OPTIMISATION AND APPLICATION OF FLUORESCENCE IN SITU HYBRIDIZATION IN SELECTED LEUKAEMIAS

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To my wife S. ESKIN

DECLARATION

I certify that this thesis does not contain material previously published or written by any other person, except where referred to in the text. The results in this thesis have not been submitted for any other degree or diploma.

Hasan ACAR

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LIST OF ABBREVIATIONS

abl	homologues DNA sequences of Abelaon's murine leukemia virus (found in the mouse)
AAF	Acetylaminofluorene
ALL	Acute lymphocytic leukaemia
AMCA	Aminomethyl cumarin
AML	Acute myeloid leukaemia
AML-M3	Acute promyeloid leukaemia
AML-M3v	Acute promyeloid leukaemia variant
AP	Alkaline phosphatase
ARS	autonomously replicating sequences
BCIP	5-Bromo-4-chloro-3 indolyphosphate
bcr	Break point cluster region
Bio	Biotin
Biotin-11-dUTP	Biotinylated deoxyuracil 5'-triphosphate
BRL	Bethesda Research Laboratories
CISS	Chromosomal in situ suppression
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
Contig	contiguous sequence
cos	cosmid
Cot	concentration over time
DABCO	Diazobicylo-octane
DAPI	4,6-diamino-2-phenyl-indole
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
Dig	Digoxigenin
DNA	Deoxyribonucleic acid
DNA pol	DNA polymerase
DNP	Dinitrophenol
dTTP	deoxythymidine 5'-triphosphate
e.g.	exempla gratia (for example)
EDTA	Ethylene diamino tetra-acetic-acid
et al.	et alia
FAB-Classification	French-American-British classification
FITC	Fluorescein isothiocyanate
G-banding	Giemsa banding
gm or gr	gram

ISH	In situ hybridisation
kb	Kilobase
Mb	Megabase
mg	milligram
MDS	Myelodysplastic syndrome
ml	millilitre
NBT	Nitroblue tetrazolium chloride
ng	nanogram
OD	Optical density
p	short arm of the chromosome
PBS	phosphate-buffered saline
PCR	Polymerase Chain reaction
Ph Chromosome	Philadelphia chromosome
PHA	phytohaemagglutinin
PNM	phosphate nonidet-P40 milk
PO	Peroxidase
q	long arm of the chromosome
Q-banding	Quinacrine banding
R-banding	Reverse banding
RARA	Retinoic acid receptor alpha gene
RNA	Ribonucleic acid
RNase	Ribonuclease
SA-Ap	Streptavidin alkaline phosphatase conjugate
SSC	Saline sodium citrate
Tm	Melting temperature
TRICT	Tetramethyl-rhodamine-isothiocyanate
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra Violet
v/v	volume per volume
w/v	weight per volume
YAC	Yeast Artificial Chromosome
µg	microgram
µl	microlitre

SUMMARY

The overall aim of this project was to optimise and then apply the technique of dual-colour fluorescence *in situ* hybridization (FISH). The first main aim of this study was optimisation of dual-colour FISH by using α-satellite centromeric probes, chromosome specific libraries and locus specific cosmid probes. The second main aim was the application of uni- and dual-colour FISH using these probes.

At the beginning, to gain experience with the non-isotopic *in situ* hybridization, a highly repetitive probe GMGY10 (DYS59) was applied by using the alkaline phosphatase technique and then the same probe was used for the fluorescence *in situ* hybridization technique.

The next step was optimisation of whole chromosome specific library probes (pBS-21 and pBS-18) for chromosome 21 and chromosome 18. Less than 600 ng (per hybridization area) of the pBS-21 probe was found to be insufficient to give complete coverage of the chromosome. With a higher concentration of pBS-21 (600-700 ng), a complete coverage was obtained. Use of this probe was discontinued since each hybridization required too much of the library probe DNA.

The whole chromosome specific library probe for chromosome 18 (pBS-18) was optimised using 600 ng (per hybridization area) of pBS-18 probe concentration for uni-colour FISH. Using different concentrations of this probe, it was consistently found that the centromeric area of chromosome 2 visualised more strongly than chromosome 18.

The study then progressed to the development of the dual-colour FISH technique. The α-satellite centromeric probes and the locus specific cosmid probes were individually labelled either with biotin by using nick translation,

or with digoxigenin using random | primed labelling or the nick translation system.

The probes to be used in the study were the α-satellite centromeric probes for chromosome 3, 8, 12 and 22, the locus specific cosmid probes for abl and bcr, and library probes 6, 8, 9, and 22.

The first step was to optimise the conditions for each probe for uni-colour FISH. The centromeric probes (for chromosomes 8 and 12) always gave sharp and specific signals.

The hybridization signal intensity of the abl cosmid probe was very specific and bright. In contrast, as reported by others, the locus specific bcr cosmid probes did not give specific sharp signals due to the instability of the probe. However, by manipulating each of the steps in the uni-colour FISH procedure, successful results were obtained with bcr probes.

Following the optimisation of uni-colour FISH using α-satellite repetitive sequence probes for chromosome 8, 12 and 22, and the locus specific cosmid probes for abl and bcr, these probes were optimised for dual-colour FISH. First, in order to overcome the technical difficulties incurred by the dual-colour FISH procedure, all steps were optimised by using α-satellite repetitive sequence probes for chromosomes 8 and 12. Dual-colour FISH results were successfully optimised with those centromeric probes.

Using uni-colour FISH specific parameters for cosmid probes such as probe and competitor DNA concentration and hybridization condition, these probes unfortunately could not tolerate some parameters described for the centromeric probe, such as detection, counterstaining and visualisation of signals. This failure may be due to the size and quality of the probe.

Therefore, these steps were refined for dual-colour FISH of the cosmid abl and bcr probes.

After optimisation of uni- and dual-colour FISH by using the α-satellite probes for chromosomes 8 and 12 , and the locus specific probes for abl and bcr , the techniques were applied to selected leukaemias. Commercial probes were also used either individually or in combination with self-prepared probes.

In this project, three different groups of leukaemias were studied. These are (1) Chronic Myeloid Leukaemia (CML), (2) Acute Promyelocytic Leukaemia (AML-M3) and (3) Chronic Lymphocytic Leukaemia (CLL).

The first group investigated was the CML group. Fourteen cytogenetically Philadelphia (Ph) positive CML cases were evaluated for the presence of the bcr-abl fusion using uni-colour FISH on metaphase spreads. Despite the low number of metaphases obtained, the FISH metaphase results showed the translocation of abl signal from chromosome 9 to the derivative chromosome 22q. These results were found to be in agreement with the cytogenetic results.

Moreover, twelve of Ph negative patients with a provisional diagnosis of CML were studied by using uni-colour FISH on metaphase spreads. Interestingly, one case proved to be bcr-abl fusion positive, while in the remaining cases cytogenetics is in agreement with FISH result. The advantages of using FISH over conventional cytogenetic techniques are: (1) With an adequate number of metaphases, the bcr-abl fusion may be analysed when the number of good quality metaphases is not available, (2) The accurate detection of the bcr-abl fusion could be analysed in case of Ph-negative CML, (3) This approach was found to be rapid and simple without requiring detailed expertise in cytogenetics.

However, since some of these Ph negative cases had extra numerical abnormalities such as trisomy 8, 9 and 21, the centromeric probe 8, the cosmid probe for the abl and the cosmid contig probe for chromosome 21 were applied and the FISH results confirmed the cytogenetic findings.

Furthermore, the abl and bcr cosmid probes were also used in dual-colour FISH on control cases and thirteen CML cases which included eight cases which were cytogenetically Ph positive, three cases which were Ph negative and two cases which had no cytogenetic studies. Dual-colour FISH confirmed the bcr-abl fusion on the eight Ph positive cases. The uni-colour FISH findings of some Ph-positive and -negative cases were confirmed by dual-colour FISH on both metaphase spreads and interphase nuclei. Two cytogenetically unknown cases revealed the bcr-abl fusion on interphase nuclei. From metaphase-FISH study, no metaphases were obtained, but the interphase-FISH study revealed the fusion of the bcr-abl fusion which were basically in agreement with the cytogenetic results.

As a final group in CML, representing a different group, two CML patients having variant translocations were analysed by using uni- and dual-colour FISH techniques with abl and bcr cosmid probes, whole chromosome specific library probes and α-satellite repetitive centromeric probes (commercial and self -prepared probes). The analysis was performed both on interphase nuclei and metaphase spreads. The FISH results were found to be in agreement with where the variant translocations were observed with cytogenetic analysis.

The second group of leukaemias in which FISH was applied was Acute Promyelocytic Leukaemia (AML-M3). This leukaemia has a very specific re-arrangement between chromosomes 15 and 17. Five control and seven AML-M3 and M3v cases were studied. In order to detect the translocation in

interphase nuclei, the dual-colour FISH technique was applied by using commercial specific cosmid probes for the chromosome 15 and 17 break point areas. Application of interphase FISH on the direct preparation showed the fusion of the red-green signals, whereas no metaphases were obtained at cytogenetics analysis. However, cytogenetics were in agreement with FISH results where the t(15;17) is observed at diagnosis. Metaphase-FISH results also agree with cytogenetics. Some cases at follow-up which are cytogenetically negative, but interphase-FISH results showed positive results.

The final group of leukaemia studied was Chronic Lymphocytic Leukaemia (CLL). In this part of the study, uni-colour FISH was applied to detect trisomy 12 in interphase nuclei using an α- satellite repetitive sequence probe for chromosome 12. Twenty six CLL patients were analysed in total and 15 % of the patients were found to have trisomy 12. Some of cases at interphase FISH study exhibited different sized signals in nuclei. Some of these findings were confirmed by applying FISH on metaphase spreads obtained from cultured cells with PHA.

Rapid detection and reliable assessment of structural and numerical abnormalities are very important in cancer genetics. Here, this study concludes that the application of dual-colour FISH on leukaemias may have a great potential in the detection of certain structural rearrangements (translocations), by analysing unstimulated interphase nuclei (when there is lack of metaphase spreads), providing useful information for the clinical cytogenetics. The uni-colour FISH results proved that locus specific cosmid probes could be used for rapid identification of chromosome rearrangements. And also the application of uni-colour FISH with α-satellite repetitive sequence probes is very useful in uncultured cells for the identification of numerical abnormalities.

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1. Outline of genomic DNA structure and theoretical background of nucleic acid hybridisation

In the human genome, deoxyribonucleic acid (DNA) carries the genetic information. This molecule consists of two long chains held together by complementary base pairs. Each chain is a long, unbranched polymer composed of only four types of sub-units. These are the deoxyribonucleotides containing the bases adenine (A), guanine (G), thymine (T) and cytosine (C). The nucleotides are linked together by covalent phosphodiester bonds that join the 5' carbon of one deoxyribose group to the 3' carbon of the next. The four different kind of bases are attached to this repetitive sugar-phosphate chain almost like beads strung on a necklace. The DNA molecule is a helical polymer composed of two strands. The two chains run in opposite directions and are held together by hydrogen bonds between A in one chain and T in the other or between G and C. This base pairing is very specific.

Under normal conditions, the two strands of DNA are held together in a stable configuration. In the DNA structure, the hydrogen bonds are weaker bonds than the covalent bonds and because of this, treating the double-stranded DNA with some chemicals (alkali and formamide) or heating under appropriate conditions causes the complementary strands of DNA to separate and form single strands without separating the base from the sugar-phosphate backbone. When the conditions return to normal such as the temperature falling below the melting temperature (Tm), the complementary strands reassociate. When homologous DNA segments are present in the reaction medium, the separated complementary DNA strands recognise one another and bind by means of

hydrogen bonds to form double stranded DNA molecules. The denaturation and reassociation of DNA strands depends on the Cot value of DNA (concentration of nucleic acid = Co and the time of reassociation =t) and the pH of the solution. By adjusting the denaturation and reassociation conditions for DNA strands, nucleic acid sequences produced as molecular probes by recombinant DNA technology can be hybridised to complementary targets in the genomic DNA. This facility can be extended for use in genomic research and diagnosis of certain genetic abnormalities.

1.2. Historical background of *in situ* hybridisation

The first report of this hybridisation method was by Marmur and Lane (1961), who showed that double stranded DNA molecules could be denatured and reassociated under controlled reaction conditions. *In situ* hybridisation between cytological targets and probes dates back to 1969, when Gall and Pardue (1969) introduced this technique to detect amplified ribosomal RNA genes in Xenopus oocytes at cellular and chromosomal levels of microscopic resolution. In this research, probes highly radioactively labelled with tritium were hybridised to denatured DNA in Xenopus laevis oocytes fixed on microscope slides and the resultant RNA-DNA hybrids formed were detected by autoradiography. On exposure, the silver grains appeared over specific chromosomal regions which presumably contained the DNA sequences complementary to the ribosomal RNA probes.

Current use of this technique has been expanded to achieve hybrids between DNA - DNA, DNA - RNA and RNA- RNA duplex molecules on nuclei, chromosomes and tissue with easily purified RNA or DNA. In the early 1970s, for other specific gene sequences, the major problem of

localisation was the lack of probes of sufficient purity. The arrival of recombinant technology meant that sequences could be prepared free of other hybridising contaminants, which resulted in major improvements in the resolution of the signal obtained. At the same time, improvements in the quality of chromosome preparations and banding have made the technique of *in situ* hybridisation sufficiently sensitive to achieve the localisation of single copy sequences.

1.2.1.1. Development of *in situ* hybridisation

1.2.1.2. Non isotopic *in situ* hybridisation (NISH)

Radioactive *in situ* hybridisation has developed into a sufficiently reliable technique for even the detection of a target sequence a few hundred base pairs in length in metaphase chromosomes (Harper et al. 1981; Rabin et al. 1984 ; Jhanwag et al. 1984). However, in spite of the high sensitivity and the wide application of radioactive *in situ* hybridisation, its use has several disadvantages, the main ones being radiation risk to the users, and the difficulty of disposing of radioactive probes, the long exposure time necessary for signal detection, and the instability of the radioactive element. To overcome these problems, non isotopic methods have been developed to replace isotopic probe labelling and auto radiographic detection. These developments have involved probe preparation and labelling, and the introduction of a variety of detection systems.

The labelling systems are classified into two groups as the direct and indirect methods. In the direct method, the reporter molecule (detectable molecule) is bound to the nucleic acid in the probe so that formed hybrids can be visualised microscopically immediately after the *in situ* hybridisation procedure. For this, nucleotides labelled with fluorochromes such as Rhodamine-dUTP (fluorored), Fluorescein-dUTP

(fluorogreen), and Coumarin-dUTP (fluoroblue) are commercially available. This method gives the result in a short time and also allows multi-colour FISH studies on the same specimen. However, the signals obtained may not be sufficiently stable to detect low copy sequences.

