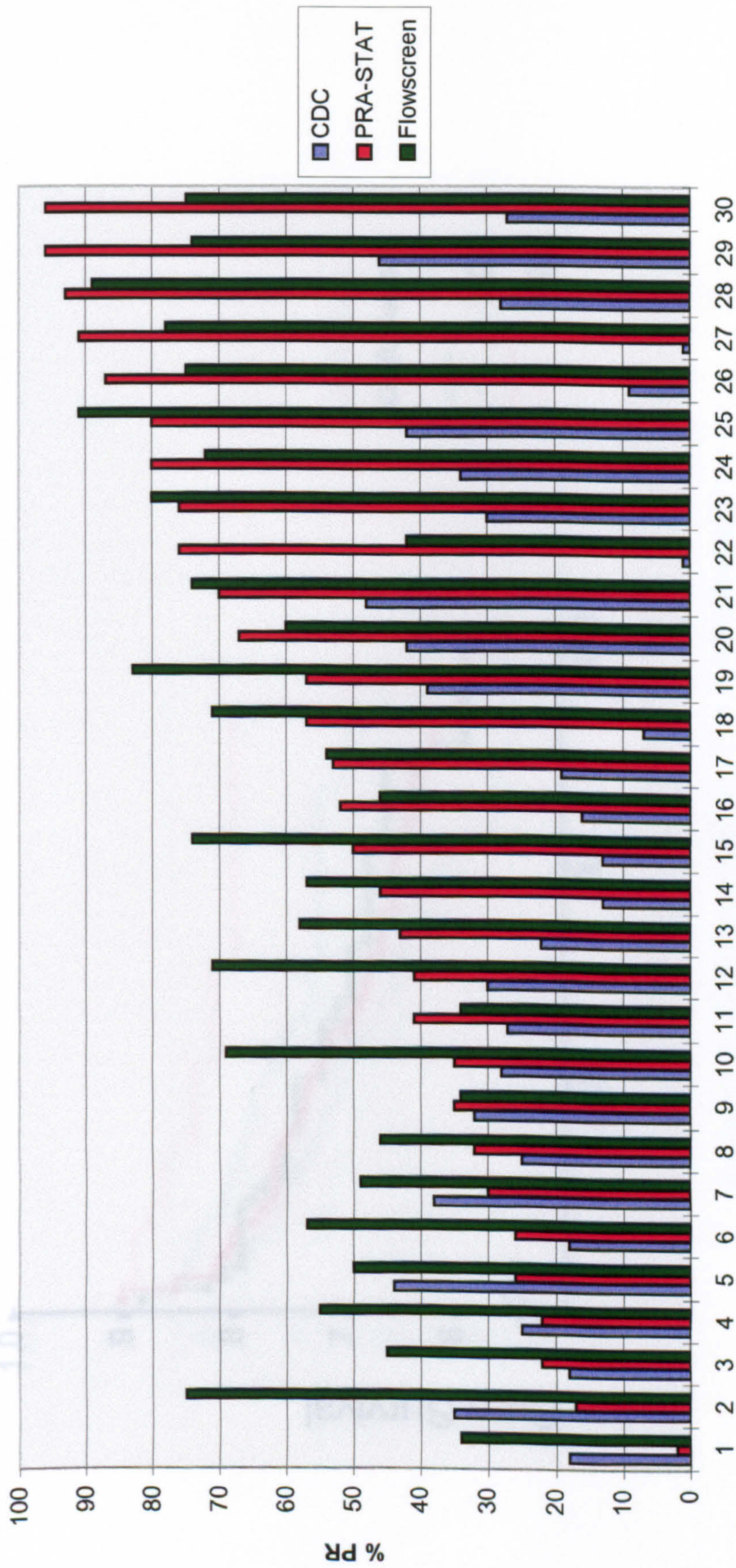


Figure 5.1 Percentage panel reactivities determined by CDC, PRA-STAT and Flowscreen testing of 30 post-graft failure samples



