THE ROLE OF THE POLYMERASE CHAIN REACTION IN THE ROUTINE HAEMATOLOGY LABORATORY

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Clearance Certificate No 30/11/88.

DECLARATION

I declare that this Research Report is my own, unaided work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) to the University of the Witwatersrand. It has not been submitted before for any degree or examination in any other University.

KAREN ELIZABETH GUNTHER

August 1993

DEDICATION

To my Gran, Alma Norbert

ABSTRACT

The Polymerase Chain Reaction (PCR) provides a means of amplifying target sequences of DNA exponentially and it is rapidly becoming an indispensable tool in the research laboratory. Many other molecular genetic techniques used for research are far too laborious and expensive to be used for routine diagnostic purposes but PCR has the potential to be different. This Research Report assesses the possible role of PCR as a routine diagnostic tool in the haematology laboratory.

In the context of haematology, PCR can be used to detect both **"pathological"** and **"physiological"** target sequences present within the genome. Pathological sequences of interest would include mutations, deletions, insertions or translocations not present within the normal genome but which may arise either as a result of an hereditary abnormality or be acquired somatically. Sensitive detection of such sequences is useful for diagnostic purposes and can also be relevant in determining prognosis, evaluating response to therapy and following up minimal residual disease in the context of haematological malignancies.

PCR detectable physiological sequences would include the immunoglobulin and T cell receptor gene rearrangements normally present within the genome of cells of the appropriate lineage. These rearrangements differ for each lymphocyte within a polyclonal population but are identical among members of a clone arising by proliferation of a single precursor cell. They can therefore be of value not only in determining cell lineage but also as markers of clonality.

In this study the practical aspects of using PCR were assessed by setting up the technique of amplification of immunoglobulin gene rearrangements. The cost of reagents and disposable equipment, as well as that of major items of equipment required which are not usually available in a routine laboratory was also determined. In addition, peripheral blood and bone marrow samples reaching the Haematology Laboratory of the Johannesburg Hospital were analysed to assess the potential demand for such investigations.

Once appropriate reaction conditions for the primers used had been established, PCR was found to be quick, technically simple and relatively inexpensive. Sufficient numbers of appropriate samples for which PCR analysis could potentially be of value were received in the Johannesburg Hospital Haematology Laboratory in the periods assessed, to indicate that diagnostic PCR, if available, would be well utilised.

Some problems were encountered, particularly with regard to variability in the extent of amplification obtained. Thus for routine diagnostic purposes, extensive research and development of each set of primers to be employed will be necessary to make the technique more reliable and consistent. Adequate quality control will also be essential if PCR is to be used for diagnostic purposes. However, once these issues have been addressed, PCR should definitely find a place as a routine diagnostic tool in the haematology laboratory

PUBLICATIONS

Parts of this research report have been presented as follows:

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1. INTRODUCTION

The enormous strides that have been made in recombinant DNA technology and the subsequent advances in the field of molecular biology have led to an improved understanding of the pathogenesis of numerous disease processes. This should facilitate not only diagnosis at a molecular level, but also more accurate assessment of prognosis and more appropriate treatment. Many of the molecular genetic techniques used in research are far too expensive and laborious to be practical for routine use, but the Polymerase Chain Reaction (PCR) has the potential to be different. PCR is already moving from the experimental to the diagnostic laboratory, and having a significant impact on many aspects of clinical medicine. This Research Report will atttempt to assess the feasibility of using PCR as a routine tool in the diagnostic haematology laboratory.

1.1 THE PCR PRINCIPLE

Originally the "brain child" of Kary Mullis (1990), PCR was initially described as a rapid and sensitive means of detection of the sickle cell gene in prenatal diagnosis (Saiki et al, 1985). In essence, it provides a means of amplifying exponentially selected "target" sequences of nucleic acids. Numerous modifications have been described to extend its utility, but in its simplest form olignonucleotides complementary to sequences of bases flanking the target region are used as "primers" to prime the enzymatic synthesis of new DNA strands complementary to both the sense and the antisense strands of the target sequence. These new strands can themselves then act as templates for the same primers. Repeated cycles of **heat denaturation** - during which the DNA is unravelled into single strands, **annealing** - during which the oligonucleotide primers hybridise specially to their complementary sequences and **extension** by a DNA polymerase in the presence of an excess of nucleotides, will thus result in exponential amplification of the target DNA sequence bounded by the primers. This constitutes the Polymerase Chain Reaction (Bell et al, 1989) (Figure 1).

1.2 ADVANCES IN PCR

Perhaps the major advance which facilitated automation of PCR was the cloning of the gene for a thermostable DNA polymerase derived from the bacterium *Thermus Aquaticus*. The use of this "**Taq polymerase**" in place of the Klenow

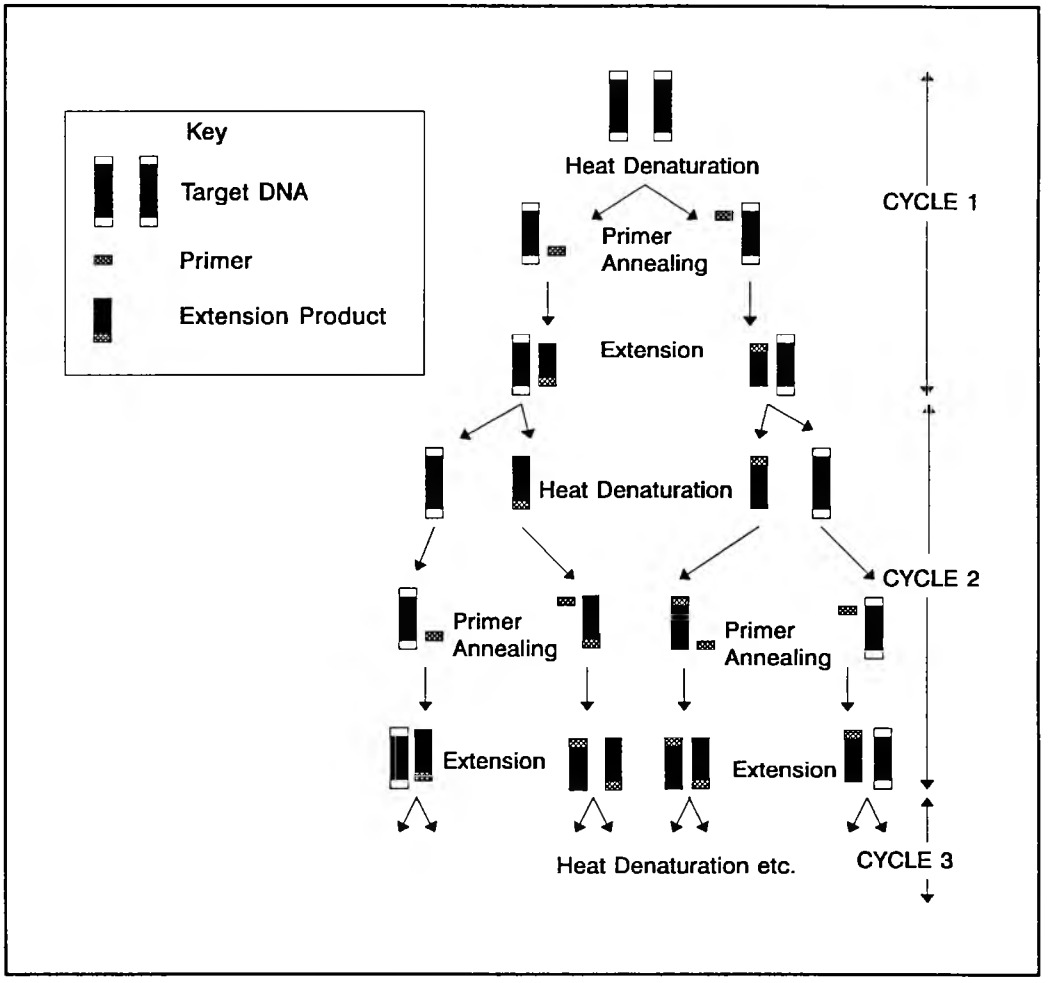

Figure 1. The PCR Principle

fragment of *E.coli* DNA polymerase obviates the need to add more enzyme after each heat denaturation step. It also allows higher annealing temperatures to be used,which improves the stringency of the reaction and reduces the amount of non-specific binding of the primers (Marx et al, 1988).

One fairly simple strategy which markedly improves the efficacy of amplification, is the use of **nested primers.** These are oligonucleotides designed to hybridize to sequences within the PCR product internal to the original set of primers. Thus a second round of PCR amplification is performed, using a small aliquot of PCR product as a template for the nested primers (with fresh deoxyribonucleotide triphosphates, buffer and enzyme). This improves not only the extent of amplification and therefore the sensitivity, but also the specificity, as no amplification will occur in the second round of PCR if the original product is artefactual. Examples of the use of such nested reactions include amplification

of the chimeric myl/retinoic acid receptor (α) gene arising from the t(15;17) translocation in acute promyelocytic leukaemia (Biondi et al, 1992), and amplification of rearranged immunoglobulin heavy chain genes (Trainor et al, 1991).

Another particularly useful adaptation has been the development of reverse transcription PCR (RTPCR), using messenger RNA (mRNA) as the starting material. Here cDNA is synthesised from the mRNA using a reverse transcriptase and conventional techniques. The cDNA is then used as the template for PCR amplification. This technique enables assessment of gene expression, for example to distinguish between an active and a latent viral infection (Hart et al, 1988). In addition, cDNA contains no introns and its use avoids the constraints placed on one step PCR amplification by the presence of long intervening sequences within the target region. (PCR amplification will only occur if the primer binding sites are less than 2-5 kilobases apart, thus limiting the size of the PCR product (Erlich et al, 1988)).

1.3 ADVANTAGES OVER SOUTHERN BLOTTING

Prior to the advent of PCR, specific genomic sequences were usually detected by Southern blotting, a process involving restriction enzyme digestion of the genomic DNA prior to hybridization with a radiolabelled probe specific to the gene sequence being assessed (Southern, 1975). PCR has numerous advantages over this laborious procedure which makes it more amenable to routine use. Results can be obtained far more rapidly (within one day as compared with several days for a Southern blot), and since no radioactivity is required, the expenses and risks of working with radioactivity are avoided. In addition, because of the small region of DNA that constitutes the target for analysis, PCR products can be derived from partially degraded DNA such as that obtained from fixed embedded pathological specimens (Wan et al, 1990). Such DNA would result in a diffuse background signal and reduced intensity of the desired signal if used for Southern blotting. The extent of amplification afforded by PCR allows its use with extremely small amount of starting DNA, compared with the 1-5 μg required for Southern blotting, and it can therefore be applied to the small numbers of cells

derived from fine needle aspiration (Wan et al, 1992; Trainor et al, 1991). Another potential advantage of PCR is its sensitivity. Southern blot analysis gives an absolute threshold of detection of specific genomic sequences of 1-5% (Wright et al, 1987; Katz et al, 1989), whereas much higher levels of sensitivity can be achieved with PCR in most contexts (e.g. Lee et al, 1987).

2. APPLICATION OF PCR TO HAEMATOLOGY

In the context of haematology, PCR has been used in association with primers directed at **"pathological"** and **"physiological"** DNA sequences. **"Pathological"** sequences are those resulting from alteration of the genome, either by a genetic or hereditary abnormality or due to an infective or malignant process. **"Physiological"** DNA sequences are those such as immunoglobulin and T-cell receptor gene rearrangements, normally present within the patient's genome.

2.1 PATHOLOGICAL SEQUENCES AS TARGETS FOR PCR.

The role of PCR in the **detection of hereditary genetic disorders** both for prenatal diagnosis and carrier screening is already well established. In the context of haematology one of the most common applications is the detection of haemoglobinopathies. In fact the first description of the use of PCR was in the diagnosis of sickle cell anaemia (Saiki et al 1985). Mutations involved in ß thalassaemia have also been characterised by sequencing of PCR amplification products (Wong et al 1987) and PCR has also been used for diagnostic purposes with the α thalassaemias (Lebo et al 1990, Bowden et al 1992). Numerous other hereditary haematological abnormalities including haemostatic defects such as Von Willebrand Disease (Peake et al 1990) are potentially amenable to detection by PCR.

In clinical medicine, PCR seems likely to make a major contribution to **detection of microbial pathogens** such as Tuberculosis (van Helden et al 1991) and HIV (Perrin et al 1990). Examples more specific to haematology would include the detection of the HTLV virus (Loughran et al, 1992). A particularly valuable possibility would be the development of a "cocktail of primers" that could be used in a single PCR reaction to provide a rapid cost effective screening technique for multiple pathogens in blood banking.

PCR is also finding a place in the **diagnosis of haematological malignancies.** The ever growing list of non-random chromosomal aberrations shown to be associated with such malignancies provides ideal targets for PCR as these are not present in normal cells but are common to all the members of the malignant clone. They can therefore act as tumour-specific markers, not only for

diagnostic purposes but also for purposes of prognostication and evaluation of therapy or follow up of minimal residual disease (MRD) (section 2.3).

2.1.1. Principles of PCR detection of non-random chromosomal aberrations.

The best studied chromosomal aberrations, which were targeted in initial PCR-MRD studies, are the translocations. The principle here is the selection of primers which will anneal to target sequences on either side of the breakpoint recombination area. Thus if the translocation is present, copies of the sense and anti-sense hybrid strands will in turn act as templates for the primers allowing exponential amplification. In the absence of the translocation no new templates would be synthesized and only linear amplification would occur (Figure 2).

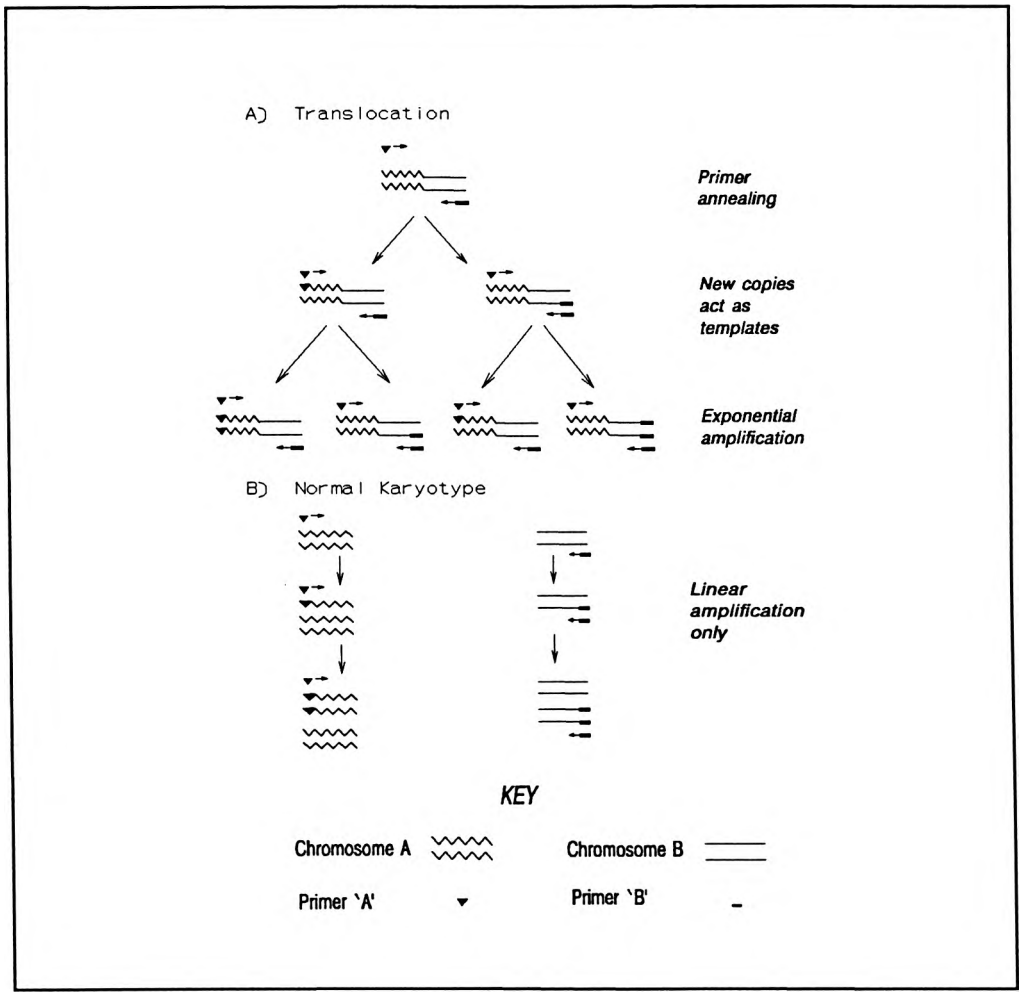

Figure 2. PCR Amplification of Chromosomal Translocations

Unfortunately, the limitation in size of the PCR product noted previously limits the application of this technique to chromosomal translocations such as the t(14;18) in which the breakpoints cluster within a small area. In the majority of translocations this is not the case and the precise breakpoint recombination site would have to be determined for each patient in order to amplify the DNA target sequence - clearly not a feasible proposition. Fortunately, in many cases a tumour specific chimeric RNA is produced which can be used as the target for PCR analysis after reverse transcription and cDNA synthesis (i.e RTPCR).

2.1.2 Examples of PCR amplification of specific chromosomal markers.

Although with improved cytogenetic techniques clonal chromosomal abnormalities are detectable in a large proportion of haematological malignancies (Williams et al 1985, Yunis et al 1984), many of these are random (i.e. identified in only a single patient). In addition, for the majority the precise breakpoints or the nature of resultant abnormal gene products is not known so they are not (yet) amenable to PCR eg. the t(8;21) in Acute Myeloid Leukaemia. However PCR has been applied successfully to the amplification of numerous non-random chromosomal aberrations in haematological malignancies (Table 1).

2.1.2.1 t(14;18).

The majority of cases of follicular non-Hodgkins lymphoma (NHL) as well as 20-30% of cases of diffuse NHL show a translocation between the bcl-2 (B-cell leukaemia/lymphoma 2) protooncogene residing on chromosome 18q21 and the immunoglobulin heavy chain gene locus on chromosome 14 (Weiss et al 1987). The breakpoints on chromosome 18q21 are usually clustered within 450 base pairs of each other in what is known as the major breakpoint cluster region (mbr) or in a minor cluster region (mcr) further downstream. Those on chromosome 14 are close to the 5′ end of one of the J segments of the immunoglobulin heavy chain locus. This consistency allows direct PCR amplification of the hybrid DNA sequence using primers flanking the mbr or mcr on chromosome 18 and a JH consensus primer complementary to the 3′ end of all J segments (Lee et al 1987, Crescenzi et al 1988).

Table 1. **Non Random Chromosomal aberrations amenable to PCR [1]**

CHROMOSOMAL ABERRATION	PUTATIVE ONCOGENE OR PCR TARGET (DNA/mRNA)	TYPE OF MALIGNANCY	INCIDENCE OF ABERRATION
t(14:18)	bcl 2/JH (DNA)	Non-Hodgkin's Lymphoma (B cell)	Follicular >90% Diffuse 10-30%
t(8:14)	c-myc - IgH (DNA)	Burkitt's Lymphoma (B-ALL)	± 90 %
t(1:19)	E2A-PBX1 (mRNA)	Precursor B-ALL	± 5-6 %
t(9:22)	bcr-abl (mRNA)	ALL CML	Children 3-8 % Adult 15-25 % >90 %
tal-1 deletion	tal-1 (DNA)	T-ALL	10-30 %
t(1:14)	tal-1-TCRα/δ (DNA)	T-ALL	1-3 %
t(10;14)	tcl3-TCRα/δ (DNA)	T-ALL	1-3 %
t(11;14)	tcl2-TCRα/δ (?DNA)	Childhood T-ALL	±10 %
t(4;11)	HRX-AF4 (?DNA)	Infant Acute Leukaemia	±75 %
p53 deletion/ mutation	p53 tumour suppressor (DNA)	Includes B-NHL, Burkitt's, CLL	?
ras mutations	N-ras (DNA)	ALL AML	Children 6 % Adult 20 % 20-30 %
t(15:17)	RARα-myl (mRNA)	AML M3	>90 %
t(6:9)	dek-can (mRNA)	AML	<1 %

[1] For references see text.

The bcl-2 oncogene has been shown to encode a protein which blocks apoptosis thus conferring a growth advantage on the lymphoma cells (Korsmeyer, 1992). However, the recent demonstration of cells carrying the t(14;18) in tissue derived from benign reactive hyperplastic lymphoid tissues (Limpens et al, 1990) suggests that the t(14;18) is necessary but not sufficient for the development of follicular lymphoma and that additional mutations are needed to render the lymphoid cells fully malignant. This concurs with the concept of malignant transformation as a multi-step process.

2.1.2.2 t(8;14).

B-cell acute lymphoblastic leukaemia (B-ALL) and Burkitt's lymphoma (characterised by extramedullary disease, FAB 'L3' cellular morphology and monoclonal surface membrane immunoglobulin gene expression) is usually associated with a translocation resulting in the relocation of the myc protooncogene from chromosome 8q24 to a site adjacent to the coding sequences of the Ig heavy chain constant region on chromosome 14 (Dalla Favera, 1982). In the remaining cases there is a translocation involving the kappa (t2;8) or lambda (t8;22) light chain genes (Taub et al, 1984; Hollis et al, 1984).

These translocations result in dysregulation of the c-myc gene possibly through removal of the promoter from the coding sequences of the myc gene or repositioning of Ig gene enhancers close to the myc gene or by causing mutations in the myc gene. Since the myc protooncogene product appears to be involved in regulating the change from a resting to a proliferative state (Alberts et al) it is not surprising that dysregulation should result in malignant transformation.

The t(8;14) is difficult to amplify using PCR because of the variability of the breakpoints but Shiramizu and Magrath (1990) have developed a method involving the use of Southern blot analysis to select a suitable pair of primers from a set of possible primers they have designed.

2.1.2.3 t(1;19).

This is found in nearly 25% of children with pre B (cytoplasmic Mu positive) ALL and ~5-6% of childhood ALL overall making it the most common chromosomal translocation in this condition (Crist et al, 1990).

The translocation results in the formation of a fusion gene comprising 5′ sequences of the E2A gene from chromosome 19 (which codes for some immunoglobulin enhancer binding factors) and 3′ sequences from the PBXI homeobox gene from chromosome 1 (Hunger et al, 1991). It is postulated that this is an oncogene coding for a protein which contributes to leukaemogenesis by alteration of expression of genes normally responsive to the PBXI gene (Kamps et al, 1990). The chimeric RNA transcript appears to be identical in all cases studied and is therefore amenable to and has been amplified by RTPCR (Hunger et al, 1991).

2.1.2.4 t(9;22).

This translocation gives rise to the well known Philadelphia chromosome which is now known to be associated not only with most cases of chronic myeloid leukaemia (CML) but also with ~5% of childhood and 15-25% of adult ALL (Pui et al, 1990). However, Ph+ve ALL and Ph+ve CML differ at a molecular level in that in CML and some adult ALL the translocation results in fusion of the ABL gene from chromosome 9 (which has a tyrosine kinase domain) to either exon II or Exon III of the BCR gene on chromosome 22. This results in production of a chimeric mRNA transcript encoding a 210 Kd protein. In contrast in most cases of childhood ALL and some adult ALL a 185 Kd protein is produced as a result of fusion of the ABL gene to exon I of BCR (Figure 3). Both of these proteins have constituent tyrosine kinase activity and are therefore likely to be involved in dysregulation of cell signalling. It has been suggested that the BCR gene segments may direct the activity of the kinase resulting in the transformation of either committed lymphoid progenitors (as in ALL) or pluripotent haemopoietic progenitors (as in CML) (Lugo et al, 1990).

The breakpoints on chromosome 9 for this translocation span a region of more than 100 kilobases and the translocation is not amenable to direct PCR amplification of the fusion gene DNA. However, despite the variation at the genomic level the fusion gene is consistently transcribed into chimeric bcr-abl mRNA which can be amplified using RPCR. (Kawasaki et al, 1988; Lee et al, 1989). The position of the primers used is shown in Figure 3.