In the indirect method, the probe(s) contain some structural element (hapten) which is introduced chemically or enzymatically and which renders the hybrids detectable by immunocytochemistry (Landegent, 1984). Recently, a number of non- isotopic reporter molecules have been developed and, at the same period, various reagents for detection of reporter molecules in the probe have been produced and have become commercially available.

The most commonly used reporter molecule is Biotin-dUTP which was synthesised as an analogue of dUTP by Langer (Langer et al. 1981). In this system, a biotin molecule covalently attaches to the C-5 position of the pyrimidine ring through a linker arm. The second most commonly used hapten molecule is digoxigenin which is linked to uridine nucleotides at the number 5 position of the pyrimidine ring through 11 C atoms (Herrington et al. 1989). A third reporter molecule is 2-acetyl-amino fluorine (AAF). This molecule binds covalently to the C-8 position of the guanine residues (Tchen et al. 1984). In addition to these molecules, several other haptens for labelling the nucleotide sequences have been introduced which are described in detail by Gray (1989).

Labelled probes are hybridised to target sequences, the probes are then detected immunologically by binding the haptens with specific antibodies. These antibodies are conjugated either with enzymes or fluorochromes.

Two enzyme systems have been used most often for the visualisation of probe hybridisation in non-isotopic *in situ* hybridisation. These are

horseradish peroxidase (PO) and alkaline phosphatase (AP). These are conjugated to antibodies or avidin. Alkaline phosphatase is a very stable enzyme which catalyses the reaction between Tetranitroblue-tetrazoliumchloride (TNBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) TNBT and BCIP. The streptavidin alkaline phosphatase (SA-AP) binds to the biotinylated target hybrid. The combination of TNBT and BCIP are used as the chromogenic substrate for the enzyme alkaline phosphatase. The colour reaction is initiated by the cleavage of the phosphate group from the BCIP by alkaline phosphatase. This reaction yields a blue colour and produces a proton which reduces NBT to yield a purple insoluble precipitate.

For detection of nonradioactive labelled probe by the fluorescence technique, a number of antibodies which have specific affinity to the reporter molecules such as biotin and digoxigenin have been produced. These antibodies are produced from different sources, and are conjugated with fluorochromes such as FITC, Rhodamine and Coumarin. These made the different combinations of antibody in use. The most commonly used fluorochromes are fluorescein-isothiocynate (FITC) (yellow-green), Rhodamine (TRITC) (red), Texas red (red) and Aminomethyl coumarin acetic acid (AMCA) (blue).

As a result of the combination of pure and specific probe preparations and the development of immunochemistry, non radioactive *in situ* hybridisation techniques have vastly improved and are widely used in different areas and different tissue specimens.

1.2.1.3. Chromosome *in situ* suppression (CISS) hybridisation

One important development is the use of chromosome specific libraries to visualise an entire chromosome from the p terminus to the q terminus.

This approach, resulting in a brightly fluorescent pair of chromosomes in metaphase spreads, is referred to as "chromosome painting" (Cremer et al. 1988; Pinkel et al. 1988).

In this technique, the probes used are cocktails of DNA sequences derived from any individual chromosome. These chromosome specific DNA libraries are constructed by cloning sequences from a single human chromosome purified by flow sorting from cultures of human chromosomes, or from somatic cell hybrids containing a single human chromosome. Each chromosome specific library contains unique sequences for that chromosome and also families of repeat sequences that are shared with other chromosomes. In order to obtain chromosome specific signal, the ubiquitous sequences in the other chromosomes are suppressed by prehybridization with appropriate competitor DNAs. The technique therefore is called "chromosome in situ suppression" (CISS) hybridisation.

Painting of individual chromosomes facilitates a rapid and striking visualisation of numerical aberrations (Lichter et al. 1988; Cremer et al. 1988; Kuo et al. 1991), structural rearrangements (Lichter et al. 1988; Hulten et al. 1991) and chromosome damage (Cremer et al. 1990), and assists in the identification of markers (Hulten et al. 1991), and studies of chromosome structure in interphase nuclei (Trask, 1989; Lawrence et al. 1990).

1.2.1.4. Multicolour *in situ* hybridisation

The developments of alternative probe labelling chemistries and the companion detection schemes described above enables the simultaneous visualisation of multiple probes each labelled with a different hapten (Nederlof et al. 1989; Nederlof et al. 1990; Reid et al. 1992). While

minimising the number of different probe haptenizations and detection systems required, | the combination of three basic reporter molecules such Biotin (Bio.), Digoxigenin (Dig.) and Dinitrophenol (DNP) and detection systems further enhances the number of unique chromosomal loci that can be enumerated within a single multi- colour hybridisation experiment (Reid et al. 1992). The detection of multicolour resulting from ratio labelling or detection seems to be difficult when using conventional fluorescence microscopy, especially for small sized probes. The main reason is that multiple exposure of colour film can not adequately display and resolve images from small sized multi-labelled probes and combinatorial labelled or detected probes.

This restriction can be overcome with computerised image processing as with the digital imaging analysis system. The development of multi-colour FISH has strongly enhanced the screening of specimens for numerical and structural chromosome aberrations. This approach is of particular importance in cases where clinical samples are limited in quantity and quality.

1.2.2. The development of interphase cytogenetics by fluorescence *in situ* hybridisation (FISH)

Chromosome banding techniques have facilitated the identification of small changes at band level in chromosome content, but this requires an adequate supply of good quality mitoses and in most instances these have to be produced by tissue culture. Sometimes it is not possible to identify the abnormality due to a small number of analysable metaphases, poor quality banding, or fuzzy morphology of the chromosomes. Moreover, chromosome analysis of most tissues by karyotyping is only possible after tissue culturing. To overcome this limitation, *in situ* hybridisation studies

have been developed for the detection of abnormalities in interphase nuclei using chromosome specific DNA probes. This is based on the fact that each chromosome is individually organised into a territorial area in the interphase nucleus. *In situ* hybridisation techniques strongly suggest that chromosome specific DNA probes can be used to detect numerical and some structural aberrations in interphase. Thus, the limitations of karyotyping assays and even the identification of sub microscopic abnormalities, may be overcome. Because of the detection of abnormalities in interphase nuclei, this technique is often referred to as "interphase cytogenetics". The technique was first introduced using FISH by Cremer et al. (1986). Since then the technique has been shown to have the potential to make a major impact on the capabilities of cytogenetic analysis in different tissues and diseases. For instance, it has been successfully applied to interphase nuclei from blood (Lichter et al. 1988; Cremer et al. 1990), amniotic fluid, chorion villus cells (Klinger et al. 1992; Christensen et al. 1992; Klever et al. 1992; Zheng et al. 1992), leukaemic cells (Dekken and Bauman 1988; Arnoldus et al. 1990; Tkachuk et al. 1990; Nederlof et al. 1990) and tumour tissues (Hopman et al. 1988; Devilee et al. 1988; Wit et al. 1992; Fletcher 1992; Giovanini et al. 1992).

Interphase cytogenetic and multicolour FISH techniques have been combined to allow detection of abnormalities using different probes such as chromosome specific libraries, locus specific and chromosome specific centromere probes (Slim et al. 1991; Reid et al. 1992; Van Dekken et al. 1990). For example, combined locus specific probes have been used for studies of terminally differentiated blood cells that are difficult to grow in culture (Tkachuk et al. 1990; Arnoldus et al. 1990).

1.3. Basic outline of fluorescence *in situ* hybridisation (FISH)

In situ hybridisation has become a fundamental technique for detecting specific nucleic acid sequences. Many laboratories have developed their own protocols for uni-colour and multicolour fluorescence *in situ* hybridisation (FISH). The general procedure of FISH is summarised as shown in **Table 1**. The success of all these steps depends on the following factors; (1) effect of tissue preparation on the retention and accessibility of the cellular target, (2) type of probe construct, (3) efficiency of probe labelling, (4) denaturation of probe and target DNA, (5) hybridisation conditions, (6) sensitivity of the method for signal detection, and (7) sensitivity of visualisation. Therefore a number of options exist and the choice of methods depends on the aforementioned criteria. Some of these variables will be described as follows;

1.3.1. Preparation of specimen

The quality of the specimen and the mitotic index of the slides are critical factors for successful uni-colour or multi-colour *in situ* hybridisation. Hybridisation can be performed on chromosomes or interphase nuclei obtained from fibroblasts, lymphocytes, bone marrow cells, tumour cells, amnion and chorion samples and other tissues. Good quality metaphase spreads and interphase nuclei preparations can be obtained by different procedures described in detail by Lawce and Brown (1991). Before hybridisation, the targets need some pre-treatment such as incubation with RNase, detergent, protease (Protease K or pepsin), acetone or Xylene. The function of RNase is to remove the endogenous RNA. This treatment improves the ratio between signals and background in the hybridisation of target DNA. Detergent treatment serves to clean the lipid membrane component around the target DNA. Protease treatment is used to increase

the accessibility of DNA by digesting the protein that surrounds the target nucleic acids, but the success of protease treatment on metaphase preparations is low because it is detrimental to chromosome and nucleus morphology. Xylene and acetone are used to clean some artefacts but since xylene is a harmful solvent, acetone is normally used.

Table 1: Schematic representation of uni-colour and dual-colour FISH procedure to interphase and metaphase spreads.

Slide Preparation

- Fixation of material on slide
- Pretreatment of specimen

Probe Preparation

- Probe labelling
- Preparation of hybridisation mixture depending on probe type

↓

Denaturation of Probe and Target
(Simultaneous or separate denaturation)

↓

Prehybridisation
(For locus specific and whole chromosome specific library probes except for repetitive)

↓

Hybridisation

↓

Washing and Detection

- Washing (e.g. with formamide, SSC)
- Blocking step
- Antibody incubation

↓

Counter staining
(With DAPI and/ or PI in mountain solution)

↓

Visualisation

1.3.2. Probe

A probe is any sequence of specific nucleic acid used to test for hybridisation. As previously mentioned, a probe is one of the most important elements for *in situ* hybridisation. The purity, size and efficiency of the probe play important roles in detecting the signals in the hybridisation technique.

1.3.2.1. Probe preparation

There are several ways to prepare probes. Small probes are usually synthesised chemically but most probes are cloned fragments of natural DNA. This is achieved by gene cloning (described in detail by Watson et al. 1983; Weatherall, 1991; and Sambrook et al. 1989).

Briefly, genomic libraries are constructed by first digesting DNA with a restriction endonuclease which cuts repeatedly at certain specific points along the genome. Partial digest libraries are constructed in a series of overlapping clones covering the genome. The DNA is then fractionated by size and the large uniformly sized DNA fragments are collected. These constructs are ligated with the DNA of a vector and are finally transferred to appropriate host cells in which they can be amplified by culturing the host cells.

1.3.2.1.1. Probe cloning

In cloning the DNA fragments, four main classes of vehicle (vectors) have been used on the basis of the size of DNA fragments to be cloned. These are plasmid, phage, cosmids and yeast artificial chromosomes (YACs)

Plasmid consists of double stranded circular DNA which contains one replication site, one or two genes for antibiotic resistance and restriction

sites. Most plasmids used are derived from the plasmid pBR322 which, when modified by the introduction and deletion of various genetic functions, become more versatile as cloning vectors. The plasmid DNA can carry up to 5 kb of foreign DNA. However large DNA insertions in a plasmid are not stable and tend to replicate slowly.

In contrast to the instability of plasmids, the **bacteriophage** has been developed to accommodate larger fragments of foreign DNA. Each phage particle consists of a head, a tail, and a protein coat surrounding a central core of DNA. It attaches to the bacterium by its tail and injects its DNA into the host cell after which the phage DNA forms a ring. It either integrates with host cellular DNA and replicates along with it, which is the lysogenic phase, or the circular DNA of the phage replicates independently of the host DNA as a lytic phase. This second phase generates large numbers of phage particles. These properties of bacteriophage have been manipulated to produce many different cloning vectors.

The most widely studied phage is bacteriophage lambda. In the middle of the genome it has lysogenic genes which can be deleted and substituted. Thus, the DNA fragments which are to be cloned can replace the lysogenic genes in the phage DNA.

The third type of vector is a **cosmid** which is designed by genetic engineering. Essentially, a cosmid vector is made up of a circular duplex of plasmid DNA containing various antibiotic resistance genes, a number of restriction sites, and "cos" sites of the phage. The "cos" sites of phage lambda have been cloned into the antibiotic resistance gene of the plasmid. The DNA fragment (up to 50 kb) to be inserted is ligated to the cloned "cos" site plasmid, which has been cleaved with a similar enzyme.

When phage lambda packing extract is added, it recognises and cleaves the insert piece of DNA flanked by "cos" sites and packages the cleaved inserts into phage heads. The appropriate host cells are then infected. The great advantage of cloning a cosmid is that it is possible to introduce larger DNA strands up to 50 kb and they can be obtained very easily because they reproduce in host cells as plasmids.

Quite recently, **yeast artificial chromosomes (YACs)** have been developed as a vector to clone very large fragments of DNA. YACs allow the stable propagation of DNA fragments ranging in size from 100 kb to 700 kb. As a result it is possible to clone a gene or a whole region of chromosome insert as one or a set of large fragments.

Basically YACs consist of two yeast telomeres, one yeast centromere and autonomously replicating sequences (ARS), selectable markers and a cloning site. The ARS sequences provide the necessary origin for replication. So, the YAC's DNA is divided into two arms by one cloning site. One arm contains a telomere and a selectable marker, the other a telomere, another selectable marker and a centromere. The large fragments of DNA are inserted between these arms.

The principle of the YAC system is similar to that used in conventional cloning of genomic DNA. Large fragments of DNA are ligated into two arms of a YAC vector, and the ligation mixture is then introduced into the yeast by transformation.

1.3.2.1.2. Probe types

Probes are classified according to their targets, origin or propagation (vector) and structure of probe DNA such as double stranded or single stranded probes. For *in situ* hybridisation (ISH), in chromosomal and interphase assignment, probes may be defined on the basis of their targets in three general classes: Locus specific probes, repeated DNA sequence probes and whole chromosome specific probes (Trask 1991; Tkachuk et al. 1991). Very recently, chromosome region or band specific probes have been developed (Deng et al. 1992; Meltzer et al. 1992).

Locus specific DNA sequences (probes) occur once or only a few times in the genome and include most genes. Therefore they are important in the diagnosis of a specific genetic disease. The probes homologous to these target sequences have to be in the range of 15 to 50 kb. Locus specific probes smaller than 15 kb may not be reliably detected in in situ diagnostic applications (Tkachuk et al. 1991).