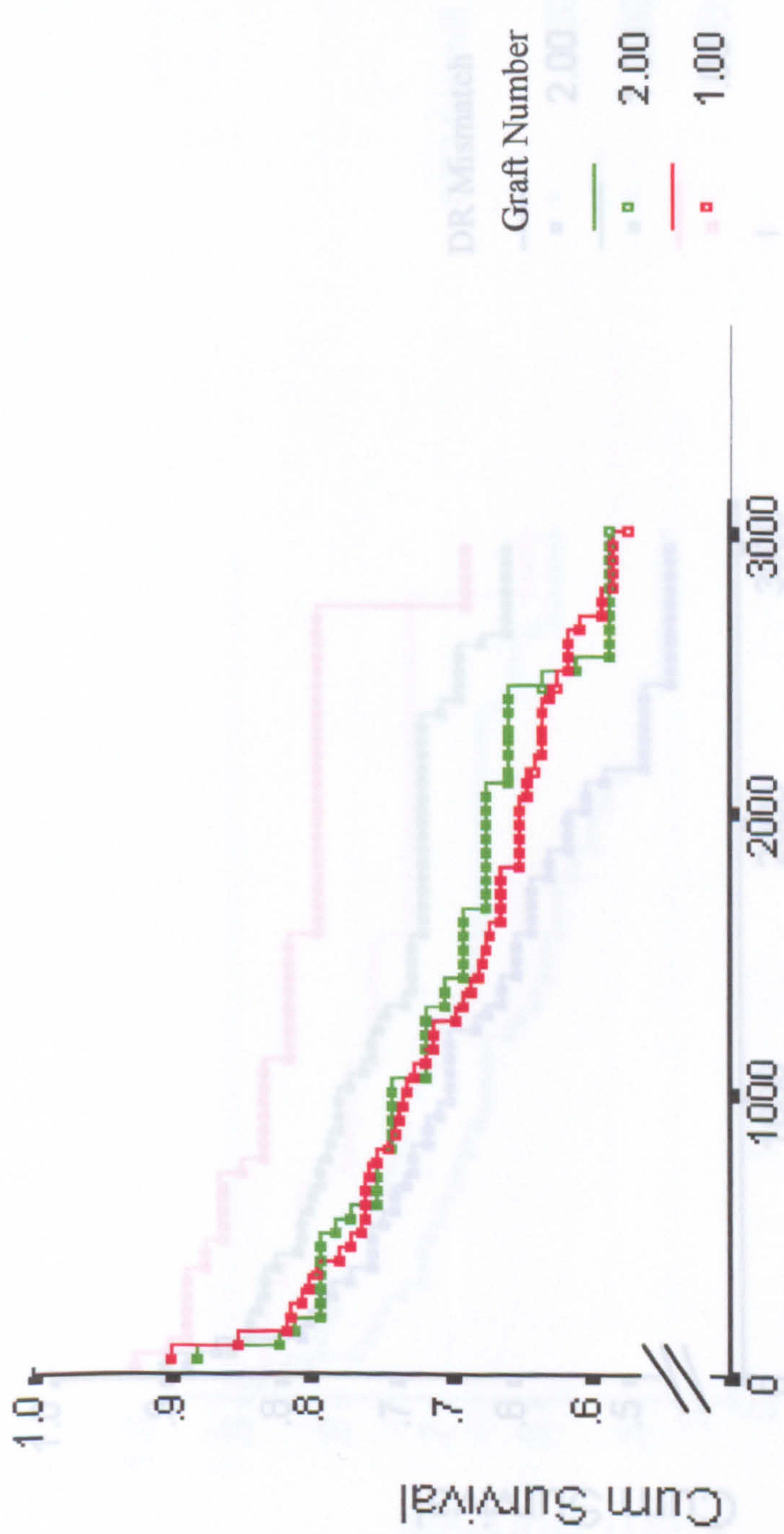


Figure 5.2 Actuarial Graft Survival for first and second transplants 1984-1993



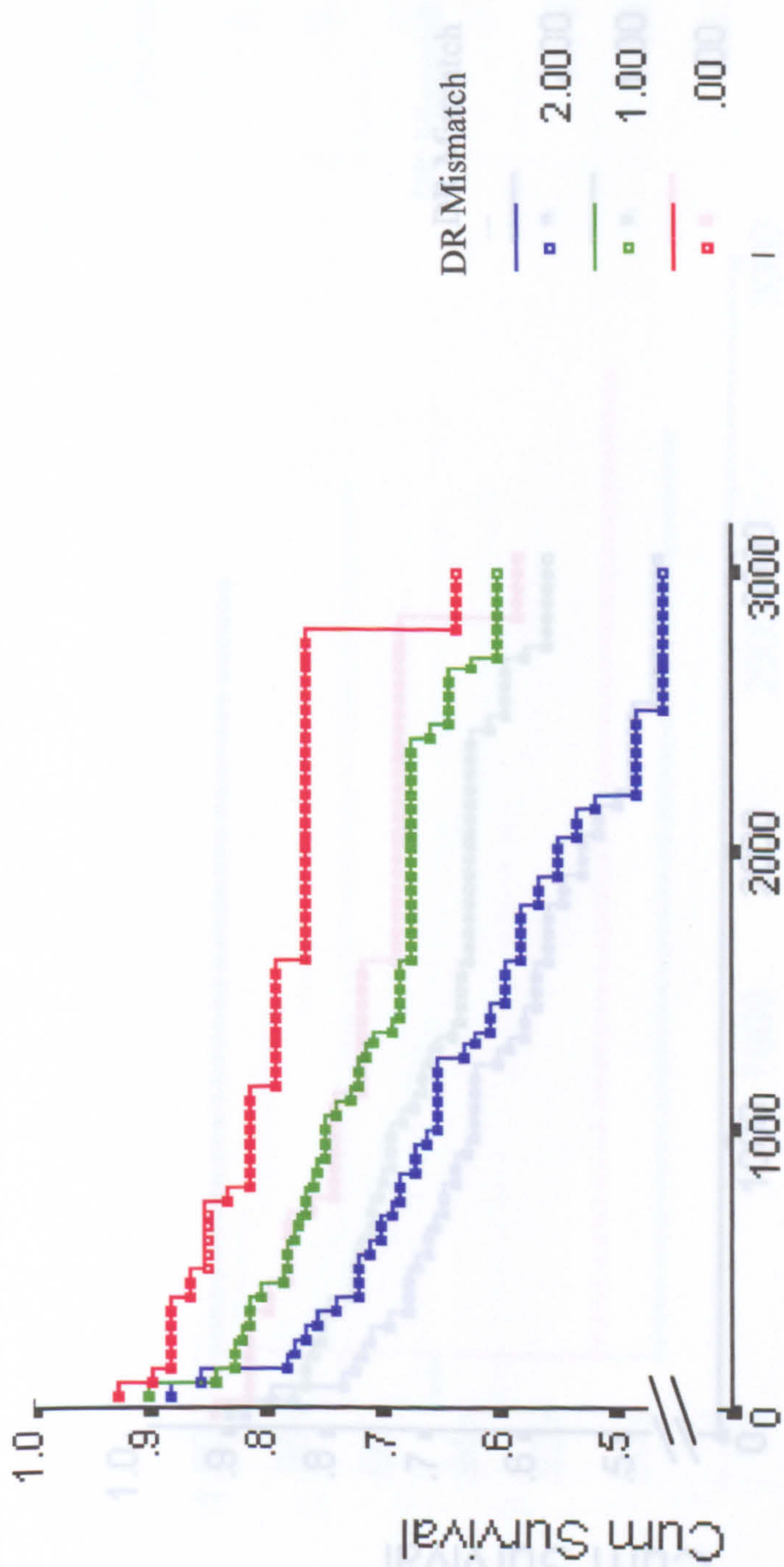


Figure 5.3 Actuarial Graft Survival and HLA-DR Matching



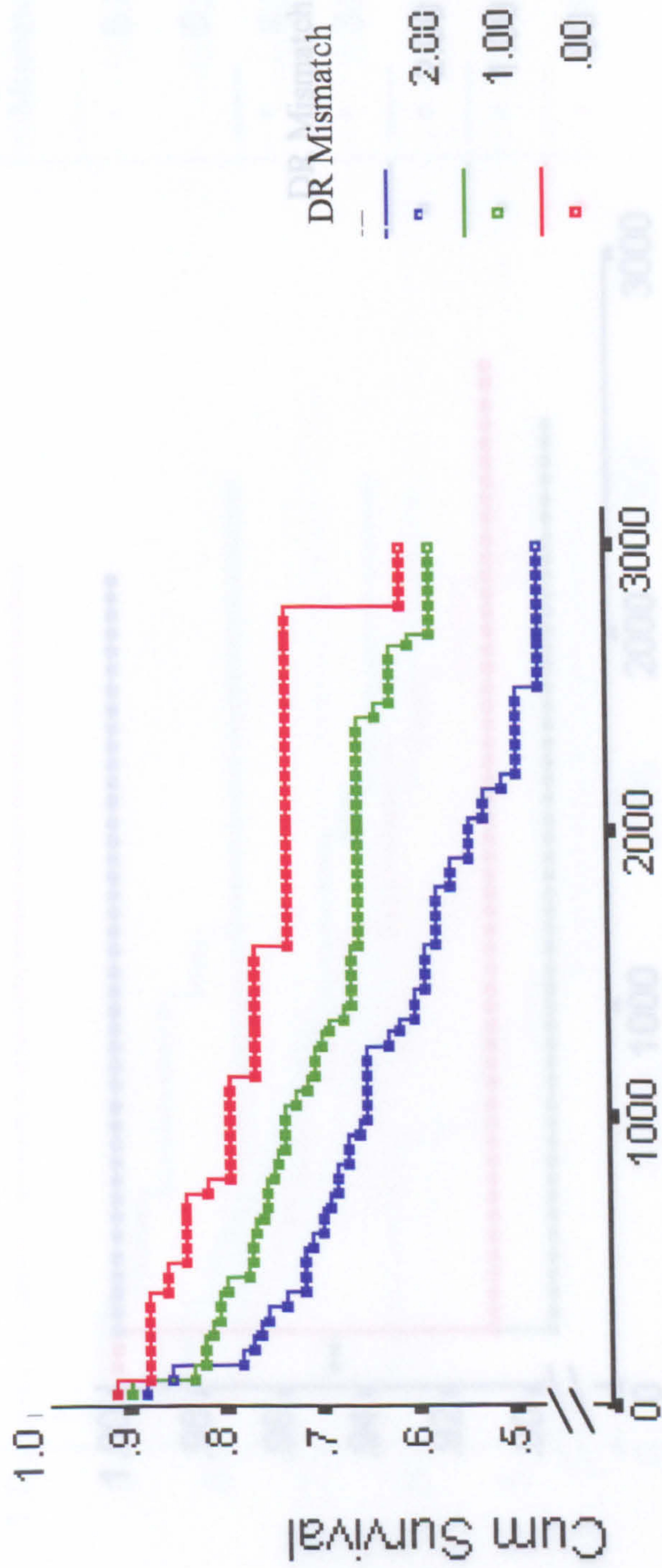


Figure 5.4 Actuarial Cadaveric Graft Survival and HLA-DR Matching



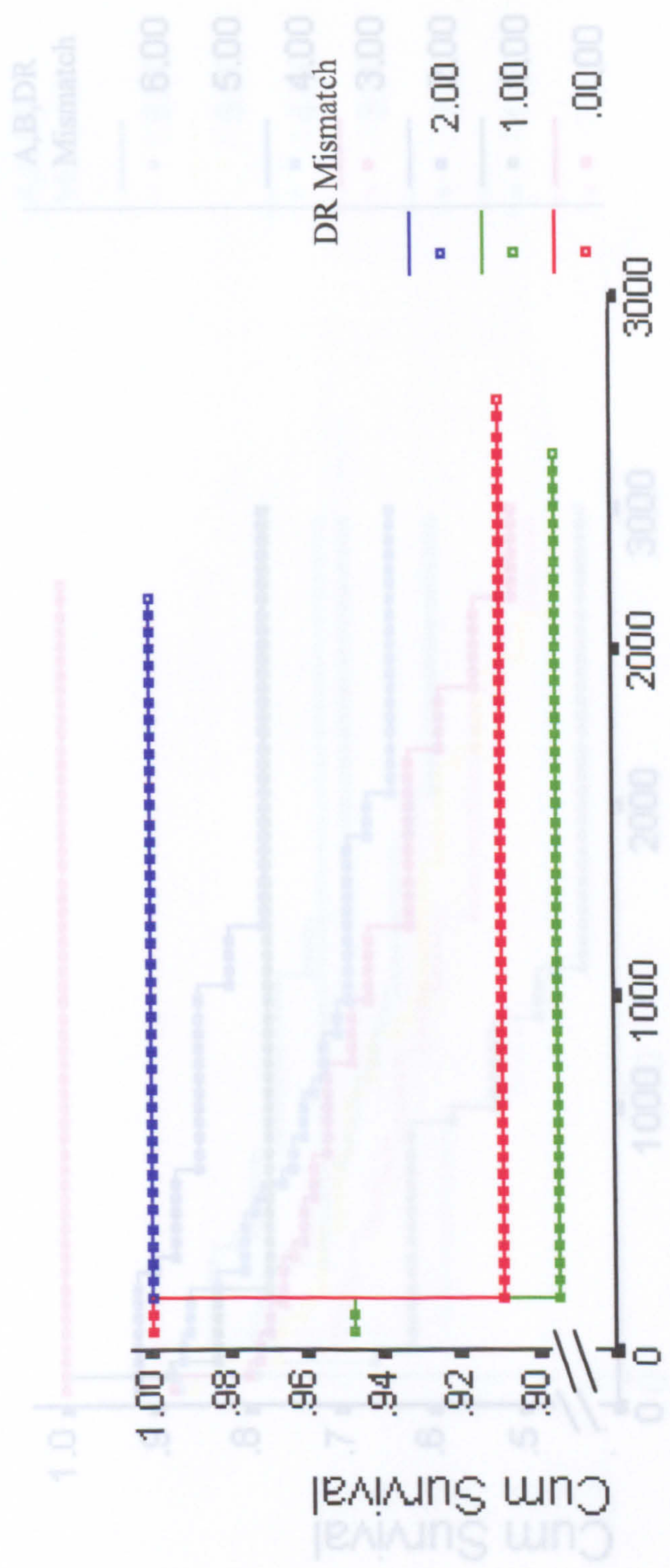


Figure 5.5 Actuarial Living Donor Graft Survival and HLA-DR Matching



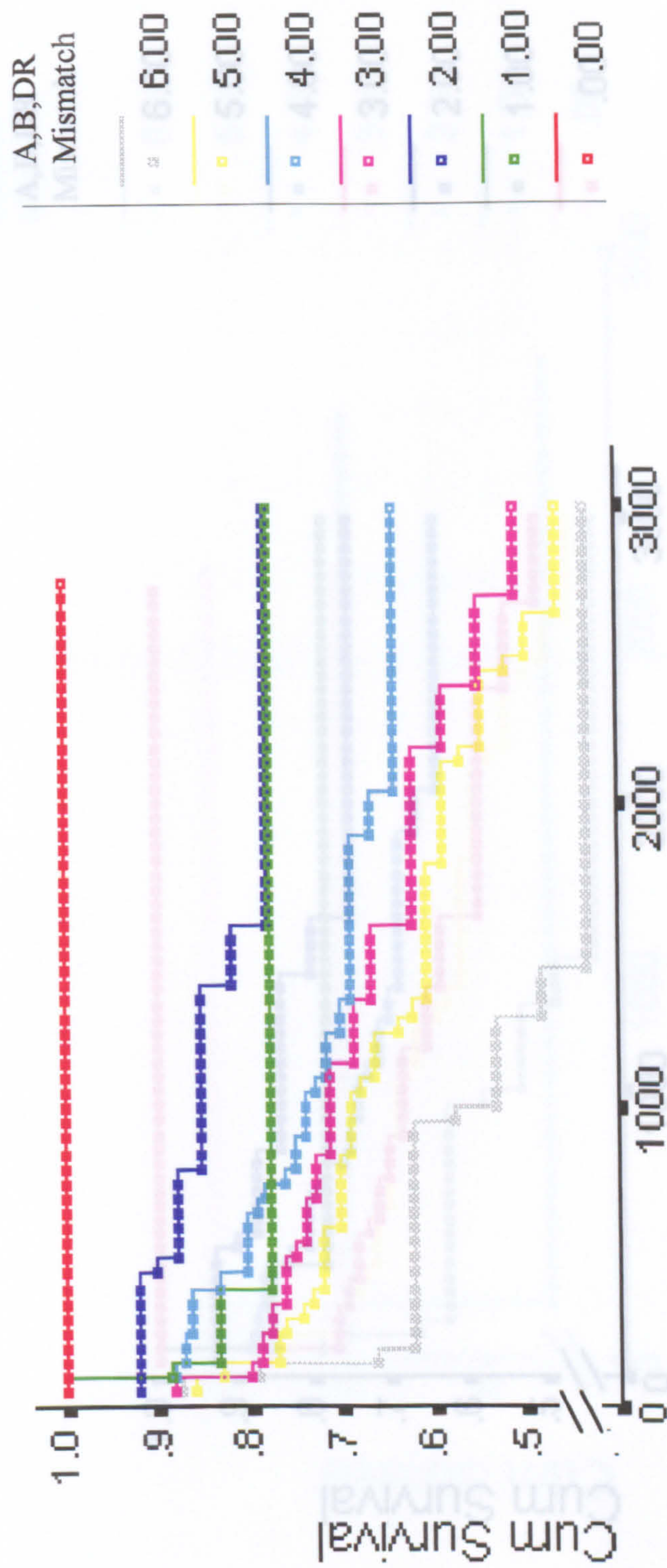


Figure 5.6 Actuarial Graft Survival and HLA-A,B,DR Matching



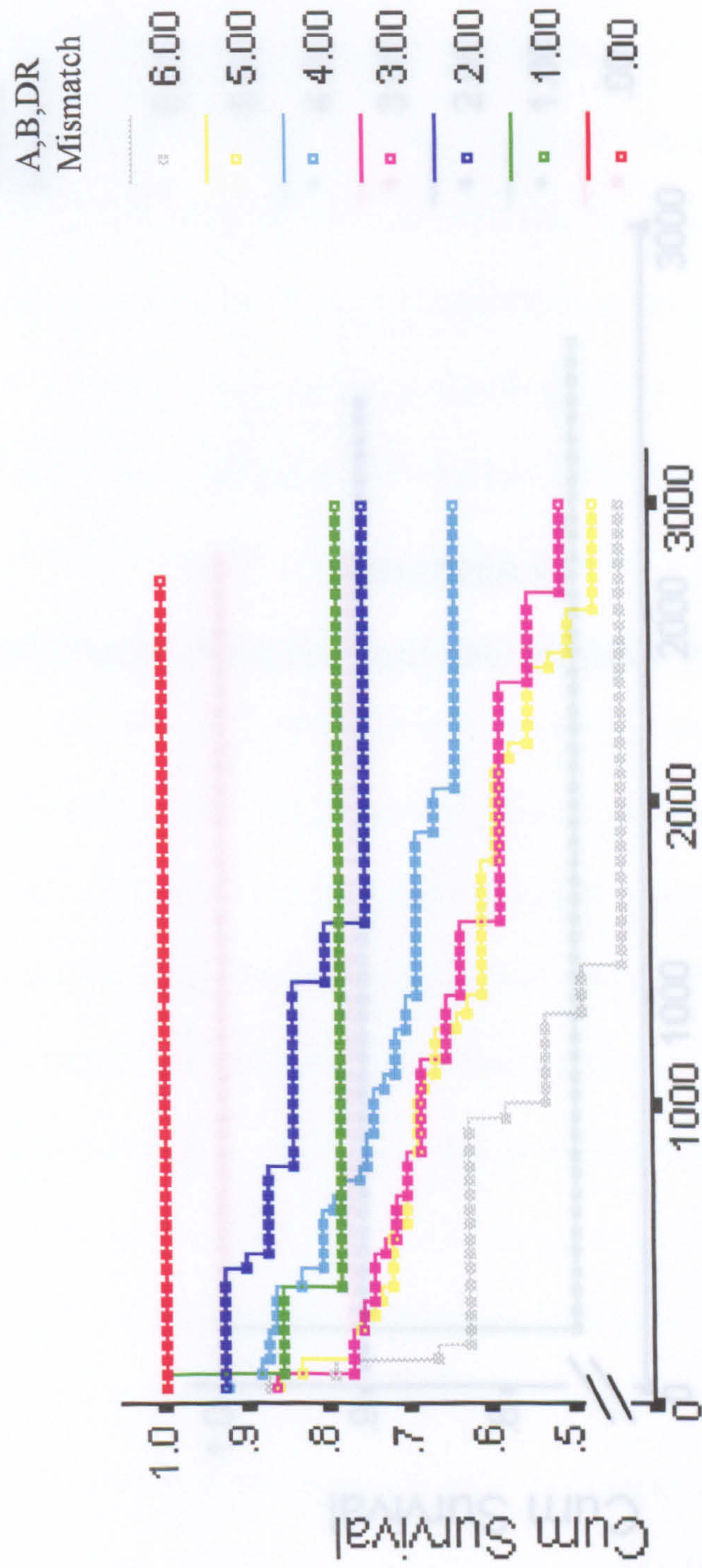


Figure 5.7 Actuarial Cadaveric Graft Survival and HLA-A,B,DR Matching



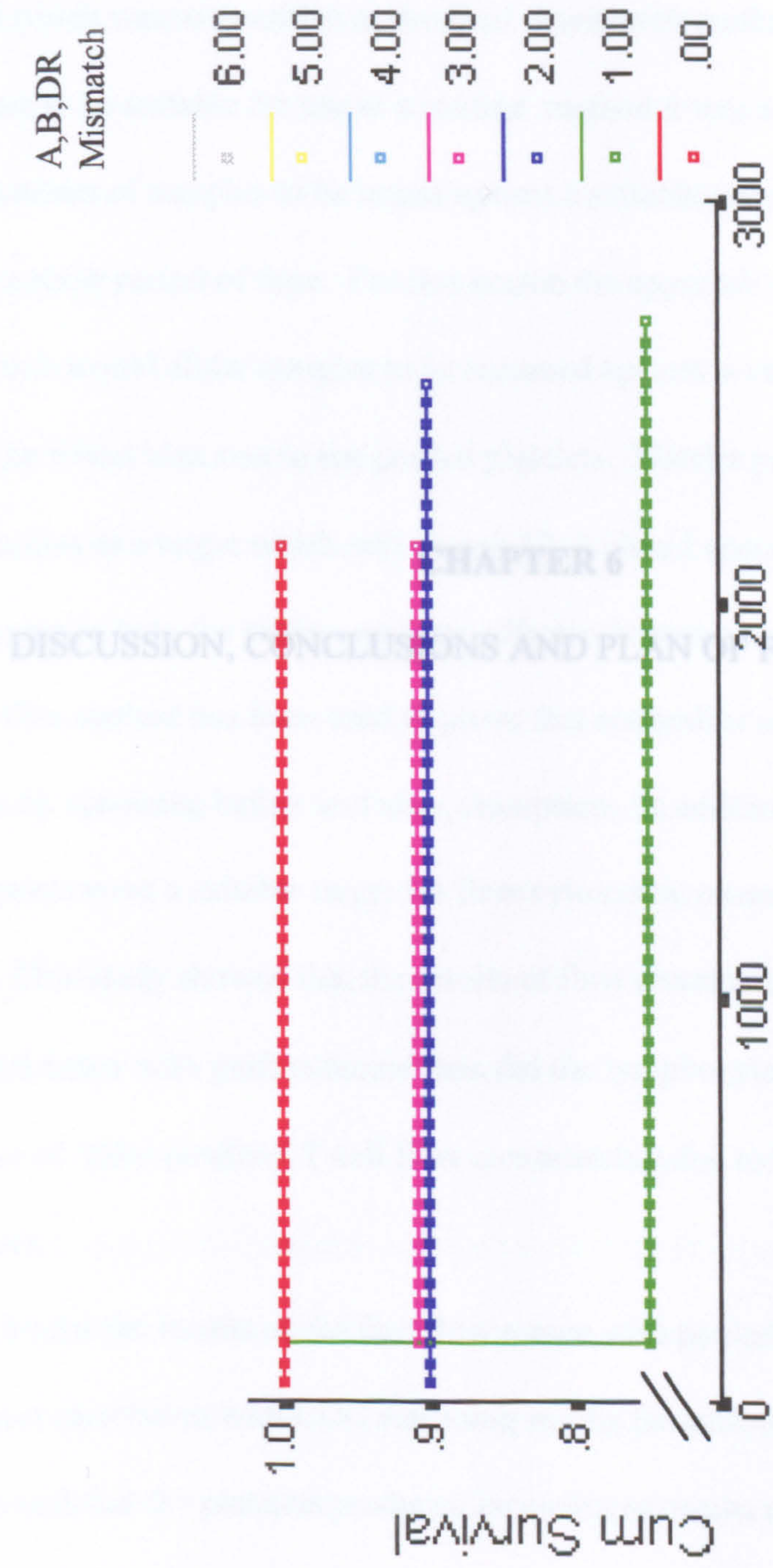


Figure 5.8 Actuarial Living Donor Graft Survival and HLA-A,B,DR Matching

## **CHAPTER 6**

### **DISCUSSION, CONCLUSIONS AND PLAN OF FUTURE STUDY**



## 6.1 Discussion

The initial aim of this study was the development of a flow cytometric technique for antibody screening which would fulfil the requirement for a screening method which was as sensitive as the final crossmatch method. In order for the technique to be suitable for use as a routine method it was necessary for relatively large numbers of samples to be tested against a suitable number of different target cells in a short