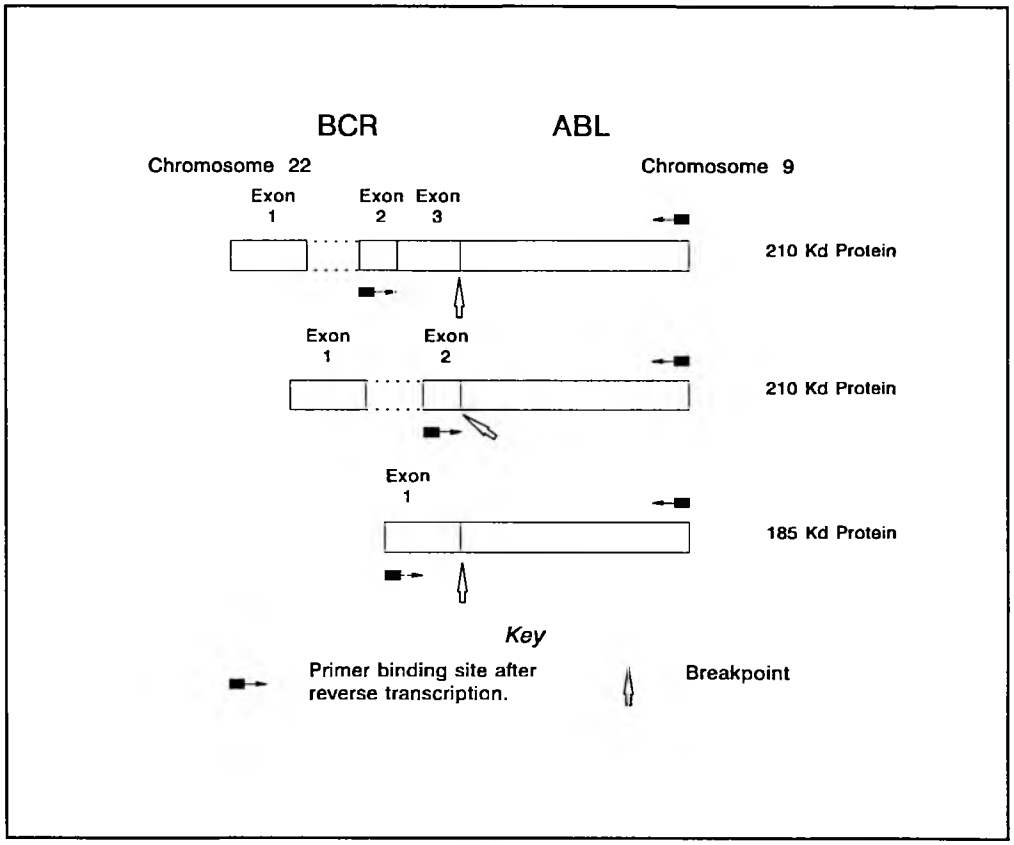

Figure 3. Ph Chimeric mRNA

2.1.2.5 tal-l.

An extremely useful chromosomal marker only recently discovered is a specific 90kb deletion involving the first exon of the tal-1 gene on chromosome 1 (Brown et al, 1990; Jonsson et al, 1991). This occurs in 10-30% of T-ALL. Sequences have been identified within this gene that potentially encode a helix-

loop-helix motif common to a number of transcriptional activators involved in the regulation of cell growth and differentiation (Chen et al, 1990).

The deletion is identical in all cases and is analogous to antigen receptor gene rearrangements (section 2.2) in that it involves junction between conserved heptamer recognition sequences and random insertion or deletion of nucleotides at the resultant junction between fragments. The deletion is not detectable by routine cytogenetic analysis but the specific nature of the deletion makes it amenable to PCR amplification by primers flanking the deleted segment (Brown et al, 1990; Jonsson et al, 1991) The random addition/deletion of bases at the junction also means that the length of the amplified product is patient-specific.

2.1.2.6 t(1-14) t(10;14) t(11;14).

Many of the non-random chromosomal rearrangements in T cell tumours involve the T cell receptor (TCR) ß and γ genes on chromosome 7 or the T cell receptor α and δ genes which are found physically interspersed on chromosome 14. In the t(1,14), present in a small number of cases of T ALL the 3′ end of the tal-1 gene is transposed into the TCR α/δ gene locus. The clustering of breakpoints in this translocation allows direct PCR amplification of the fusion gene product (Chen et al, 1990).

In the t(10;14) it is the putative oncogene tcl3 on chromosome 10 that is juxtaposed to the TCR α/δ gene locus. This translocation has also been amplified directly using DNA PCR (Kagan et al, 1990). The t(11;14) which is found in $\sim$10% of childhood T-ALL (Raimondi et al, 1988) also involves the TCR α/δ locus this time in association with a region on chromosome 11 termed the T-ALL bcr locus containing the putative tcl-2 oncogene. Once again there appears to be clustering of the breakpoints and this translocation may also prove amenable to PCR.

2.1.2.7 t(4;11)

One other non-random chromosomal translocation associated with leukaemia in which breakpoint clustering is present indicating a potential for PCR detection,

is the t(4;11) (q21;q23) (Chen et al, 1991). This is commonly associated with infant acute leukaemias characterised by a high white cell count and splenomegaly which carry a poor prognosis (Pui et al, 1990). The gene at 11q23 (known by various names including ALLI and HRX) has also been shown to be involved in other translocations, e.g. the t(9;11) and the t(11;19). It has recently been characterised as having homology to sequences within the Drosophila trithorax gene which encodes a protein with DNA binding motifs including zinc fingers (Gu et al, 1992; Tkachuk et al, 1992)

2.1.2.8 p53 mutations/deletions.

Alterations in the p53 gene are now known to be associated with numerous malignancies. The gene which resides on chromosome 17, appears to be a suppressor of cell proliferation and inactivation of it by mutations or deletions is associated with neoplastic transformation. A relatively simple and rapid technique called PCR mediated single strand conformational polymorphism analysis (PCR-SSCP analysis) has been used to demonstrate p53 alterations in lymphoid malignancies in particular B cell lymphoma, Burkitt lymphoma and chronic lymphocytic leukaemia (Gaidano et al, 1991; Ichikawa et al, 1992). The principle of this technique is that changes in the nucleotide sequence will cause variation in the mobility of amplified PCR product in a non-denaturing gel. This variation can be used to screen for DNA alterations including point mutations (Orita et al, 1989).

2.1.2.9 ras mutations.

The ras family of protooncogenes encode 'G' proteins which bind and hydrolyse GTP and are thought to be involved in coupling growth factor receptors to effector proteins in the cell (Alberts et al). Mutations in these protooncogenes could therefore easily lead to dysregulation of cell growth and subsequent neoplastic proliferation. Point mutations in N-ras occur in approximately 20% of adult ALL, 6% of childhood ALL (Lubbert et al, 1990) and 20-30% of AML. (Toksoz et al, 1989). Using PCR and sequence specific oligonucleotide probes, low levels of cells with ras mutations can be detected (Lubbert et al, 1990; Farr

et al, 1988). However, changes in ras mutations at relapse compared to presentation, limits their utility in following up leukaemia (Terada et al, 1990).

2.1.2.10 t(15;17).

In contrast to ALL, very few molecular markers have been described for AML and molecular analysis of most AML specific chromosomal aberrations has not yet proceeded far enough to make them amenable to PCR amplification. One notable exception is the t(15;17) in acute promyelocytic leukaemia (APL). This is a reciprocal translocation and results in the production of chimeric genes comprising the myl gene (also designated PML) from chromosome 15 and the Retinoic acid receptor α gene (RAR-α) from chromosome 17 (RAR-α/myl and myl/RAR-α). These are present in 100% of APL cases (Biondi et al, 1992).

The majority of breakpoint sites on chromosome 17 are within the second intron of the RARα gene but the breakpoints on chromosome 15 are variable thus DNA PCR is not practical. However, RTPCR of the chimeric mRNA transcripts has been used to amplify the fusion product for diagnosis and monitoring of the APL clone (Chang et al, 1992; Biondi et al, 1992; Borrow et al, 1992).

2.1.2.11 t(6;9).

The chimeric mRNA transcript of the dek-can fusion gene arising from the t(6;9) which is associated with a specific subgroup of myeloid leukaemia has also been amplified using reverse transcriptase PCR (von Lindern et al, 1990).

The chromosomal abnormalities described above are ideal as tumour specific markers particularly as they are probably related to the oncogenic event and therefore tend to remain constant during the disease course. Unfortunately those currently investigated are only applicable to a minority of haematological malignancies. The potentially far wider applicability of "physiological markers" makes the development of ways to amplify them using PCR of particular interest.

2.2 PHYSIOLOGICAL SEQUENCES AS TARGETS FOR PCR.

The enormous diversity of immunoglobulins (Ig's) and T-cell receptors (TCR's) that make up the human immune repertoire is the result of rearrangements of the Ig and TCR genes which occur during B and T cell ontogeny (Tonegawa, 1983; Leder, 1982). These rearrangements differ for each lymphocyte within a polyclonal population but are identical between members of a clone arising by proliferation of a single precursor cell. They can therefore be of value as markers, not only of cell lineage, but also of clonality when distinction is not possible on morphological, immunological or cytogenetic grounds. Gene rearrangements were originally studied by Southern blotting but the development of techniques to amplify these gene rearrangements using PCR has made analysis of them far simpler so that they are potentially accessible to routine investigation.

2.2.1 Immunoglobulin gene rearrangements.

2.2.1.1 "Hierarchy".

Immunoglobulins are comprised of two heavy chains encoded by the Ig heavy chain (IgH) gene locus on chromosome 14 (Croce et al, 1979) and two light chains which are either kappa (Igκ) encoded by a locus on chromosome 2 or lambda (Igλ) encoded on chromosome 22 (McBride et al, 1982) (Figure 4).

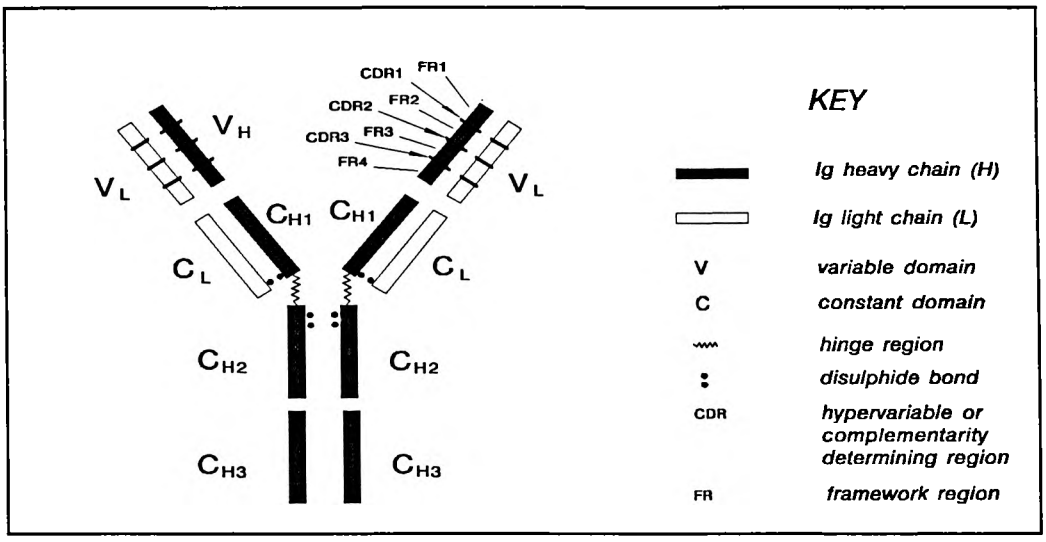

Figure 4. Immunoglobulin Structure

It has been suggested that the various types of lymphoid malignancies are clonal neoplastic counterparts of the normal stages of lymphocyte ontogeny and they have been used to study the hierarchic order of Ig (and TCR) rearrangements (Korsmeyer et al, 1981; Greaves et al, 1986).

These studies suggest that Ig heavy chain genes rearrange first at a very early stage of B cell ontogeny. A successful, rearrangement is reflected by the production of cytoplasmic μ heavy chains which are first seen at the pre-B cell stage. Subsequently rearrangement of the κ light chains occurs. If this results in a functional gene, immunoglobulins comprising Ig heavy chains and κ light chains are expressed at the cell surface. If κ rearrangement is unsuccessful on both alleles the Ig λ genes rearrange (usually accompanied by deletion of at least part of the κ genes) and IgH/λ immunoglobulin is expressed at the cell surface.

2.2.1.2 Incidence.

Approximately 98% of precursor B ALL (i.e. null ALL, common ALL and pre-B ALL) and all mature B cell malignancies have rearranged their IgH genes and in the majority of B-cell malignancies rearrangement or deletion has occurred on both alleles. In contrast only 5-25% of precursor B ALL have rearranged either of the Ig light chain genes (van Dongen and Wolvers-Tettero, 1991). This implies that analysis of IgH gene rearrangements will be the most widely applicable and only these will be discussed in detail here.

2.2.1.3 Germline configuration of the IgH gene.

In its germline form the IgH gene consists of 100-200 V (variable) gene segments grouped into six families on the basis of homology, at least 10 D (Diversity) gene segments and approximately six J (joining) gene segments. (Ravetch et al, 1981). These encode the variable domain of the Ig heavy chain which includes the antigen binding site. They are associated with a number of C (constant) genes (at least one for each Ig H isotype) each of which comprises one exon for each C domain and one or more exons for the hinge region (Flanagan and Rabbitts, 1982) (Figure 5). The constant domains are responsible for effector functions such as complement fixation and FC receptor binding.

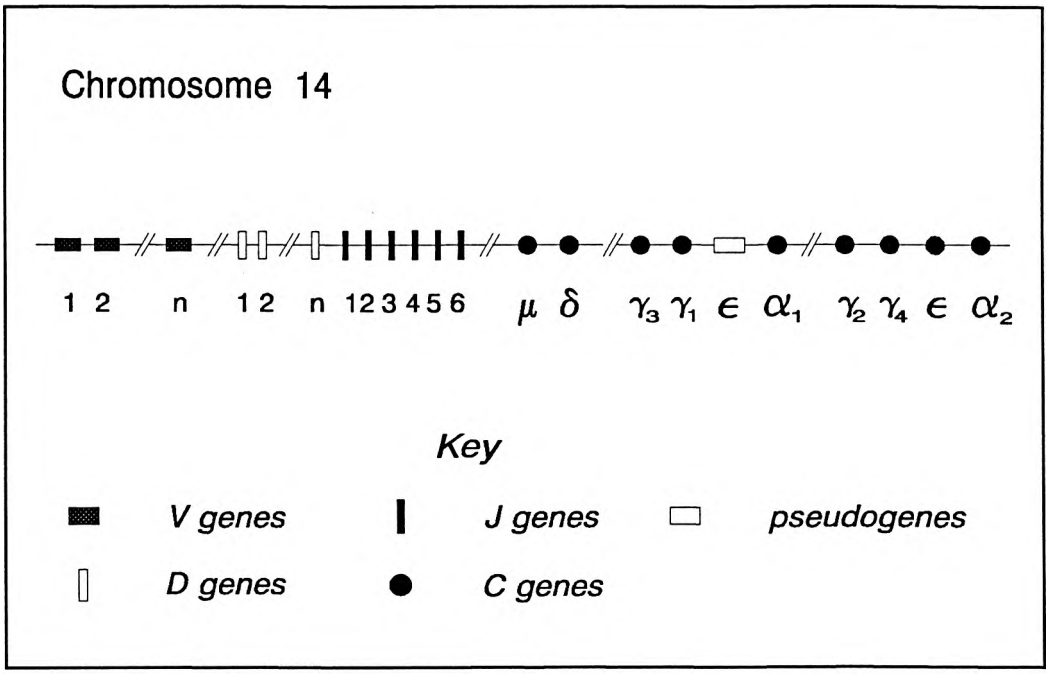

Figure 5. Germline Configuration of the IgH Gene

2.2.1.4 IgH gene rearrangement.

The rearrangement process involves the selection of one of each of the V,D and J segments from the germline cluster. Initially one of the D segments is joined to one of the J segments with deletion of all intervening segments. Subsequently one of the V segments is joined to the D-J fusion product thus formed again with deletion of intervening sequences (Figure 6) (Tonegawa, 1983). The selection of the gene segments used during the rearrangement process may not be entirely random and there appears to be preferential usage of specific gene segments in the immunoglobulin or T cell receptor gene rearrangements of some conditions. (Alt et al, 1987; Yokota et al, 1991; Deane et al, 1991). Alternatively this may merely reflect clonal expansion due to some growth advantage conferred by specific receptor specificities.

Junction between segments depends on the presence of highly conserved palindromic heptamer and nonamer sequences which lie downstream of (3′ to) each V segment and D segment and upstream of (5′ to) each D segment and J segment. These sequences are separated by non-conserved spacer segments of 12 or 23 base pairs (bp) and functional recombination can only occur between

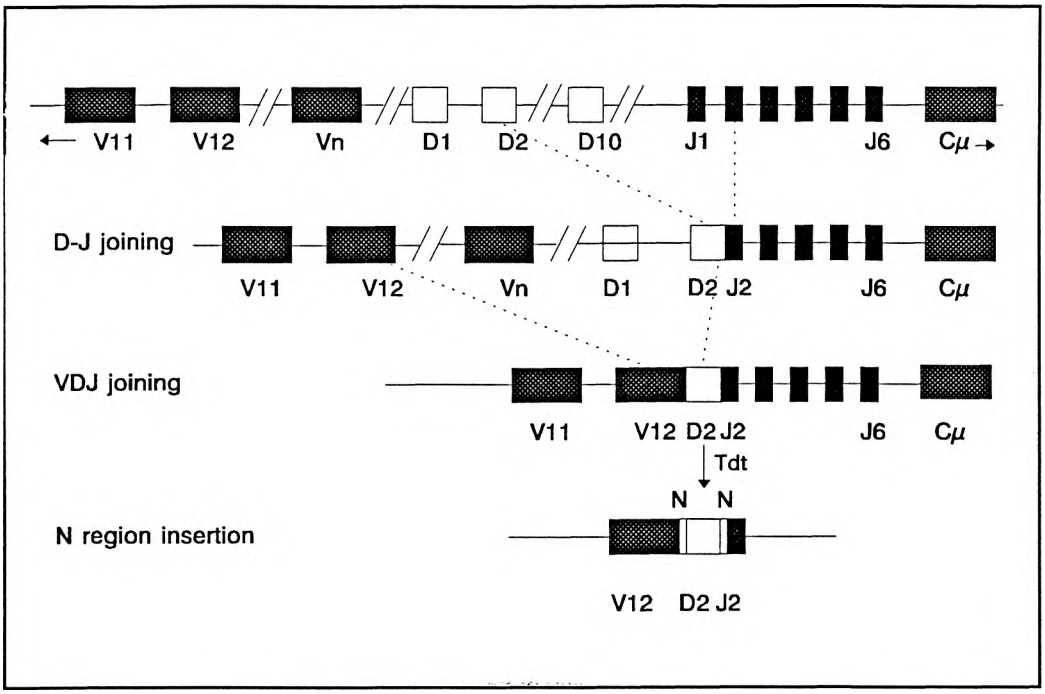

Figure 6. Immunoglobulin Gene Rearrangement

segments if one has the 12 bp spacer and the other a 23 bp spacer. During recombination, fusion of these heptamer and nonamer sequences occurs followed by deletion of the sequences themselves as well as the intervening DNA (Figure 7) (Tonegawa,1983). The recombinase enzyme responsible for this process has not yet been identified but it seems likely that it is common to B and T cells and it has been postulated that it comprises two DNA binding proteins recognising the heptamer - nonamer sequences in conjunction with a 12 or 23 bp spacer respectively (Tonegawa, 1983). Junction between two segments may also arise (rarely) by inversion without deletion of gene segments (Alt et al, 1987).

2.2.1.5 Generation of antibody diversity.

As noted previously the VDJ gene product encodes the variable domain of the immunoglobulin gene which includes the antigen receptor site. Within this domain three hypervariable regions - the complementarity determining regions (CDR's) are recognised. The first two of these regions (CDR 1 and 2) are encoded by the V gene segment while the third (CDR3) is encoded by the junctional region of the V,D, and J segments (Davies and Metzger, 1983). The CDR's are separated by

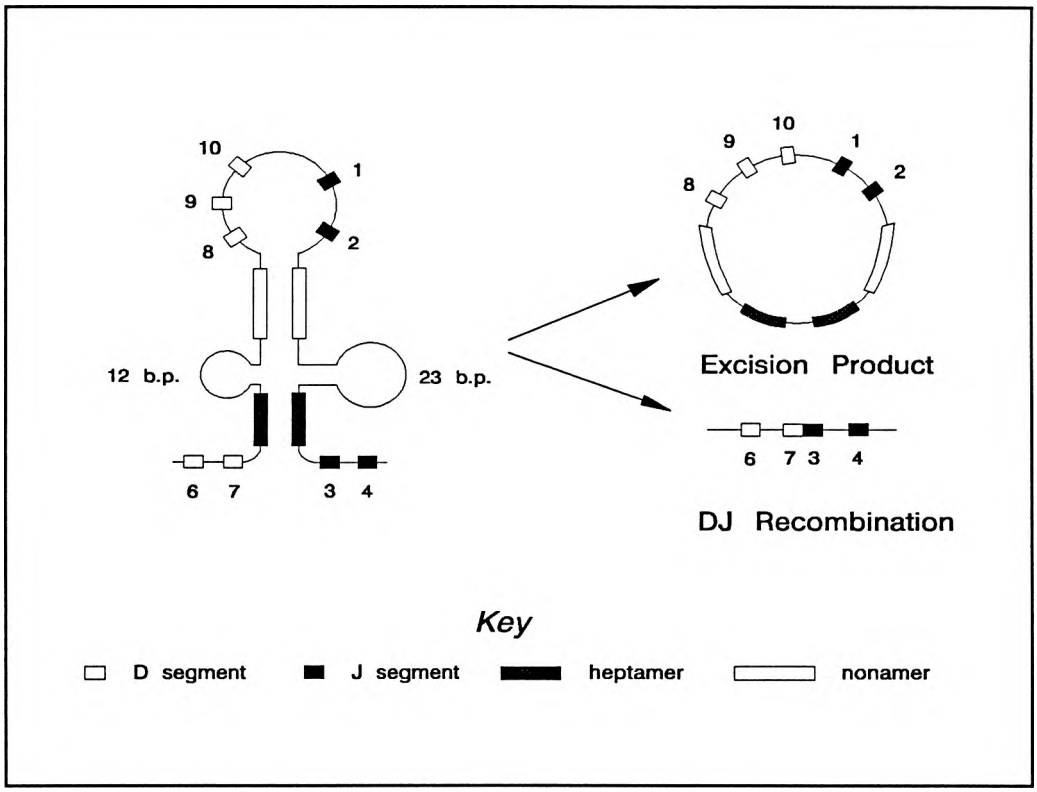

Figure 7. Junction Between Gene Segments

framework regions (FR'S) within the V gene segment in which the sequences of nucleotides is fairly well conserved between segments (Figure 4).

The enormous diversity of antigen specificity of the immunoglobulins is a result of variations in the antigen receptor sites arising due to the large number of potentially participating gene segments in the germline genome (germline diversity), the various possible V.D.J. recombinations (combinatorial diversity) and the presence of junctional diversity. Junctional diversity is the result of imprecise joining of the D-J and V-DJ segments due not only to the loss of some of the nucleotides present in the germline gene segments but also to the random insertion of nucleotides at the junctional region known as N region insertion (Figure 6). This occurs in a non-template dependent fashion mediated by the enzyme terminal deoxynucleotidyl transferase (TdT) which is present in all precursor lymphocytes (both B and T) (Desiderio et al, 1984). The N region can vary in size up to as many as nine nucleotides thus greatly increasing junctional diversity.

2.2.1.6 Secondary gene rearrangements

Because of the existence of the triplet reading frame, many of the immunoglobulin gene rearrangements that occur will result in an out of frame rearrangement prohibiting transcription and translation of the DNA into functional heavy chains. However there appears to be a mechanism for a secondary gene rearrangement to "rescue" the non-productive gene. Two types of secondary rearrangements have been described for IgH genes. Firstly replacement of the entire DJ. segment by combination of an upstream D sequence with a downstream J sequence is possible. This may in fact occur repeatedly on the same allele until all flanking D and J segments have been used (Reth et al, 1986(a)).