Repeated DNA sequences (probes) present in many copies per genome. This class also includes some genes (e.g. those for ribosomal and transfer RNA and histones). Most repeat sequences are in the α-satellite (Waye and Willard 1987) or satellite-III families (Fowler et al. 1989). The α-satellite DNA, located at all human chromosome centromeres, is a tandemly repeated unit of 171 bp (Choo et al. 1991). Specificity of probes for these regions comes from the fact that there is significant variation in the repeated monomer among chromosome types. On the basis of these variations, chromosome specific repeat sequence probes have now been isolated and cloned for human chromosomes. Consequently, they are

widely used in *in situ* hybridisation for the detection of chromosomal abnormalities.

Whole chromosome specific probes are made from the DNA of one specific chromosome. Individual chromosome probes can be derived from somatic cell hybrids carrying the desired chromosome as its human material or from a suspension of chromosomes purified by flow sorting. Partial chromosome libraries are derived from microdissection of chromosomes. After obtaining the specific chromosome, chromosomal DNA is extracted and this DNA is then cloned in the appropriate vector by digesting with restriction enzyme and ligation (Van Dilla et al. 1986; Fuscoe 1987; Perlman and Fuscoe 1986; Fuscoe et al. 1986). The resulting recombinant DNA cocktail in the vectors contains both the chromosome specific and repeat sequences (Alu and Kpn) which are shared with other chromosomes, and consequently unlabelled competitor DNA is added during hybridisation to block any cross hybridisation with other chromosomes.

Other recently developed probes are chromosome region or band specific probes. These probes are generated from chromosome spreads by the microdissection technique (Meltzer et al. 1992). A dissected chromosome region or band specific area of DNA is amplified and labelled by the polymerase chain reaction or other labelling system (Deng et al. 1992). Alternatively the dissected chromosome material may be cloned into a vector and this recombinant DNA is transformed into an appropriate host cell to produce more probe DNA. Both products can be used as library probe constructs. The use of this approach allows not only the identification of structural abnormalities at the band level, but also the identification of cytogenetically unrecognisable marker chromosomes.

1.3.3. Probe labelling

Two major alternatives exist for the nucleotide or nucleic acid sequence labelling to be incorporated into the probe: radioactive labels which are detected by autoradiograpy, or nonradioactive labels which are detected by immunochemistry. For non radioactive labelling, two alternative systems exist : direct and indirect labelling. In direct labelling the target-specific probes are directly and covalently linked with the signal-generating reporter group : thus, the detection of nucleic acids in a direct system consists of hybrid formation between target and labelled probe; and signal generation via the covalently coupled reporter molecule. Frequently used direct reporter groups are fluorescent dyes like fluorescein and Rhodamine (Lichter et al. 1990).

In contrast, the indirect system first requires the modification of the target-specific probe by the introduction of a particular modification group. This modification group binds through an additional, noncovalent interaction to a universal reporter group. Thus, the signal generating reporter group is linked indirectly to the hybrid by an additional interaction between the modification group and high affinity binding partner coupled with the reporter group.

The incorporation of a labelled molecule into a probe can be achieved in a number of different ways. However, the most useful labelling procedures for probes which are to be used in *in situ* hybridisations are enzymatic labelling procedures because they result in highly labelled nucleic acid probes. These are nick translation, random priming and polymerase

chain reaction (PCR). In these enzymatic labellings, analogous nucleotides modified with particular haptens like biotin, digoxigenin, or fluorescein are substituted for their non-modified counterparts.

1.3.3.1. Nick translation

The most popular technique for labelling DNA is nick translation, which was first developed for labelling hybridisation probes with radioisotopes by Rigby et al. (1977). Subsequently the incorporation of nonradioactive groups (biotin) Langer et al. 1981) (digoxigenin) (Höltke et al. 1990) into DNA by nick translation was reported. In the nick translation system, two enzymes, DNase I (pancreatic deoxyribonuclease) and DNA polymerase I (E. coli DNA pol I), are used. DNase I is an endonuclease that randomly creates nicks in the phosphodiester backbone of double stranded or single stranded DNA. However, DNA polymerase has three associated activities; 5'----->3' polymerase; 5'----->3' exonuclease; and 3'----->5' exonuclease. When Mg^{2+} ions are present in vitro, DNase I attacks each strand of DNA independently, resulting in single stranded nicks. After the formation of the single stranded nick in the DNA strands, the exonuclease activity of DNA polymerase I eliminates nucleotides from the 5'-phosphoryl terminus of the nick in a 5` ------->3` direction. At the same time its polymerase activity sequentially incorporates nucleation residues in the same direction to the 3` hydroxide terminus of the fragment 5` to the nick exactly complementary to the template strand in the presence of all four deoxyribonucleoside triphosphates or their analogues.

When one of the deoxyribonucleoside triphosphate conjugates with a reporter molecule such as a non radioactive, or radioactive molecule is used to label the probe, the reaction will result in the formation of labelled

molecules due to the replacement of original nucleation residues by nucleations conjugated with either isotopes such a P^{32} or non- isotopic reporters such as biotin-dNTP and digoxigenin-dNTP.

1.3.3.2. Random prime labelling

This method of DNA labelling was introduced by Feinberg and Vogelstein (1984). By contrast with nick translation only the Klenow fragment of E. coli DNA polymerase (Pol I) and primer are used and DNase is not needed in the reaction. The type of DNA dependent polymerase used depends on the nature of the template, but it is essential to use polymerase lacking 5' -------> 3' exonuclease activity, otherwise degradation of the primer will occur.

In a typical random primed labelling reaction, the DNA sample is first denatured by heating and each DNA strand can then be used as a template in a subsequent synthesis reaction. The initial event in the synthesis reaction is binding of hexonucleotide, which is either derived from DNase I digests of DNA or produced by oligonucleotides synthesis, to the denatured DNA strand templates. In this way, these short oligoncleotides bind to the template in a random fashion because they have heterogeneity of sequence. In the presence of all deoxyribonucleoside triphosphates, or their analogues, the Klenow fragment incorporates nucleotide residues to the primer in a 5'---->3' direction complementary to the template strands. The newly synthesised strands become labelled by incorporation of the labelled nucleotides when a labelled nucleotide is used. This reaction is called random primed labelling, since the synthesis of the complementary strand is primed at random sites of template strands. The concentration of primer determines the mean probe size; the

higher concentration of primers, the shorter the resulting probes. This can be controlled by varying the ratio of primer to template.

1.3.3.3. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR), first used diagnostically by Saiki et al (1985), is a powerful technique for the labelling and amplification of a large amount of specific DNA sequences from a small amount of complex genome in vitro. A PCR system contains target nucleotide sequences to be amplified, oligonucleotides which are homologous flanking regions to both DNA strands, free dNTP or labelled dNTP (radioactive or nonradioactive), reaction buffer and Taq DNA polymerase. The basic PCR cycle involves thermal denaturation of double stranded target molecules, followed by annealing of the amplification primers to each strand and finally polymerase controlled DNA synthesis. During the PCR, the newly synthesised strands of DNA also function as templates for the reaction. The number of target sequences multiples exponentially resulting in large amounts of targeted DNA segments defined by the 5′ ends of the amplification of primers.

By including nucleotides which are conjugated either with radioactivity or with non-radioactive compounds such as Biotin during the PCR reaction, the amplified DNA can be specifically labelled on a particular strand or both strands of PCR products with very high specificity. The incorporation of a modification group into the PCR product has been described for biotin (Lo et al. 1988) as well as digoxigenin (Seibl et al. 1990). This property has also been used for the labelling of microdissected probe pools (Deng et al. 1992), phage (Lengauer et al. 1990; Isa, 1991) and YACs probe pools (Baldini et al. 1992 and Lengauer et al. 1992)

PCR may also be used as a labelling system for metaphase spreads (Koch et al. 1991). This alternative method, termed *primed in situ (PRINS)* labelling, is based on the sequence-specific annealing *in situ* of unlabelled DNA (Koch et al. 1989), in which the probe is digested with a specific restriction enzyme to generate a 3′ end. This then serves as a primer for chain elongation *in situ* catalysed by DNA polymerase. The DNA synthesised during chain elongation will be labelled when labelled nucleotides are used as substrates for DNA synthesis. Recently, fluorescein labelled dUTP was incorporated into DNA synthesised *in situ* by primed *in situ* labelling (PRINS) by Koch et al. (1993)

1.3.4. Hybridisation

Molecular hybridisation in *in situ* is a reaction in which single stranded target nucleic acid sequences complementary to molecular labelled probes form double stranded molecules under controlled conditions.

Hybridisation procedures for uni-colour or multicolour experiments are more or less the same. The only difference is that by using combinations of different hapten molecules labelled probes such as digoxigenin and biotin, multiple simultaneous hybridisations can be performed to localise different regions of chromosomes and nuclei Thus in this section, both techniques will be described under the same title.

For *in situ* hybridisation, target and probe DNAs must be denatured. The denaturation of target sequences can be achieved with extremes of alkalinity or by heat. Normally such treatments may lead to loss of morphology, thus in practice, compromise must be found between hybridisation signal and morphology. A widely used method of denaturation is to use formamide as an organic solvent. Formamide reduces the thermal stability of double stranded nucleic acid sequences, so

that denaturation and hybridisation can be performed at lower temperatures. For this reason, formamide is added to the hybridisation buffer (mixture) described below.

After denaturation, the target DNA sequence is applied with a hybridisation mixture that contains formamide, SSC, dextran sulphate, labelled and denatured probe(s), and competitor DNA (in the case of locus specific and whole chromosome specific DNA probes) This is then incubated in a moist chamber at a stable temperature which depends on the concentration of formamide in the hybridisation mixture. Successful hybridisation relies on the probe length, probe concentration, competitor DNA if necessary, and salt concentration in the hybridisation mixture as well as hybridisation time. Thus, all these parameters can be optimised depending on the probe being used.

1.3.5. Post washing and detection

After hybridisation, post hybridisation washing is performed at various stringencies to remove non specific binding and non - hybridised probe. For this aim, a number of washing solutions have been described such as SSC, PBS, PN. The stringency can be manipulated by varying the salt concentration, and formamide concentration if used, in the washing solution as well as by varying the temperature at which the washes are carried out.

1.3.5.1. Uni-colour detection system

Following post hybridisation washing, the detection of hybridised sequences depends on the type of labelling. If a probe is labelled with a type of dNTP conjugated with a fluorochrome such as fluorescein or

Rhodamine, hybridisation can be visualised directly using a fluorescence microscope. This is called direct detection.

There is also an indirect detection system which involves labelling a probe with a hapten molecule without conjugation with a fluorochrome. An immunochemical detection system is required to visualise the probe localisation on the target. Thus, fluorescence detection systems have been developed.

Various conjugated fluorochromes, non-conjugated reagents (antibodies) and amplification reagents are now commercially available for use in fluorescence detection systems. The most commonly used fluorochromes are fluorescein isothiocynate (FITC), tetramethylrhodamine (TRITC), Texas red and amino-methyl coumarin acetic acid (AMCA). For unicolour fluorescence detection, the most commonly used detection systems use biotin and digoxigenin since they allow a flexibility of choice in final reporter molecule. In principle biotin can be detected by antibiotin antibodies; however avidin is more frequently used because of the high binding capacity of the interaction.

1.3.5.2. Multicolour detection system

By using different antibodies conjugated with reporter molecules and fluorochromes, several probes labelled with different hapten molecules have simultaneously been detected in different colours (Raap et al. 1989; Nederlof et al. 1989; Nederlof et al. 1990). In this way, very recently, a combination of probe labelling and detection has been developed to produce multiple colour signals for a number of simultaneous probe detections (Reid et al. 1992). The basis of detection is schematized in **Figure 1.**

1.3.6. Visualisation

A probe detected using fluorescein is visualised as a (green) signal. Chromosomesor nuclei can be counterstained using propidium iodide (PI) which gives red fluorescence, DAPI which gives blue fluorescence, or a combination of both when using uni-colour FISH. However when using dual colour FISH , the probes detected with Rhodamine or Texas red (red) and Fluorescence (yellow-green) are visualised as red and green signals on metaphase spreads and interphase nuclei. The visualazation of dual-colour FISH on metaphase spreads and interphase nuclei is schematized by representing abl and bcr probes in CML in **Figure 1.** They can be counterstained using only DAPI which gives blue fluorescence. DAPI allows the visualisation of red and green signals, but the use of the PI may be suppressed especially red signal because both of them give red fluorescence.

An anti-fading agent, such as diazobicylo-octane (DABCO) or n-propylgallete should be added to the embedding medium to reduce fading. The embedding solution is 9 parts glycerol containing 2.3 % (w/v) 1,4-diazobicylo-(2,2,2)-octane (DABCO), dissolved by warming to 70°C) and 1 part 0.2 M Tris-HCI, 0.02% NaN_3, pH 8.0. Presently, commercial embedding solutions are available for this purpose. (Enzymatic detection systems do not need counter stain and can be visualised under the phase contrast by covering with conventional mounting solution.)

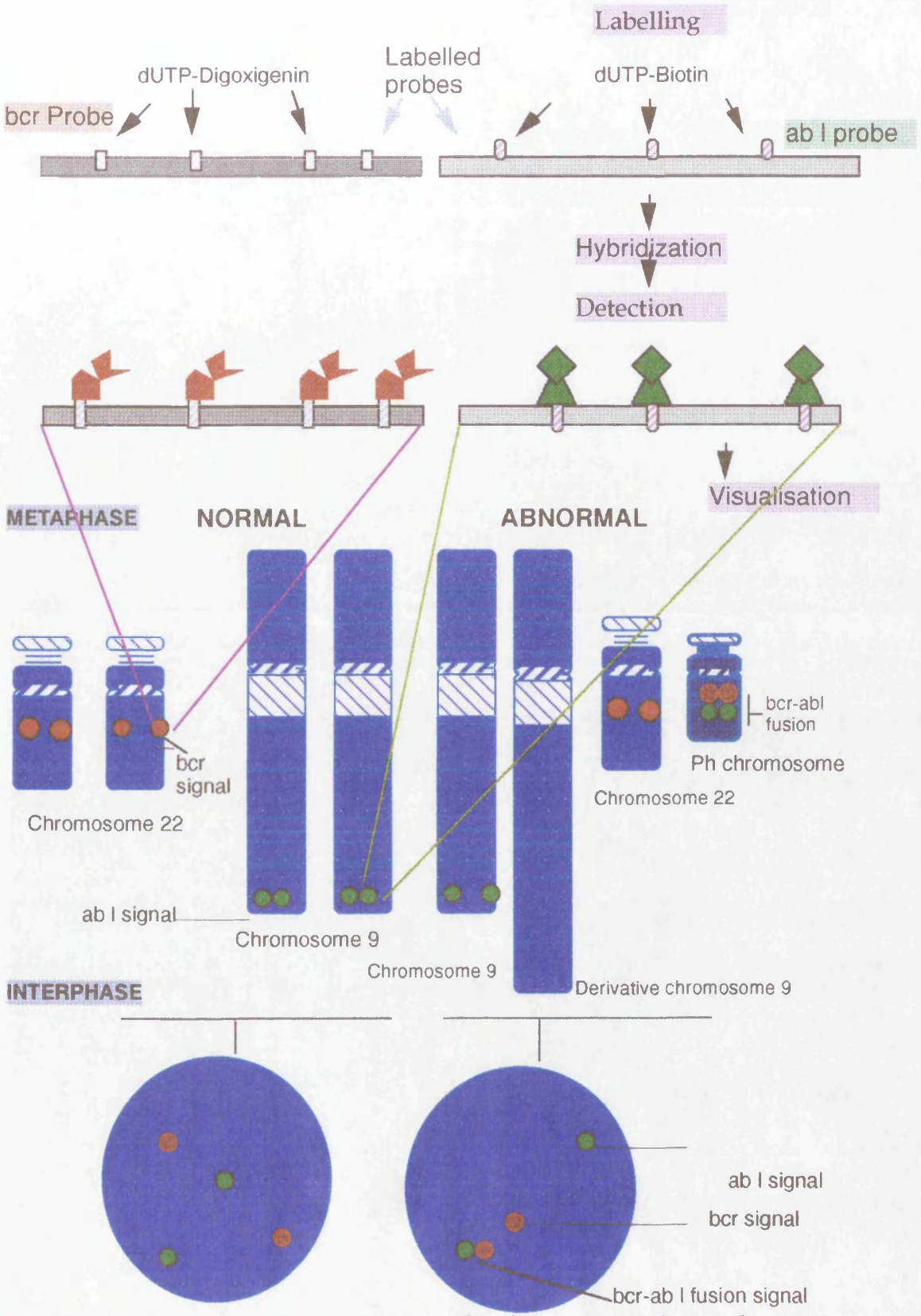

Figure 1 : Illustration demonstrating the detection and visualisation patterns in dual colour FISH procedure, with abl and bcr probes, for normal and abnormal interphase nuclei and metaphase spreads.