period of time. For this reason the approach taken was to use pooled cells which would allow samples to be screened against several different cells at one time. The initial idea was to use pooled platelets. Platelet pools have been used in many studies as a target which will absorb HLA class I specific antibodies present in serum samples onto the surface antigen, effectively removing the antibody from the serum. This method has been used to prove that antibodies are specific for class I antigens by screening before and after absorption. In addition it had been suggested that platelets were a suitable target for flow cytometric crossmatching by Wang et al (1989). This study showed that the results of flow crossmatching with donor platelets correlated better with graft outcome than did the lymphocyte FCXM, possibly due to a number of 'false positive' T cell flow crossmatches due to non-HLA specific antibodies.

Whilst the results of the first flow screen with pooled platelets did show a significant correlation with CDC screening results for antibody detection subsequent tests showed that the platelets produced inconsistent results and that there was an unacceptable number of false negative results with the platelets.. Therefore this method failed to fulfil the requirement for a more sensitive method than CDC to correspond with the more sensitive FCXM crossmatching technique. In addition there

was little correlation between the percentage binding to platelets and the CDC panel reactivities. The use of CLL cell pools produced more satisfactory results with a strong correlation between the flow cytometric method and CDC techniques but with an apparent greater degree of sensitivity of the flow cytometric method as shown by the increasing number of flow positive CDC negative results obtained with higher serum dilutions. The use of CLL pools was thus confirmed as a suitable method for screening with a sensitivity to match that of the flow crossmatch.

Although the CLL cells had proved an ideal target the supply of cells was limited and it was decided to investigate the use of cultured cells which could be stored in liquid nitrogen and then grown up in large numbers whenever required. This also provided the opportunity to choose cell lines with specific HLA types from the large catalogue of such cells which are available. It was therefore possible to construct pools so that most HLA antigens were represented. Testing of EBV cell pools showed that these were also suitable for screening samples for both HLA class I and class II specific antibodies using the technique developed with the CLLs.