The second type of secondary rearrangement is the replacement of the V gene segment involved in the VDJ junction by an upstream V gene, i.e. V to VDJ rearrangement. This is mediated by a heptamer joining sequence found in the 3′ region of most VH genes (Reth et al, 1986(b); Wasserman et al, 1992). This type of secondary rearrangement has also been demonstrated in a previously functionally rearranged gene (Kleinfeld et al, 1986).

2.2.1.7 Somatic hypermutation.

In addition to alterations in the rearranged IgH gene brought about by secondary rearrangements, somatic mutations may arise in the V segment during the immune response (Tonegawa, 1983; Wysocki et al, 1986). These point mutations which usually involve the portions of the V gene encoding the CDR1 and CDR2 are antigen driven and only occur in antigenically stimulated B cells resulting in a greatly improved affinity for the causative antigen. The frequency of mutations at these sites appears to be more than 1000 times greater than that of other genes (Teillaud et al, 1983).

2.2.1.8 Immunoglobulin light chain gene rearrangements.

The germline configurations of the Igκ and Igλ genes are shown in Figure 8 (Hieter et al, 1980, 1981). Rearrangements of the immunoglobulin Kappa and lambda light chain genes occur in a similar fashion to those of the IgH genes with some minor exceptions. The absence of D gene segments (Figure 8) or the limited or absent insertion of N regions (Desiderio et al, 1984) mean that the

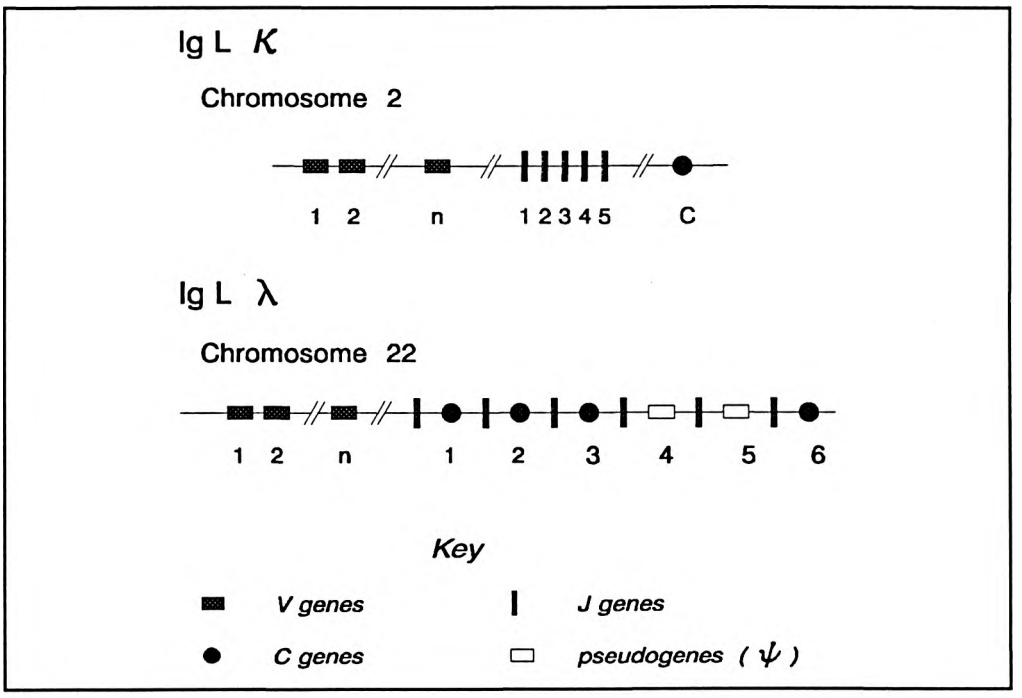

Figure 8. Germline Configuration of the Ig Light Chain Genes

overall diversity of the rearranged genes is somewhat reduced. Secondary rearrangements (involving V-J replacements) and somatic hypermutation have also been demonstrated in the IgL genes (Reth et al, 1986 (a); Tonegawa, 1983; Wysocki et al, 1986).

2.2.2 T cell receptor gene rearrangements.

2.2.2.1 Structure of the T cell receptor.

T cell receptors (TCR's) are the membrane bound antigen receptor molecules of T cells. They are heterodimers comprised of two chains either α and ß as in the great majority of cases (Marrack and Kappler, 1987) or γ and δ in ~10-15% of cases (Brenner et al, 1986). These are associated on the cell surface with a set of constant molecules making up the CD3 complex which appears to be involved with signal transduction (Figure 9) (Alberts et al).

T-cell receptors differ from immunoglobulins in that antigen recognition is major histocompatibility complex (MHC) restricted. This means that the corresponding antigen is only recognised if presented in association with one of the MHC molecules on the surface of another cell either MHC class II for CD4 positive T

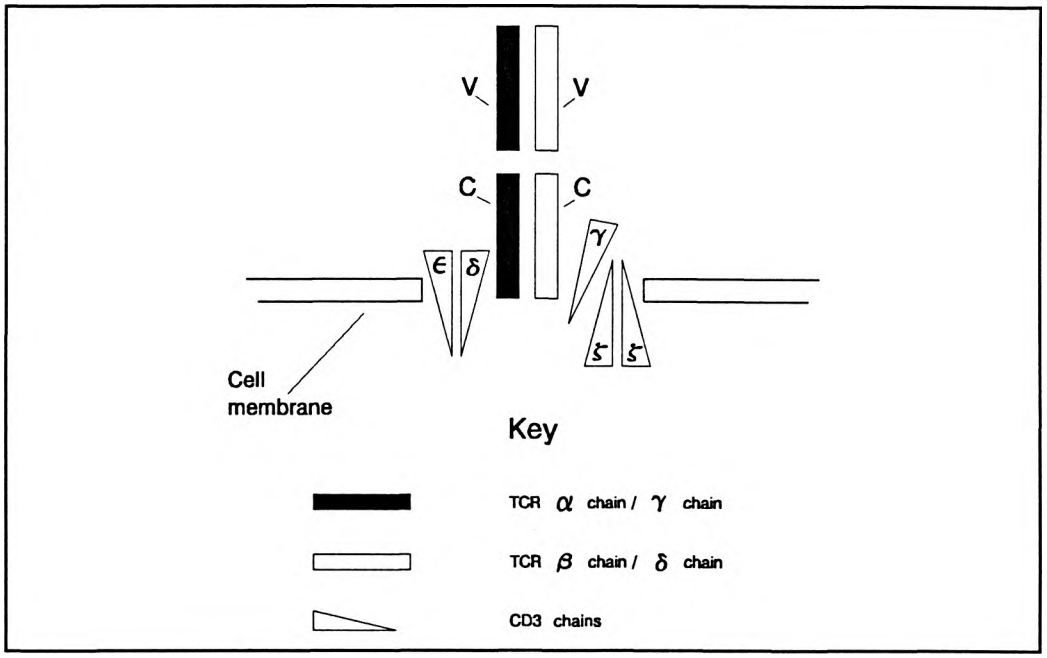

Figure 9. The T Cell Receptor - CD3 Complex

helper/inducer cells or MHC class I for CD8 and positive T suppressor/cytotoxic cells (Swain, 1983).

2.2.2.2 T cell receptor genes.

The genes for the TCR ß and γ chains are on chromosome 7 while those for the α and δ chains are interspersed on chromosome 14 (Rabbitts et al, 1985). Like immunoglobulins, TCR's comprise variable and constant domains. The variable domain is encoded by VD and J segments for the ß and δ chains but only V and J segments for the α and γ chains. The constant domains are encoded by either a single C gene in the case of the α and δ chains or two C genes in the case of the ß and γ chains (Figure 10) (Yoshikai et al, 1985; Toyongaga et al, 1985; Quertermous et al, 1986; Loh et al, 1988).

2.2.2.3 "Hierarchy" and incidence of TCR gene rearrangements

As with the immunoglobulin genes, TCR gene rearrangements within neoplastic clones of T cells have been studied to determine the hierarchic order of the gene rearrangements. These data indicate that the TCR δ genes rearrange first very early during T cell ontogeny as virtually all T cell malignancies have rearranged or deleted TCR δ genes (van Dongen et al, 1989). This is followed by

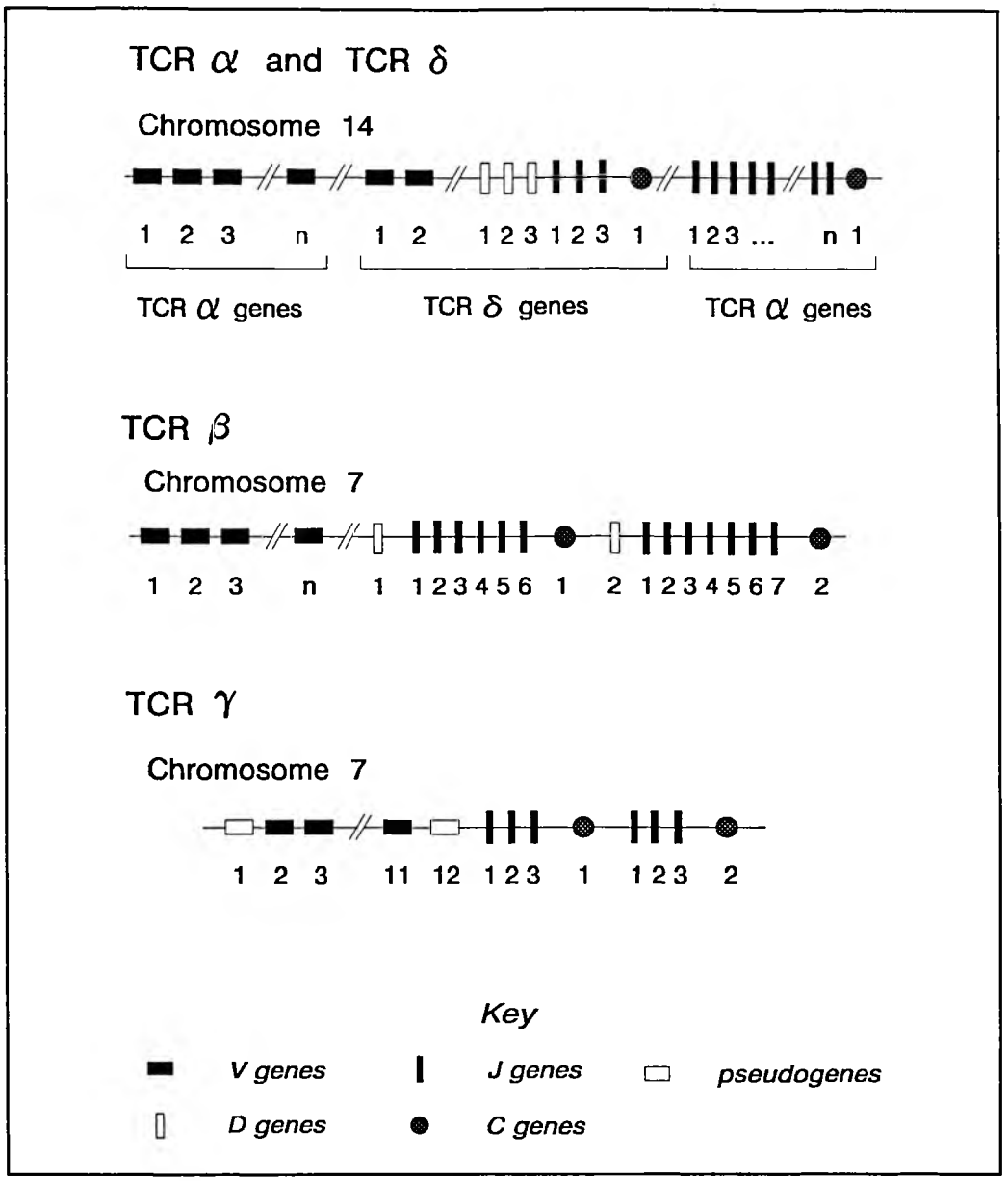

Figure 10. Germline Configuration of the T Cell Receptor Genes

rearrangements of the TCR γ and ß genes. Thus ~85% CD3-ve (immature) T cell malignancies as well as the great majority of CD3+ve (mature TCR expressing) T cell malignancies (both γδ and αß TCR+ve) have rearranged TCR γ and ß genes (van Dongen and Wolvers Tettero, 1991). The TCR α genes are the last to be rearranged and this is usually associated with deletion of the TCR δ genes (van Dongen et al, 1989). As a result, approximately 80% of TCR αß malignancies have deleted both TCR δ genes while the remaining 20% have only one TCR δ allele still present either in the germline or a rearranged configuration.

2.2.2.4 Mechanisms of TCR gene rearrangements.

TCR gene rearrangements occur in a manner analogous to that of the IgH gene except that as noted above, the TCR α and γ genes have no D segments. An interesting variation in TCR δ gene rearrangements is the possibility of using multiple D segments to create not only VDJ but also VDDJ and VDDDJ complexes (Loh et al, 1988). TCR chains also tend to have particularly large N region insertions (Loh et al, 1988) thus although there is limited germline variation of V and J segments there is marked junctional diversity between differently rearranged cells or clones. This has been exploited in the development of a technique to follow minimal residual disease in ALL using PCR (Hansen-Hagge et al, 1989) - (section 2.3.2).

Secondary rearrangements involving VJ replacements have been described with TCR α genes (Marolleau et al, 1988) and V to VDJ rearrangements of TCR ß genes have also been sited as the cause of changes in antigen specificity of T lymphocytes (Epplen et al, 1986). However, somatic hypermutation in TCR gene V segments is not well described (e.g. Ikuta et al, 1985).

2.2.3 Application of PCR to antigen receptor gene rearrangements.

The above mechanisms result in an enormous diversity of rearranged antigen receptor genes - particularly around the V(D) J junctions. This makes them unique to each cell and therefore potentially usable as clonospecific markers for cells arising by proliferation of a single neoplastic precursor. Fortunately the variability does not preclude the use of PCR to amplify the junctional regions.

For IgH genes, this is because the sequences of the V and J segments involved in the recombination process show sufficient homology to allow the design of a pair of "consensus" primers capable of binding to the great majority of V and J genes respectively. The primer binding to the V gene is designed to hybridize to one of the framework regions (usually FR3) of the V gene. That hybridizing to the J gene is directed toward a consensus region at the 3′ end of the gene which shows considerable homology between the six germline J segments. (Brisco

et al, 1990). A pair of such primers will amplify the CDR3 region which is the area of greatest variability.

The greater sequence variability of the component gene segments of the various T cell receptors makes the design of consensus primers far more difficult in this context. However the more limited germline repertoires allow this to be overcome by using "cocktails" of different primers to cover all possible participating gene segments for example to amplify rearranged TCR γ genes (Trainor et al, 1991). Alternatively, primers designed to hybridize to preferentially used gene segments can be used, for example Vδ1 and Jδ1 in T-ALL (Hansen-Hagge et al, 1989) or Vγ1 in ALL (Taylor et al, 1991). Other workers have used a preliminary Southern blot analysis of gene segment utilisation to guide primer selection (Macintyre et al, 1990).

The limitation in size of target sequences which can be successfully amplified means that in the germline configuration the V and J segments are too far apart to support PCR amplification. Successful amplification is therefore indicative of the presence of rearranged genes and by extrapolation, the presence of a lymphoid population.

The size of the PCR product generated by amplification using primers flanking the V(D)J junctions will depend on the exact combination of gene segments utilised and the amount of base pair deletions or "N region insertions" at the junction between segments. Thus the presence of a monoclonal population of lymphoid cells all derived from a single neoplastic precursor and thus sharing a single gene rearrangement could be inferred by the presence of an amplified PCR product comprising fragments of identical length. A polyclonal population would be characterised by fragments of different lengths (Trainor et al, 1990).

PCR amplification of immunoglobulin and T cell receptor gene rearrangements could therefore potentially be used in the determination of lineage as well as for the detection of clonality in a broad spectrum of lymphoproliferative conditions.

However background information about the significance of the results of Ig and TCR gene analysis in these contexts is essential for meaningful interpretation.

2.2.4. Lineage determination - cross lineage rearrangements.

It was originally thought Ig and TCR gene rearrangements were lineage specific. However this does not appear to be the case and cross lineage rearrangements including TCR gene rearrangements in B cell malignancies, Ig gene rearrangements in T cell malignancies and both TCR and Ig gene rearrangements in acute non-lymphoblastic leukaemia are well described (e.g. Williams et al, 1987; Davey et al, 1986; Goorha et al, 1987; Cheng et al, 1986).

2.2.4.1 Incidence.

The frequency of such cross lineage rearrangements depends on the nature of the malignant process and the specific gene rearrangement involved. Table 2 (compiled from the data of Van Dongen and Wolvers-Tettero, 1991) shows the frequency of different gene rearrangements in various conditions. Cross lineage rearrangements appear to be far less frequent in mature B and T cell malignancies than in the precursor ALL's (occurring in only ~0-7%). The incidence of IgH gene rearrangements in T-ALL and acute myeloblastic leukaemias is about the same (~14%) while cross lineage IgK and Igλ rearrangements are extremely rare. Cross lineage TCR gene rearrangements occur fairly frequently in precursor B cell leukaemias and (as with TCR rearrangements in AML's) the hierarchical order and hence relative incidence appears to be in line with that seen in normal lymphoid differentiation. Thus TCRδ rearrangements/deletions are the most common (occurring in ~80% precursor B-ALL) followed by TCR γ(~55%) then TCRß rearrangements (~33%).

The age of the patient also seems to affect the frequency of cross lineage rearrangements with TCR rearrangements being more common in adult precursor B-ALL than childhood precursor B-ALL. (Nuss et al, 1988).

One feature of practical importance is that the specific configuration may differ between "lineage appropriate" and cross lineage rearrangements. For example,

the preferential usage of the Vδ2 rather than the Vδ1 segment in cross lineage TCRδ rearrangement in common (B) ALL necessitates the use of different sets of primers to amplify the rearranged gene in B and T cell acute lymphoblastic leukaemia respectively (Yokota et al, 1991).

2.2.4.2 Mechanism of cross lineage rearrangement

The explanation for the existence of cross lineage rearrangements has caused

Table 2. Frequency of Ig and TCR Gene Rearrangements (R) and Deletions (D) [a]

Gene		Disease- Percentage frequency				
		Precursor B ALL	Mature B cell Malignancy	T ALL	Mature T cell Malignancy	AML
IgH	(R)	98	100	14	0 - 7	14
Igκ	(R/D)	45	95 - 100 [c]	0 [d]	0 [d]	2
Igλ	(R)	20	0 / 100 [b]	0	0	0
TCR δ	(R)	54	0	68	0	9
TCR δ	(D)	26	0	28	100	9
TCR γ	(R)	55	0 - 6	91	100	5
TCR β	(R)	33	0 - 7	89	93 - 100	7

Notes
a. Data compiled from van Dongen + Wolvers-Tettero 1991.
b. Ig λ genes are rearranged in all mature B cell malignancies expressing surface membrane immunoglobulin (Smig) with λ light chains but none expressing Smig with κ light chains.
c. Ig κ genes are rearranged in all mature B cell malignancies expressing Smig with κ light chains and rearranged or deleted in those expressing Smig with λ light chains.
d. Rearranged κ genes have been found in two cases of peripheral T NHL and 2 T cell lymphoblastic leukaemia/lymphoma (Sheibani et al. 1987).
e. Due to the complexity of the TCR α gene, data on TCR α gene rearrangements is limited and these have not been included.

some debate. McCulloch (1982) postulates that co-expression of markers such as gene rearrangements normally found in cells belonging to different lineages, arises due to misprogramming of the differentiation of leukaemic cells - so called

"Lineage Infidelity". However, the mechanism by which multipotential stem cells adopt a particular lineage is as yet not clearly understood. Greaves et al (1986) therefore postulate that co-expression of certain lineage associated genes coding for proteins which may participate in lineage commitment (such as TdT or growth factor receptors) may occur during a transient phase in the maturation of normal multipotential progenitors, so called **"Lineage Promiscuity"**.

Commitment to a particular lineage may then be the result of interaction between environmental factors and the products of such gene expression. Once irreversible lineage commitment occurred, down or up regulation of expression of the appropriate genes would give rise to a "lineage appropriate" phenotype. However, if a leukaemia arose in the pre-commitment stage the mixed lineage phenotype would be preserved within the blast population. In terms of this model, Greaves suggests that cross lineage gene rearrangements can be attributed to events occurring during a period of lineage promiscuity which is part of normal differentiation. However, once present gene rearrangements would of necessity persist within the cell throughout maturation thus cross lineage gene rearrangements could be expected within the majority of cells (both normal and leukaemic) if this explanation were entirely correct.

Since the process of Ig and TCR gene rearrangement is probably mediated by the same recombinase enzyme it is not surprising that both types of genes could be rearranged in the same cell. The presence or absence of such gene rearrangements may be determined by the actual chromatin configuration around the gene which in turn regulates access of the recombinase enzyme (van Dongen and Wolvers-Tettero, 1991). The increased incidence of cross lineage rearrangements in precursor B and T cell malignancies compared with mature lymphoid malignancies is an interesting finding. Chen et al (1987) suggest that the presence of cross lineage rearrangements may somehow limit the capacity of the cell to differentiate and proliferate making them a rare finding in mature cells.

2.2.4.3 Implications.

However they arise, the presence of cross lineage gene rearrangements precludes the use of analysis of gene rearrangements in isolation to determine lineage for example in cases of acute undifferentiated leukaemia with ambiguous phenotype. However in many cases such information is useful for lineage assignment particularly when used in association with morphology and immunophenotyping. Moreover, cross lineage gene rearrangements are not usually expressed and it has been suggested that detection of mRNA transcripts of rearranged genes may be a more reliable marker of lineage (Potter et al, 1992). One advantage of such gene rearrangements is that they may broaden the scope of various sets of primers used to detect monoclonality by amplification of gene rearrangements. For example amplification of rearranged TCRδ genes will be of relevance in the great majority of both B and T lineage ALL. The absence of all antigen receptor gene rearrangements can itself be useful in lineage determination for example to confirm myeloid lineage (Meyers et al, 1986).

2.2.5 Detection of clonality.

The potential to detect clonality by PCR amplification of antigen receptor gene rearrangements has several diagnostic applications:-

2.2.5.1 Differentiation between reactive and malignant states

Malignancy is the result of clonal proliferation of a single neoplastic precursor. Thus the demonstration of clonality is an important marker of malignancy. Analysis of gene rearrangements is particularly useful in this regard in cases where malignancy cannot be diagnosed unequivocally on morphological grounds and where immunological markers of clonality are unhelpful, e.g. in B cell tumours without IgL expression where the κ/λ ratio cannot be used, or mature TdT-ve T cell malignancies. (TdT+ve T cells normally only occur in the thymus. Thus the presence of TdT+ve T cells in peripheral blood, bone marrow or lymph node biopsy material is a sensitive indicator of an immature (TdT+ve) T cell malignancy) (van Dongen and Wolvers-Tettero, 1991).

However, it is important to note that clonal proliferations can be found in the absence of an overt malignancy. For example clonal B cell proliferations are found in association with the "monoclonal gammopathies of uncertain significance". These occur fairly commonly in patients with immunodeficiencies, autoimmune disease and haematological malignancies but also in uncompromised patients in whom the incidence increases with age. Many are transient and appear to be associated with impaired T cell function but long term follow up shows that 10-30% progress to overt malignancies. (Kyle and Lust, 1989).

The lymphoproliferations associated with Ebstein Barr Virus (EBV) in immunocompromised individuals have also been shown to have clonal IgH rearrangements (Zutter et al, 1988) but at least in transplant patients, they may regress on withdrawal of the immunosuppression indicating they are not yet overtly malignant. (Starzl et al, 1984).

Clonal rearrangement of IgH genes, (with and without evidence of EBV infection) has also been found in the systemic form of Castleman's disease. This is a rare condition characterised by generalised adenopathy hepatosplenomegaly and severe systemic symptoms which is known to be associated with an increased risk of neoplasia but has not as yet been recognised as a malignancy in its own right. (Hanson et al, 1988).

Analysis of TCR ß gene rearrangements has been used to demonstrate clonality in some of the clinically benign cutaneous T cell lymphoproliferations such as lymphoid papulosis (Kadin et al, 1987). These may represent one end of a spectrum of T cell cutaneous lymphoproliferations which have the potential to evolve into overt lymphoma such as mycosis fungoides

Clonal T cell proliferations have also been demonstrated by TCRß gene rearrangement analysis in some of the autoimmune diseases such as Rheumatoid Arthritis (Miltenburg et al, 1990). Unlike many of the other benign clonal lymphoproliferations mentioned above these conditions are not known to be associated with progression to malignant lymphoma.