1.4. Cancer cytogenetics

The first spectacular discovery in cancer cytogenetics came in 1960 when Nowell and Hungerford discovered a small karyotypic marker, the Philadelphia (Ph) chromosome, in patients with chronic myeloid leukaemia (1960). This was the first consistent chromosome abnormality to be found in a human cancer. Later, chromosome banding techniques were developed which completely revolutionised cytogenetic analysis. Further developments in high resolution techniques, tissue culture, and improved molecular studies have all meant that an increasing number of aberrations can be characterised in greater detail. In the last decade, FISH studies have had a very important impact on detection of abnormalities in metaphase and interphase cells in leukaemia. In this study, some specific haematological malignancies will be highlighted.

1.4.1. Leukaemia

Leukaemia is a cancer of the white blood cells. Human leukaemia is a heterogeneous group of disorders characterised by the malignant expansion of a clone derived from a cell of a lineage that normally populates the blood and bone marrow. Blood cells are derived from pluripotent bone marrow stem cells which have the capacity to progressively limit their expansion of differentiation to a single cell lineage. They go through a progressive maturation characterised by distinct stages, each with their own associated morphological and functional features. According to this progressive development, two main groups of bone marrow stem cells have been described; the myeloid and the lymphoid stem cells.

Both myeloid and lymphoid leukaemias are found in either chronic or acute forms (Rowley 1990). Acute leukaemia is characterised by the

proliferation of undifferentiated or poorly differentiated (blast) cells. Chronic leukaemia reflects massive overgrowth of well differentiated (mature) cells.

In addition to this classification, leukaemias are classified into a number of subgroups. Acute Myeloid Leukaemias are defined by the FAB (French / American / British) collaboration into seven subtypes, M1 to M7, and lymphoid leukaemias are classified from L1 to L3. The FAB classification uses morphological, immunological and cytochemical criteria of the prominent cells.

There is a wide range of structural and numerical chromosome abnormalities in malignant bone marrow cells. The various aberrations can be subdivided into primary and secondary abnormalities. Primary aberrations are frequently found as the sole abnormality in malignancies; they are also often specifically associated with a particular disorder. Secondary changes occur in addition to the primary abnormality and consist of further numerical or structural abnormalities which are added to those found in the primary line. This is called karyotypic evolution and is associated with disease progression.

The recurring chromosome abnormalities in leukaemia are summarised in **Table 2.**

In this study, chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL) and acute promyelocytic leukaemia (APL) which is a subgroup of acute myeloid leukaemia (AML), will be investigated.

1.4.1.1. Chronic Myeloid Leukaemia (CML)

Chronic Myeloid Leukaemia is the most common of the myeloproliferative disorders (MPDs) and characterised by neoplastic overproduction of

myelocytes. Patients with CML have a specific chromosome abnormality in some of their blood cells, even in the chronic phase of the illness. CML is characterised by the presence of the Philadelphia (Ph) chromosome. This usually arises from a translocation between chromosomes 9 and 22. In this translocation, breakpoints consistently occur on the long arm of chromosome 9 at band q34 and on the long arm of chromosome 22 at band q11, which leads to the formation of a derived chromosome and chimaeric gene. A schematic representation of this translocation, which is described in cytogenetic nomenclature as t (9;22) (q34;q11), is shown in **Figure 2 a.**

Molecular analysis of DNA from CML patients provided genetic evidence for the reciprocal nature of this translocation (de Klein et al.1982). It was demonstrated that the cellular oncogene of the Abelson murine sarcoma virus (abl), normally located at 9q34, is translocated to a position adjacent to a region of the bcr gene at 22q11 known as the breakpoint cluster region (bcr) (de Klein et al. 1984). Analysis of the translocation breakpoint on chromosome 22 shows it to be restricted to a small region of the 5.8 kb breakpoint cluster region (bcr), which lies within the large bcr gene (Groffen et al. 1984). Specifically, the translocations were found to lie between exons 2 and 3 or between exons 3 and 4, creating two molecular types of Ph chromosome in CML, one with bcr sequences up to exon 2 followed by abl sequences, the other with bcr sequences up to exon 3 followed by abl sequences (Westbrook et al. 1988). In contrast, the translocation breakpoints on chromosome 9 have been found to be scattered widely within the very large 175 kb first intron of abl (Westbrook et al. 1988).

The consequence of this t(9;22) is the formation of a chimaeric gene (**Figure 2 b**), which contains protein coding sequences from both abl and

bcr genes to produce a larger fusion abl product (250 kilo daltons protein) that has high tyrosine kinase activity.

By cytogenetic analysis, 90-95% of patients with a clinical diagnosis of CML are found to have the Ph chromosome. Some of these Ph-positive patients have variant translocations involving another chromosome t(C;22) where C can be any chromosome except the Y chromosome. At the microscopic level the involvement of chromosome 9 cannot be seen (Hooberman et al. 1990). These variant translocations are described more fully in section 1.4.1.1.2.

In patients who have a clinical picture of CML but no Philadelphia chromosome, some may present with other chromosome abnormalities (Kurzrock et al. 1986). In other cases no Ph can be detected by cytogenetics but it has been demonstrated that abl sequences are adjacent to the bcr region on chromosome 22.

Table 2: Common chromosomal changes in leukaemia (Hooberman et al. 1990).

Type	Gains	Losses	Rearrangements
Myeloid Leukemia			
CML			
Chronic phase			t(9;22)(q34;q11)
Blastic crisis	+8, +Ph	Rare; -7	t(9;22)(q34;q11)
ANLL			
AML (M2)	18	-7; less -5	t(8;21)(q22;q22)
APL (M3)	-	-	t(15;17)(q22;q12-21)
AMMoL (M4Eo)	+8, +22	-7	inv(16)(p13q32) t(16;16),del(16q)
AMol (M5)	-	-	t(9;11)(p22;q23), t(11q), del(11q)
M2/M4(incr.basophils	-	-	t(6;9)(p23;q34)
M4 (incr.platelets)	-	-	t(3;3)(q21;q26), inv(3)
Lymphoid Leukeamia			
CLL			
B-cell	+12	-	14q+(q32)
T-cell	-	-	t(8;14)(q24;q11) inv(14)(q11;q32)
ALL			
B lymphoid/myeloid			t(4;11)(q21;q23)
Precursor	+21, +6	Rare	t(9;22), del(6q)(q15-q21) nearhaploid
Pre-B	-	-	t(1;19)(q23;p13)
B-cell			t(8;14)(q24;q32), t(2;8)(p13;q24), t(8;22)(q24;q11) t(9p), del(9)(p21-22)
Early T-cell precursor			inv(14)(q11;q32), t(8;14)(q24;q11) t(10;14)(q24;q11) t(11;14)(p13;q11) t(7;7)(p15;q11) t(7;9)(q36;q34) t(7;14)(q35-36;q11)

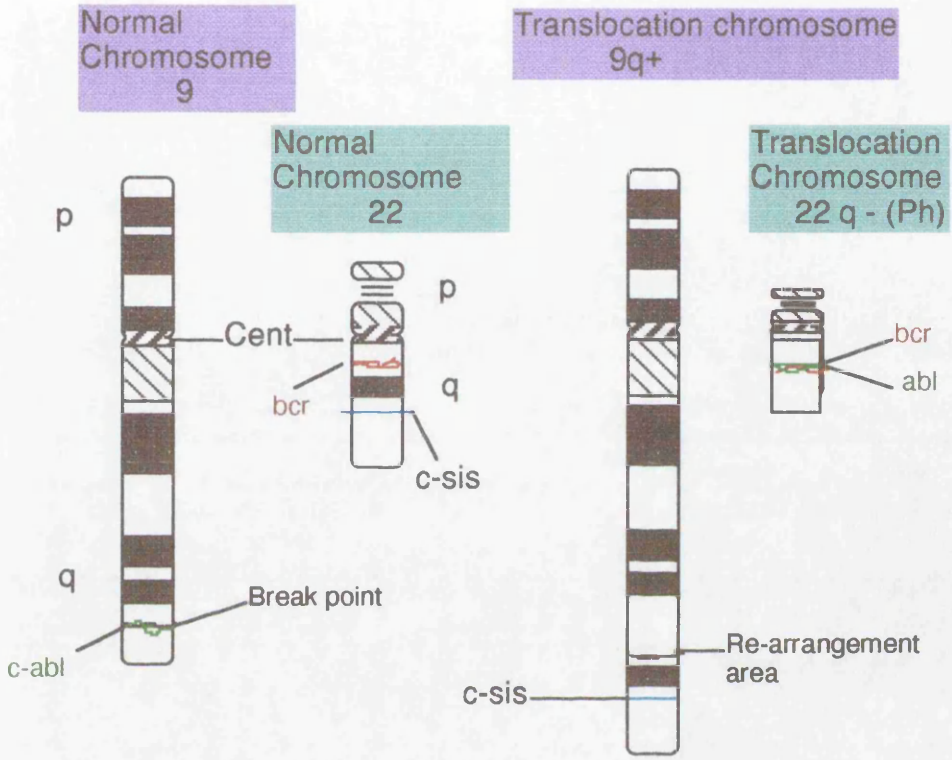

Figure 2 a: Schematic drawing of chromosomes 9 and 22, illustrating the chromosomal translocation that produces the 9q+ and 22q- (Ph) chromosomes. The abl gene is moved to chromosome 22 adjacent to bcr gene. And localisation of other genes (by modifying from Altman, 1988).

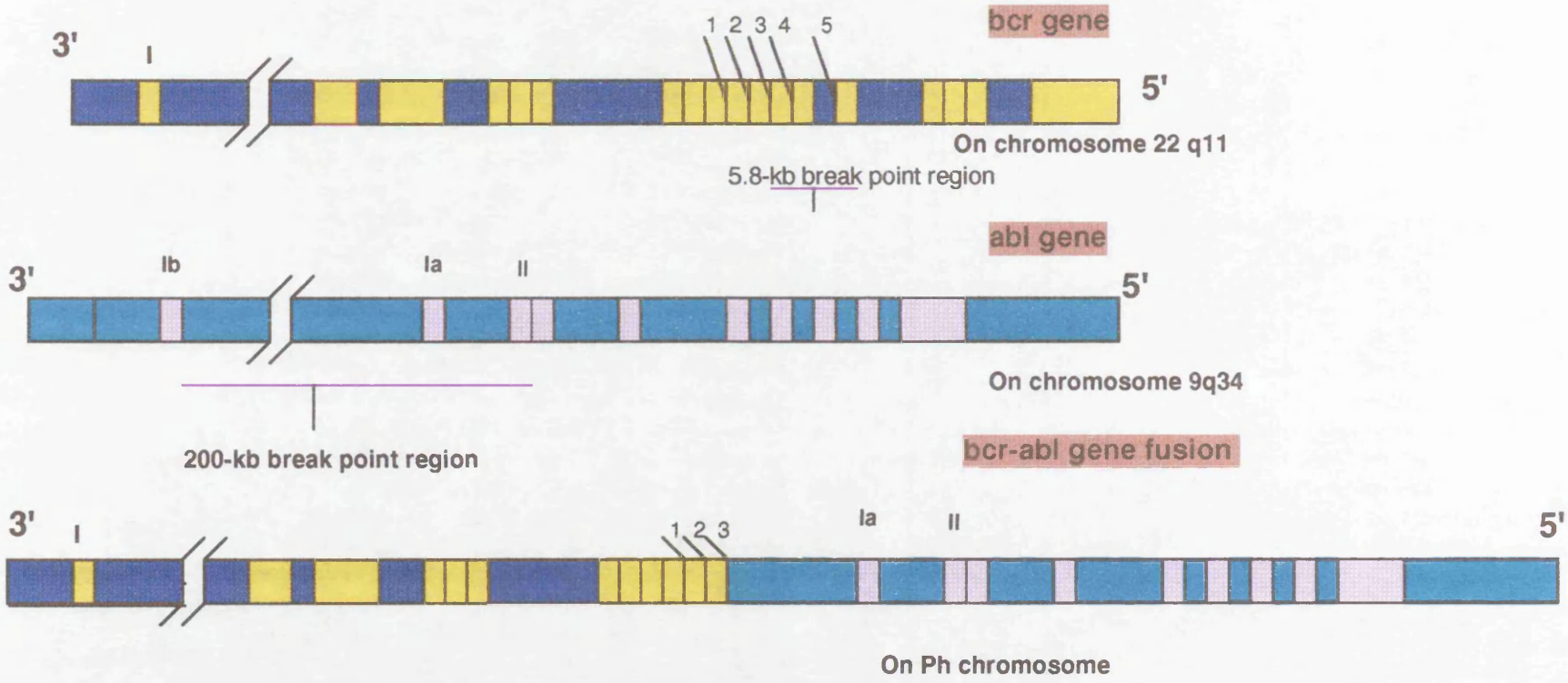

Figure 2 b: Schematic representation of the abl and bcr genes.The exons of bcr gene are depicted as yellow boxes in the bold blue horizontal bar. The exons of abl gene are depicted as pink boxes on the turquoise horizontal bar. The bcr-abl fusion is depicted as a juxtaposition of yellow and pink boxes in which located the bold blue and turquoise horizontal bar, representing translocation between abl and bcr genes (From Tkachuk et al. 1990).