The flow method was found to be able to detect antibodies which CDC had shown to be directed against 'uncommon' HLA antigens which were represented on only 1 of the cell lines contained in the pool. It was also found that positive results were sometimes obtained even when the antigen to which the antibody was thought to be directed (from the CDC screening results) was not present in the pool. The reason for this could not be clearly demonstrated but it is possible that antibodies which appear to be monospecific or directed at a limited number of discrete HLA antigens by CDC techniques are in fact more widely reactive when a more sensitive technique is used. It may be that the antibody detected by CDC is present in the serum at a



higher titre than additional antibodies, directed at other antigens. These could be antigens within recognised crossreactive groups, sharing epitopes with the principle target antigen. Lower titre antibodies could be detected by the more sensitive flow cytometric technique.

Another possibility is that the antibodies detected by the flow cytometric technique may be of a non-complement fixing IgG sub-type which would not therefore be detected by the complement dependant technique. Sumitran-Karuppan et al (1992) had investigated IgG subclasses in positive flow cytometric crossmatches. Patients with negative CDC crossmatches but positive flow crossmatches were found to have an increased risk of rejection and the subclass analysis showed a mixture of different combinations of subclasses with sometimes high titres of non-complement fixing IgG4 together with lower titre IgG1 and IgG3. This analysis suggests that both antibody titre and subclass may be factors in the observed sensitivity of the flow cytometric screen.

The results had shown flow cytometric analysis of cell pools to be a suitable method for antibody detection which met the requirements set at the start of the study. Confirmation that the development of this particular technique as an approach to screening was appropriate was given by the concurrent development of similar techniques by other workers. Cicciarilli et al (1992) used a pool of 10 peripheral blood lymphocytes for screening by flow cytometry, allowing the detection of HLA class I antibodies. The method was used to investigate antibody status of the pre-transplant serum of re-graft recipients. The majority of samples were positive by both CDC and the flow technique, flow cytometry detected antibody in some samples which were CDC negative and CDC detected IgM antibodies in some flow negative

samples. These results are similar to those seen when developing the Flowscreen technique with CLL pools and with the EBV pools.

Shroyer et al (1995) used a pool of 6 peripheral blood lymphocytes selected such that antigens within each of 14 cross reactive group were represented. The results of this study showed a strong correlation for antibody detection between CDC and the flow cytometric method, with flow cytometry detecting a significant number of extra positives not detected by CDC. The mean PR was higher by flow cytometry than by CDC for the samples which were positive. This finding of higher reactivity with the flow cytometric screening with the use of a limited pool where only CREGs were represented rather than individual antigens would support the hypothesis that flow cytometry detects cross reactive antibodies which are not detected by CDC. This would suggest that in a large majority of cases the reactivity of the antibodies is far broader than is indicated by the results of CDC screening and monospecific antibody production elicited by recognition of a single private epitope is rare.

ELISA screening was introduced as a commercially available screening method by SangStat. Buelow et al (1995) published details of the development of the method and of a comparison with CDC screening techniques carried out in 5 laboratories. The reported concordance between PRA-STAT and CDC screening for detection of HLA class I specific antibodies was good with inter-laboratory agreement and very high levels of reproducibility of results. A number of reports appeared in the literature shortly after the method became available with varying views on the ability of the method to detect HLA class I specific antibodies. Kerman et al (1996) reported good correlation between PRA-STAT and CDC for the detection of antibodies. In this study the antibody status of patients prior to transplant was examined and it was



shown that there was a stronger correlation between patients with PRA-STAT detectable antibody and graft rejection than with CDC detectable antibody. However no distinction between IgG and IgM antibodies detected by CDC was made when the clinical significance of the antibodies was investigated despite the identification of IgM antibodies being commented on when the correlation between the 2 methods for antibody detection was made. The comparison of the methods for clinical significance is therefore flawed as IgM autoantibodies are included in the CDC detectable group despite the well documented evidence that these antibodies are not clinically significant. A later study by Monteiro et al (1997) did distinguish between IgG and IgM antibodies detected by CDC in making a similar comparison and found that patients with PRA-STAT detectable antibody pre-transplant did have a higher proportion of graft losses compared with patients with CDC detectable IgG antibody.

Zachary et al (1995) whilst finding the method to be reliably reproducible and correlating well with CDC for detection of antibody did not find that the specificity determination by PRA-STAT corresponded well with the CDC determined specificities. In only 12 of the 66 sera which were positive by both methods were the antibody specificities found to be identical. There were examples of samples which were CDC positive/PRA-STAT negative, PRA-STAT positive/CDC negative and many where the specificities simply did not agree. Bryan et al (1995) compared PRA-STAT with CDC screening and an in house ELISA method which utilised solubilised HLA antigens from platelet pools. The local ELISA method was found to correlate closely with an AHG CDC screening technique whereas PRA-STAT had a lower correlation.

PRA-STAT was investigated as a part of this study initially in the hope that the method could be used to determine HLA class I specificities in samples which were known to contain IgG antibodies from the results of the Flowscreen. Initial testing showed a good correlation for antibody detection in most cases, although there appeared to be a problem with PRA-STAT being unable to reliably detect IgG antibodies in sera which also contained high titre IgM antibody. This had also been commented on in the report by Zachary et al (1995). Although antibody detection appeared to correlate well there were considerable differences in the specificities found by PRA-STAT and CDC. The very strong correlation between Flowscreen and PRA-STAT was the first indication that the ELISA technique was detecting antibodies other than those found by the conventional CDC technique for class I screening. In 15 of 16 patients with serial samples the results of Flowscreen and PRA-STAT for PR mirrored each other exactly. It was therefore not surprising when it was discovered from the manufacturers that rather than having isolated HLA class I antigen captured on the plates there was also class II antigen present.

An important factor in establishing the presence of class II antigens on the ELISA plates was the HLA typing, by PCR-SSP, of the cell lines. Whilst providing the details of the class II types the results also showed that there were some mistakes in the original class I types of the cells which had been used in all the analysis software provided by the manufacturers for use with the kits. The veracity of the genotypes rather than the provided phenotypes was shown by analysis of the class I specificities found in some of the samples in this study. The incorrect class I types used in all the previous studies, including the first reports of the technique and the fact



that HLA class II antigens were present make the strong correlations found in some studies somewhat surprising.

Kerman et al reported an 82% agreement in antibody specificity determination between CDC and PRA-STAT, whereas Zachary found identical specificities in less than 20% of the samples tested. Zachary found that there were a number of cases where PRA-STAT detected additional class I specificities, an observation also made in this study. However there was complete disagreement on specificity between CDC and PRA-STAT for almost 50% of the samples. This could be explained by the presence of HLA class II specific antibodies in the samples. The results of this analysis have shown that class II specific antibody will cause many positive reactions which could be wrongly interpreted if the analysis was performed using only the class I types as was the case in the studies published prior to the discovery of class II antigen on the PRA-STAT plates. Whilst Zachary's results can easily be reinterpreted in the knowledge of the class II component of the test it is less clear why Kerman et al should have found such close agreement on antibody specificity, although a large contribution to the good correlation was made by the inclusion of over 300 samples (out of the 495 tested) which were antibody negative. It could be possible that few of the samples which tested positive contained any class II specific antibody, the majority of the samples test came from patients awaiting a first transplant although the study did show PRA-STAT positivity to be more common in patients with failed first grafts. The results of this study have found that the majority of patients with failed grafts who were tested by PRA-STAT do have class II specific antibody.

Bryan et al (1995) concluded that PRA-STAT was not an appropriate method for the detection of class I antibody when compared with a locally produced ELISA

plate which used solubilised antigen from platelets. This is not surprising as comparison was not, as believed at the time, between equivalent methods but of a method where class I antigen was present on the plate with one where both class I and class II antigens were present.