2.2.5.2 Detection of multiple clones.

Analysis of the immunoglobulin genes sometimes reveals the presence of multiple rearrangements the pathogenesis of which differs in different diseases.

The lymphomas occurring as a result of Epstein Barr infection in immunocompromised patients have been shown to represent **multiple tumours** with unrelated Ig gene rearrangement patterns (Cleary and Sklar, 1984). This is in keeping with the theory that they arise as a result of a multi-step process in which stimulation by the infecting virus causes polyclonal proliferation of B cells. In the absence of adequate T cell regulation this can result in the emergence of clones of B cells some of which may undergo malignant transformation (for example by a translocation involving the c-myc gene) leading to the development of multiple lymphomas. (List et al, 1987).

In follicular lymphomas, multiple rearranged IgH genes probably reflect the emergence of subclones as a result of the **somatic hypermutation** discussed previously (section 2.2.1.7) (Cleary et al, 1986). The fact that follicular lymphomas are considered to arise from germinal centre cells (de Jong et al, 1989) and that the process of somatic hypermutation is associated with antigenic stimulation and the generation of memory B cells which most probably occurs in the germinal centres would support this idea.

In precursor B cell acute lymphoblastic leukaemias, multiple IgH gene rearrangements (from three to six or more) are more common than in other B cell tumours, occurring in between 15-45% of cases (Kitchingman et al 1986; Bird et al, 1988; Katz et al, 1989; Beishuizen et al, 1991(a)). The pathogenesis here appears to be somewhat different: Glucose-6-phosphate dehydrogenase enzyme analysis indicates that at least the great majority of ALL's are monoclonal arising from a single parent cell (Dow et al, 1985). The multiple rearrangements are therefore probably not indicative of multiple tumours.

Similarly, somatic hypermutation does not appear to be involved as when assessed by Southern blotting, most multiple rearranged bands are detectable in all restriction enzyme digests indicating the alteration of all restriction sites - an unlikely occurrence due to somatic mutations alone (Cleary et al, 1986; de Jong et al, 1989). The absence of significant somatic hypermutation has subsequently been confirmed by cloning and sequencing the CDR3 regions in cases of ALL with multiple rearrangements (Bird et al, 1988; Wasserman et al, 1992).

Because of the relatively high incidence of chromosomal abnormalities in ALL particularly involving the Immunoglobulin genes (Pui et al, 1990) it is necessary to exclude duplications of, or translocations involving, chromosome 14 as a possible cause of multiple gene rearrangements. However, most studies have shown that although this may be contributory, it cannot explain the majority of cases. (Beishuizen et al, 1991(a); Bird et al, 1988; Kitchingman et al, 1986). Also in order to give rise to additional gene rearrangements, these cytogenetic events would have to occur prior to gene rearrangement.

It appears therefore that the presence of multiple rearrangements in ALL reflects subclone formation during **clonal evolution.** Bird et al (1988) postulate that this is due to **ongoing gene rearrangements** continuing for at least some interval after transformation in tumours arising from B cell precursors prior to the rearrangement process. In support of this, they demonstrated different VDJ and DJ sequences in multiple rearranged bands sequenced from 2 patients.

In a larger series of cases Wasserman et al (1992) demonstrated VH alteration but **conserved DJH** joining in sequenced CD3 regions from patients with multiple gene rearrangements at diagnosis and in patients with altered patterns of gene rearrangement between diagnosis and relapse. This would support the idea that **secondary gene rearrangements** with VH-VH replacements (section 2.2.1.6) or alternatively "primary" VH to DJH joining events may also be responsible for clonal evolution. It was also noted in this study that the likelihood of detecting clonal evolution appears to increase with time.

The persistence of new clones arising as a result of ongoing or secondary gene rearrangements would depend on their conferring some growth advantage or arising in association with further mutations which confer a growth advantage. This could explain why multiple rearrangements and clonal evolution are not seen in all cases and would tend to increase as a function of time. Although alteration of TCRß gene rearrangements have been described between presentation and relapse specimens (Raghavachar et al, 1987), the frequency of clonal evolution of TCR gene rearrangements appears to be lower.

There is some debate in the literature as to the clinical significance of finding multiple rearranged genes in ALL. Kitchingman et al (1986) suggested that it may be associated with resistance to therapy and a greater tendency to relapse while Katz et al (1989) found no evidence that it conferred a poor prognosis. In a more recent study Beishuizen et al (1991) (a) could not demonstrate a statistically significant increase in relapse rate but did show a correlation between multiple rearrangements and other bad prognostic indicators such as a high white cell count.

The occurrence of multiple rearrangements as a result of clonal evolution does however carry significant diagnostic implications. The incidence appears to be fairly high (Beishuizen et al, 1991(b)) and subclone formation with regression of the original clone may result in false negative results during follow up of precursor B-ALL in some situations, e.g. when follow up involves PCR mediated amplification of tumour specific junctional (CD3) regions or use of junction specific probes (see below). Wasserman et al (1992) have suggested that the DJ joining sequences may be more stable than the VD joining region and thus provide a better target for follow up. Alternatively, since the frequency of clonal evolution of TCR gene rearrangements appears to be lower than that of IgH genes, and the cross lineage TCRγ and TCRδ rearrangements found in a large proportion of B ALL have not been shown to undergo clonal evolution (van Dongen and Wolvers-Tettero, 1991) it has been suggested that PCR amplification of TCR junctional regions be used (Beishuizen et al, 1991(b)).

2.2.5.3 Assessment of the relationship between malignant clones.

Distinguishing between the development of a second malignancy and relapse or progression of pre-existing disease can be relevant to treatment decisions. This can be difficult on morphological grounds as phenotypic changes may occur in a tumour population during the course of the disease (de Jong et al, 1989). However, the demonstration of identical or partly identical gene rearrangements can be used to confirm the clonal relationship between tumour populations. Clonal evolution of Ig gene rearrangements although common, often only involves one IgH allele (Raghavachar et al, 1987) and is unlikely to cause a completely unrecognisable gene rearrangement pattern although this possibility must be borne in mind.

Some interesting work has been done in this context comparing patterns of gene rearrangement in Richter's syndrome. This is a condition characterised by the development of a high grade large cell non-Hodgkins lymphoma (NHL) in a patient with CLL. Van Dongen and Wolvers-Tettero (1991) showed that the same clinical picture could arise either as a result of disease progression (as in one case which showed identical gene rearrangement patterns between the CLL and NHL cells), or as a result of the development of a second malignancy (as in two cases which showed different gene rearrangement patterns)

2.2.5.4 Sensitive detection of small numbers of malignant cells.

The ability to detect the presence of small numbers of clonal / malignant cells has made PCR amplification of antigen receptor gene rearrangements a powerful tool in the follow up of haematological malignancies and the detection of "minimal residual disease".

2.3 MINIMAL RESIDUAL DISEASE IN HAEMATOLOGICAL MALIGNANCIES

Despite the improvement in prognosis associated with new approaches to therapy of haematological malignancies, relapse remains a problem even in patients in whom routine methods of analysis such as light microscopy reveal no evidence

of persistent disease. Presumably this arises from residual malignant cells present in small numbers undetectable by such methods - so called minimal residual disease (MRD). Much work has therefore been done to improve methods of detection of such cells.

Sensitive detection of MRD may well prove to be of value not only in predicting recurrences or allowing the early detection of relapse but also in assessing response to therapy and determining the duration of therapy. It may also allow more detailed staging at diagnosis and ultimately lead to the individualization of treatment protocols. In the field of bone marrow transplantation the ability to detect minimal residual disease would be valuable in assessing the quality of marrow used for autologous transplants and the efficacy of marrow purging as well as in the follow up of patients after marrow transplant.

2.3.1 Techniques for the detection of MRD.

Various methods have been used to look for residual malignant cells. **Marrow morphology**, one of the simplest techniques, is limited to a sensitivity of around 5% particularly in acute leukaemias as blasts may be present as part of normal haemopoiesis. Likewise the potential to track minimal residual disease cells by conventional **cytogenetic analysis** is limited by the need for large chromosomal aberrations for visibility and the laborious procedure involved. This includes in vitro culture of the malignant cells as non-dividing cells cannot be analysed. The relatively recently developed technique of fluorescence in situ hybridisation (FISH) involves the use of biotin labelled single stranded DNA probes for specific chromosomes or loci. These are hybridized to the denatured chromosomal DNA and then visualised with fluorescent-labelled avidin. This allows analysis of non-dividing cells and may well prove of great value in follow up of MRD in malignancies with chromosomal aberrations (Gray et al, 1991).

In vitro culture of leukaemic cells has also been used to detect occult clonogenic cells. Although this has the advantage of allowing assessment of growth requirements and drug sensitivities, the difficulties associated with culture limit the applicability of this technique (Lowenberg and Touw, 1991)

The potential for detection of MRD in acute leukaemia by **immunological marker analysis** using fluorochrome-conjugated antibodies has been studied by Campana et al (1990). Since few immunological markers are really leukaemia specific the pattern of antigen expression using dual marker analysis is assessed (for example co-expression of nuclear TdT and CD1,CD3 or CD5 in T ALL). It appears that MRD is detectable at levels between 0,02-5% of mononuclear cells but there is a high false negative rate and the sensitivity is also operator dependent. **Southern blotting** to detect MRD at a molecular genetic level has also been used but again the detection limit of between 1-3% of malignant cells (Katz et al, 1989) is probably far above that at which residual cells can cause relapse.

In contrast to the above methods, PCR provides a potentially extremely powerful tool to detect minimal residual disease depending on the genotype of the malignant cells. PCR amplification of chromosomal aberrations such as the t(14;18) in follicular lymphomas or the t(9;22) in CML has been shown to be able to detect frequencies of leukaemic cells as low as 1 in 100,000 (Lee et al, 1987; 1988). Unfortunately, as noted previously, the majority of tumours do not have an appropriate "pathological" target sequence and physiological targets such as gene rearrangements must be used. The presence of background normal cells limits the sensitivity of PCR detection of leukaemic cells in these contexts to at best 1 in 100 (Trainor et al, 1990; Potter et al, 1992). This is adequate for determining clonality but not for detecting MRD. In order to overcome this limitation numerous methods for increasing the sensitivity of detection of clonal gene rearrangements have been developed.

2.3.2 Methods to increase sensitivity of detections of clonal rearrangements.

Many of these techniques involve sequencing of the original PCR product from the presentation marrow either directly or by cloning. This information is then used to synthesize a clone-specific probe complementary to the areas of maximum variability. This may be the VDJ junctional region within the CDR 3 sequence of rearranged IgH genes (Yamada et al, 1990) or the V-J junctional region of

rearranged TCR γ genes (Macintyre et al, 1990). After radio labelling, such probes have been shown to detect the presence of identical sequences in subsequent "follow up" marrow samples (after PCR amplification using the original consensus primers) to a sensitivity of 1 in 10,000 to 1 in 100, 000. Similar sensitivities have been achieved using clone specific probes for TCRδ and TRCß gene rearrangements (Neale et al, 1991).

An alternate strategy is to use the information provided by sequencing of amplified junctional regions to generate a pair of leukaemia/tumour specific amplimers which are used as primers to amplify any identical sequences present in subsequent marrows. Sensitivities between 1 in 1000 (Brisco MJ et al 1990) and 1 in 100,000 (Jonsson et al, 1990; Billadeau et al, 1991) have been achieved in this way.

Since sequencing of PCR products for each patient is unlikely to be practical on a routine basis, methods which avoid this are of particular interest. Hansen-Hagge et al (1989) have developed a method for amplifying a clone specific fragment from rearranged TCRδ gene junctional regions. This can then be eluted, labelled and used directly as an individual specific probe for screening of "follow up" marrow samples for minimal residual disease. Nizet et al (1991) describe a similar method applicable to IgH gene rearrangements. Here the PCR product derived from amplification of the rearranged IgH genes in the presentation specimen is modified by length reduction (by restriction digestion) and labelling and then used as a direct clone specific probe for follow up of MRD in the same patient. The sensitivity of these techniques is comparable to those involving sequencing.

Veelken et al (1991) have used a completely different approach involving a PCR mediated ribonuclease protection assay to detect clonal TCR γ gene rearrangements to a similar level of sensitivity. In this case one of the PCR primers has an attached RNA polymerase promoter facilitating transcription of the PCR product. The resulting RNA is labelled and mixed with the PCR product from subsequent follow up marrow in conditions allowing hybridisation. After

RNAse digestion, any RNA which has hybridised to complementary DNA strands in the amplified product will persist, indicating the presence of residual disease.

A less technically complex method which can still increase the sensitivity of detection of clonal rearrangements to the order of 1 in 1000 has been developed by Deane and Norton (1990) who advocate the use of a panel of family specific VH region primers in place of a single consensus V region primer to "fingerprint" the spectrum of clonal B cells present in lymphoproliferative disorders.

2.3.3 Significance of detection of minimal residual disease

The potential for such highly sensitive detection of small numbers of malignant cells raises numerous questions about their significance. In the context of acute lymphoblastic leukaemias the persistence of MRD in patients thought to be in morphological remission is well described. Detailed studies suggest that persistence until late in treatment (15-20 months post-diagnosis for B-ALL but possibly less for T-ALL) is common even in patients remaining in remission (Yamada et al, 1990; Nizet et al, 1991; Neale et al, 1991; Potter et al, 1993) However, patients who remain in complete remission do not have detectable MRD at the end of therapy, while persistence of MRD at this stage appears to correlate with a subsequent relapse although this may be considerably delayed (Potter et al, 1993; Nizet et al 1991). A rising level of detectable MRD during treatment may also be predictive of impending relapse (Yamada et al, 1990; Nizet et al 1991; Neale et al 1991) thus it is the pattern of evolution of MRD rather than just its presence that is important. Although these data suggest that serial monitoring of patients for MRD using PCR is a useful predictor of relapse, it should be borne in mind that at present there is no strong evidence that treating leukaemic relapse at a very early stage improves the clinical outcome (Fey et al, 1991). It has also not been determined if there is a level below which residual cells can be dealt with by the immune system, or lost by stochastic attrition.

In the context of bone marrow transplantation (BMT) a similar picture is emerging. PCR detection of residual bcr-abl mRNA transcripts after BMT in patients with CML is not uncommon within the first six months even in patients

who achieve long lasting remission. However, PCR positivity persisting longer after BMT may be indicative of an increased risk of relapse (Hughes et al, 1991). In contrast, Delage et al (1991) describe patients showing persistent PCR positivity who are in continuing clinical and haematological remission. Although this may reflect the presence of long living memory lymphocytes which have no proliferative potential, it makes the interpretation of PCR positivity in this context difficult.

PCR detection of bcr-abl mRNA has also been used to follow up patients post-transplantation for Ph +ve ALL (Gehly et al, 1991). Here persistently negative PCR findings post-transplant did appear to correlate with long lasting remission. In contrast to the situation in CML, the t(9;22) translocation is restricted to the leukaemic blasts in Ph+ve ALL and the finding of PCR positivity may be more significant in this context.

As mentioned earlier, PCR detection of MRD has a potential role in assessing the quality of marrow used for autologous transplants and the findings appear to be clinically significant. For example, Gribben et al (1991) present strong evidence that the absence of residual cells showing the t(14;18) following immunologic purging of autologous marrow is associated with a significant increase in disease free survival and a reduced relapse rate. In a different setting, the significance of finding persistent cells carrying the t(14;18) has been questioned. Price et al (1991) found that cells with this translocation frequently persist in the peripheral blood of patients in long term remission of advanced follicular lymphoma. This again demonstrates the fact that the significance of PCR positivity may be determined by the context.

2.4 CHOICE OF PCR TARGET IN HAEMATOLOGICAL MALIGNANCIES

The choice of target sequence for PCR will ultimately be determined by factors such as the presence or absence of chromosomal aberrations and Ig and/or TCR gene rearrangements, the possibility of clonal evolution and the level of sensitivity required. Amplification of antigen receptor gene rearrangements (without

addition of the techniques mentioned above) is not specific to the malignant cells and therefore does not make use of the potential power of PCR for sensitive detection of a neoplastic population. Thus if a pathological sequence, such as a chromosomal translocation for which the breakpoints are known, is present this would be the target of choice, especially if the intention is to use PCR to detect MRD. Failing this, amplification of rearranged Ig or TCR genes would be indicated in the case of lymphoid malignancies.

The high incidence of cross lineage TCR gene rearrangements in B lineage leukaemias makes these seem a particularly attractive target as they would be widely applicable and could be used for screening purposes in the diagnosis of lymphoid neoplasia in general. However, the lack of homology between gene segments precludes the use of consensus primers, except possibly for the Vγ1 family genes commonly used in the TCRγ gene rearrangements in ALL (Taylor et al, 1991). This necessitates the use of "cocktails" of primers for all possible gene segments (Trainor et al, 1991) or the use of various means to select appropriate primers (Macintyre et al, 1990). In addition, TCRδ gene deletion is a common event (see Table 2), precluding study of significant numbers of T and B-ALL by amplification of this gene.

Although clonal evolution of TCR $\gamma\delta$ alleles has been observed (Macintrye et al, 1990), the incidence appears to be somewhat lower than that observed for IgH gene (van Dongen et al, 1991). They may therefore represent a more stable target. However, the reduced combinatorial diversity resulting from the more limited germline repertoire of gene segments means that the PCR amplification products of both TCR γ and δ rearrangements are less varied in size, making detection of the presence of a clone within a population of background lymphocytes more difficult (Trainor et al, 1990).

2.5 AIMS OF THIS STUDY

The aim of this study, is to assess the role of PCR in the routine haematology laboratory. To investigate the practical aspects of using PCR, I have chosen to set up the technique of amplification of immunoglobulin gene rearrangements

because of the potentially wide application afforded by a single set of primers. Obviously a far wider selection of primers chosen with regard to factors mentioned above will be necessary for a comprehensive diagnostic service. The second part of the study involves analysis of the costs incurred, and the potential demand for such investigations, as these will be important in deciding if routine use of PCR is a realistic proposition.

3. MATERIALS AND METHODS

The first part of this study (3.1) concerns establishing the technique of PCR detection of immunoglobulin heavy chain gene rearrangements and determining the sensitivity of detection. The second part (3.2) concerns determining the feasibility of routine use of PCR in the diagnostic haematology laboratory in terms of the costs involved and the potential demand for such investigations.

3.1 ESTABLISHING THE TECHNIQUE

3.1.1 Samples

Specimens were obtained from a "DNA Bank" stored at the Johannesburg Hospital Haematology Laboratory of the South African Institute for Medical Research. The "Bank" consists of samples of peripheral blood and bone marrow from patients with various haematological malignancies, as well as patients with non-malignant haematological disorders and normal individuals. Sixty-five samples were analysed; 40 from patients with malignancies of B-lineage cells at presentation; 15 from patients with non-B-lineage cell haematological malignancies at diagnosis; and 10 from normal individuals.

3.1.2 DNA extraction

For the purposes of establishing the technique, DNA was extracted from the above samples by the Jeffreys' Extraction Method of phenol chloroform extraction and ethanol precipitation (Jeffreys, 1985). The sample to be extracted was thawed and transferred to a 15 ml polypropylene tube. Triton-saline solution (0.2% (v/v) Triton X-100; 0.9% (w/v) NaCl) was added to fill the tube, and the sample mixed by shaking prior to centrifuging at 2000 x g for 15 minutes. The supernatant was discarded and the pellet resuspended in 5 ml lysis buffer (0.3 M Na Acetate; 10 mMol Tris HCl; 1 mM EDTA pH 7.5). Two-hundred and fifty μl 10 % (w/v) sodium dodecyl sulphate (SDS) was then added to lyse the cells and after shaking 5 ml of phenol/chloroform/isopropyl alcohol/8-hydroxy-quinolone (50:50:1:0,1) was added. This was shaken vigorously for 5 minutes to extract the lysate prior to separation of the phases by centrifugation at 2000 x g for 10 minutes.

The aqueous phase was transferred to a clean tube with a wide bore pipette and a second phenol.chloroform extraction performed as above. The aqueous phase was subsequently transferred to a clean tube and extracted a third time with an equal volume of chloroform/isopropyl alcohol (24:1). This was again centrifuged at 2000 x g for 10 minutes and the supernatant (aqueous phase) transferred to a clean tube in which the DNA was precipitated by addition of 2 volumes of cold absolute ethanol (-20°C) and gentle inversion.

The DNA was recovered by "spooling" on to the tip of a Pasteur pipette which was then rinsed in 70% ethanol and air dried for approximately 30 minutes. The pipette tip was then broken off into a 1.5 ml Eppendorf tube and the adherent DNA dissolved in 200 μl of TE buffer (1mM EDTA; 10 mM Tris/HCl pH 7.5) overnight. The DNA concentration and purity was determined by spectrophotometry (section 3.1.3) and the samples stored at -20°C until required.

In addition, two shorter methods of sample preparation for PCR were assessed by comparison of the results obtained after PCR amplification with those obtained from the same samples after conventional DNA extraction. For the first method described by Nordvag et al (1992), 100 μl of thawed anticoagulated blood or marrow was placed in a 0.5 ml Eppendorf tube. This was washed twice in 400 μl of 10 mM EDTA, 10 mM NaCl, and pelleted by spinning for 4 minutes in a microcentrifuge. The pelleted cells were resuspended in 50 mM Tris-HCl (pH 8.0) and boiled for 3 minutes prior to being used directly for PCR.

The second method followed a rapid DNA extraction protocol (Talmud et al, 1991). Four hundred microlitres of freshly prepared 0.17M NH_4Cl was added to 100 μl of thawed anticoagulated blood or marrow in a 1.5 ml Eppendorf tube and mixed well by inversion. This was left at room temperature for 20 minutes before being spun in a microcentrifuge for 30 seconds. After discarding the supernatant, the pellet was washed 3 times with 0.9% NaCl until almost colourless, then resuspended (by vortexing) in 200 μl 0.05 M NaOH. This was boiled for 10 minutes and neutralised by addition of 25 μl 1M Tris HCl pH 8.0.

The DNA was quantitated and the purity determined by spectrophotometry and the samples were stored at -20°C until required.

3.1.3 DNA quantitation

To determine the concentration and purity of DNA extracted by phenol chloroform extraction and ethanol precipitation or the rapid extraction method described above, an aliquot of 20 μl of DNA containing solution was diluted to 1 ml with 980 μl water and the optical density (OD) at 260 and 280 nm read. An OD_{260} of 1 corresponds to approximately 50 μg/ml for double stranded DNA (Maniatis et al, 1982) thus the concentration of DNA in μg/ul of the original sample could be calculated from the formula:

$$DNA\ conc\ (\mu g/\mu l) = \frac{OD_{260} \times 50 \times dilution\ (50)}{1000}$$

$$= OD_{260} \times 2.5$$

The purity was assessed from the OD_{260}/OD_{280} ratio (Maniatis et al, 1982).

3.1.4 PCR reaction

Primers designed by Brisco et al (1990) to amplify the CDR3 region of rearranged IgH genes were used. To locate a region sufficiently conserved to allow design of a consensus V region primer, they compared 17 published immunoglobulin gene DNA sequences. Primer FR3A corresponds to a stretch of 20 bases representing codons 88-95 of the highly conserved third framework region (FR3) of the V genes. The sequences of the J region primers LJH and VLJH were designed to be complementary to a highly conserved region shown to be present at the 3′ end of 6 germline J segments (Ravetch et al, 1981). Since homology between the 3′ end of a primer and its target sequence appears to be of major importance (Sommer and Tautz, 1989), all primers were designed to have completely homologous 3′ ends. At positions within the primers corresponding to areas of the target sequence showing some variation, the most commonly occurring base was used. If two bases occurred at fairly equal frequency, a redundancy was introduced in the primer. Figure 11 shows the

position of the above primers in relation to the rearranged IgH gene and the primer sequences.