1.4.1.1.1. Other chromosome abnormalities in CML

As well as being Ph positive or negative, some patients with CML may have, in addition, other visible karyotypic abnormalities. These abnormalities commonly include a gain of chromosome 8, i(17q), gain of a second Ph and loss of the Y chromosome. Trisomy 19 is also seen although this is not as common as the abnormalities noted above. The fifth most common additional abnormality is a gain of an extra chromosome 21. However other abnormalities can be seen. A second Ph chromosome usually results from non-disjunction of the derivative chromosome 22 and does not result from a second translocation. Additional abnormalities are rare at diagnosis. Karyotypic changes usually accompany the metamorphosis of CML to the acute blastic crisis and are associated with disease progression.

1.4.1.1.2. Variant Philadelphia translocations in CML

Variant translocations are frequently seen between any of the other chromosomes (except the Y chromosome) and chromosome 22. Three different types of Ph translocations are recognised. About 3-4 % of CML patients have either simple or complex variant translocations where chromosome 22 is involved. The simple variant translocation involves two chromosomes, the deleted segment of 22q is translocated onto a chromosome other than 9, whereas a complex variant translocation involves more than two chromosomes (Heim and Mitelman 1989). Three-, four- and five way variant translocations have been reported (Hagemeijer 1984). The breakpoints in the complex variant translocations are located at the interstitial bands, however in simple translocations the break points are in terminal part of chromosomes. However, the distribution of

breakpoints in complex translocations is mainly localised on light bands according to G banding (Heim and Mitelman, 1989).

1.4.2. Acute Myeloid Leukaemia (AML)

An array of specific cytogenetic abnormalities has been linked to the morphologic characteristics of leukaemic blasts in acute myeloid leukaemia. When the leukaemia appears as de novo (primary) AML, the most common abnormalities are trisomy 8, t(15;17), t(8;21), abnormalities the break points 16q22, 11q23, 9q34 and 12p and loss of all or part of chromosome 5 and/or 7 (Machniki et al. 1990).

According to the FAB classification (French -American -British Cooparative Group), although some of these abnormalities are strictly non-random, some of these specific rearrangements are closely associated with a particular subtype of AML (e.g. t(8;21)(q22;q22) typically occurs in AML - M2 and t(15;17)(q22;q21) in acute promyelocytic leukaemia (AML-M3), and del(10)(q22) or inv(16)(p13q22) in AML-M4.

1.4.2.1. Acute Promyelocytic Leukaemia (AML-M3)

Acute promyelocytic leukaemia (APL; FAB M3) is a sub type of acute myeloid leukaemia. The translocation t(15;17)(q22;q21) is diagnostic for this disorder. It is present in almost 100 percent of cases (Borrow et al. 1990). The fact that this translocation has not been found in patients with any other type of leukaemia or solid tumour, and is generally the only karyotypic aberration present, is evidence that it plays a crucial role in the pathogenesis of AML-M3 (Alcalay et al. 1991).

The breakpoints of this translocation occur on the long arm of chromosome 15 at band q22 and on the long arm of chromosome 17 at band q11 **(Figure 3).** Molecular studies show the break points in AML-M3

cases to cluster in a 12 kb region of chromosome 17, containing two CpG-rich islands (Borrow et al. 1990). The region is the first intron of the retinoic acid receptor alpha gene (RARA) (Borrow et al. 1990). The chromosome 15 break point falls within a gene, which was originally designated myl, but then renamed PML, for promyelocytes (Grignani et al. 1993).

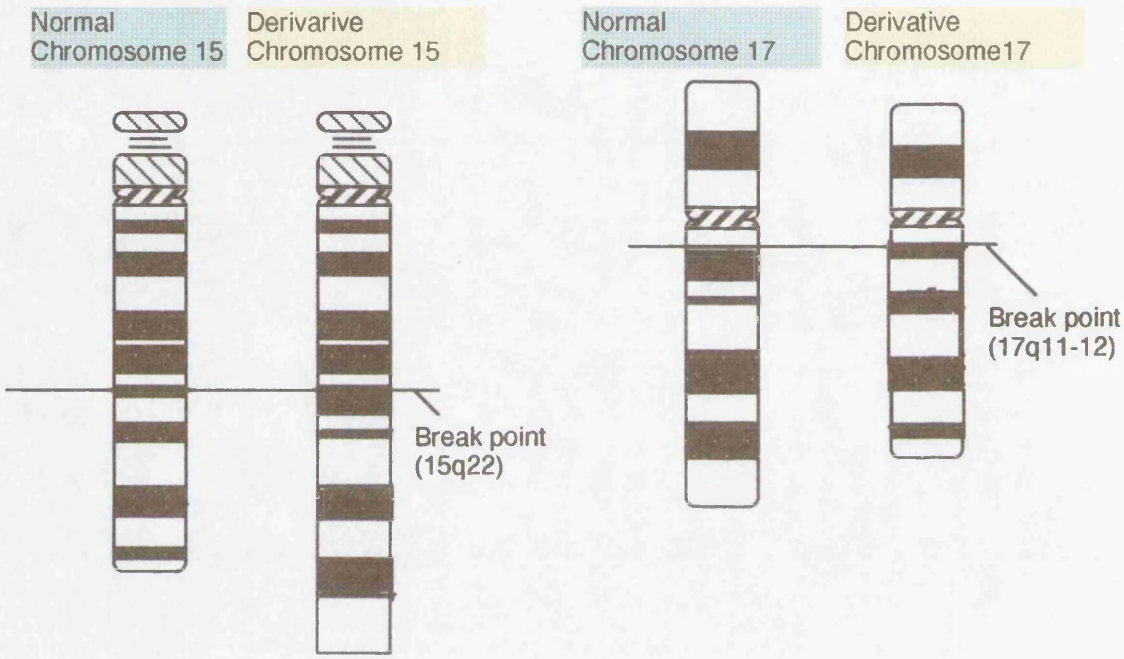

Figure 3. Schematic representation of chromosomes 15 and 17 translocation, illustrating the 15q+ and 17q- chromosomes.

1.4.3. Chronic Lymphocytic Leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) is the most common human leukaemia. The overwhelming majority of CLLs are of B-cell origin. T-CLL accounts for less than 5 % of all cases (Heim and Mitelman 1987).

Trisomy 12 is the most frequently reported chromosome abnormality. This abnormality occurs only in the neoplastic B- cells, not in the patient's T-cells. An extra chromosome 12 has been found both as the sole cytogenetic abnormality and together with other changes.

T-CLL is a rare disorder. However, patients with T-CLL also have multiple chromosome aberrations. These aberrations are more complicated than in B-CLL (Heim and Mitelman, 1987)

1.5. Application of uni-colour and multicolour FISH in leukaemias

Fluorescence *in situ* hybridisation techniques have been widely applied to several researches and clinical genetic studies (**Table 3**). This technique has particular potential application on human cytogenetics and in leukaemia studies. Application of FISH in the analysis of samples has allowed the evaluation of chromosome aberrations not only in mitotic cells, but also non dividing interphase cells, and in terminally differentiated cells. Both uni-colour and multi-colour FISH techniques using chromosome libraries, centromeric probes and unisequences probe have been successfully applied in leukaemias.

For instance, chromosome specific repetitive probes have been applied to determine numerical chromosome aberrations in different diseases. In chronic lymphocytic leukaemia (CLL), trisomy 12 was successfully detected by Losada et al. (1991), Cuneo et al. (1992), Que et al. (1993), Beinz et al. (1993) and Escudier et al. (1993). Nakagawa et al.(1992) detected the deletion of one p arm of chromosome 17 in case of CML, which reflects iso chromosome 17 (i(17q)), using chromosome specific α-satellite centromeric probe for chromosome 17. This technique also revealed differently sized signals in the centromeric region of chromosome 17 also indicating i(17q) in metaphase and interphase cells in CML (Shi et al. 1994).

Table 3: Application for fluorescence in situ hybridisation (taken by combination of two tables described by Lichter, 1990 and Le Beau, 1993).

Clinical	Research
• In clinical cytogenetics, prenatal and Cancer diagnostics	• Chromosomal localization of genes and DNA sequences
-Detection of numerical and strucral chromosomal abnormalities	• Gene expression
-Identification of marker chromosomes (rearranged chromosomes of uncertain origin)	• Developmental Biology
-Detection of early relapse in leukemia	• Tumor Biology
-Identification of the origin of bone marrow transplantation	• Microbiology and virology (Detection of viral sequence in cells)
-Identification of the lineage of neoplastic cells after bone marrow transplantation	• Examination of the organization of DNA/chromosomes in interphase nuclei (Nuclear topography).
-Examination of the karyotypic pattern of nondividing or interphase cells	• Cell sorting
-Detection of gene amplification	• Somatic cell hybrid analysis
-Carrier status disease genes	• Detection of amplified genes
-Infectious disease diagnostics	• Analysis of chromosome aberation in genetic toxicology studies
	• Preparation of cytogenetic maps (including determining the order of and distance between sequence

Locus specific probes have been used to detect structural aberrations such as translocations, deletions, insertions and duplications. For instance, chromosome rearrangements have been studied by using uni-colour and multi-colour FISH (Kibbelaar et al. 1992). The fusion of bcr and abl genes was determined in metaphase spreads and interphase nuclei obtained from CML cases by using dual-colour FISH (Tkachuk et al. 1990; Arnoldus et al. 1990; Zhang et al. 1993). Stilgenbauer et al. (1993) demonstrated the deletion of a tumour suppression gene in CLL in both metaphase spreads and interphase nuclei. Nacheva et al (1993) demonstrated that; (1) in one patient, metaphase spreads showed the 3' region of the abl gene to be deleted from one chromosome 9 and inserted into chromosome 22, (2) in a second patient, 5' bcr sequences were deleted from one copy of chromosome 22, and co-localised with 3' abl sequences on chromosome 9. The technique has also been used for assessing the success of bone marrow transplantation especially for the detection of residual malignant cells in blood and bone marrow (Anastasia et al. 1991).

Whole chromosome specific DNA library probes have significant advantages for metaphase spreads (but not for interphase nuclei), since many malignancies show highly complex rearrangements and it is difficult to produce good quality metaphase preparations. Interpretation of these abnormalities is very difficult with classical cytogenetic techniques. For this reason, chromosome painting using whole chromosome specific DNA library probes is commonly used to interpret the abnormalities in haematological malignancies (Wessels et al. 1991; Dauwerse et al. 1992; Kibbelaar et al. 1992; Zhao et al. 1993).

1.6. Aims of the project

The first aim of this study was to optimise the technique of dual-colour *in situ* hybridisation (FISH) using whole chromosome specific library (chromosome painting) probes, chromosome specific α-satellite repetitive centromeric probes, and locus specific cosmid probes, and apply them to patients with leukaemia using interphase and metaphase preparations. Whenever necessary, commercial probes were used.

Specifically the aims are as follows:

1. Preparation of DNA probe from chromosome specific library constructed from Bluescript plasmids and optimisation of whole chromosome specific DNA library for chromosomal *in situ* suppression hybridisation,

2. Optimisation of uni-colour and dual-colour fluorescence *in situ* hybridisation on metaphase spreads and interphase nuclei using centromeric probes (PJM 128, D12Z1 and D22Z2).

3. Optimisation of the locus specific cosmid probes (abl-18 and bcr-19 and bcr-51) for uni-colour and dual-colour *in situ* hybridisation on interphase nuclei and metaphase spreads from normal (control) cases and patients with leukaemia.

4. Aims in leukaemia studies:

A) CML:

i) Investigate the reliability of dual-colour FISH using abl/bcr probes in both metaphase spreads and interphase nuclei as an improved diagnostic

test for the presence of the Ph chromosome, and to investigate its value in detection of minimal residual disease.

ii) Use both locus specific probes and chromosome library probes in further study of Ph negative and variant Ph cases.

B) AML - M3:

i)Investigate the capability of the probe set for chromosomes 15 and 17 to improve the diagnosis of the specific translocation t(15;17).

ii) To investigate the reliability, and the comparability of the results of, interphase studies using the probe set for chromosomes 15 and 17.

C) CLL:

Detection of trisomy 12 in interphase nuclei. A reliable and clinically useful test ?

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and methods

2.1. Materials

2.1.1. General

All solutions were prepared in deionized water (Millipore "Milli RQ" water purification system). When necessary, solutions were sterilised in an autoclave (Denly) at 121 °C for 15 minutes.

All glassware, most plastics and all tips for micro pipetting were sterilised in an autoclave (Denly) at 121°C for 15 minutes.

Plastic tubes 50 ml were supplied by Sarstedt.

Eppendorf tubes (1 and 1.5 ml) were used.

The glass microscope slides were cleaned by soaking in Decon (Decon Lab.) overnight and then rinsed in running tap water for at least 2 hours, and stored in 70 % ethanol. Before use, slides were rinsed through running tap water 1.5-2 hours to remove alcohol from slide's surface.

The coverslips were stored in 70% ethanol until use and then dried by wiping with Kimwipes tissue (Kimberly-Klark).

2.1.2. Solutions and reagents

2.1.2.1. General solutions

Basic reagents and buffers were prepared according to Sambrook et al (1989) unless stated in the text.

Luria - Bertoni (LB) Medium (500ml)

NaCl	5.0 gr.
Tryptone (Difco Bacto)	5.0 gr.
Yeast Extract (Difco Bacto)	2.5 gr.

Adjusted to 500 ml with distilled water. Sterilised by autoclaving at 121 °C for 15 minutes. Cooled and added appropriate antibiotic, which gives 10 mg/ml in the medium, under sterile conditions.

20xSSC (500 ml)

NaCl	87.6 gr.
Na Citrate	44.1 gr.

Dissolved in 250 ml distilled water and then adjust the pH to 7.0 - 7.3 with concentrated HCI. Adjust volume 500 ml. Autoclaved at 121 °C for 15 minutes .

Hypotonic Solution (500 ml)

Two types of hypotonic solution were used depending on cell preparation: For interphase nuclei, the hypotonic used: 2.79 g KCI dissolved in 500 ml distilled water and kept at 37 °C until use.

For chromosome preparations obtained by culturing cells of peripheral blood or bone marrow in Iscoves medium, the hypotonic solution used was: 2.5 g tri-sodium citrate and 1.39 g KCI was dissolved in 500 ml distilled water and kept at 37 °C until use.