The reanalysis of the PRA-STAT data already collected showed that inclusion of the class II types into the analysis software allowed reliable detection of class II antibodies in addition to class I antibodies. One problem in the detection of class II antibodies has been the difficulty of distinguishing them from class I antibodies also present in the same samples. This can only be done by carrying out absorptions to remove the class I antibodies or by blocking antibody binding using monoclonal antibodies. This is a more lengthy process than conventional screening and a technique which allows detection of antibodies to both class I and class II at the same time would obviously be advantageous. It was found that in many samples this was possible. The analysis of antibody binding by measuring optical density provides detailed information on the strength of each individual reaction, especially when compared with the scoring systems used in CDC screening which are not only very subjective but also limited to only 5 or 6 broad categories for strength of reaction. The software allows presentation of the results sorted according to strength of reaction. This will often show that the reactions to one antigen produce similar optical density measurements so that positive results for an individual antigen will be grouped together when sorted according to strength. This has allowed determination of both class I and class II specificities in the same sample in many cases although there are some samples where this is still impossible, particularly in some of those with very high PRs. There are inevitable limitations associated with fixed panels



which are also encountered in CDC screening techniques. Some antigens are very highly represented across the panel, especially the broad DQ antigens. There are also some antigens where linkage disequilibrium has not been overcome, e.g. B8 is only found in association with DR3 and B57 with DR7.

Despite the limitations of the technique PRA-STAT was shown to be a very good complimentary method to the Flowscreen already developed, allowing specificity determination for both the class I and class II specific antibodies detected by Flowscreen. The original testing of the Flowscreen had shown that the method was more sensitive than CDC testing, producing more positive results even when compared with CDC screening by CLLs which detects both class I and class II antibodies. The strong similarities in the antibody profiles of the patients tested post-transplant between PRA-STAT and Flowscreen suggested that PRA-STAT may have a similar level of sensitivity and could therefore be used to identify antibodies which are not detected by CDC in some samples which are Flowscreen positive.

The development of the Flowscreen technique was intended to provide a method which was more sensitive than CDC screening and to use this method to investigate antibody production in patients with failed transplants. With little published data specifically relating to patients with failed grafts, especially with regards class II specific antibodies it was hoped that the Flowscreen method would provide more information about these patients than the existing CDC techniques used. The approach taken was to look at antibody development following graft failure in all patients over a ten year period. As well as looking at differences in the antibodies detected by various techniques in these patients the antibodies produced were also to be related to the degree of HLA matching of the failed grafts as previous studies had

produced conflicting results on the influence of matching on subsequent antibody production. As matching was to be investigated in this way it was also decided to compare matching in the functioning and failed transplants.

Before commencing with the testing of samples from patients with failed grafts the results of the CDC screening were reviewed in detail. A number of patients produced HLA class I specific antibodies immediately following graft failure which were reactive with a very high proportion of the CDC panels. It was decided that many of these samples would not benefit from Flowscreening as the results of the routine Flowscreen, now well established in the laboratory, had shown that these patients invariably had Flowscreen PRs over 80%. It was felt that this would not provide any substantial additional information on these patients. All samples with CDC PRs of less than 50% were to be screened by Flowscreen, in addition Flowscreen was also to be used for a proportion of those with CDC PRs over 50% where the specificity was not entirely clear. The subsequent testing of PRA-STAT and its identification as a method for the determination of both HLA class I and class II specificity provided a further screening technique for analysing the samples already tested by Flowscreen.

The results of the testing showed that both Flowscreen and PRA-STAT detected HLA specific antibodies in samples which were negative by conventional CDC screening. The samples were tested by Flowscreen before PRA-STAT and it had been initially thought that the Flowscreen positive samples which were CDC negative may contain HLA class II specific antibodies in many cases although the possibility of greater sensitivity for class I detection was also acknowledged, especially where samples subsequently developed CDC detectable class I specificity.



The PRA-STAT screening showed that in most cases both these explanations held true. The majority of samples tested by all 3 methods were shown to contain both HLA class I and class II specific antibodies, directed towards mismatched donor antigens. In addition PRA-STAT detected HLA class I specific antibodies which were not found by CDC screening. The study therefore confirmed the view that screening techniques with greater sensitivity than the conventional CDC screen could provide additional information about the antibodies produced following transplant failure. The ability of PRA-STAT to define antibody specificity, even in many of the samples with high PR, indicates that samples with CDC PRs of greater than 50% would benefit from PRA-STAT screening. This has not been done in this study but future studies should utilise PRA-STAT screening for these samples.

In many of the cases tested where Flowscreen and PRA-STAT detected antibodies not shown by CDC, cytotoxic testing of subsequent samples did show an increase in the PR and in the specificities detected. This suggests that the antibodies detected by the newer methods could be low titre antibodies, although the strength of many of the reactions as measured by optical density in PRA-STAT would not altogether support this idea. It is also possible that the antibodies could be non-complement fixing as discussed earlier.

Although PRA-STAT showed higher sensitivity than conventional CDC, Flowscreen appears to be the most sensitive technique. The high Flowscreen PRs could not always be accounted for by the specificities of the antibodies detected by PRA-STAT. This suggests that Flowscreen is detecting additional antibody. It is possible that this could be due to very low titre antibodies to additional HLA antigens,

particularly those within cross reactive groups as discussed earlier, or the antibodies detected may be directed at non-HLA antigens.

There were 3 cases where Flowscreen was the only method to detect any antibody with no evidence of any HLA specific antibody being detected by CDC or PRA-STAT in any subsequent samples. In all cases the Flowscreen PRs were relatively low, less than 40%. Low Flowscreen PRs were also found in only 2 of 46 patients with functioning grafts. The fact that the majority of patients with Flowscreen detectable antibodies do eventually develop cytotoxic antibody suggests that the low levels of antibody binding which are detected in a small proportion of patients may not be HLA specific and may not have any detrimental effect on transplanted kidneys as they have been found in patients with stable graft function.

A number of studies have investigated antibody production following transplantation and its association with graft function. It has been shown that HLA specific antibody production often accompanies rejection episodes in the patients studied (Martin et al 1987, Tang et al 1988, Scornik et al 1989, Halloran et al 1992). It has been suggested that rejection episodes which are accompanied by antibody production may be more severe than those where there is no evidence of antibody (Al-Hussein et al 1994) and the eventual outcome of these rejection more likely to be graft failure (Lobo et al 1995). The comparison of the Flowscreen and PRA-STAT methods involved investigation of antibody production in transplanted patients. It was found that production of antibodies which were specific for mismatched donor HLA antigens accompanied irreversible rejections in this small group of patients whereas a number of patients with no detectable antibody experienced reversible rejection episodes.



A recent study using PRA-STAT has found that rejection episodes which are accompanied by antibody production do not respond standard therapy whereas those with no PRA-STAT detectable antibody do (Nanni-Costa et al 1997) confirming earlier observations of the poor prognosis for rejections which are associated with antibody. If antibody production, whilst not predicting the onset of a rejection episode, is predictive of the severity of the rejection and the response to treatment it may be possible to use such information to modify the anti-rejection therapy accordingly. Routine monitoring of antibody production post transplant would be a time consuming, and possibly expensive, procedure which may be of use in only a minority of cases. The techniques developed and assessed in this study can be used to produce results quickly. Flowscreen can be used to rapidly screen large numbers of samples at very little cost. PRA-STAT can provide the essential information about antibody specificity. A combination of these two methods could be used to gain information on antibody status in patients at the onset of rejection even if regular monitoring were not considered possible.

Analysis of the influence of HLA mismatching on subsequent antibody production tended to support the view that the greater the number of mismatched antigens the higher the resulting panel reactivity. This was shown to be significant when matching for A.B.DR was analysed but did not achieve statistical significance when DR matching was considered alone. This could be due to the very small number of fully DR matched grafts which failed. Overall 7 complete DR matched grafts failed, but no post transplant samples were available for screening in 2 cases (1 cadaveric and 1 living donor transplant). Of the 5 patients who were tested 3 produced

no detectable antibody at any time following graft failure, the remaining 2 produced antibodies with PRs of 35 and 96 respectively.