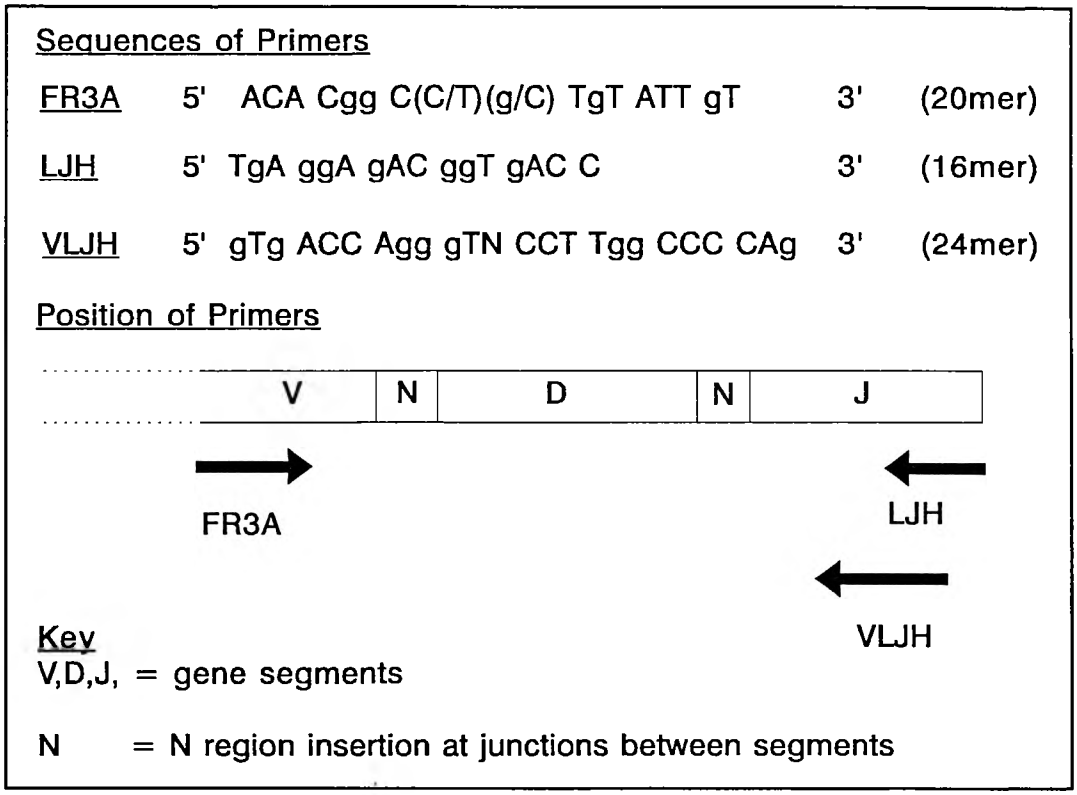

Figure 11. Primers Used to Amplify the CDR3 Region of the Rearranged IgH Gene

PCR was performed as described by Saiki et al (1985). Initially a reaction volume of 100 μl was used and the amount of genomic DNA as well as the primer concentration, magnesium concentration, deoxyribonucleotide triphosphate (dNTP) concentration and the annealing temperature were varied until optimal reaction conditions were established. The effect of reducing the reaction volume to 50 or 25 μl was then determined. The use of primer FR3A in conjunction with primer VLJH or LJH alone was studied, as was the semi-nested reaction utilising the product of PCR amplification with primers FR3A and LJH as the substrate for a second round of amplification using primers FR3A and VLJH.

As a result of the above investigations, the following reaction conditions were adopted to study the samples described in section 3.1.1 : the reaction mixture of

50 μl volume contained 0.5 μg of sample DNA which had been heated to 94°C for 7 minutes prior to addition of a "master mix" containing:
50 picomoles each of primers FR3A and LJH; deoxyadenosine 5′ triphosphate, deoxycytidine 5′ triphosphate, deoxyguanidine 5′ triphosphate and deoxythymidine 5′ triphosphate each at a final concentration of 150 μM; 1.25 units of Taq DNA polymerase (Promega enzyme, SA Scientific Products); 5 μl of the reaction buffer supplied with the above enzyme and $MgCl_2$ to a final concentration of 1,5 mM. This was covered with light mineral oil to prevent evaporation and subjected to 30 cycles of PCR (1 minute each at 94°C, 52°C and 72°C). A semi-nested PCR reaction was then performed using 2 μl of a 1 in 20 dilution of the initial PCR product as the substrate for a second round of PCR using primers FR3A and VLJH in an otherwise identical reaction mixture. The primers used would be expected to generate amplified fragments of approximately 70-140 bases in length (Liang et al, 1991; Potter et al, 1992).

3.1.5 Visualisation of amplified fragments

Reaction products were visualised by electrophoresis through an agarose gel stained with ethidium bromide using TAE buffer (Maniatis et al, 1982). As a result of the small size of the amplified fragments it was found that a high percentage gel (3% (w/v) agarose) was required to give optimal separation of reaction products.

3.1.6 Controls

Negative controls consisting of a sample containing no DNA and a sample containing DNA known not to have rearranged IgH genes as well as a positive control consisting of a sample of DNA known to give a monoclonal rearranged band on PCR amplification were included with each set of samples run. Each specimen was run on at least two separate occasions.

In addition, the amplification of exon 11 of the cystic fibrosis gene as an internal control was assessed. The rationale behind the use of an internal control is that failure of amplification of an isolated sample within a set may occur for technical reasons even though appropriate amplification is obtained from control samples

and other test samples run at the same time. This could give rise to false negative results. To overcome this, an unrelated gene constantly present within the genome (the 'internal control') is co-amplified with the target gene (in this case the IgH gene) for each specimen run (e.g. Deane & Norton, 1990). The amplified product from the internal control gene should therefore be present in every sample following PCR. Its absence from a single tube would be indicative of inhibition of PCR in that tube precluding the interpretation of failure of amplification of the target gene as a true negative result.

Exon 11 of the cystic fibrosis gene was chosen as an internal control in this study as the primers used for amplification of this exon (which is always present within the genome) had been shown to be effective under similar reaction conditions to those used for the IgH gene (A. Goldman, personal communication). Figure 12 shows the sequences of the primers used which would be expected to give rise to a homogeneous amplified fragment of approximately 440 base pairs from all samples.

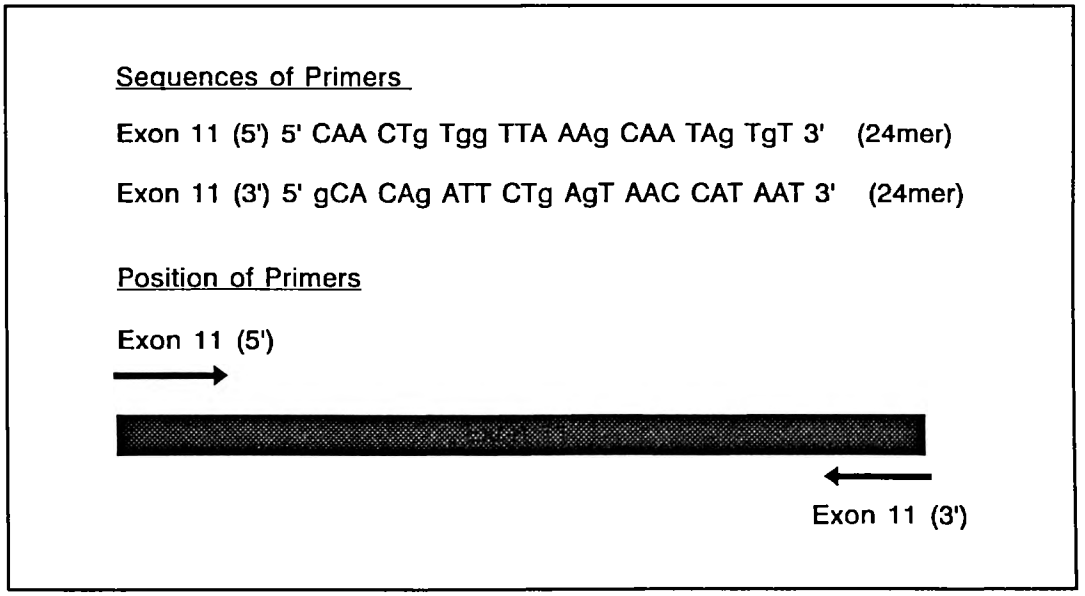

Figure 12. Primers Used to Amplify Exon 11 of the Cystic Fibrosis Gene.

The usefulness of these primers as an internal control was assessed by amplifying 12 samples with the exon 11 primers alone, the IgH CDR3 primers alone and both sets of primers together under the reaction conditions described above.

Seven of the samples were from patients with B-lineage leukaemias and would be expected to give a discrete amplified band with both sets of primers. The remaining five samples were from patients with ANLL and normal individuals and would be expected to give a discrete amplified band only with the exon 11 primers.

3.1.7 Sensitivity

The sensitivity of this PCR technique for detecting a clonal population of B lymphocytes within a background of normal polyclonal cells was tested using samples from 3 different patients with malignancies of B-lineage cells (2 with C-ALL, 1 with CLL). The samples chosen for these sensitivity experiments were selected from marrow aspirates obtained at diagnosis when over 90% of abnormal cells were present as assessed by marrow morphology. Serial dilutions of the patient DNA into DNA extracted from normal bone marrow or peripheral blood lymphocytes were amplified using PCR and the lowest dilution at which clonal bands could still be observed was determined.

3.2 ASSESSING THE FEASIBILITY OF ROUTINE USE OF PCR

3.2.1 Cost Analysis

The cost of a single PCR reaction was determined by evaluating the cost of reagents and disposable equipment required for DNA extraction, the PCR reaction itself and visualisation of the PCR product on an agarose gel. Where a choice existed, the cheapest possible source was used and the cost of bulk rather than small scale purchase of reagents was used in all calculations, as this would most accurately reflect the costs incurred by a routine laboratory. Commercially available customised PCR "kits" were also priced.

The major items of equipment required which are not normally present in a routine haematology laboratory are the thermal cycler and equipment for gel electrophoresis including a power pack, gel trays and an electrophoresis trough. The costs of these items was also assessed.

3.2.2 Analysis of potential utilisation of PCR technology

In order to assess the potential for utilising PCR in the routine haematology laboratory, marrow aspirates reaching the Haematology Laboratory of the Johannesburg Hospital for a period of one month, from August 1 to 31, 1992, were analysed to determine the number of cases in which PCR amplification of either pathological or physiological markers of clonality would potentially be of value.

Blood samples received from the two specialist Haematology/Oncology Units at the Johannesburg Hospital over a period of 1 week from March 22 to 29, 1993, were also analysed for this purpose. It was assumed that the majority of appropriate blood samples would come from these units, although other units, particularly the general medical wards, may also be a source of smaller number of appropriate samples.

The time periods selected for these analyses were chosen at random and the number and nature of samples obtained did not subjectively appear to be in any way unusual.

4. RESULTS

4.1 PCR AMPLIFICATION OF IMMUNOGLOBULIN GENE REARRANGEMENTS

4.1.1 Use of primers FR3A, VLJH and LJH

When amplification occurred, bands were seen in the expected size range of 70-140 base pairs. Figure 13 shows the result of agarose gel electrophoresis of the PCR amplification products from a selection of patients with B and non B lineage haematological malignancies and normal individuals. As expected, clonal B lineage populations (lanes 1-4) gave rise to one or more discrete amplified bands, whilst the polyclonal B lineage populations found in normal individuals (lanes 7 and 8) gave rise to a diffuse smear. No amplification was obtained from non B lineage populations (lanes 5 and 6), with the exception of one sample from a patient with T-ALL.

Variations in reaction conditions were found to have a significant effect on the extent of amplification achieved. For example, Figures 14 and 15 show the effect of altering the primer concentration and DNA concentration respectively. Alterations of the magnesium concentration and annealing temperatures were also found to affect amplification (not shown). The reaction conditions selected to give optimal PCR amplification as a result of these studies are given in the Materials and Methods section.

Figure 16 shows the difference in length of amplified product obtained using primer FR3A in conjunction with the two different J region primers in a single stage PCR reaction. As expected, the amplification product obtained using primer VLJH was slightly shorter. The additional small band of approximately 50 base pairs noted in lane 1a was a constant feature of amplification using primers FR3A and VLJH. It probably reflects the formation of "primer dimers". No difference in efficacy of amplification was noted between the two J region primers, except in one case of C-ALL which give rise to a homogenous amplified product using primers FR3A and VLJH but could not be amplified using primers FR3A and LJH.

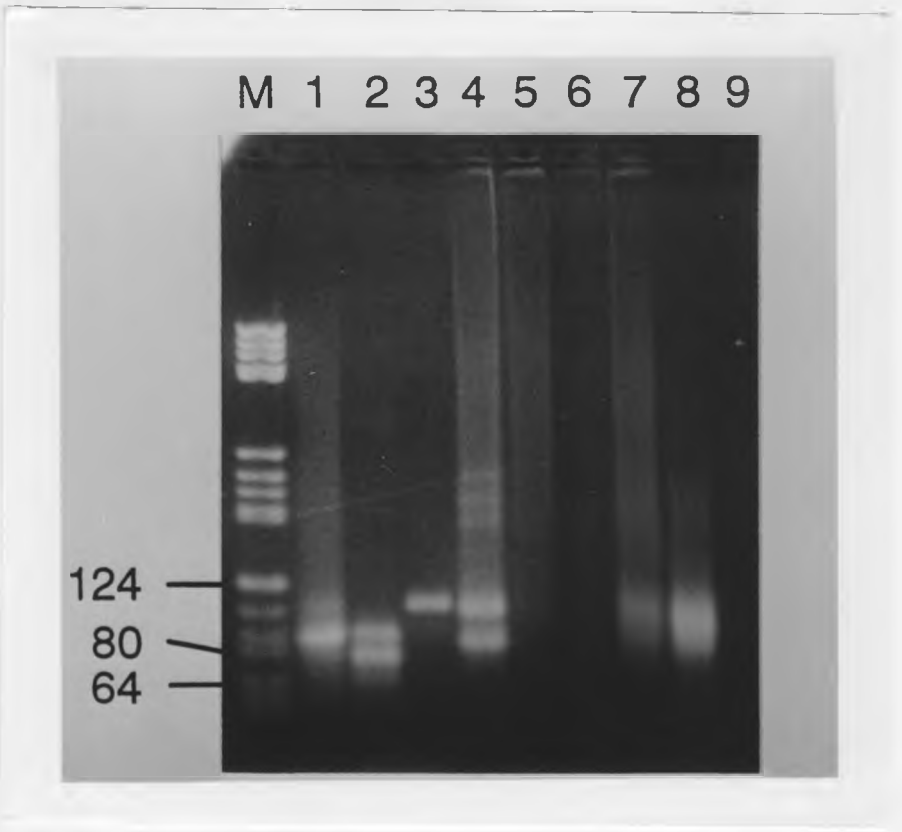

Figure 13. PCR amplification of IgH gene rearrangements using a semi-nested reaction with primers FR3A, LJH and VLJH.

Agarose gel showing PCR amplification of samples from patients with common ALL (lanes 1 and 2), CLL (lanes 3 and 4), ANLL (lane 5), T-ALL (lane 6), normal bone marrow (lane 7) and normal peripheral blood (lane 8). Lane 9 is a negative control containing no DNA. M is DNA molecular weight marker V (Boehringer Mannheim). Note the presence of discrete bands reflecting monoclonal rearrangements of one or both alleles in the patients with B lineage leukaemias, the absence of any amplified product in the patients with ANLL and T-ALL and the diffuse smear reflecting the presence of polyclonal rearrangements in normal peripheral blood and bone marrow.

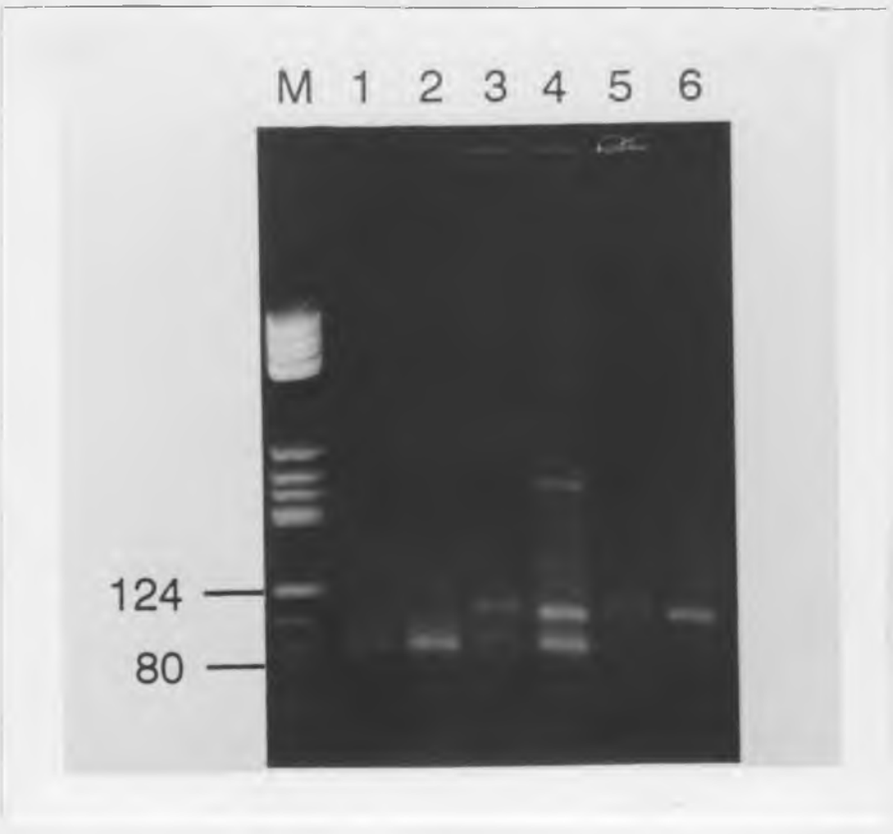

Figure 14 The Effect of Primer Concentration.

Agarose gel showing improved amplification of clonal IgH gene rearrangements from 3 patients with C-ALL using 50 picomoles of each primer (lanes 2, 4, 6) as compared with 25 picomoles of each primer (lanes 1, 3, 5) in a 50 ul reaction volume. Note one of the patients had two clonal rearrangements probably reflecting rearrangement of both IgH alleles (lanes 3 and 4) while the other two patients each showed only a single monoclonal rearrangement (lanes 1 and 2, 5 and 6). An additional large discrete band outside the expected size range is also seen in lane 4. Such 'spurious' bands should not interfere with interpretation of results. M is DNA molecular weight marker V (Boehringer Mannheim).

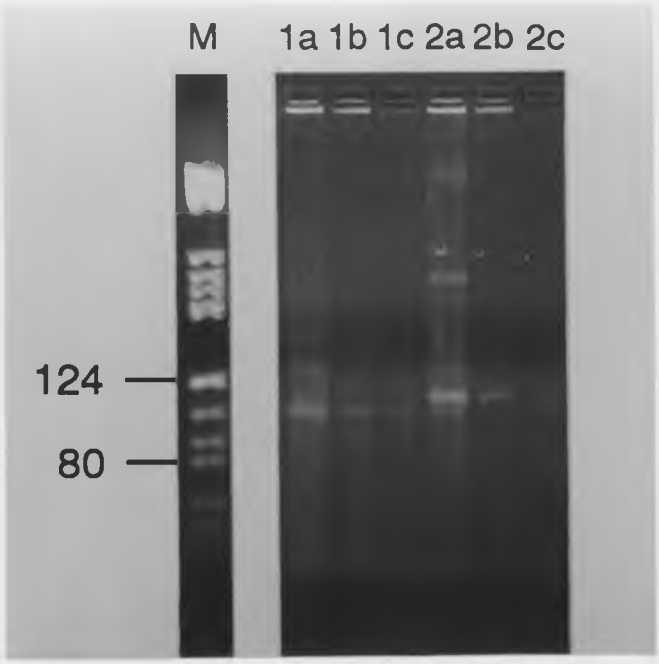

Figure 15. The effect of DNA concentration.

Agarose gel comparing PCR amplification of clonal B cell populations from 2 patients with C-ALL (1 and 2) using 0.5 ug template DNA (lanes 1a and 2a), 0.25 ug template DNA (lanes 1b and 2b) and 0.05 ug template DNA (lanes 1c and 2c) in a 50 ul reaction volume. Optimal amplification is seen using 0.5 ug template DNA (lanes 1a and 2a). M is DNA molecular weight marker V (Boehringer Mannheim).

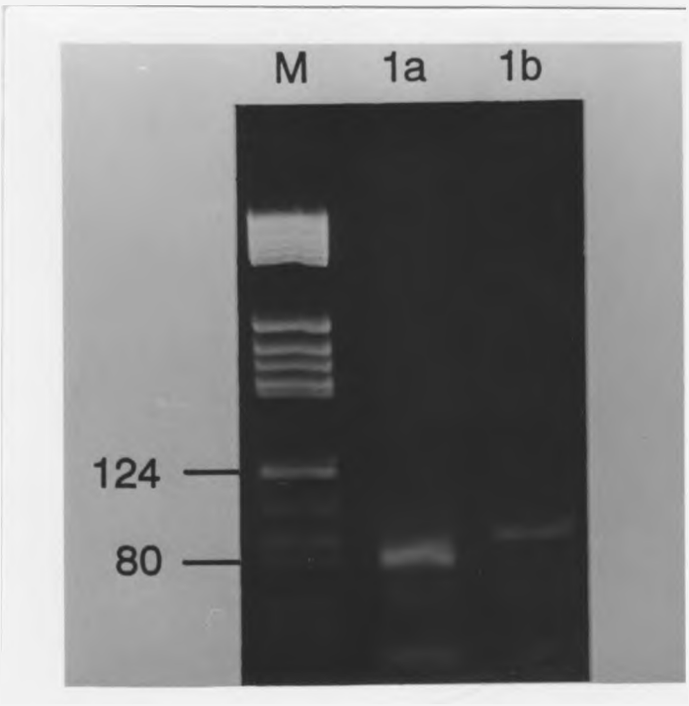

Figure 16. Comparison of Primers LJH and VLJH.

Comparison of amplification products obtained using primer FR3A and VLJH (lane 1a) and FR3A with LJH (lane 1b) in a single stage PCR reaction on a clonal B-lineage cell population from a patient with C-ALL. M is DNA molecular weight marker V (Boehringer Mannheim). The amplified product obtained using primer VLJH is slightly shorter. The faint band at the end of lane 1a probably reflects 'primer dimer' formation.

Clonality of B lymphocytes as evidenced by DNA fragment length homogeneity was detected in samples from 34 out of 40 cases of B lineage neoplasia (85%). This included 23 out of 27 cases of C-ALL (85%), 8 out of 8 cases of CLL (100%), and 3 out of 5 cases of multiple myeloma (60%) tested. As noted previously, one case of T-ALL (12.5%) also gave rise to a clonal amplified product. A diffuse smear indicative of B cell polyclonality was obtained from all samples from normal individuals studied, using the semi-nested reaction, but no amplified product was obtained after a single stage reaction on these samples. No amplification at all was obtained using DNA from patients with acute non-lymphoblastic leukaemia and from 7 out of the 8 patients with T-ALL studied (Table 3).

Table 3. Detection of Monoclonality by PCR Amplification of Ig Gene Rearrangements

DIAGNOSIS	NATURE OF SPECIMEN	CLONALITY DETECTED
B-ALL	Presentation Marrow	23/27 (85%)
CLL	Blood Marrow	4/4 (100%) 4/4 (100%)
MM	Presentation Marrow	3/5 (60%)
T-ALL	Presentation Marrow	1/8 (12.5%)
ANLL	Presentation Marrow	0/7 (0%)
NORMALS	Blood Marrow	0/4 (0%) 0/6 (0%)

Details of patients studied and results obtained on PCR amplification of IgH gene rearrangements:

C-ALL: Common Acute Lymphoblastic Leukaemia
CLL: Chronic Lymphocytic Luekaemia
MM: Multiple Myeloma
T-ALL: T-Lineage Acute Lymphoblastic Leukaemia
ANLL: Acute Non-Lymphoblastic Leukaemia

More than one clonal band was detected in 8 cases of B lineage malignancy. Two cases of CLL and 4 cases of C-ALL gave rise to two clonal bands, whilst 2 cases of C-ALL gave rise to 3 clonal bands after PCR amplification. The significance of these multiple bands is discussed in section 5.1.1 .