1 % Ethidium Bromide

Ethidium Bromide (EtBr)	1 gr
Distilled water	100 ml

Dissolved by stirring and wrapped with aluminium foil to protect from direct light. Stored at room temperature, or +4 °C for further use

7.5 M Ammonium Acetate (500 ml)

Dissolved 278.7 gr Ammonium acetate in 500 ml distilled water. Dispensed in aliquots. Sterilised by autoclaving at 121 °C for 15 minutes .

5 M NaCI (500 ml)

Dissolved 186.1 gr NaCI in 500 ml distilled water. Aliquoted in 100 ml each. Sterilised by autoclaving at 121 °C for 15 minutes .

1 M Tris-HCI pH 8.0 (250 ml)

Dissolved 30.27 gr Tris in 200 ml distilled water. Adjusted to pH 8.0 by adding concentrated HCI. Adjusted the volume to 250 ml total volume with distilled water. Dispensed into aliquots . Sterilised by autoclaving at 121 °C for 15 minutes .

Tris -NaEDTA Buffer (TE Buffer)

10 mM Tris - HCI pH 8.0

20 mM NaEDTA pH 8.0

Mixed in ratio 1 to 1 (v/v) and stored at room temperature

Electrophoresis Buffer (EB)

40 mM Tris Acetate

20 mM Potassium (or Sodium) acetate

1 mM Na EDTA

RNase (DNase-free)

Added 80 µl concentrated stock (25 mg/ml) (Boehringer) in 20 ml 2xSSC. Boiled for 10 mins to destroy any contaminating DNase. Stored in 1.5 ml centrifuge tubes at -20 °C.

2.1.2.2. Buffers for alkaline phosphatase *in situ* hybridisation

a. Buffer 1 (B1)

50 ml 1 M Tris pH 7.6

10 ml 5 M NaCI

0.2 gr Magnesium chloride

100 µl Triton-X-100 (Sigma)

Made the volume up to 500 ml with distilled water. Stirred for 10 mins.

b. Buffer 2 (B2)

75 ml Buffer B1

2.25 gr Bovine Serum albumin (Sigma). Mixed by stirring.

c. Buffer 3 (B3)

3.63 gr Tris

3.04 gr Magnesium Chloride

6 ml 5 M NaCI.

Adjusted the final volume to 300 ml with distilled water and pH to 9.5 with concentrated HCI.

d. Stop Buffer

2 ml 1 M Tris pH 7.6

1 ml 0.5 M EDTA pH 8.0

Adjusted the final volume to 100 ml with distilled water.

2.1.2.3. Solutions for dual-colour and uni-colour fluorescence *in situ* hybridisation

a) Preparation of Competitor DNA (Salmon Sperm and Human Placental DNA)

200 mg salmon sperm DNA (Sigma) was completely dissolved in 10 ml of distilled water to give 20 mg / ml by incubating in a waterbath at 70 °C. The salmon sperm DNA (SSDNA) was then subjected to sheering by repeatedly passing it through a needle attached to a 10 ml syringe. It was placed on ice and then sonicated for 4 mins three times (at 4 voltage). The fragment size of sonicated DNA was checked by gel electrophoresis in 0.8% agarose gel and then stored at 4 °C.

Human placental DNA was prepared by the same method as salmon sperm DNA during the first year of the project. Thereafter, commercial human placental DNA (10 mg/ml) (Sigma) was used, and also commercial human Cot-1 DNA (10 mg/ml) (Sigma) was also used.

b). Hybridisation Buffer

1 g Dextran sulphate (Pharmacia) was dissolved in 5 ml of formamide (99 %) (Fluka) and 1 ml of 20xSSC by heating at 70 °C for several hours and then cooled. The pH was adjusted to 7.0 with concentrated HCI and the volume brought to 7 ml with distiled water.

c) Biotin 11- dUTP (0.4 mM)

Added 0.1 gr Biotin 11-dUTP (5 (N-Biotinyl-Aminocaproyl)-3-Aminoally-2' Deoxyuridine 5' triphosphate Ammonium salt) (Sigma- B7645) in 290 mM Tris / HCI and 0.1 mM EDTA pH 7.5, and stored at -20 °C.

d) Digoxigenin dUTP (0.35 mM)

10x digoxigenin 11-dUTP(Boehringer)

e) Denaturation buffer (70 % Formamide)

Added 15 ml 2xSSC (pH=7.0) to 35 ml formamide (99 %) (Fluka) and kept it at +4 °C under dark conditions until use.

f) Washing Solution (50 % Formamide)

Added 25 ml 2xSSC (pH 7.0) to 25 ml formamide (Fluka) (v/v) and stored it at +4 °C in the dark condition. It can be used effectively for 1 to 1.5 months.

g) Washing Solution (2xSSC and 4xSSC)

Prepared them by diluting 20xSCC with distilled water.

Tween-20 (Sigma) was added to 4xSSC (0.05%).

h) Washing Solution (0.1xSSC)

Prepared it by diluting 20xSSC with distilled water (1part of 20xSSC plus 190 part distilled water).

h) Washing Solution (4xSSC with 5% nfdm and 0.05% Tween-20)(500ml)

Diluted 20xSSC to give 4xSSC and added 25 mg of non-fat dried milk (nfdm) (Marvel) and 250 µl of Tween-20, mixed, centrifuged and retained supernatant for use.

i) Phosphate-Nonidet-P40 non fat dry milk Buffer (PNM Buffer)

500 ml of 0.1 M Na_2HPO_4 was titrated to pH 8.0 with 0.1 M NaH_2PO_4. Then 500 µl of Nonidet -P40 (Sigma) was added to make a 0.1% concentration. 5 g of non fat dried milk (Marvel) and 50 µl of 1% sodium azide were added and mixed thoroughly until completely dissolved, then centrifuged and supernatant was removed and stored at +4 ^{o}C for use as a washing solution.

j) Antifade medium (100 ml)

There are different types of antifade medium recipe and some are commercially available.

The antifade medium used is a mixture of diazobicyclo-octane (1,4-diazobicylo-(2,2,2)-octane) (DABCO) and the mounting solution. Mounting solution is 9 parts of glycerol and 1 part of 0.2 M Tris-HCI, 0.02% NaN_3, (pH 8.0). 2.3 g of DABCO is dissolved in the embedding solution by warming to 70 ^{o}C for 2-3 hours, and then stored at 4 ^{o}C.

After one yearon this project, Citiflour (ONCOR) was used as an antifade medium by mixing with counter stains (Propodium iodide and DAPI).

k) Propodium iodide (20 µg /ml)

20 µl of propodium iodide (1 mg / ml; Sigma) was diluted in 980 µl of distilled water and mixed very well. Wrapped with aluminium foil and stored at 4 °C.

l) DAPI (4,6-Diamidino-2-Phenenyl-Indole) (40 µg / ml)

40 µl of DAPI (1 mg / ml) (Sigma) was diluted in 960 µl of distilled water and mixed very well. Wrapped with aluminium foil and stored at 4 °C.

2.1.2.4. Detection reagents for uni-colour and dual-colour FISH

The following detection reagents were used for this study:

a) Fluorescein isothiocyanate (FITC) avidin (Vector Lab)

b) Rhodamine avidin (Vector Lab)

c) Biotinylated goat anti avidin (Vector Lab)

d) Biotinylated goat anti avidin FITC (Vector Lab)

e) Sheep antidigoxigenin fluoresceinated FAB fragment (Boehringer)

f) Sheep antidigoxigenin Rhodamine FAB fragment (Boehringer)

g) Anti-sheep Isothiocyanate IgG (H+L) (Boehringer)

2.1.3. Probes

In this project, the probes used were from different sources as detailed below:

I. Probes which were grown up and labelled in the department;

a) Unique sequence highly repetitive probe DYS59, (GMGY10). This probe is localised on the short arms of the Y chromosome and has an

insert size of 4.5 kb (Affara et al. 1986). This probe is available as a glycerol stock in the department.

b) Centomeric probes used were PJM128, D12Z1 and D22Z2 detecting chromosomes 8, 12 and 22 respectively. These were obtained commercially from the ATCC company and were grown up and labelled in the department.

The PJM 128 is an α-satellite repetitive probe, 2.25 kb, and localised on centromere of chromosome 8 (Donlon et al, 1986).

The D12Z1 is an alpha satellite repetitive probe, 1.35 kb and localised on centromere of chromosome 12 (obtained from ATCC).

The D22Z2 is an alpha satellite repetitive probe, 2.1 kb, and localised on the centromere of chromosome 22 (Mc Dermid et al, 1986).

c) Whole chromosome specific DNA libraries for chromosomes 18 and 21 (pBS-18 and pBS-21) were grown up and labelled in the laboratory (gift from Dr.J.Gray, San Francisco).

d) Locus specific (or unique sequence) cosmid (cos) probe abl-18 for chromosomes 9 at band q34, and cos bcr-19 and cos bcr-51 specific for chromosome 22 at band q11, which were grown up and labelled in the department.

The cos abl-18 and cos bcr-19 were obtained as a generous gift from Professor G.C. Grosveld, The University of Rotterdam, Holland. The cos bcr-51 was kindly sent by Dr. Guida Boavida, Institute of National de Saude, Portugal.

The abl-18 is a cosmid probe. It contains a 40 kb fragment insert representing the 3' coding and 3' flanking sequences of the human abl

oncogene (chromsome 9q34) **(Figure 4)**. This probe contains the complete Abelson murine leukaemia virus (Abelson MuLV) (Heistterkamp et al, 1983).

The bcr-19 is a cosmid probe. It contains a 34 kb fragment representing most of the exon and 5' half of the first intron of bcr gene on chromosome 22 at band q11 **(Figure 4)** (Hermans et al., 1989). The bcr-51 is also a cosmid probe and contains a 35.5 kb fragment.

II) Commercial Probes:

a) t(15;17) DNA Probes (P5119-D/B) were obtained from ONCOR company. This composes two cosmid probes, the first for the MYL oncogene which is located at 15q21 and the second for the retinoic acid receptor alpha (RARA) genes on the 17q12. The cosmid probes for chromosmes 15 and 17 break points were already labelled with biotin-dUTP and digoxigenin-dUTP respectively (ONCOR).

b) Whole chromosome specific DNA library of chromosomes 3, 6, 8, and 22 were obtained from CAMBIO and were already labelled with Biotin. A chromosome 9 library probe obtained from BRL had been directly labelled with spectrum orange fluorophore.

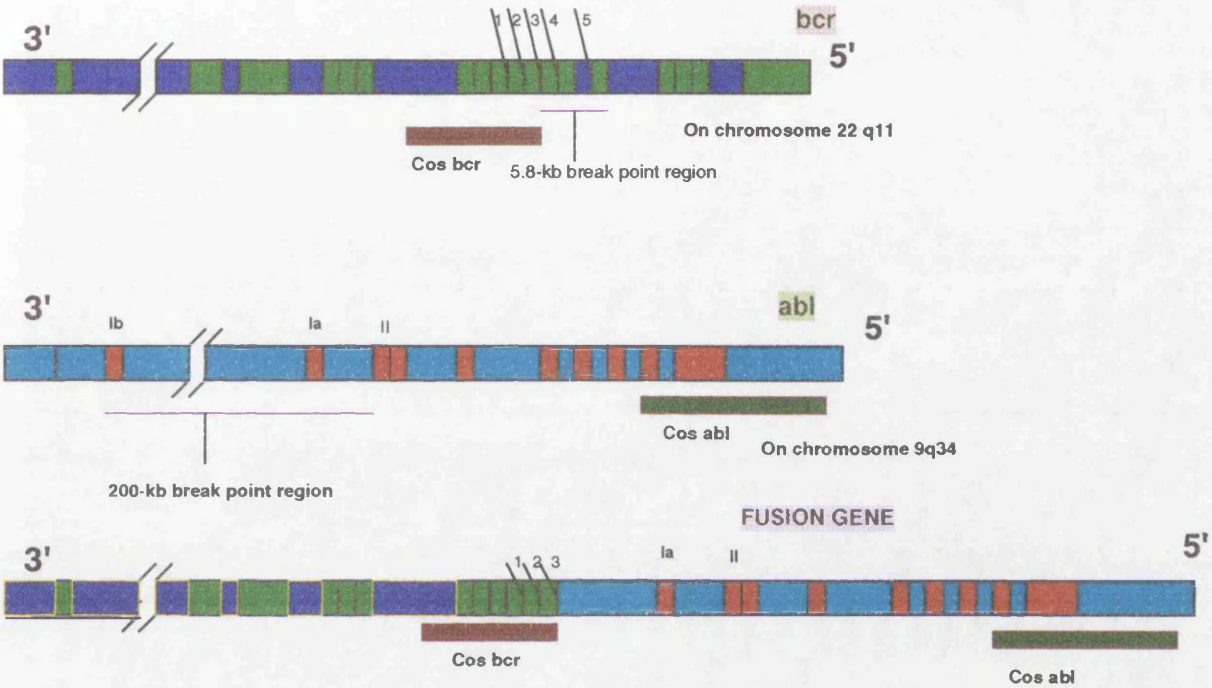

Figure 4: Schematic representation of the bcr, abl and the bcr-abl fusion genes (not scale). Exons of bcr gene are depicted as black boxes. I refers to the first exon; the numerals 1 to 5 refer to the exons the breakpoint cluster region. The bcr probe is indicated by horizontal red bar. The exons of the abl gene are depicted as dark pink boxes. The numerals Ia and Ib refer to the alternative first exon, and II to second exon. The abl probe will always be away from the fusion junction. Therefore, on the bcr-abl fusion gene the bcr probe will always lie at the junction and the abl will be separated from it.

2.1.4. Patient material

Three main groups of diseases were studied in this project -Chronic Myeloid Leukaemia (CML), Chronic Lymphocytic Leukaemia (CLL) and Acute Promyelocytic Leukaemia (AML-M3) which is a subgroup of acute myeloid leukaemia (AML). In CML and AML-M3 the studies involved both mitotic chromosomes and interphase nuclei, and were mainly carried out on fixed material provided by the leukaemia cytogenetics section of the DGI. (Duncan Guthrie Institute) In CML patients some interphase preparations were made from fresh peripheral blood samples. The CLL studies were performed on interphase preparations and mitotic chromosomes produced from peripheral blood samples kindly provided by the Haematology Department at the Victoria Infirmary.

Normal cases for optimisation studies and controls were obtained from the diagnostic cytogenetics laboratory.

2.2. Methods

2.2.1. Probe isolation

2.2.1.1. Culture and preparation of the host bacterial strains containing specific probe

The isolation of whole chromosome specific DNA library probes (pBS-18 and pBS-21), cosmid probes (abl-18, bcr-19 and bcr-51) and centromeric probes (D12Z1, PJM 128 and D22Z2) from their host cells was carried in the same way as following.