Two previous studies have produced conflicting conclusions in this area. Scornik et al (1987) investigated the effect of HLA-A and B match on subsequent antibody production following graft failure and found that there was a significantly higher peak PR in patients with 3 or 4 mismatched antigens than in those with 2 or less. Matas et al (1990) however, when looking at HLA-A,B,DR mismatching did not find any significant effect on subsequent antibody production. The results presented in Matas' study make pairwise comparisons of PR according to mismatch and there is no data presented which indicates whether an analysis for trend was carried out. The results presented in this study have shown that a trend for increasing PRs can be shown if the appropriate statistical analysis is performed. The study by Matas et al limited its investigation of antibody production to antibodies detected by CDC screening with peripheral blood lymphocytes, therefore it is unlikely that any class II specific antibodies would have been detected.

As shown by the studies commented on above very little attention has been paid to the production of HLA class II specific antibodies following graft loss. This may be due to the lack of agreement on the clinical significance of B cell positive crossmatches. It has been suggested that positive B cell crossmatches which are due to class II specific antibodies are associated with inferior graft outcome whereas those which are not a result of HLA specific antibody are not (Russ et al 1987, Scornik et al 1992, ten Hoor et al 1993, Lazda et al 1993). This study has shown that the majority of patients screened for class II specific antibody following graft failure were found to produce donor specific class II antibodies, directed at both HLA-DR and HLA-DQ.



This finding supports the idea that positive B cell crossmatches in patients with a previous failed transplant may well be a result of HLA class II specific antibody.

The significance of the antibodies produced following graft failure on the outcome of subsequent transplants has been investigated. As with previous studies (Sanfilippo et al 1987, Matas et al 1990) it was found that a higher proportion of patients who never produced antibody following a failed graft were retransplanted compared with those who do produce detectable antibody. In the group of patients studied the success of second grafts was significantly higher in patients with no history of antibody production compared with those patients who did produce antibody, although the numbers analysed were small. Although the policy of the unit during this period had been to avoid transplanting a patient with a mismatched antigen if there was any evidence of an antibody specific for that antigen at some time prior to the crossmatch the results of the new screening techniques used suggest that the CDC results which were the basis of such decision taking may not have given sufficient information about the patients antibodies. Although there were only 3 cases where the PRA-STAT had clearly shown that an antibody specific for an antigen which the patient was subsequently mismatched for had been produced prior to that transplant all 3 cases failed. One of these was a class I antibody and 2 were specific for HLA-DQ. Whilst antibodies specific for HLA-DR have been infrequently studied, DQ specific antibodies have received even less attention. One report of a positive crossmatch due to DQ specific antibody suggested that this was not associated with any deleterious effects on the transplant (Taylor et al 1987). This study has shown that patients frequently produce both DR and DQ specific antibodies following graft

loss, although it is sometimes difficult to distinguish DR and DQ specificity separately due to the strong linkage disequilibrium between the 2 loci.

It has been suggested that repeat class I mismatches are not harmful in regrafts if it can be shown that the patient has never produced an antibody specific to that mismatched antigen (Welsh et al 1988). This study has not found any significant association between repeat mismatches and reduced graft outcome which supports the view that kidneys with repeat mismatches can be successfully transplanted. However when comparing HLA class II repeat mismatches these did appear to have a higher proportion of failures than the class I repeat mismatches, 5 of 10 class II repeat class II mismatches compared with 1 of 6 with a class I repeat mismatch. Cecka and Terasaki (1994) found that patients with repeat class II mismatches had lower 3 year graft survival rates than those with class I repeat mismatches. The important factor in the study by Welsh et al was to determine whether any antibody had been formed which was specific for the repeat mismatch but which was no longer detectable in the serum samples which were used for crossmatching. Due to the screening strategy used in this study not all the patients receiving repeat class II mismatches have been screened by PRA-STAT and so it is not clear whether there had been any class II specific antibody produced following the failure of the first graft in all the cases. This question should be studied in more detail in a larger group of regrafts comparing class II repeat mismatch and no repeat mismatch groups as the evidence would suggest that there may be an important effect of class II specific antibody on regraft outcome.

A recent report has shown an association between HLA-DP matching and graft outcome in retransplants (Mytilineos et al 1997). The association between DP mismatches and poor graft survival was particularly strong in patients with high levels



of CDC detectable antibodies. This data suggests that it may be of interest to investigate HLA-DP specific antibodies in patients with failed transplants and to correlate this information with data on repeat DP mismatches. The cell lines used in the PRA-STAT kits have been HLA-DP typed and the details of the DP antigens present on the plate are to be released by the manufacturers in a replacement for the SOFTSTAT programme used in this study. It should therefore be possible to carry out an analysis of HLA-DP specific antibodies at the same time as investigating the HLA-DR and DQ antibodies in more detail.

In addition to investigating the impact of HLA matching on antibody production and on retransplants by way of repeat mismatching an analysis of HLA matching and graft survival was performed on the data collected for the study. January 1984 was chosen as the start date for the 10 year study as this was the first full year in which transplanted patients received Cyclosporin immunosuppression. It was also at this time that HLA class II typing was becoming an established technique in the laboratory, with cadaveric donors being typed at the time of donation for HLA class I and class II antigens.

There were a small number of failures in class II typing in the early years of the study, especially before the introduction of magnetic beads allowed purified B cell preparations to be used, however class II types were available for over 90% of donors. A slightly higher proportion of recipients were found not to have class II types, although these were generally patients with grafts which were still functioning or those who had died at some time following transplantation. Patients with failed grafts would have been class II typed when they were returned to the waiting list for a subsequent transplant. In order to obtain more complete matching records samples

were requested from patients with functioning transplants who had not been class II typed. Samples were taken when patients attended for a routine clinic appointment. A small number of patients with stable function were seen at such infrequent intervals that samples were not obtained during the course of the study, in addition patients who had died with functioning grafts could not be class II typed. Overall there was complete matching data available on 395 of 460 transplants.

The matching analysis performed considered matching only for broad antigens rather than splits although it has been shown that matching for antigen splits is more advantageous than matching for broad antigens (Opelz 1988, Mytilinoeos et al 1997). However this approach requires relatively high resolution typing and for the population studied the HLA class II types were often only given at the level of the broad antigen, especially in the case of donors typed during the early years of the study. The results of the analysis show that within the population studied there was a significant effect of matching on graft survival, both for HLA-DR and for HLA-A,B,DR. These results are in agreement with many of the large studies which have been published.

Failure to find a significant effect of matching has often been associated with single centre studies (Matas et al 1990, Hayes et al 1993), although this has not always been the case (Dyer et al 1989, Washburn et al 1995). In such studies the results may have been affected by the matching policy in operation at the centre. Some centres finding matching to be associated with graft survival have actively attempted to match donor, recipient pairs whereas other centres finding no effect of matching have had very few cases which were well matched for comparison with the large proportion of poorly matched grafts. Throughout the 10 years which are



considered in this study there was no active matching policy at the transplant unit, with grafts being allocated primarily on the basis of negative crossmatch. Other factors, including HLA match, donor and recipient age, recipient waiting time and clinical status of the potential recipients were also taken into account. This resulted in a majority of patients receiving mismatches in the middle of the range from complete match to complete mismatch with smaller numbers at each extreme. However the spread was sufficient to provide a comparison which yielded a significant result. Thus a policy which took only slight account of the evidence regarding the benefits of HLA matching has produced data which confirm the view that matching confers a significant advantage in graft outcome. The results of the small number of living donor grafts performed were in agreement with some studies which have found that HLA matching does not provide any advantage in this group of patients (Terasaki et al 1995, Jones et al 1994). The largest published study did however show a significant effect of matching in live donor grafts (Opelz 1997). With the very small number of live donor grafts in this study the results of the matching analysis cannot be regarded as significant.

The beneficial effect of HLA matching which was found in this group of patients supports the practice of allocating cadaveric organs on the basis of HLA matching. In addition the analysis of antibody production following graft failure has shown that higher levels of panel reactive antibodies will result from the failure of poorly matched grafts compared with well matched grafts. This increased antibody production significantly affects both the chance of a patient receiving a regrant and the success of any subsequent graft. The small number of cases where HLA class II mismatches were repeated on re-grafts provides some evidence that class II

mismatching may be particularly detrimental. The use of new and more sensitive techniques for antibody screening have demonstrated that class II specific antibodies are produced in many patients following failure of a class II mismatched graft. Routine screening for HLA class II antibodies is not undertaken in many laboratories, a practice which this study suggests may lead to a gap in the understanding of patient's antibodies.