4.1.2 Use of Exon 11 of the cystic fibrosis gene as an internal control.

Figure 17 shows the results of PCR amplification of DNA from two patients with B lineage leukaemias (lanes 1 and 2) and one patient with ANLL (lane 3) with a) the IgH CDR3 primers (FR3A and LJH) alone, b) the exon 11 primers alone and c) both sets of primers together. As expected, all 3 samples gave rise to a discrete amplified band of approximately 440 base pairs when amplified using the primers for Exon 11 of the cystic fibrosis gene either alone (lanes 1b, 2b, 3b) or in combination with the IgH CDR3 primers (lanes 1c, 2c, 3c). This was found to be the case with all 12 samples tested. In addition, comparison of lanes 1a and 1c and lanes 2a and 2c in Figure 17 shows that for the two samples from patients with B lineage leukaemias identical discrete bands in the correct size range (70-140 base pairs) representing amplified clonal Ig gene rearrangements were obtained both when using the IgH CDR3 primers alone and when using them in conjunction with the exon 11 primers.

These findings would suggest that amplification of exon 11 may be useful as an internal control. However some problems were encountered. Firstly additional amplified bands of approximately 150 base pairs (arrowed in Figure 17) and larger were frequently obtained on co-amplification of samples with both sets of primers. The origin of these spurious bands is uncertain but once recognised they need not interfere with interpretation of results. Of greater concern was the occurrence of competition between the two sets of primers. In one sample from a patient with B lineage leukaemia, strong amplification of the rearranged IgH gene occurred at the expense of amplification of exon 11. Although this would not be clinically relevant, in two other samples from patients with C-ALL, amplification of the rearranged IgH gene appeared to be reduced in the presence of a strongly amplified Exon 11 band. Such an effect could potentially give rise to a false negative result.

4.1.3 Sensitivity.

Using a single stage PCR reaction with primers FR3A and VLJH, clonality was detected down to a dilution of 5%, 10% and 2.5% respectively for the 3 samples

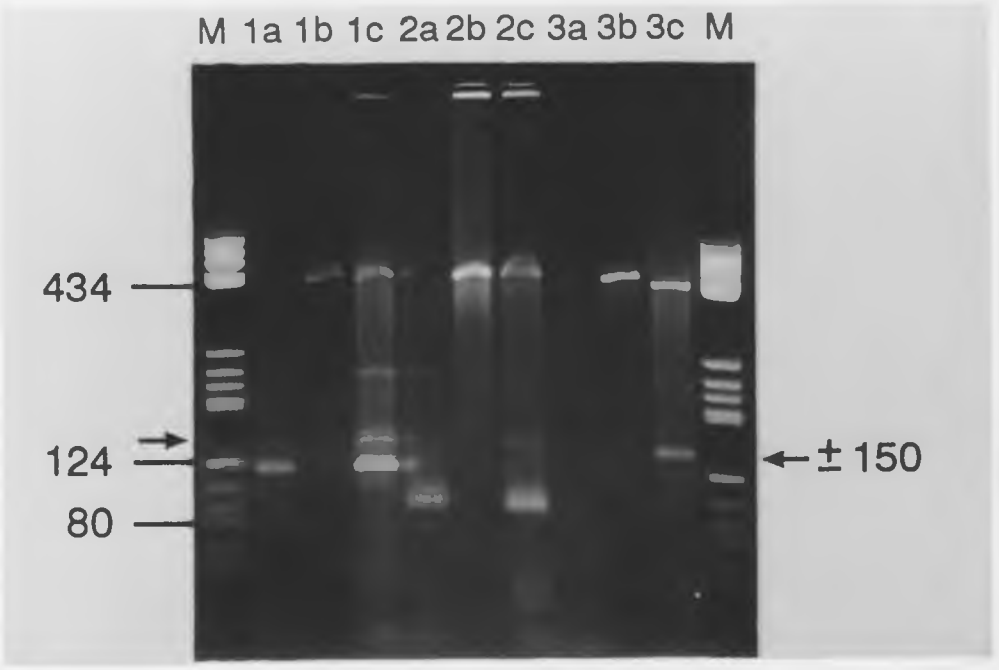

Figure 17. Use of Exon 11 of the Cystic Fibrosis Gene as an Internal Control.

Agarose gel showing the effect of amplification of DNA from patients with C-ALL (1), CLL (2) and ANLL (3) with a) primers for the IgH CDR3 (FR3A and LJH) alone; b) primers for exon 11 of the cystic fibrosis gene alone; and c) both sets of primers together. An amplified band of ± 440 base pairs corresponding to Exon 11 is seen in all samples when amplified using the Exon 11 primers alone (lanes 1b, 2b and 3b) or in combination with the IgH CDR3 primers (lanes 1c, 2c and 3c). Identical discrete bands of between 70 and 140 base pairs corresponding to amplified clonally rearranged IgH genes are seen in lanes 1a and 1c and lanes 2a and 2c respectively, indicating that co-amplification of Exon 11 has not interfered with amplification of the rearranged IgH gene in these samples. As expected no amplification is seen with the IgH CDR3 primers in the patient with ANLL (lanes 3a and 3c). Additional bands of ± 150 base pairs (arrowed) and larger are seen in lanes 1c, 2c and 3c. These are discussed in the text (section 4.1.2). M is DNA molecular weight marker V (Boehringer Mannheim).

tested. Figure 18 shows the results of a dilution study using a single stage PCR. The sensitivity of detection of a clonal population from a background of polyclonal lymphocytes using the semi-nested PCR reaction was found to be similar, viz. 2.5%, 10% and 2.5% respectively. Figure 19 is an example of a dilution study using a semi-nested PCR. The extent of amplification as judged by the brightness of amplified bands on an agarose gel was not always proportional to the degree of dilution.

4.1.4 Comparison of methods of sample preparation.

Direct PCR amplification of washed blood cells subsequent to boiling for 3 minutes was not found to be a satisfactory method of sample preparation. Although in 3 cases of C-ALL prepared in this way amplification did occur, in the majority of cases there was failure of amplification although high molecular weight DNA could be seen sitting in the wells of the gel.

Table 4. Spectrophotometric analysis of DNA prepared using two different methods of DNA extraction.

METHOD	AVERAGE PURITY (OD_{260} / OD_{280})	AVERAGE DNA CONCENTRATION (μg/ul)
Phenol / Chloroform extraction (Jeffrey et al)	1.8	0.42
Short method (Talmud et al)	1.4	0.23

Spectrophotometric analysis of the DNA extracted from 4 samples by phenol/chloroform extraction (Jeffrey's method) and by the short method described by Talmud et al (1991), showed that both the average purity (as assessed by the OD_{260}/OD_{280} ratio) and the average concentration of DNA obtained was lower for the samples prepared by the short method (Table 4). However, provided the same quantity of DNA was used, the efficacy of PCR amplification did not appear to be affected by the method of DNA extraction and the short method gave satisfactory results (Figure 20).

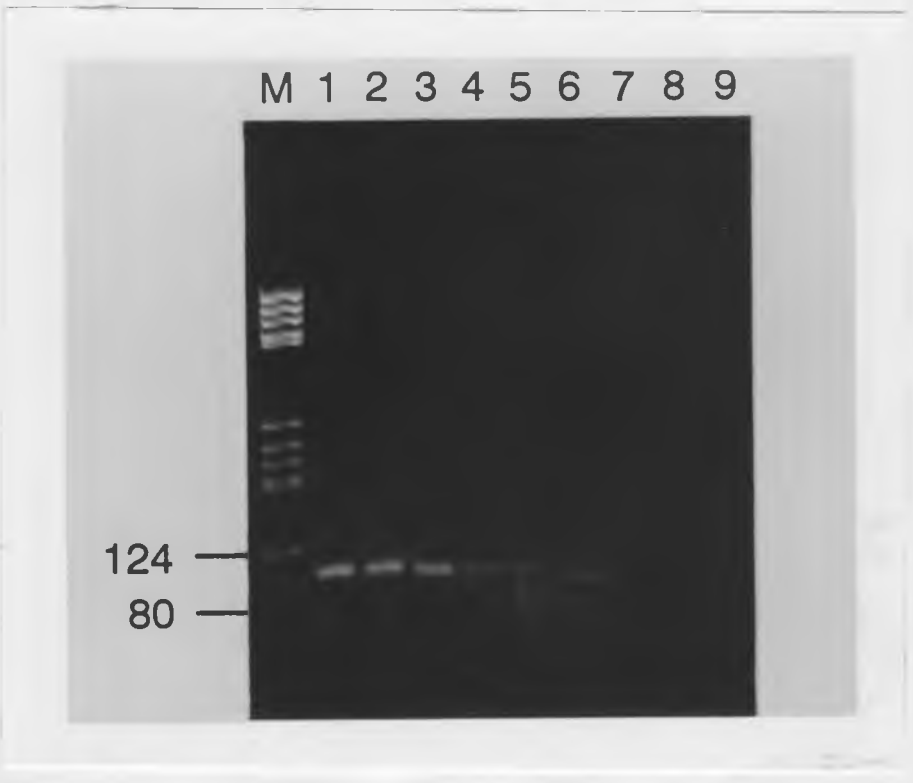

Figure 18. Sensitivity of Single-stage PCR.

Agarose gel showing sensitivity of detection of a clonal population of B-cells (from the marrow of a patient with C-ALL at presentation) after serial dilution into normal bone marrow using a single stage PCR:
Lane 1 - 100% patient marrow. Lane 2 - 50%. Lane 3 - 25%. Lane 4 - 20%. Lane 5 - 10%. Lane 6 - 5%. Lane 7 - 2.5%. Lane 8 - 1%. Lane 9 - 0%.
A clonal band is detectable down to a dilution of 5% (lane 6). M is DNA molecular weight marker V (Boehringer Mannheim).

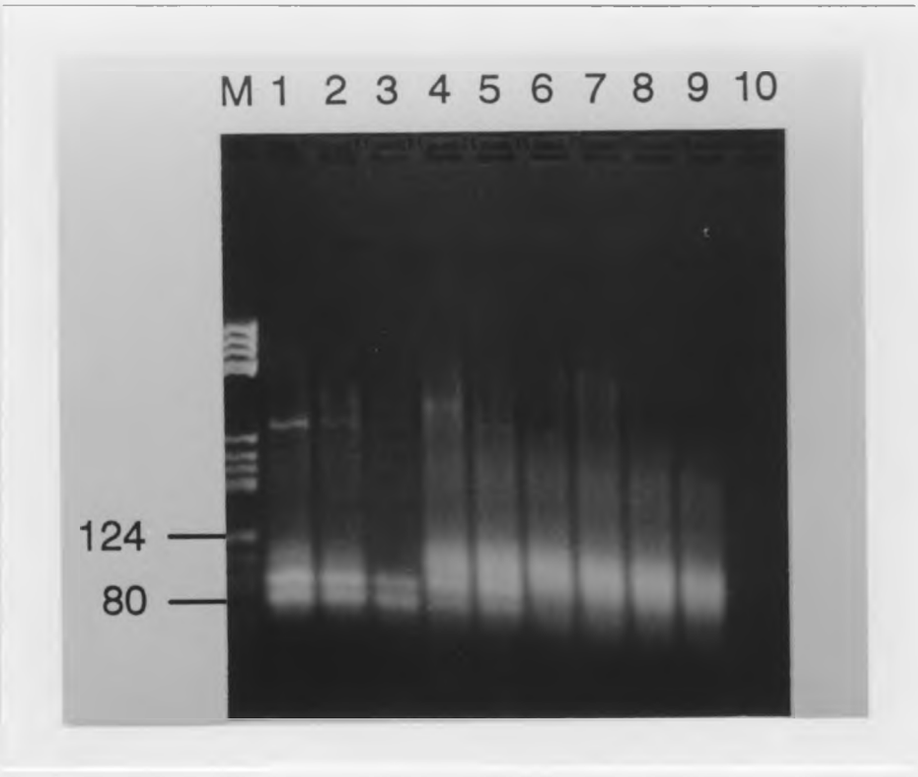

Figure 19: Sensitivity of Semi-nested PCR

Agarose gel showing sensitivity of detection of a clonal population of B cells (from the marrow of a patient with C-ALL at presentation) after serial dilution into normal bone marrow using a semi-nested PCR reaction.
Lane 1 - 100% patient marrow; Lane 2 - 50%; Lane 3 - 25%; Lane 4 - 20%; Lane 5 - 10%; Lane 6 - 5%; Lane 7 - 2.5%; Lane 8 - 1%; Lane 9 - 0%. Lane 10 is a negative control with no DNA.
Clonal bands are detectable down to a dilution of 10% (lane 5). At greater dilutions the monoclonal bands are obliterated by a polyclonal smear. M is molecular weight marker V (Boehringer Mannheim).

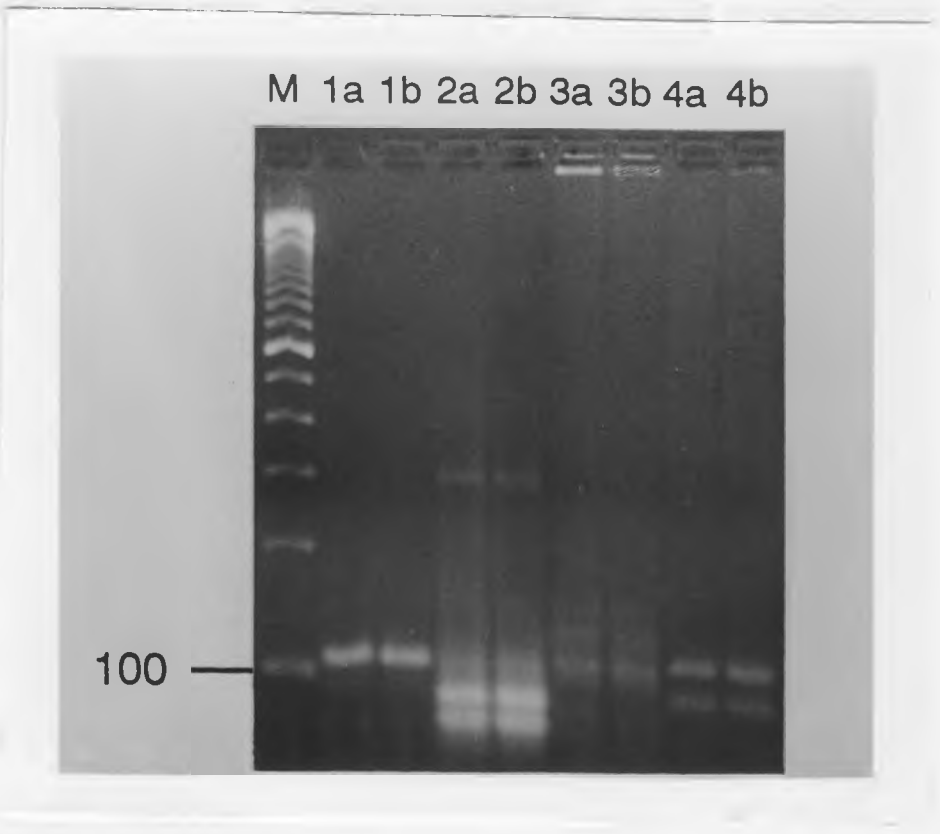

Figure 20. Comparison of methods of DNA extraction.

Agarose gel comparing PCR amplification of DNA from clonal B-lineage populations of 4 patients with C-ALL after phenol/chloroform extraction (Lanes 1a to 4a) and after extraction by the short method of Talmud et al (Lanes 1b to 4b). M is DNA molecular weight marker BRL (100 bp ladder). The extent of amplification does not appear to be affected by the method of DNA extraction used.

4.1.5 Variability of amplification

All specimens were run on at least two occasions. Despite apparently identical reaction conditions, comparable amplification was not always obtained on each occasion that the specimen was run. Sometimes 'blank runs' were recorded in which no amplification occurred in samples or controls. These data were ignored and the samples rerun. Of greater significance was the occasional failure of an isolated tube, within an otherwise successfully amplified set of samples, to give an appropriate result.

4.2 FEASIBILITY OF ROUTINE USE OF PCR.

4.2.1 Cost analysis.

4.2.1.1 Cost of each PCR reaction.

The cost of disposable equipment and reagents for a single unreplicated PCR reaction from extraction of DNA using Jeffrey's method to visualisation of reaction products by agarose gel electrophoresis is approximately R8.00 (Table 5). This is calculated for a single stage PCR reaction using a single set of primers in a reaction volume of 100 μl. Batch processing of samples so that 9 samples are run together on each agarose gel is assumed. The cost of running positive and negative controls with each batch of samples and the cost of amortization of equipment and of salaries are not included. A detailed breakdown of the cost analysis is available in Appendix 1.

4.2.1.2 Cost of commercially available PCR kits.

Customised diagnostic kits for the detection of selected pathogens using PCR have recently become available in South Africa. The kits provide all necessary reagents for the process, from sample preparation to visualisation of amplified PCR products. The cost to the laboratory is variable, from R30 per test for detection of chlamydia to R120 per test for the detection of the Human Immunodeficiency Virus (HIV). The suggested charge out tariff is R210 per test. These prices are as quoted by the Roche Diagnostic Department as at April 1, 1993. No such kits are yet available for the diagnosis of conditions specifically relevant to haematology.

Table 5. Cost of Consumables required per PCR Test (as at April 1993).

STAGE	COST
DNA EXTRACTION (Jeffrey's Method) Reagents: Triton-Saline, Lysis buffer, Phenol/Chloroform, etc Disposables: Pasteur Pipettes, Polypropylene tubes, etc.	± R1.10c
PCR REACTION (Reaction volume 100 μl) Reagents: Taq, dNTP's, Primers, etc. Disposables: Pipette tips, etc	± R5.35
AGAROSE GEL ELECTROPHORESIS (9 samples/gel) Reagents: Agarose, DNA size markers, etc. Disposables: Parafilm, Pipette tips	± R1.31
TOTAL	**± R7.96**

4.2.1.3 Major items of capital expenditure.

The major items of equipment required for PCR not normally present in a routine haematology laboratory are the thermal cycler and equipment for gel electrophoresis. Table 6 gives examples of approximate prices of such equipment as at April, 1993. As can be seen, there is an extremely wide variation in the cost of currently available thermal cyclers. Factors which should be taken into account in choosing a thermal cycler for a diagnostic laboratory would include the accuracy of temperature control, the uniformity of temperature achieved across the heating block, the cycling speed which would determine the "through time", the capacity of the heating block, the ability to process samples in microtitre plates as well as in microcentrifuge tubes and the programming facilities present.

Table 6. Cost of PCR Equipment (as at April 1993).

EQUIPMENT	COST
THERMAL CYCLERS:	
Hybaid Thermal Cycler (54 well)	±R16 000.00
Hybaid Omnigene (96 well)	±R22 000.00
Perkin Elmer 9600 (96 well)	±R42 000.00
GEL ELECTROHORESIS EQUIPMENT:	
Power Pack - Consort E321 (Max 250V 100 mA)	±R 1 530.00
8 x 10 cm Horizontal Gel Casting Tray (SA Scientific Products)	±R 55.00
Triple Place Mini-Gel Tank (SA Scientific Products)	±R 1 230.00

4.2.2 Analysis of potential for utilisation of PCR technology.

Table 7 shows the number of marrow aspirates received by the Haematology Laboratory of the Johannesburg Hospital in August, 1992, in which PCR analysis of either physiological or pathological markers of clonality could potentially have been of value. Only the more frequently occurring PCR detectable markers are included. Others such as the t(1;14) and t(10;14) found in small numbers of T-ALL have been omitted although they could potentially be of interest within a restricted group of cases. Marrow aspirates submitted for assessment of the presence of metastatic tumour are not included although PCR detection of mutations of the p53 gene may well prove to be a useful marker of malignancy in these and other conditions. Likewise, aspirates submitted for assessment of thrombocytopenia have not been included although exclusion of clonal Ig gene rearrangements using PCR may be relevant in such cases. Aspirates where the use of PCR to detect pathogens or hereditary abnormalities may have been valuable have also not been included.

Table 8 shows a similar analysis of peripheral blood samples obtained from the two Haematology Oncology Units (wards 294 and 495) of the Johannesburg Hospital over a period of one week, from March 22 to 29, 1993. As noted in

Table 7. Marrow Aspirates Suitable for PCR

(Johannesburg Hospital, August, 1992).

DIAGNOSIS	NO OF CASES (NEW CASES)	PCR TARGET
ALL	24 (4)	Ig gene, TCR gene, t(9;22), t(1;19), tal-1
CLL	2 (1)	Ig gene, (p53)
Hairy Cell Leukaemia	1 (1)	Ig gene
Multiple Myeloma	3 (1)	Ig gene
APL	2 (1)	t(15;17)
CML	7 (2)	t(9;22)
NHL	16 (9)	t(14;18), Ig gene, (p53)
Burkitts	1 (1)	Ig gene (t(8;14))

Table 8. Peripheral blood samples suitable for PCR

(Wards 495 and 294, Johannesburg Hospital, 22-29 March, 1993).

DIAGNOSIS	NO OF CASES	PCR TARGET
ALL	41	Ig gene, TCR gene, t(9;22), t(1;19), tal 1
CLL	3	Ig gene, (p53)
HCL	3	Ig gene
MGUS *	2	Ig gene
Multiple Myeloma	6	Ig gene
APL	2	t(15;17)
CML	4	t(9;22)
(B) NHL	30	t(14;18), Ig gene, (p53)
T-cell Lymphoma	3	TCR gene
Burkitts	2	Ig gene, t(8;14)

* MGUS - monoclonal gammopathy of uncertain significence

the Materials and Methods section, potential appropriate blood samples from other units are not included. Where more than one sample was obtained from one patient during the week, only the first sample was included.

Overall PCR could potentially have been used to detect markers of clonality in 56 of the marrow aspirates received in 1 month and 96 of the peripheral blood samples received in 1 week by the Haematology Laboratory of the Johannesburg Hospital. The Johannesburg Hospital is a large (833 bed) tertiary referral centre with two specialist Haematology/ Oncology Units which together with Baragwanath, J.G. Strijdom and Hillbrow Hospitals draws patients from most of the greater Johannesburg and some outlying areas.

5. DISCUSSION

5.1 DETECTION OF IMMUNOGLOBULIN GENE REARRANGEMENTS USING PCR

5.1.1 Specificity

These results show that clonal immunoglobulin gene rearrangements are detectable by PCR in a high percentage of B cell neoplasms. Further cases of multiple myeloma would have to be assessed in order to determine the significance of the apparently lower incidence of clonality detected in this setting. The failure to detect clonal rearrangements in around 20% of cases of B lineage malignancy using these consensus primers is a consistent find (Trainor et al, 1990, 1991; Jonsson et al, 1990; Liang et al 1991; Potter et al 1992). There is no obvious relationship between the occurrence of failure to detect clonality and the type of B lineage malignancy in these studies.

Failure of amplification may arise as a result of suboptimal priming because of inadequate homology between primer and target sequences due to deletions or other base mismatches within the B gene segments (Deane et al, 1991). As noted previously, homology between the three 3′ nucleotides of the primer and the target sequence appears to be of particular importance (Sommer and Tautz, 1989). Alternatively complete failure of priming may occur as a result of translocations involving the IgH gene locus, e.g. the t(14;18) or inversion of gene segments during gene rearrangement. Either of these would result in wide separation of primer binding sites preventing amplification (Trainor et al, 1991).

To compensate for these problems, Ramsamay et al (1992) advocate the use of a "repertoire" of primers homologous to the FR2 and FR3 regions. Alternative solutions would be the use of family specific primers for the FR1 region of the VH genes (Deane et al, 1991) or where applicable, combined use of primers amplifying the t(14;18) and rearranged IgH genes (Corbally et al, 1992).

The occurrence of more than one clonal rearrangement was a fairly frequent finding. In most cases not more than 2 rearranged bands were present, probably reflecting rearrangement on both of the IgH alleles. However, in 2 cases of C-ALL, 3 rearranged bands were detected. The pathogenesis of multiple (i.e.

3 or more) rearrangements has been discussed in Chapter 2. In the context of ALL they are believed to reflect clonal evolution arising as a result of ongoing gene rearrangements (Bird et al, 1988) or secondary gene rearrangements (Wasserman et al, 1992). The incidence of detection of multiple rearrangements in this study was approximately 7.5%, which is lower than the incidence of 15-45% quoted in the literature (Kitchingman et al, 1986; Bird et al, 1988; Katz et al, 1989; Beishuizen et al 1991(a)). This may reflect a reduced sensitivity of detection of minor subclones. Alternatively it may result from the exclusive use of marrows obtained at the time of presentation as the incidence of clonal evolution and subclone formation increases with time (Wasserman et al, 1992).