The Luria- Bertoni (LB) medium prepared according to Sambrook et al (1989) was aliquoted into 10 and 40 ml after adding an appropriate antibiotic (Ampicilin or Kanamycin) under sterile conditions. 100 µl of the host bacterial cells containing specific DNA probe was added to 10 ml medium and incubated for 2- 3 hours in an orbital shaker at 37 °C. After this period, the volume was adjusted to 50 ml with LB medium prewarmed to 37 °C. Host cells containing probe were grown in 50 ml of LB medium overnight at 37 °C in the orbital shaker.

2.2.1.2. Rapid isolation of probe DNAs from host cells

For isolation of probe DNA (vector DNA plus insert DNA), the CIRCLEPREB KIT (Bio 101, Inc.) was used. The isolation was done according to the manufacturer's protocol with a few modifications.

I. Cleared - Supernatant Preparation

1) Cultured cells were transferred to a 50 ml plastic conical tube, and were spun down at 10,000 rpm for 8 min at 4 - 7 °C. Supernatant was discarded and tube was drained for 1 to 2 minutes.

2) 4 ml of prelysis buffer (Tris / EDTA / glucose solution) was added onto the pellet which was then re-suspended by vortexing. This is important for obtaining uniform lysis during the next step.

3) 4 ml of alkaline lysis reagent (0.2 N NaOH and 1 % SDS) was then added and mixed until all cells were uniformly lysed. When larger pellet occurred, if necessary, it was placed in a waterbath at 45 to 60 ^{o}C for a few minutes to help attain uniform lysis.

4) 4 ml of neutralising solution (3 M Potassium acetate) was added, mixed thoroughly for 1 to 5 minutes until contents became essentially two phases, consisting of a white precipitate suspended in clear liquid of water - like viscosity.

5) The mixture was centrifuged at 10,000 rpm for 8 mins at 4-7 ^{o}C and the supernatant (approximately 12 ml) transferred to a clean 50 ml centrifuge tube.

6) Following that, an equal volume of propanol (~12ml) was added to the supernatant.

7) After centrifugation at 10,000 rpm at 4-7 ^{o}C, the supernatant was discarded and the pellet was drained by inverting the tube. The inside wall was wiped carefully with a soft tissue to remove most of the residual liquid.

8) The pellet was dissolved in 0.5 ml water with the aid of vortex mixing. The mixture was transferred to a microcentrifuge tube. At this point, the mixture was either stored at 4 ^{o}C until use or the next step was followed immediately.

The mixture at this stage consists of RNA, plasmid- or cosmid- DNA containing specific insert DNAs, and some cellular chromosomal DNA.

II. Lithium Chloride (LiCl) Precipitation of Ribosomal (rRNA) and Single Stranded DNA (ssDNA)

1) The microcentrifuge tube containing the cleared supernatant was incubated in a hot block at 100 ^{o}C for 3-5 minutes. The object was to heat the tube long enough to denature linear cellular DNA without nicking supercoiled vector DNA. Then immediately, it was cooled in an ice bath for at least 1 minute.

2) 300 µl of LiCI was added to the tube and mixed well and then allowed to stand for 5 minutes at room temperature, followed by centrifugation for 2 minutes at 12,000 rpm to pellet down the ribosomal RNA (rRNA) and single stranded DNA (ssDNA).

3) The supernatant was transferred to a new centrifuge tube and 600 -700 µl of isopropanol were added to it.

4) The tubes were centrifuged for 2 minutes at 12,000 rpm. The supernatant was discarded, and the pellet was drained and then dissolved in 0.5 ml distilled water by mixing very well. 300 µl of LiCI was added to the mixture.

III. Purification of Probe DNA on CIRCLEPREP GLASSMILK

1) Following the previous step, 75 µl of homogenous glassmilk solution was added, mixed well and incubated at room temperature for 5 mins. Then it was centrifuged for a few seconds to pellet the CIRCLEPREP GLASSMILK / DNA complex.

2) The supernatant was removed and the pellet was washed 2-3 times with 1 ml binding buffer (KBr, NaI and Tris mixture) which selectively maintains binding of DNA to CIRCLEPREP GLASSMILK, but remove RNA, protein and polysaccharides from solution.

3) After centrifugation and removing the last binding buffer wash from pellet, the pellet was washed twice with washing solution. After pouring off second wash, the tube was spun for a second to remove traces of liquid from bottom of tube.

4) Probe DNA was eluted from CIRCLEPREP GLASSMILK by resuspending the pellet in 100 to 300 µl distilled water or T.E (1 mM Tris, 1 mM EDTA pH 7.6) buffer, and incubating for 5 mins in a 45 to 60 ^{o}C waterbath. The tube was centrifuged at 12,000 rpm for 30 seconds to form a tight pellet and then the supernatant containing the probe DNA was transferred to a new sterile centrifuge tube.

2.2.1.3. Estimation of DNA concentration

The concentration of probe DNA was estimated by measuring the optical density (O.D.) at 260 nm in a spectrophotometer.

The optical density of 1000 µl distilled water was measured at 260 nm and then the spectrophotometer settled to zero. 10 µl of aliquot probe DNA was added to 990 µl distilled water, vortexed and then estimated at 260 nm. The concentration of DNA in the suspension as µg/ml was calculated with following formula;

Dilution Factor x O.D. of DNA suspension x 50 = µg / ml.

where 50 = 1 O.D. gives 50 µg DNA at 260 nm.

2.2.1.4. Restriction enzyme digestion of whole chromosome specific DNA probe (pBS-18 and pBS-21)

The digestion reaction was carried out for whether the purified plasmid DNAs contains the chromosome specific inserted library DNA or not. 3-4 μg of DNA (pBS-18 or pBS21) was digested individually with Hind III (15 U / μl) in the presence of 4 mM spermidine hydrochloride and enzyme buffer in a 20 μl volume. The mixture was vortexed and spun briefly and then incubated at 37 °C for at least 3 hours or overnight (16 hours).

Digestion reaction was stopped by adding 5 μl of loading mix. The digested DNA sample was either loaded onto 0.8% agarose gel or stored at -20 °C until use.

2.2.1.5. Gel electrophoresis

The digested DNAs were separated into the insert chromosome specific library DNA and the plasmid DNA by agarose gel electrophoresis. For this, 0.8 g agarose (Sea, Kem GTG, FMC) was dissolved in 100 ml of 1 x electrophoresis buffer (1xEB) and thawed in a microwave oven for 3 mins. When the temperature reached less than 60 °C, 2 μl of 1 % Ethidium bromide (EtBr) (Sigma) was added to the mixture to enable visualisation of DNAs. The gel was poured onto the electrophoresis gel casting unit. When the agarose solidified, DNA samples were loaded. In order to determine the size of the DNA fragments, 1 kb ladders of standard length DNA fragments were loaded as well. The gel was run in the 1xEB at 50 mA for 3 hr. After electrophoresis, the gel was photographed using a Polaroid CU-U hand camera fitted with a red filter and black & white Polaroid type 667 film.

2.2.1.6. Digestion of the probe and electrophoresis

The size of probe is critical for efficient hybridisation. The optimum size of probe DNA fragments for in situ hybridization is approximately 500 base pairs (bp) (Lichter et al 1988). Therefore, the human chromosome genomic libraries 18 and 21 (pBS-18 and pBS-21) individually were first digested into fragments using DNase because untreated probes did not result in complete coverage on chromosomes. The DNase concentration (10,000 IU / µl) (Pharmacia) was empirically established to yield fragments in the desired size range.

The DNA sample, which was precipitated with standard DNA precipitation to obtain desired concentration, was dissolved in the DNase buffer (40 mM Tris-HCI, pH 8.0; 6 mM MgCI) and warmed in a 37 °C waterbath. 1 µl DNase solution (10 IU/ul) was added and incubated at 37 °C for a few minutes. The DNA sample was immediately transferred to a freezer at -20 °C for storing until the gel has been run. In order to check the fragment size, the digested probes were run in 0.8 g agarose gel in 100 ml 1xEB. When the required DNA fragment size of about 500 -700 nucleotides was achieved, the reaction was terminated by heating the mixture at 70 °C for 8 mins.

The digested DNA probe was labelled by nick translation. The nick translation reaction was the same as in Section 2.2.2.1.

2.2.2. DNA probe labelling

Two labelling systems were used. These are random primed labelling and nick translation labelling systems. With nick translation system, DNA probes were labelled with either biotin -11-dUTP (Sigma) or Digoxigenin

dUTP (Boehringer) reporter molecules by enzymatic incorporation. However, with random primed labelling system, only Digoxigenin dUTP as a reporter molecule was used to label the probe.

2.2.2.1. Probe labelling with nick translation

Probes pBS-18, pBS-21, DYS59, PJM 128, D12Z1, D22Z2, cos abl-18, cos bcr-19 and cos bcr-51 were labelled with biotin- 11-dUTP or Digoxigenin-11-dUTP by Nick Translation (BRL Kit). The reaction was performed in a 50 µl volume as follows; In an ependorf centrifuge tube the following were added sequentially: 5 µl of solution A4 (BRL Kit) (0.2 mM Nucleotides G, A and C in 500 mM Tris-HCI (pH 7.8), 50 mM Magnesium chloride, 100 mM 2-Mercaptoethanol, and 100 µg / ml Bovine serum albumin (BSA)), 1-3 µg probe DNA to be labelled, 2.5 µl of 0.4 mM Biotin-11-dUTP (Sigma, UK.) and solution E (distilled water) to make final volume 45 µl. To this mixture 5 µl solution C (0.4 Units / µl BRL DNA polymerase I, 40 pg/µl DNase I, 50 mM Tris-HCI (pH 7.5), 5 mM Mg-acetate, 1 mM 2- mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 % glycerol and 100 µg/ml nuclease-free BSA) was added. The solution was mixed gently and then incubated at 14-15 °C for 60-90 minutes. After this period, the reaction was stopped by adding 5 µl solution D (300 mM disodium EDTA pH 8.0, stop buffer). Unincorporated nucleotides were removed by adding 4.6 µl of 3 M Sodium acetate (pH 5.2), 1 µl of 20 mg/ml Glycogen (Boehringer) and 122 µl cold ethanol, and was then vortexed briefly. The mixture was then centrifuged for 30 minutes at 12,000 rpm. The supernatant was aspirated and the pellet was vacuum - dried and then dissolved in TE buffer to get desired concentration and vortexed to mix properly. It was left either at room temperature for 2 - 3 hours or placed in the waterbath at 37 °C for 20-30 minutes, and then stored at -20 °C until use.

Above probes were also labelled with digoxigenin - dUTP using the nick translation labelling system. The nick translation was carried out as described for Biotin 11-dUTP except for that the 50 µl of labelling mixture contained 2 µl of digoxigenin DNA labelling mixture (dATP, 1mmol/l; dATP, 1 mmol/l; dGTP, 1 mmol/l; dTTP, 0.65 mmol/l; and Dig - dUTP, 0.35 mmol / l pH 7.5) (Boehringer) instead of solution A4 (Gibco - BRL) and 2.5 µl of 0.4 mM Biotin - dUTP (Sigma). The probe precipitation and following steps are same as for biotin labelling with the nick translation (above).

2.2.2.2. Probe labelling with random prime labelling

Probes PJM 128, D12Z1, abl-18, bcr-19 and bcr-51 were labelled with digoxigenin-11-dUTP by random prime labelling system (Boehringer). The reaction was performed in 20 µl total volume. Firstly, 1-3 µg DNA in T.E-buffer or distilled water was added to 1.5 ml micro centrifuge tube, and denatured to obtain single stranded DNA by heating at 80-90 °C for 5-8 mins and chilled quickly on ice. Complete denaturation is essential for efficient labelling. Then the following reagents were added to the micro centrifuge tube containing the denatured DNA to be labelled on ice: 2 µl of hexanucleotide mixture (10x concentrated) (1mM ATP, 1 mM dCTP, 1 mM dGTP and 0.65 mM dTTP, pH 7.0) and 2 µl of Digoxigenin-dUTP (10x concentrated) (0.35 mM) were added. The volume adjusted to 19 µl with distilled water and then 1 µl of Klenow enzyme (Boehringer) was added. The mixture was centrifuged at 12.000 rpm for 30 seconds and incubated at 37 ^{o}C for at least 60 minutes or overnight because longer incubation can increase the yield of labelled DNA. The reaction was stopped by adding 2 µl of EDTA solution (0.2 M, pH 8.0). The labelled DNA was precipitated by adding 1.9 µl of 3 M Sodium acetate (pH 5.2), 1 µl of 20 mg/ml Glycogen (Boehringer) and 98 µl cold ethanol, and vortexed briefly. After incubation

at -20 °C for 30 mins, the mixture was centrifuged at 12,000 rpm for 30 mins. The supernatant was removed, the pellet was dried in the vacuum drier machine and then dissolved in TE-buffer at 37 °C for 30 mins. It was stored at -20 °C until use.

2.2.3. Specimen preparation

For fluorescence *in situ* hybridisation, metaphase spreads and interphase nuclei were prepared. Metaphase spreads from bone marrow and peripheral lymphocytes were prepared according to conventional method by bone marrow section in the department.

2.2.3.1. Preparation of chromosome spreads from peripheral lymphocytes

Culture medium (Iscoves-Imperial) for peripheral lymphocytes contained thefollowing:1% heparin (5.000 U/ml) (Leo Lab.), 2 % phytohemaglutinin (M-form) (Gibco) and 1% antibiotics (10.000 IU/ml Penicillin / 10.000 µg/ml Streptomycin solution (Gibco)) . 10 ml aliquots were dispensed into the 15 ml sterile plastic centrifuge tubes. To each tube , 1 ml of peripheral blood was added and the culture incubated for 72 hours at 37 °C.

Prior to harvesting the peripheral lymphocytes, 0.1 ml of 3.2 µg/ml Colcemid (Gibco) was added and then incubation continued for 45 minutes at 37 °C to stop the cell cycle at metaphase stage by inhibiting spindles formation. The cells were harvested by centrifugation at 1000 rpm for 7 mins. The supernatant was discarded and the cell pellet was gently re-suspended in 10 ml of prewarmed to 37 °C hypotonic solution (0.075 M KCl) and left at 37 °C for 8 -15 mins. Following centrifugation at 1000 rpm for 7 mins, the supernatant was removed and the cells were re-suspended in 10 ml fresh fixative (3:1; Methanol : Acetic acid). This step was repeated twice. If the pellet was not clean, washing with fixative was repeated until

clear pellet obtained. The tubes were sealed and left at 4 °C for 30 mins. The fixed cells then were centrifuged at 1000 rpm for 7 mins and fresh fixative was added. The cell pellet was either stored at 4 °C until use or used directly. The slides were prepared as follows; 2-3 drops of cell suspension was dropped from a height of 30-40 cm onto a clean wet microscope slide. The slides were dried at room temperature overnight or incubated at 42 °C overnight. Some slides were stored at +4 °C for long time storage till use.