The use of sensitive crossmatching techniques, particularly flow cytometry, has meant that many transplants are not performed because of a positive crossmatch when the standard CDC test is negative. This practice probably detects a large proportion of the antibodies which this study has shown sensitive screening techniques will detect. However reliance on the crossmatch to detect these antibodies is dependant on the samples which are used for crossmatching. Whilst some centres will use all the stored samples from a patient many do not because of the practical difficulties associated with crossmatching very large numbers of samples from patients who have been on the waiting list for a considerable length of time. These laboratories will make a selection of sera for crossmatching based on the information screening results have supplied about the panel reactivities, specificities and antibody class for each sample. The results of this study suggest that the standard screening techniques may underestimate antibody levels in some samples and that a more sensitive screening technique may be advantageous. It has been stated that a screening technique which is as sensitive as the final crossmatch test should be employed (Fuller 1991). The results of this study strongly support this view and by using such a method indicate that the extra information provided may prove important in setting up and interpreting crossmatches for clinical renal transplantation.



## **6.2 Conclusions**

Flow cytometric analysis of antibody binding to pools of CLL cells or EBV transformed cell lines is a sensitive and reliable method for the detection of HLA class I and class II specific antibodies.

The commercially produced ELISA kit, PRA-STAT, detects both HLA class I and class II specific antibodies with greater sensitivity than standard lymphocytotoxicity screening. Antibody specificity can be determined from the PRA-STAT results.

Flow cytometric and PRA-STAT screening of serum samples from patients with failed transplants provides information about antibody specificity and panel reactivity which conventional CDC screening does not provide.

The majority of patients with failed transplants produce both HLA class I and class II specific antibodies.

HLA matching for primary cadaveric renal transplantation is related to graft survival and failure of a poorly matched graft results in greater sensitisation as measured by panel reactive antibody than does failure of a well matched graft.

There is evidence that repeating HLA class II mismatches and that pre-formed HLA class II specific antibodies may be detrimental for regrafts. This requires further investigation to clarify the importance of HLA class II antibodies and matching for second and subsequent grafts.

## **6.3 Plan of Future Study**

Figure 6.1 illustrates a screening strategy for detecting antibody production and defining specificity in regularly collected samples. Using this strategy the impact

of antibody production following graft failure on subsequent grafts will be further investigated in a retrospective study. The most sensitive technique will be used as the first line screen. Samples which are positive by Flowscreen will be screened by both CDC and PRA-STAT. The use of both techniques is necessary to confirm HLA class I specificity as this may be difficult to do using PRA-STAT only in samples with both class I and class II specific antibodies because of the limitations of the fixed panel. The antibody specificities determined for each serum sample can be analysed by computer programme and additional specificities can be predicted based on epitope analysis. These additional specificities can be confirmed by screening selected samples from each individual concerned on a cell panel constructed to contain the antigens of interest, which are not found in PRA-STAT or the CDC panels already used.

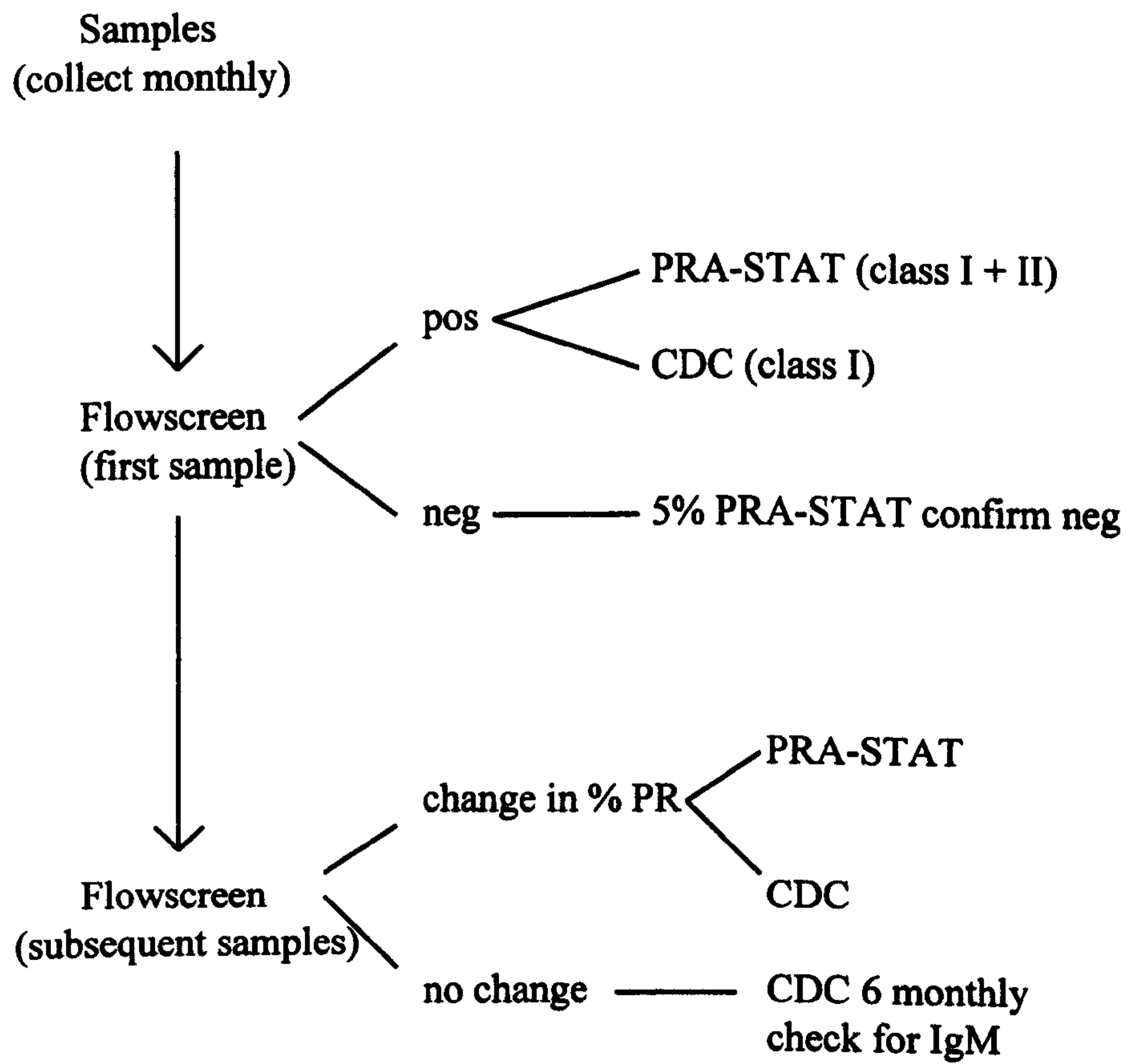
This comprehensive technique should provide detailed information on antibodies specific for HLA A,B,C,DR,DQ and DP. It will also be possible to investigate the importance of immunoglobulin class and subclass using this strategy. Monoclonal antibodies to the IgG subclasses, IgM and IgA are available and can be used in both flow cytometric and ELISA techniques. High resolution HLA typing of both donor and recipient may be necessary to provide sufficient detail on matching for a complete analysis of the data.

The results of the study will be used to determine, in greater detail than the present study, the specificity, immunoglobulin classes and relationship to previous mismatches of antibodies which may be detrimental to regraft outcome.

The relationship between antibody production and rejection episodes post-transplant will be investigated in a larger group of patients. The results will be



analysed to determine whether information on antibody status, available at the time of diagnosis of rejection, may be helpful in determining the appropriate anti-rejection treatment to be administered.



**Figure 6.1** Screening strategy for detecting antibody production and defining HLA class I and class II specificity



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