As expected, none of the samples from patients with ANLL or normal individuals gave rise to clonal bands on amplification. The occurrence of a clonal IgH rearrangement in one of the 8 cases of T-ALL studied is in keeping with the expected incidence of ± 14% for such cross lineage gene rearrangements (see Table 2). Cross lineage rearrangements are discussed in Chapter 2 and as noted their existence precludes the use of PCR analysis of gene rearrangements in isolation to determine lineage.

5.1.2 Sensitivity

PCR is an extremely powerful tool for detecting very small amounts of target DNA. However, in the context of immunoglobulin gene rearrangements background normal lymphocytes act as competing IgH targets. This not only makes the detection of a clonal band difficult because of the presence of a background 'smear' of amplified polyclonal product, but also reduces the actual extent of amplification of the specific target by substrate competition (Sykes et al, 1992). These factors significantly reduce the sensitivity of PCR in this context.

Some authors using a single stage PCR reaction with primers FR3A and VLJH claim to be able to consistently detect a clonal population within a background of normal lymphocytes to levels as low as 1% (Potter et al, 1992), or even 0.1% (Liang et al, 1991). However, in this study, the sensitivity of detection was between 2.5% and 10%, with comparable sensitivity being obtained using a single

stage and a semi-nested reaction. This concurs with the finding of Trainor et al (1990), who demonstrated a sensitivity of 2-5% using a single stage reaction and Wan et al (1992) who detected clonal populations to a sensitivity of 10% using a semi-nested reaction. This level of sensitivity could be adequate to distinguish between reactive and malignant states and PCR should be more accurate than marrow morphology in this regard. In the context of B lineage neoplasms other techniques such as detection of light chain restriction are available for this purpose however for T cell malignancies a simple test to demonstrate clonality would be extremely useful.

Although, as discussed in Chapter 2, the level at which detection of minimal residual disease holds clinical significance is uncertain, it is unlikely that the sensitivity of straight forward PCR amplification of gene rearrangements will be adequate for this purpose. However, the techniques described by Hansen-Hagge et al (1989) and Nizet et al (1991) to generate "clone-specific" probes to increase the sensitivity (see section 2.3.2) may be amenable to use on a fairly routine basis. In addition, improved techniques for DNA sequencing may eventually make even the methods involving sequencing of the CDR3 region to synthesise a clone specific probe, a practical proposition (section 2.3.2).

5.1.3 Speed and simplicity.

One of the great advantages of PCR as a routine tool is its speed and simplicity. Understanding the underlying principles is certainly not a prerequisite to its use and once reaction conditions have been established it does not require high levels of technical expertise. The customised kits developed for some of the more common applications have been mentioned previously. Use of these obviates the need to establish reaction conditions making the routine use of PCR still simpler. Unfortunately suitable kits are not as yet available for routine haematological diagnosis.

Once the DNA has been extracted, the results of the PCR can be obtained rapidly: Setting up the PCR reaction takes ± 30 minutes, depending on the number of samples tested, the PCR reaction itself takes ±3 hours for 30 cycles of

amplification and loading the reaction products onto a gel followed by electrophoresis for the purposes of visualisation takes ±1½ hours. However, conventional DNA extraction involving phenol/
chloroform extraction is a laborious procedure and it was decided to investigate two of the many 'rapid' methods of sample preparation now described.

Simple boiling of washed cells, as described by Nordvag et al (1992), gave poor results in the majority of cases. This may be the result of inhibition of the PCR by the presence of the remaining cellular debris. In support of this, the presence of biological contaminants such as blood has been shown to inhibit PCR, probably due to entrapment of target DNA by the organic material present. (Panaccio and Lew, 1991). Alternatively the poor amplification obtained may reflect the difficulty in regulating the amount of DNA present using this method. The cell count and hence DNA content of a fixed volume of blood or marrow is open to wide variation and since the DNA concentration is important in determining the efficacy of PCR this variation is likely to have a significant effect on outcome.

In contrast, the rapid method of DNA extraction described by Talmud et al (1991) proved to be as good as Jeffrey's method for the purposes of PCR. The procedure is simple and quick, taking just over 30 minutes. Thus, using this method, it would definitely be possible to process a specimen and obtain a PCR result within one day.

Although agarose gel electrophoresis is a relatively simple procedure, alternative methods of visualisation of PCR products may be more practical for routine use. The customised kits available from Roche make use of biotin-labelled primers, thus any amplified product obtained will be biotinylated. Subsequent to amplification the PCR product is denatured and added to microtitre plates containing bound 'capture probes'. These are single stranded DNA probes designed to hybridize to regions within the target sequence. They will therefore bind any PCR product containing the appropriate sequence. After washing to remove any unbound DNA strands and unused primers an avidin-enzyme complex is added. This will bind to any biotin-labelled PCR product present and produce

a colour change when a suitable substrate is added. The occurrence of a colour change is therefore indicative of the presence of amplified PCR product with a sequence homologous to the target DNA, i.e. a positive result. The occurrence of normal background lymphocytes with amplifiable IgH rearrangements precludes the use of this technique for the detection of clonal IgH rearrangements unless a clone-specific capture probe is used. However, in other contexts such colour change reactions to determine the outcome of PCR would be ideal for routine diagnostic purposes.

5.1.4 Problems with PCR.

The major problem encountered in this study was the inconsistency of the outcome of PCR. Despite apparently identical conditions, amplification was not always obtained on each occasion that a 'positive' specimen was run. Failure of amplification probably usually arises as a result of **technical problems**. These may include the use of **substandard reagents** such as impure or inconsistently sequenced primers, or poor quality enzyme. Also in view of the large number of variables present, **optimisation of reaction conditions** may be difficult. The effect of the **presence of biological contaminants** and the problems associated with **primer design** in the context of IgH gene rearrangements have already been discussed.

Inclusion of a 'known positive' sample as a control with each batch of samples run should avoid misinterpretation of results by allowing identification of a completely blank run (i.e. one in which no amplification of either samples or controls occurs). However, this would not help to detect the occurrence of failure of amplification in an isolated sample within an otherwise successfully amplified batch. For this purpose an internal control comprising primers amplifying an unrelated but constant gene would have to be included with each sample run. In this study co-amplification of exon 11 of the cystic fibrosis gene as an internal control was assessed. As noted in Chapter 4, it was not ideal. The generation of the spurious extra bands seen on co-amplification of samples with both sets of primers need not interfere with the interpretation of results. However, the occurrence of competition between the sets of primers was a significant finding

as reduced amplification of the target (IgH) gene as a result of such competition could give rise to a false negative result.
Such competition between primers may also prove to be a problem when 'cocktails' of diagnostic primers are used together.

Although not applicable to this study, it should be noted that false negatives may arise due to the nature of the disease itself: clonal evolution (discussed in section 2.2.5.2) may cause false negatives during the follow up of minimal residual disease in ALL and non-random distribution of a tumour may cause it to be missed on sampling. For example, extramedullary relapse has been missed when patients with ALL are followed up by PCR analysis of bone marrow samples alone (Yamanda et al, 1990).

The extreme sensitivity of PCR means that false positive results caused by contamination with even minute amounts of DNA can be a problem particularly when using nested PCR. This is more likely to affect PCR reactions using primers which give rise to standard length products (e.g. bcr-abl cDNA in CML) than those which result in clone specific products, such as the junctional regions of different antigen receptor gene rearrangements. In addition, the relative insensitivity of PCR detection of clonal Ig gene rearrangements is actually an advantage in this context. Guidelines on practical ways of limiting contamination have been published (Kwok and Higuchi, 1989) and more recently a method has been developed which allows for specific hydrolysis of the DNA produced by PCR amplification to prevent carry-over contamination (Lango et al, 1990). This involves the use of deoxyuridine triphosphate (dUTP) in place of deoxythymidine triphosphate (dTTP) in the PCR reaction mixture. After analysis of the PCR product, nucleic acids containing dUTP are hydrolysed by reaction with uracil-DNA glycosylase (UNG). Naturally occurring DNA which contains dTTP is not affected by the enzyme. Problems with contamination were not experienced during this study, but the use of appropriate negative controls, including a sample containing known 'negative' DNA and one containing no DNA with each batch of samples run, would have made the presence of contamination easy to recognise.

5.2 ROUTINE USE OF PCR

5.2.1 Costs incurred

The capital expenditure required to purchase the major items of equipment needed to set up PCR in a routine laboratory is small. In addition, the actual cost of a single PCR reaction is very low, comparing favourably with a routine full blood count. However, although the cost per reaction was calculated for a 100 μl reaction volume and volumes of 50 or even 25 μl can be used, in the short term the cost of establishing PCR is much higher. This is because the research and development required to determine optimal reaction conditions for each pair of primers and for each combination of primer pairs used, can be very expensive. In addition, the cost of including adequate controls and of rerunning specimens when failure of amplification occurs, would have to be included. The extensive research that has gone into optimising reaction conditions for the customised PCR kits currently available for the diagnosis of chlamydial or HIV infection makes them far more consistent and reliable and in part justifies the considerably higher cost. Even at this price the speed, simplicity and diagnostic potential of PCR should give it a place in a routine laboratory. One other factor which may have to be considered is the cost of any licence fee payable if the technique is to be used for diagnostic purposes.

5.2.2 Extent of utility of PCR in haematological diagnosis

The methods used to determine the potential for utilisation of PCR are only adequate to give a rough guideline. Peripheral blood samples from wards other than the two Haematology/ Oncology Units were not included for practical reasons, even though the general medical wards would definitely be a source of small numbers of appropriate samples. In addition, wax embedded pathological specimens and fine needle aspirates represent another source of suitable material not analysed. Conversely, PCR analysis of all the samples included may not have been appropriate, for example repeated analysis of bone marrow obviously involved by leukaemia is unlikely to provide further valuable information. The

sensitivity of the PCR technique being used would also have to be considered to determine whether analysis of a particular sample is justified.

However, within these constraints the large numbers of potentially suitable samples would definitely warrant the availability of PCR for routine use in the diagnostic laboratories of larger referral centres. If advantage is to be taken of the rapidity with which results can be obtained using PCR, batching of samples will probably only be possible for analysis of immunoglobulin and TCR gene rearrangements and possibly for the detection of the t(14;18) in NHL. Other markers of clonality would have to be analysed on an individual basis. This would prevent the use of shared controls and increase costs.

Marrow aspirates and peripheral blood samples were not analysed to determine the number of cases for which the use of PCR to detect the presence of pathogens or hereditary abnormalities may have been valuable.

5.3 CONCLUSION

PCR is a simple, rapid and extremely powerful technique. It is also relatively inexpensive. With increasing knowledge of the human genome and improved understanding of the molecular basis of blood diseases, more and more potential applications for PCR are being discovered. However, for routine diagnostic purposes it needs to be made more reliable and consistent. This will necessitate extensive research and development of each set of primers to be employed and, at least for the developmental phase, its use should probably be confined to a few major centres, so that appropriate quality control measures can be applied and equipment and reagents can be optimally utilised. In addition, because of the power of PCR to detect small amounts of target DNA, research is still needed to assess the significance of positive findings. However, once these issues are addressed, it should definitely find a place as a routine diagnostic tool in the haematology laboratory.

APPENDIX I

BREAKDOWN OF COST ANALYSIS FOR A SINGLE PCR REACTION

1. DNA EXTRACTION (Jeffrey's method)	Rand
a) Disposable Equipment:	
4 x 15 ml Polypropylene Tubes @ R4.95 per 50	0.40
2 x Disposable Pasteur Pipettes @ R21 per 250	0.17
1 x 1.5 ml Eppendorf Microcentrifuge Tube@ R234 per 1000	0.23
b) Reagents:	
10 ml Triton-saline (0.2% v/v Triton in 0.9% NaCl)	0.02
5 ml Lysis Buffer (0.3 M Na Acetate, 20 mM Tris, 1 mM EDTA)	0.03
250 µl 10% SDS	0.03
10 ml Phenol/Chloroform/Isoamyl Alcohol/ 8-hydroxyquinolone	0.08
5 ml Chloroform/Isoamyl Alcohol	0.04
10 ml Absolute Ethanol	0.10
200 µl TE Buffer (1 mM EDTA, 10 mM Tris)	0.01
Total cost of DNA Extraction per sample	**1.10**

2. PCR REACTION (Using 100 µl Reaction Volume)	
Disposable Equipment:	**Rand**
1 x 0.5 ml Mini-Eppendorf Microcentrifuge Tube @ R640 per 2000	0.32
5 x Pipette Tips @ R23.95 per 1000	0.12
Reagents:	
2.5 U Taq DNA Polymerase (Promega Enzyme -SA Scientific) @ R881.22 per 500 U	4.40
10 µl 10 x Reaction Buffer(included with enzyme)	
6 µl 25 mM $MgCl_2$ (included with enzyme)	
50 picomoles of each primer @ R353 per 0.2 µmole (24 mer) @ 0.09c each	0.18
0.05 µmoles of each dNTP @ R885.78 per set of 40 µmoles of each of dATP, dCTP, dTTP and dGTP	0.33
50 µl Light Mineral Oil @ R25 per 500 ml	0.21
Total cost of PCR Reaction per sample	**5.35**

3. GEL ELECTROPHORESIS

a) **Disposable Equipment:**	**Rand**
1 x Pipette Tips @ R23.95 per 1000	0.03
20 cm Parafilm @ R56.98 per 40 metres	0.28
b) Reagents (assuming gel with 12 wells used with one well containing Molecular Weight Marker V, one positive and one negative control and 9 sample wells);	
1.5 g Agarose @ R2066 per 500 g - R6.20 per 9 samples	0.68
400 ml 1 x TAE Buffer - 58c per 9 samples	0.06
3 μl Ethidium Bromide (conc 10 mg/ml) @ R114 per 2 g	0.01
2 μl DNA Molecular Weight Marker V (Boehringer Mannheim) @ R421 per 50 μg (conc 250 ug/ml) R4.20 per 9 samples	0.46
Total cost of Gel Electrophoresis per sample	**1.51**

TOTAL COST OF PCR ANALYSIS, INCLUDING DNA EXTRACTION AND VISUALISATION BY AGAROSE GEL ELECTROPHORESIS **7.96**

NOTE:

1. Prices quoted for disposable equipment and standard reagents, such as absolute ethanol, are those obtained by the Central Buying Department of the SAIMR from routine suppliers.
2. Prices of specially prepared solutions and buffers are calculated from the cost of their constituent components as quoted in the Boehringer Mannheim Biochemica Catalogue for 1993 (calculations not shown).
3. Prices of reagents for the PCR reaction itself, with the exception of the primers, are those quoted for Promega products, currently the cheapest available. The cost of primers is that charged by the Biochemistry Department of the University of the Witwatersrand (R65 basic charge plus R12 per coupling). The primers are used unpurified.

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REFERENCES

ALBERTS B, BRAY D, LEWIS J, RAFF M, ROBERTS K, WATSON JD (Editors). Molecular Biology of the Cell. 2nd Edition. Garland Publishing Inc. New York and London.

ALT FW, BLACKWELL TK, YANCOPOULOS GD. Development of the primaryantibody repetoire. Science 1987; 238: 1079-1087.

BEISHUIZEN A, HAHLEN K, HAGEMEIJER A, VERHOEVEN MJ, HOOIJKAAS H, ADRIAANSEN HJ. Multiple rearranged immunoglobulin genes in childhood acute lymphoblastic leukaemia of precursor B cell origin. Leukaemia 1991 (a); 5: 657-667.

BEISHUIZEN A, HAHLEN K, VAN WERING ER, VON DONGEN JJM. Detection of minimal residual disease in childhood leukaemia with the polymerase chain reaction. New Engl J Med 1991 (b); 324: 772-773.

BELL J. The polymerase chain reaction. Immunology Today 1989; 10: 351-355.

BILLADEAU B, BLACKSTADT M, GREIPP P, KYLE RA, OKEN MM, et al. Analysis of B lymphoid Malignancies using Allele Specific Polymerase Chain Reaction: A Technique for sequential quantitation of residual disease. Blood 1991: 78: 3021-3029.

BIONDI A, RAMBALDI A, PANDOLFI P, ROSSI V, GIUDICI G, ALCALAY M, LOCOCO F. Molecular monitoring of the myl/retinoic acid receptor α fusion gene in Acute Promyelocytic Leukaemia by Polymerase Chain Reaction. Blood 1992; 80: 492-497.

BIRD J, GALILI N, LINK M, STITES D, SKLAR J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukaemia. J Exp Med 1988; 168: 229-245.

BORROW J, GODDARD A, GIBBONS B, KATZ F, SWIRSKY D, FIORET OST, et al. Diagnosis of acute promyelocytic leukaemia by RT-PCR: Detection of PML-RARA and RARA-PML fusion transcripts. Br J Haematol 1992; 82: 529-540.

BOWDEN DK, VICKERS MA, HIGGS DR. A PCR based strategy to detect the common severe determinants of α thalassaemia. Br J Haematol 1992; 81: 104-108.

BRENNER MB, McLEAN J, DIALYNAS DP, STROMINGER JL, SMITH JA, OWEN FL, et al. Identification of a putative second T cell receptor. Nature 1986; 322: 145-149.

BRISCO MJ, TAN LW, OSBORN AM, MORLEY AA. Development of a highly sensitive assay based on the polymerase chain reaction, for rare B-lymphocyte clones in a polyclonal population. Br J Haematol 1990; 75: 163-167.

BROWN L, CHENG J-T, CHEN Q, SICILIANO MJ, CRIST W, BUCHANAN G, BAER R. Site specific recombination of the tal-1 gene is a common occurrence in human T cell leukaemia. EMBO 1990; 9: 3343-3351.

CAMPANA D, COUSTON-SMITH E, JANOSSY G. The detection of minimal residual disease in acute leukaemia. Blood 1990; 76: 163-171.

CHANG KS, LU J, WANG G, TRUJILLO JM, ESLEY E. The t(15;17) breakpoints in Acute Promyelocytic leukaemia cluster within two different sites of the myl gene: Targets for detection of Minimal Residual Disease by the Polymerase Chain Reaction. Blood 1992; 79: 554-558.

CHEN CS, MEDBERRY PS, ARTHUR DC, KERSEY JH. Breakpoint clustering in t(4;11) (q21;q23) acute leukaemia. Blood 1991; 78: 2498-2504.

CHEN Q, CHENG J-T, TSAI L-H, SCHNEIDER N, BUCHANAN G, CARROLL A, CRIST W, OZANNE B, SCILIANO MJ, BAER R. The tal-1 gene undergoes chromosomal translocation in T cell leukaemia and potentially encodes a helix-loop-helix protein. EMBO 1990; 9: 415-424.

CHEN Z, LE PASTIER D, DAUSSET J, DEGOS L, FLANDRIN G, COHEN D, SIGAUX F. Human T cell γ genes are frequently rearranged in B-lineage acute lymphoblastic leukemias but not in chronic B cell proliferations. J Exp Med 1987; 165: 1000-1015.

CHENG GY, MINDEN MD, TOYONAGA B, MAK TW, MCCULLOCH EA. T cell receptor and immunoglobulin gene rearrangements in acute myeloblastic leukaemia. J Exp Med 1986; 163: 414-424.

CLEARY ML, SKLAR J. Lymphoproliferative disorders in cardiac transplant recipients are multiclonal lymphomas. Lancet 1984; ii: 489-493.

CLEARY ML, MEEKER TC, LEVY S, LEE E, TRELA M, SKLAR J, LEVY R. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. Cell 1986; 44: 97-106.

CORBALLY N, GROGAN L, DERVAN PA, CARNEY DN. The detection of specific gene rearrangements in non-Hodgkin's lymphoma using the polymerase chain reaction. Br J Cancer 1992; 46: 805-809.

CRESCENZI M, SETO M, RAFFELD M, COHEN P, COSSMAN J. Thermostable DNA polymerase chain amplification of the t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 1988; 85: 4869-4873.

CRIST WM, CARROLL AJ, SHUSTER JJ, BEHM FG, WHITEHEAD M, VIETTE TJ, LOOK AT, MAHONEY D, RAGAB A, PULLEN DJ, LAND VJ. Poor prognosis of children with pre B acute lymphoblastic leukaemia is associated with the t(1;19) (q23;p13): A Paediatric Oncology Group Study. Blood 1990; 76: 117-121.

CROCE CM, SHANDER M, MARTINIS J, CICUREL L, DIANCONA GG, DOLBY TW, KOPROWSKI H. Chromosomal location of the genes for human immunoglobulin heavy chains. Proc Natl Acad Sci 1979; 76: 3416-3419.

DALLA FAVERA R, BREGNI M, ERIKSON J, PATTERSON D, GALLO RC, CROCE CM. Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitts lymphoma cells. Proc Natl Acad Sci USA 1982; 79: 7824-7827.

DAVEY MP, BONGIOVANNI KF, KAULFRESCH W, QUERTERMOUS T, SEIDMAN JG. Immunoglobulin and T cell receptor gene rearrangement and expression in human lymphoid leukaemia at different stages of maturation. Proc Natl Acad Sci USA 1986; 83: 8759-8763.

DAVIES DR, METZGER H. Structural basis of antibody function. Annu Rev Immunol 1983; 1: 87-117.

DE JONG D, VOETDIJK BMH, VAN OMMEN GHB, KLUIN PM. Alterations in immunoglobulin genes reveal the origin and evolution of monotypic and bitypic B cell lymphomas. Am J Pathol 1989; 134: 1233-1242.

DEANE M, NORTON JD. Detection of immunoglobulin gene rearrangement in B lymphocyte malignancies by polymerase chain reaction gene amplification. Br J Haematol. 1990; 74: 251-256.

DEANE M, McCARTHY KP, WEIDEMAN N, NORTON JD. An improved method for detection of B lymphoid clonality by Polymerase Chain Reaction. Leukaemia 1991; 5: 726-730.

DEANE M, NORTON JD. Immunoglobulin gene 'finger printing': An approach to analysis of B lymphoid clonality in lymphoproliferative disorders. Br J Haematol 1991; 77: 274-281.

DELAGE F, SOIFFER RJ, DEAR K, RITZ J. Clinical significance of bcr-abl gene rearrangement detected by polymerase chain reaction after allogeneic bone marrow transplantation in chronic myelogenous leukaemia. Blood 1991; 78: 2759-2767.

DESIDERIO SV, YANCOPOULOS GD, PASKIND M, THOMAS E, BOSS MA, LANDAU N, ALT FW, BALTIMORE D. Insertion of N regions into heavy chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984; 311: 752-755.

DOW LW, MARTIN P, MOOHR J, GREENBERG M, MACDOUGALL L. Evidence for clonal development of childhood acute lymphoblastic leukaemia. Blood 1985; 66: 902.

EPPLEN JT, BARTELS F, BECKER A, NERZ G, PRESTER M, RINALDY A, SIMON MM. Change in antigen specificity of cytotoxic T lymphocytes is associated with the rearrangement and expression of a T cell receptor B chain gene. Proc Natl Acad Sci USA 1986; 83: 4441-4445.

ERLICH HA, GELFAND DH, SAIKI RK. Specific DNA amplification. Nature 1988; 331: 461-462.

FARR CJ, SAIKI RK, ERLICH HA, MCCORMICK F, MARSHALL C. Analysis of ras gene mutations in acute myeloid leukaemia by polymerase chain reaction and oligonucleotide probes. Proc Natl Acad Sci USA 1988; 85: 1629-1633.

FEY MF, KULOZIK AE, HANSEN-HAGGE TE, TOBLER A. The Polymerase Chain Reaction: A new tool for detection of Minimal Residual Disease in Haematological Malignancies.
Eur J Cancer 1991; 27: 89-94.

FLANAGAN JG, RABBITTS TH. Arrangement of human immunoglobulin heavy chain constant regions implies evolutionary duplication of a segment containing γ, E and α genes. Nature 1982; 300: 709-713.

GAIDANO G, BALLERINI P, GONG J, INGHIRAMI G, NERI A, NEWCOMB EW, MAGRATH IT, KNOWLES DM, DALLA FAVERA R. P53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukaemia. Proc Natl Acad Sci USA 1991; 88: 5413-5417.

DEANE M, NORTON JD. Immunoglobulin gene 'finger printing': An approach to analysis of B lymphoid clonality in lymphoproliferative disorders. Br J Haematol 1991; 77: 274-281.

DELAGE F, SOIFFER RJ, DEAR K, RITZ J. Clinical significance of bcr-abl gene rearrangement detected by polymerase chain reaction after allogeneic bone marrow transplantation in chronic myelogenous leukaemia. Blood 1991; 78: 2759-2767.

DESIDERIO SV, YANCOPOULOS GD, PASKIND M, THOMAS E, BOSS MA, LANDAU N, ALT FW, BALTIMORE D. Insertion of N regions into heavy chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984; 311: 752-755.

DOW LW, MARTIN P, MOOHR J, GREENBERG M, MACDOUGALL L. Evidence for clonal development of childhood acute lymphoblastic leukaemia. Blood 1985; 66: 902.

EPPLEN JT, BARTELS F, BECKER A, NERZ G, PRESTER M, RINALDY A, SIMON MM. Change in antigen specificity of cytotoxic T lymphocytes is associated with the rearrangement and expression of a T cell receptor B chain gene. Proc Natl Acad Sci USA 1986; 83: 4441-4445.

ERLICH HA, GELFAND DH, SAIKI RK. Specific DNA amplification. Nature 1988; 331: 461-462.

FARR CJ, SAIKI RK, ERLICH HA, MCCORMICK F, MARSHALL C. Analysis of ras gene mutations in acute myeloid leukaemia by polymerase chain reaction and oligonucleotide probes. Proc Natl Acad Sci USA 1988; 85: 1629-1633.

FEY MF, KULOZIK AE, HANSEN-HAGGE TE, TOBLER A. The Polymerase Chain Reaction: A new tool for detection of Minimal Residual Disease in Haematological Malignancies.
Eur J Cancer 1991; 27: 89-94.

FLANAGAN JG, RABBITTS TH. Arrangement of human immunoglobulin heavy chain constant regions implies evolutionary duplication of a segment containing γ, E and α genes. Nature 1982; 300: 709-713.

GAIDANO G, BALLERINI P, GONG J, INGHIRAMI G, NERI A, NEWCOMB EW, MAGRATH IT, KNOWLES DM, DALLA FAVERA R. P53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukaemia. Proc Natl Acad Sci USA 1991; 88: 5413-5417.

HIETER PA, HOLLIS GF, KORSMEYER SJ, WALDMAN TA, LEDER P. Clustered arrangement of immunoglobulin lambda constant region genes in man. Nature 1981; 294: 536-540.

HIETER PA, MAX EE, SEIDMAN JG, MAIZEL JB, LEDER P. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 1980; 22: 197-207.

HOLLIS GF, MITCHELL KF, BATTEY J, POTTER H, TAUB R, LENOIR GM, LEDER P. A variant translocation places the λ immunoglobulin genes 3′ to the c-myc oncogene in Burkitt's lymphoma. Nature 1984; 307: 752-753.

HUGHES TP, MORGAN GJ, MARTIAT P, GOLDMAN JM. Detection of Residual Leukaemia after Bone Marrow Transplantation for Chronic Myeloid Leukaemia: Role of Polymerase Chain Reaction in Predicting Relapse. Blood 1991; 77: 874-878.

HUNGER SP, GALILI N, CARROLL AJ, CRIST WM, LINK MP. CLEARY ML. The t(1;19) (q23;p13) results in consistent fusion of E2A and PBXI coding sequences in acute lymphoblastic leukaemia. Blood 1991; 77: 678-693.

ICHIKAWA A, HOTTA T, TAKAGI N, TSUSHITA K, KINASHITA T, NGAI M, MURAKAMI Y, HAYASHI K, SAITO H. Mutations of p53 gene and their relation to disease progression in B-cell lymphoma. Blood 1992; 79: 2701-2707.

IKUTA K, AGURA T, SHIMIZU A, HONJO T. Low frequency of somatic mutation in B chain variable region genes of human T cell receptors. Proc Natl Acad Sci USA 1985; 82: 7701-7705.

JEFFREYS AJ. Method for extraction of genomic DNA from blood. In: Experimental Manual in Molecular Biology (Leicester University Press). 1985 (Addendum).

JONSSON OG, KITCHENS RL, BAER RJ, BUCHANAN GR, SMITH RG. Rearrangements of the tal-1 locus as clonal markers for T cell acute lymphoblastic leukaemia. J Clin Invest 1991; 88: 2029-2035.

JONSSON OG, KITCHENS RL, SCOTT FC., GRAHAM-SMITH R. Detection of Minimal Residual Disease in Acute Lymphoblastic Leukaemia using Immunoglobulin Hypervariable Region Specific Oligonucleotide Probes. Blood 1990; 76: 2072-2079.

KADIN ME, VONDERLEID EC, SAKO D, CLAYTON LK, OLBRICHT S. Clonal composition of T cells in lymphoid papulosis. Am J Pathol 1987; 126: 13-17.

KAGAN J, FINGER LR, BESA E, CROCE CM. Detection of minimal residual disease in leukaemic patients with the t(10;14) (q24;q11) chromosomal translocation. Cancer Res. 1990; 50: 5240-5244.

KAMPS MP, MURRE C, SUN X-H, BALTIMORE D. A new homeobox gene contributes to the DNA binding domain of the t(1;19) translocation protein in pre B ALL. Cell 1990; 60: 547-552.

KATZ F, BALL L, GIBBONS B, CHESSELLS J. The use of DNA probes to monitor minimal residual disease in childhood acute lymphoblastic leukaemia. Br J Haematol 1989; 73: 173-180.

KAWASAKI ES, CLARK SS, COYNE MY, SMITH SD, CHAMPLIN R, WITTE ON, McCORMICK FP. Diagnosis of chronic myelogenous leukaemia and acute leukaemia by detection of leukaemia-specific mRNA sequences amplified in vitro. Proc Natl Acad Sci USA 1988; 85: 5698.

KITCHINGMAN GR, MIRRO J, STASS S, ROVIGATTI V, MELVIN SL, et al. Biologic and prognostic significance of more than two μ heavy chain genes in childhood acute lymphoblastic leukaemia of precursor B cell origin. Blood 1986; 67: 698-703.

KLEINFELD R, HARDY RR, TARLINTON D, DANGL J, HERZENBERG LA, WEIGERT M. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly1+ B cell lymphoma. Nature 1986; 322: 843-846.

KORSMEYER SJ, HIETER PA, RAVETCH JV, POPLACK DG, WALDMAN TA, LEDER P. Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pre B cells. Proc Natl Acad Sci USA 1981; 78: 7096-7100.

KORSMEYER SJ. Bcl-2 Initiates a New Category of Oncogenes: Regulators of cell death. Blood 1992; 80: 879-886.

KWOK S, HIGUCHI R. Avoiding false positives with PCR. Nature 1989; 339: 237-238.

KYLE RA, LUST JA. Monoclonal gammopathies of undetermined significance. Semin Hematol 1989; 26: 176-200.

LANGO MC, BERNINGER MS, HARTLEY JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene 1990; 93: 125-128.

LEBO RV, SAIKI RK, SWANSON K, MONTANO MA, EHRLICH HA, GOLBUS MS. Prenatal diagnosis of α thalassaemia by polymerase chain reaction and dual restriction enzyme analysis. Human Genet 1990; 85: 293-299.

LEDER P. The genetics of antibody diversity. Sci Am 1982; 246: 102-115.

LEE MS, CHANG KS, FREIREICH J, KANTARJIAN HM, TALPAZ M, TRUJILLO JM, STASS SA. Detection of minimal residual bcr\abl transcripts by a modified polymerase chain reaction. Blood 1988; 72: 893-897.

LEE MS, LE MAISTRE A, KANTARJIAN HM, TALPAZ M, FREIREICH EJ, TRUJILLO JM, STASS SA. Detection of two alternative bcr/abl mRNA junctions and minimal residual disease in Philadelphia chromosome positive chronic myelogenous leukaemia by Polymerase Chain Reaction. Blood 1989; 73: 2165-2170.

LEE MS, CHANG KS, CABANILLAS F, FREIREICH EJ, TRUJILLO JM, STASS SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 1987; 237: 175-178.

LIANG R, CHAN V, CHAN TK, WONG T, CHIU E, TODD D. Detection of immunoglobulin gene rearrangements in Acute and Chronic Lymphoid Leukaemias of B-cell Lineage by Polymerase chain reaction gene amplification. Am J Hematol 1991; 38: 189-193.

LIMPENS J, DE JONG D, VOETDIJK A. Translocation t(14;18) in benign B lymphocytes. Blood 1990; 76: 237a (abstract).

LIST AF, GRECO FA, VOGLER LB. Lymphoproliferative disease in immunocompromised hosts: The role of Epstein-Barr virus. J Clin Oncol 1987; 5: 1673-1689.

LOH EY, CWIRLA S, SERAFINI AT, PHILIPS JH, LANIER LL. Human T-cell receptor δ chain: Genomic organisation diversity and expression in populations of cells. Proc Natl Acad Sci USA 1988; 85: 9714-9718.

LONG R, CHAN V, CHAN TK, WONG T, CHIU E, TODD D. Detection of Immunoglobulin Gene Rearrangements in Acute and Chronic Lymphoid Leukaemias of B-cell Lineage by Polymerase Chain Reaction Gene Amplification. Am J Hematol 1991; 38: 189-193.

LOUGHRAN TP Jr, COYLE T, SHERMAN MP, STARKEBAUM G, EHRLICH GD, RUSCETTI FW, POIESZ BJ. Detection of human T-cell leukaemia/lymphoma virus, type II in a patient with large granular lymphocyte leukaemia. Blood 1992; 80: 1116-1119.

LOWENBERG B, TOUW IP. Practical aspects and diagnostic significance of in vitro manipulation of progenitors in human acute myeloid and lymphoid leukaemia. Bailliere's Clinical Haematology 1991; 4: 637-653.

LUBBERT M, MIRRO J, MILLER CW. N-Ras point mutations in childhood acute lymphoblastic leukaemia correlate with a poor prognosis. Blood 1990; 75: 1163-1169.

LUGO TG, PRENDERGAST A-M, MULLER AJ, WITTE ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 1990; 247: 1079.

MACINTYRE E, D'AURIOL L, DUPARC N, LEVEREGER G, GALIBERT F, SIGAUX F. Use of oligonucleotide probes, directed against T cell antigen receptor Gamma Delta variable-(diversity)-joining junctional sequences as a general method for detecting minimal residual disease in acute lymphoblastic leukaemia. J Clin Invest 1990; 86: 2125-2135.

MANIATIS T, FRITSCH EF, SAMBROOK J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1982.

MARRACK P, KAPPLER J. The T cell receptor. Science 1987; 238: 1073-1079.

MAROLLEAU JP, FONDELL JD, MALISSEN M, TRUCY J, BARBIER E, et al. The joining of germline Vα to Jα genes replaces pre-existing Vα to Jα complexes in a T cell receptor α, B positive T cell line. Cell 1988: 55: 291-300.

MARX JL. Multiplying genes by leaps and bounds. Science 1988; 240: 1408-1410.

MCBRIDE OW, HIETER PA, HOLLIS GF, SWAN D, OTEY MC, LEDER P. Chromosomal location of human kappa and lambda immunoglobulin light chain constant regions. J Exp Med 1982; 155: 1480-1490.

MCCULLOCH EA. Stem cells in normal and leukemic hemopoiesis. Blood 1983; 62: 1-8.

MEYERS FJ, MILLER CH, LEWIS JP. The resolution of phenotypic ambiguity in a case of human leukaemia using cytogenetics and immunoglobulin gene rearrangement. Am J Clin Path 1986; 85: 372-374.

MILTENBURG AMM, VAN LAAR JM, DAHA MR, DE VRIES RRP, VAN DER ELSEN PJ, BREEDVELD FC. Dominant T cell receptor B chain gene rearrangements indicate clonal expansion in the rheumatoid joint. Scand J Immunol 1990; 31: 121-125.

MULLIS KB. The unusual origin of the Polymerase Chain Reaction. Sci Am 1990; April: 36-43.

NEALE GAM, MENARGUEZ J, KITCHINGMAN G, FITZGERALD TJ, KOEHLER M, MIRRO J, GOORHA RM. Detection of Minimal Residual Disease in T-cell acute lymphoblastic leukaemia using Polymerase Chain Reaction predicts impending relapse. Blood 1991; 78: 739-747.

NIZET Y, MARTIAT P, VAERMAN JL, PHILIPPE M, WILDMANN C, STAELENS JP. Follow up of residual disease (MRD) in B lineage acute leukemias using a simplified PCR strategy: Evolution of MRD rather than its detection is correlated with clinical outcome. Br J Haematol 1991; 79: 205-210.

NORDVAG B-Y, HUSBY G, EL-GEWELY MR. Direct PCR of Washed Blood Cells. Bio Techniques 1992; 12: 490-493.

NUSS R, KITCHINGMAN G, et al. T cell receptor gene rearrangements in B precursor acute lymphoblastic leukaemia correlates with age and stage of B cell differentiation. Leukaemia 1988; 2: 722-727.

ORITA M, SUZUKI Y, SEKIYA T, HAYASHI K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989; 5: 874-879.

PANACCIO M, LEW A. PCR based diagnosis in the presence of 8% (v/v) blood. Nucleic Acids Res 1991; 19: 1151.

PEAKE IR, BOWEN D, BIGNELL P, LIDDELL MB, SADLER JE, STANDEN G, BLOOM AL. Family studies and prenatal diagnosis in severe von Willebrand's Disease by Polymerase Chain Reaction amplification of a variable number tandem repeat region of the von Willebrand factor gene. Blood 1990; 76: 555-561.

PERRIN LH, YERLY S, ADAMI N. Human immunodeficiency virus DNA amplification and serology in blood donors. Blood 1990; 76: 641-645.

POTTER MN, STEWARD CG, OAKHILL A. The significance of detection of minimal residual disease in childhood acute lymphoblastic leukaemia. Br J Haematol 1993; 83: 412-418.

POTTER MN, STEWARD CA, MAITLAND NJ, OAKHILL A. Detection of clonality in childhood B-lineage acute lymphoblastic leukaemia by the polymerase chain reaction. Leukaemia 1992; 6: 289-294.

PRICE CGA, MEERABUX J, MURTAGH S, COTTER FE, ROHATINER AZS, et al. The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 1991; 9: 1527-1532.

PUI CH, CRIST W, LOOK AT. Biology and clinical significance of cytogenetic abnormalities in Childhood Acute Lymphoblastic Leukaemia. Blood 1990; 76: 1449-1463.

QUERTERMOUS T, MURRE C, DIALYNAS D, DUBY AD, STRAMINGER JL. Human T cell γ chain genes: Organisation, diversity and rearrangement. Science 1986; 31: 252-255.

RABBITTS TH, LEFRANC MP, STINSON MA, SIMS JE, SCHRODER TS, STEINMETZ M. The chromosomal location of T cell receptor genes and a T cell rearranging gene: Possible correlation with specific translocation in human T cell leukaemia. EMBO 1985; 4: 1461-1465.

RAGHAVACHAR A, THIEL E, BARTRAM CR. Analysis of phenotype and genotype in acute lymphoblastic leukaemias at first presentation and in relapse. Blood 1987; 70: 1079-1083.

RAIMONDI SC, BEHM FG, ROBERSON PK, PUI CH, RIVERA GK, MURPHY SB, WILLIAMS DL. Cytogenetics of childhood T cell leukaemia. Blood 1988; 72: 1560-1567.

RAMASAMY I, BRISCO M, MORLEY A. Improved PCR method for detecting monoclonal immunoglobulin heaving chain rearrangement in B cell neoplasms. J Clin Pathol 1992; 45: 770-775 (no 110).

RAVETCH JV, SIEBENLIST U, KORSMEYER S,S WALDMANN T, LEDER P. Structure of the human immunoglobulin μ locus: Characterisation of embryonic and rearranged J and D genes. Cell 1981; 27: 583-591.

RETH ME, JACKSON S, ALT FW (a). VHD JH formation and DJH replacement during pre-B differentiation: non-random usage of gene segments. EMBO 1986; 5: 2131-2138.

RETH M, GEHRMANN P, PETICE E, WIESE P (b). A novel VH to VHDJH joining mechanism in heavy chain negative (null) pre-B cells results in heavy chain production. Nature 1986; 322: 840-842.

SAIKI RK, SCHARF S, FALOONA F, et al. Enzymatic amplification of B Globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 1985; 230: 1350-1354.

SHEIBANI K, WU A, BEN EZRA J, STROUP R, RAPPAPORT H, WINBERG C. Rearrangement of K chain and T-cell receptor B-chain genes in malignant lymphomas of T-cell phenotype. Am J Pathol 1987; 129: 201-207.

SHIRAMIZU B, MAGRATH I. Localisation of breakpoints by polymerase chain reactions in Burkitt's lymphoma with 8;14 translocations. Blood 1990; 75: 1848-1852.

SOMMER R, TAUTZ D. Minimal homology requirements for PCR primers. Nucleic Acids Res 1989; 17: 6749.

SOUTHERN EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 1975; 98: 503-517.

STARZL TE, PORTER KA, IWATSUKI S, ROSENTHAL JT, SHAW BW, ATCHISON RW. Reversibility of lymphomas and lymphoproliferative lesions developing under cyclosporin-steroid therapy. Lancet 1984; i: 583-587.

SWAIN SL. T cell subsets and the recognition of MHC class. Immunol Rev 1983; 74: 129-142.

SYKES PS, NEOH SH, BRISCO MJ, HUGHES E, CONDON J, MORLEY AA. Quantitation of targets for PCR by use of limiting dilution. Biotechniques 1992; 13: 444-449.

TAKIHARA Y, TKACHUK D, MICHALOPOULOS E, CHAMPAGN E, REIMANN J. Sequence and organisation of the diversity, joining and constant region genes of the human T cell δ-chain locus. Proc Natl Acad Sci USA 1988; 85: 6097-6101.

TALMUD P, TYBJAERG-HANSEN A, BHATNAGR D, MBEWU A, MILLER JP, DURRINGTON P, HUMPHRIES S. Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolaemia. Atherosclerosis 1991; 89: 137-141.

TAUB R, KELLY K, BATTEY J, LATT S, LENOIR GM, TU Z, LEDER P. A novel alteration in the structure of an activated c-myc gene in a variant t(2,8) Burkitt lymphoma. Cell 1984; 37: 511-516

TAYLOR JJ, ROWE D, WILLIAMSON IK, CHRISTMAS SE, PROCTOR SJ, MIDDLETON PG. Detection of T cell receptor γ chain V gene rearrangements using the Polymerase Chain Reaction: Application to the study of clonal disease cells in acute lymphoblastic leukaemia. Blood 1991; 79: 1989-1995.

TEILLAUD J-L, DEGAYMORD C, GIUSTI AM, HASELTINE B, POLLOCK RR, YELTON DE, SACK DJ, SCHARFF MD. Monoclonal antibodies reveal the structural basis of antibody diversity. Science 1983; 222: 721-726.

TERADA N, MIYOSHI J, KAWATLA K. Alteration of N-Ras gene mutation after relapse in acute lymphoblastic leukaemia. Blood 1990; 75: 453-457.

TKACHUK DC, KOHLER S, CLEARY ML. Involvement of a homolog of Drosophila trithorax by 11q23 chromosomal translocation in acute leukaemias. Cell 1992; 71: 691-700.

TOKSOZ D, FARR CJ, MARSHALL CJ. ras genes and acute myeloid leukaemia. Br J Haematol 1989; 71: 1-6.

TONEGAWA S. Somatic generation of antibody diversity. Nature 1983; 302: 575-581.

TOYONGAGA B, YOSHIKAI Y, VADAZ V, CHIN B, MAK TW. Organisation and sequences of the diversity joining and constant region genes of the human T-cell receptor ß chain. Proc Natl Acad Sci USA 1985; 82: 8624-8628.

TRAINOR KJ, BRISCO MJ, STORY CJ, MORLEY AA. Monoclonality in B lymphoproliferative disorders detected at the DNA level. Blood 1990; 75: 2220-2222.

TRAINOR J, BRISCO MH, WAN JH, NEOH S, GRIST S, MORLEY AA. Gene rearrangement in B and T lymphoproliferative disease detected by the Polymerase Chain Reaction. Blood 1991; 78: 192-196.

VAN DONGEN JJM, WOLVERS-TETTERO ILM. Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative disease and related disorders. Clinica Chimica Acta 1991; 198: 93-174.

VAN DONGEN JJM, WOLVERS-TETTERO ILM, WASENAAR F, BORST J, VAN DEN ELSEN P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukaemia. Blood 1989; 74: 334-342.

VAN HELDEN PD, TOIT R, JORDAAN A, et al. The use of the polymerase chain reaction in the diagnosis of tuberculosis. S Afr Med J 1991; 80: 616-619.

VEELKEN J, TYCKO B, SKLAR J. Sensitive detection of clonal antigen receptor gene rearrangements for the diagnosis and monitoring of lymphoid neoplasms by a polymerase chain reaction-mediated ribonuclease protection assay. Blood 1991; 78: 1318-1326.

VON LINDERN M, POUSTKA A, LERACH H, GROSVELD G. The (6;9) chromosome translocation, associated with a specific subtype of non-lymphocytic leukaemia leads to aberrant transcription of a target gene on 9 q34. Mol Cell Biol 1990; 10: 4016-4026.

WAN JH, SYKES PJ, ORELL SR, MORLEY AA. Rapid method for detecting monclonality in B cell lymphoma in lymph node aspirates using the polymerase chain reaction. J Clin Pathol 1992; 45: 420-433.

WAN JH, TRAINOR KJ, BRISCO MJ, MORLEY AA. Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. Clin Pathol 1990; 43: 888-890.

WASSERMAN R, YAAMDA M, ITO Y, FINGER LR, REICHARD BA. VH Gene rearrangement events can modify the immunoglobulin heavy chain during progression of B lineage acute lymphoblastic leukaemia. Blood 1992; 79: 223-228.

WEISS LM, WARNKE RA, SKLAR J, CLEARY ML. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. N Engl J Med 1987; 317: 1185-1189.

WILLIAMS DL, RAIMONDI S, RIVERA G, BERARD CW, GEORGE S, MURPHY SB. Presence of clonal chromosome abnormalities in virtually all cases of acute lymphoblastic leukaemia. N Engl J Med 1985; 313: 640.

WILLIAMS ME, INNES DJ, BOROWITZ MH, LOVELL MA, SWERDLOW SH, HURTUBISE PE. Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukaemia. Blood 1987; 69: 79-86.

WONG C, DOWLING CE, SAIKI RK, et al. Characterisation of B thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 1987; 320: 384-386.

WRIGHT JJ, POPLACK DG, BAKHSHI A, REAMAN G, COLE D, JENSEN JP, KORSMEYER SJ. Gene rearrangements as markers of clonal variation and minimal residual disease in acute lymphoblastic leukaemia. J Clin Oncol 1987; 5: 735-741.

WYSOCKI L, MANSER T, GEFTER ML. Somatic evolution of variable region structures during an immune response. Proc Natl Acad Sci 1986; 83: 1847-1851.

YAMADA M, WASSERMAN R, LANGE B, REICHARD BA, WOMER RB, ROVERA G. Minimal residual disease in childhood B-lineage lymphoblastic leukaemia. New Engl J Med 1990; 323: 448-455.

YOKOTA S, HANSEN-HAGGE TE, LUDWIG WD, REITER A, RAGHAVCHAR A, KLEIHAUER E. Use of Polymerase Chain Reaction to Monitor Minimal Residual disease in Acute Lymphoblastic Leukaemia Patients. Blood 1991; 77: 331-339.

YOSHIKAI Y, SLARK SP, TAYLOR S, SOHN U, WILSON BI, MINDEN MD, et al. Organisation and sequences of the variable, joining and constant region genes of the human T cell receptor α chain. Nature 1985; 316: 837-840.

YUNIS JJ, BRUNNING RD, HOWE RB, LOBELL M. High resolution chromosomes as an independent prognostic indicator in adult acute non lymphocytic leukaemia. N Engl J Med 1984; 311: 812.

ZUTTER MM, MARTIN PJ, SALE GE, SHULMAN HM, FISHER L, THOMAS ED, DURNAM DM. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988; 72: 520-529.

4. RESULTS