2.2.3.2. Preparation of interphase nuclei from peripheral blood of CLL and CML cases

Preparation of interphase nuclei from patients with CLL and control groups was performed in two ways. One is direct preparation (alternative I) and the other (alternative II) is by separating of mononuclear cells. CML samples, peripheral interphase nuclei, were prepared according to alternative I.

a) Alternative I: 12-15 ml of hypotonic solution (0.075 M KCl) was added to 1- 1.5 ml of whole blood, mixed very well and directly centrifuged at 900 -1000 rpm for 7 minutes. Supernatant was removed, and to pellet 1ml of distilled water was added to help to remove any clots. Cell suspension was fixed in methanol : acetic acid (3:1; v/v) and then centrifuged at 900 - 1000 rpm. Supernatant was removed and the pellet was washed with fixative twice as described in the previous step except that no water was added. Slide preparation and storage were carried out exactly as mentioned in the previous section.

a) Alternative II: Firstly, Histopaque-1077 (Sigma) in 15 ml centrifuge tube is brought to room temperature, and 2 ml of whole peripheral blood was added onto the histopaque (1/1; v/v) (Sigma,1077-1) very carefully. It was centrifuged at 1500-1800 rpm for 30 minutes. After centrifugation, the opaque interface layer containing the mononuclear cells within its upper layer was transferred to another centrifuge tube. The cells were washed twice with cold phosphate buffered saline (PBS) (Sigma) solution by mixing very gently. After the last washing, the supernatant was aspirated and discarded. Hypotonic solution (0.5 % KCI and 0.28 % tri-sodium citrate) was added to homogenated pellet, and incubated for 5 mins at 37 ^{o}C. Following the centrifugation, the pellet was washed two to three times with methanol : acetic acid (3:1; v/v). The pellet was dropped onto clean wet glass slides. They were aged at 42 ^{o}C or room temperature overnight.

2.2.4. *In situ* hybridisation

2.2.4.1. Hybridisation and detection of DYS59 with alkaline phosphatase

Normal male samples were used in this section. Lipsol banding or trypsin banding methods were applied to different aged slides (one day to three months) to allow chromosome identification before *in situ* hybridisation and then hybridisation and detection were performed.

2.2.4.1.1. Lipsol banding

For chromosome identification, the lipsol banding technique (Garson et al. 1987) was used prior to the hybridisation. The chromosomes were banded by treatment in a solution of the detergent lipsol (1 %) for 7-15 seconds. The slides were rinsed with saline solution and stained using Leishman's stain in pH 6.8 buffer for 1-4 mins. The slides were washed with buffer (Gurr) (-BDH) pH 6.8 and covered with a 24x64 mm clean

coverslip to prevent direct contact between oil and metaphase spreads and nuclei. Well spread and banded metaphases were photographed and recorded to enable the same metaphases to be localised after hybridisation when banding is no longer clear and therefore chromosome identification is difficult. Immersion oil was wiped off with soft tissue and the slides immersed in phosphate buffer (PBS)(-Sigma) or 2xSSC, and the coverslips were removed automatically. The slides were then destained through an alcohol series (50%, 70%, 90%, and 100%) and air-dried (Garson et al, 1987). Alternatively, destaining was carried out using a modification of Klever and Co-workers' method (1991). According to this method, the slides were washed for 1 minute in xylol / ethanol (1/1, v/v) and then washed twice for 5 mins in methanol / acetic acid (3/1,v/v) and air-dried at room temperature. The area of slide containing the photographed metaphases was marked with a diamond pen.

2.2.4.1.2. Trypsin banding

For trypsin banding, Garson and co-workers' (1987) technique was used, because routine trypsin banding technique may remove too much DNA from the chromosomes. In this method the slides were incubated at 37 °C for 15-120 seconds in phosphate buffer saline (PBS)(Sigma) 7.4 containing $1x10^{-7}$ % (w/v) trypsin (Gibco). Followed by further 1 hour incubation in PBS at 60 °C, G-banding was completed by staining the slides for 1 to 3 seconds in Leismann which diluted in the buffer (pH 6.8) (1:4). After metaphases were localised and photographed, slides were destained as stated in section 2.2.4.1.1.

2.2.4.1.3. Biotinylated *in situ* hybridisation with the probe DYS59

The probe DYS59 (GMGY10) was used to assess with the alkaline phosphatase biotinylated technique developed by Garson et al. (1987). This method was modified as follows; after destaining, 150 µl RNase (100 µg/ml in 2xSSC) was applied to each pre- marked area of the slides and incubated under 24x32 mm coverslip on a metal tray floating in waterbath at 37 °C for 1 hour. The coverslip was then removed by soaking the slides in PBS or 2xSSC. The slides were then washed in fresh PBS or 2xSSC. The slides were dehydrated in an alcohol series (50 %, 70 %, 90 % and 100 %), and air-dried. 1 µl (100 ng / µl) of the probe DYS59 was added to 9 µl hybridisation buffer and mixed briefly. The hybridisation mixture was applied to the pre-marked area on the slides without competitor DNA. A 24x24 mm coverslip was placed over the area to which the probe has been applied, and the edge of coverslip was sealed with cow gum. The slides were then placed in an oven at 80 - 90 °C for 10 minutes in order to denature both the chromosomal and probe DNAs. The slides were then incubated in a waterbath at 37-43 °C overnight (15-18 hours).

Next day, the cow gum was peeled off very gently and slides washed in 2xSSC or PBS to remove the coverslip, and briefly rinsed in fresh PBS or 2xSSC. The slides were then washed as follows: 2xSSC for 15 minutes; the buffer B2 (75 ml buffer B1 and 2.25 g bovine serum albumin) for 15 mins. All washes were carried out at room temperature, except for the wash in 0.1xSSC which was prewarmed to 42 °C, submerged washed with shaking for 30 minutes.

2.2.4.1.4. Detection of the probe DYS59 with alkaline phosphatase technique

The probe DYS59 is a highly repetitive probe and was detected with the alkaline phosphatase technique as follows; after the B2 wash, the area of the slides surrounding the marked area was dried with soft tissue. 1 µl streptavidin alkaline phospatase conjugate (SA-AP) (1 mg/ml-BRL Blue GENE KIT) in 99 µl filtered sterilised B2 buffer was applied onto the slides and then incubated for 20 minutes at 37 °C. SA-AP was flushed off with the buffer B1 and the slides washed with fresh buffer B1 for 5 minutes followed by a 10 mins wash in the B3 buffer. The surrounding area was dried with soft tissue and 150 µl substrate was applied to each marked area and incubated for 7-20 mins in dark. The substrate comprised 1 ml of filtered B3 buffer, 4.4 µl Nitrobluetetrazolium (NBT) (Blue GENE KIT), 3.3 µl Bromochloroindolyphosphate (BCIP) (BlueGENE KIT) and 10 µl of 10 mM Levamisol. If inadequate signal was detected, the incubation time was extended. After the wash with the buffer B3, the slides were immersed in the Stop buffer for 5 minutes to terminate colour development, and air dried. The slides were mounted with coverslips using the glycergel (Dakopatts).

2.2.4.2. Biotinylated *in situ* hybridisation with unique sequence DYS59 Probe, using fluorescence detection system

Hybridisation was accomplished by using a modification of the procedure described by Pinkel et al (1986). Slides prepared according to section 2.2.3.1 were treated with RNase (100 µg/ml) in 2xSSC at 37 °C for 1 hour. After treatment the slides were washed in PBS (pH 7.0) (Sigma) or 2xSSC (pH 7.0) twice and then dehydrated in a 50 %, 70 %, 90% and 100 % ethanol series, and air -dried. The chromosomal DNA on the slide was denatured in 70 % formamide/ 2xSSC (v/v), pH 7.0 at 70 C for 3-5 mins and then

immediately dehydrated in a cold ethanol series of increasing concentration (70%, 90% and 100%) and dried at room temperature. While drying the slides, 10 µl hybridisation mixture containing 50 % formamide, 2xSSC - pH 7.0, 10 % dextran sulphate, 1 mg of sonicated salmon sperm DNA/ml and 1 ng of biotinylated chromosome DYS59 unique sequence probe (GMGY10) was denatured at 70 °C for 5 mins. After denaturation the hybridisation mixture was applied on the marked area of the slide and then covered with 24x24 mm coverslip and sealed edges of the coverslip with cow gum.

Alternatively, after washing the RNase in alcohol series, the hybridisation mixture was directly applied on the marked area of the slides and then covered with 24x24 mm coverslip and sealed as above. Both chromosomal and probe DNAs were simultaneously denatured in the oven at 80-90 °C for 7-10 mins. Both types of hybridisation were carried out in a waterbath at 37-42 °C for at least 16 hours.

2.2.4.2.1. Detection of biotinylated unique sequence probe DYS59

After hybridisation the cow gum was peeled off very gently. The slides were then immersed in three changes 3 minutes each of 50 % formamide in 2xSSC pH 7.0 at 45 °C, washed twice in PBS pH 7.0 or 2xSSC pH 7.0 at room temperature. Thereafter, protein binding sites were blocked using 4xSSC 3% bovine serum albumin and then washed twice with 2xSSC. All subsequent steps were performed at room temperature.

Two hundred microliters of the fluoresceinated avidin (FITC-Avidin) (Vector Laboratory) (2-5 µg/ml in 5% non fat dried milk (nfdm) in 4xSSC (Lichter et al. 1988, Lawrence et al 1988) was applied onto slides and incubated for 30 mins, covered with aluminium foil to protect the effect of lights on the fluorescence. After draining FITC- avidin, the slides were

then washed in three minutes changes of phosphate Nonidet-p40 buffer (0.1 M NaH2PO4 /0.1 M NaHPO4 pH 8.0 / 0.1 % Nonidet-P40 (BDH)) with gentle agitation on a shaker.

Alternatively, the washing was carried out in the following solutions; 4xSSC, 4xSSC with 0.1 % Triton X-100 (BHD) and then 4xSSC for ten minutes each, respectively (Lawrance et al. 1988). The intensity of biotin linked fluorescence was amplified by incubation for 30 minutes with biotinylated goat anti avidin antibodies (Vector Laboratory) (200 µl of 2-5 µg/ml in 5 % nfdm in phosphate buffer pH 8.0 or 4xSSC, followed by washing as above and incubation with layer of fluoresceinated-avidin. For the highly repetitive probe DYS59, the layer was applied one to three times. The slides were later washed in same washing solutions as above.

Ten microliters of DABCO antifade solution, containing 2 µg/ml propidium Iodide as chromosome counter stain, was applied to the slide and covered with a coverslip.

The slides were screened under the epifluorescence microscope (Zeiss Axioplan) at excitation wave length 450-490 nm.

2.2.5. Chromosome suppression *in situ* hybridisation with the pBS-18 and pBS-21 probes and detection

The undigested and digested whole chromosome specific DNA libraries, pBS-21 and pBS-18, were labelled with biotin-11-dUTP by nick translation.

Prior to hybridisation, slides were treated with acetone for ten minutes and air dried. The chromosome *in situ* suppression hybridisation with whole chromosome 18 and 21 libraries were individually carried out with slight modifications described by Pinkel et al (1988) and Lichter et al (1988).

Hybridisation and washing conditions were tested for each digested and undigested pBS-18 and pBS-21 probes.

Thus, total hybridisation mixture consisted of 200-700 ng biotinylated whole chromosome specific DNA library probe pBS-21, 50 % formamide in 2xSSC pH 7.0, 10 % dextran sulphate and either 0-2000 ng of sonicated salmon sperm DNA, or sonicated human placental DNA, or combination of salmon sperm and human placental DNAs. Then, prehybridisation mixture containing the probe was left to denature at 65-70 °C for 30 minutes and then incubated at 37 °C for 30 minutes to 1.5 hours.

The target DNAs were denatured by incubation in 70 % formamide in 2xSSC (v/v) at 65 -70 °C for 2 - 5 mins, chilled and dehydrated in a cold ethanol series (70 %, 85 % and 100 %) and then air-dried at room temperature. Simultaneous hybridisation of whole chromosome specific probe and target DNAs was also tested as described in section 2.2.1.4.3 the denaturation for unique sequence probe DYS59.

The hybridisation mixture (total volume 10-13 µl) was applied onto the slides containing denatured metaphase and nuclei area, covered with a coverslip and sealed around of the coverslip with cow gum in order to prevent evaporation of hybridisation mixture and allowed to hybridise at 37-42 °C overnight to 24 hrs. After hybridisation, the cow gum was peeled off very gently and detection was carried out as follows: First, coverslips were removed by soaking slides in 2xSSC at 40 - 44 °C for 3 - 5 minutes. Second, the slides were washed twice by incubating in 50 % formamide washing solution at 37 - 44 °C for 3-5 minutes and followed by two washes in 2xSSC at same temperatures as above. Next, after incubation with 4xSSC containing 5 % nfdm and 0.05% Tween-20, detection of the probes was accomplished with fluorescein labelled avidin DCS and anti avidin

system according to Pinkel (1988) and Lichter (1988) with slight modifications. The slides were incubated with 100 µl of fluoresceinated avidin DCS (2.5 :1000 dilution, solution containing 4xSSC, 5 % nfdm and 0.05 % Tween-20, or in PNM solution) at room temperature or at 37 °C for 20 - 30 minutes. Amplification of signal was achieved by incubation with 100 µl of biotinylated anti avidin (1.5 mg / ml) (2.5 :1000 as above) as first layer. Finally, last layer was carried out exactly as the first layer. Between each layer, the slides were washed in either 4xSSC wash solution with or without nfdm, or in PNM wash solution. The slides were then washed in 4xSSC containing 0.05 % Tween-20 and then dehydrated in an alcohol series (70 %, 90 % and 100 %) and air-dried. The slides were stained with counter stain solution including propodium iodide in DABCO. The slides were finally screened using fluorescence filters (Excitation 450-490 nm) for FITC under the epifluoresceince microscope (Zeiss Axioplan), or stored in dark at 4 °C until the screening.

2.2.6. Optimisation of the abl and bcr cosmid probes for uni- and dual-colour fluorescence *in situ* hybridisations

Prior to optimisation of dual-colour fluorescence in situ hybridisation, cosmid abl and bcr probes, which are locus specific probes, were optimised for uni-colour FISH using the biotinylated probes and the digoxigenin labelled probes. Then the optimised uni-colour FISH technique was modified to dual-colour FISH technique.

2.2.6.1. Optimisation of uni-colour fluorescence *in situ* hybridisations for abl and bcr cosmid probes

For both abl and bcr cosmid probes, the following steps were optimised on cytogenetically normal case samples: preparation of hybridisation mixture (such as probe and competitor DNA concentration), denaturation of probe and target DNA, post hybridisation washing, immunochemical detection and counterstaining.

a) Preparation of hybridisation mixture:

The labelled abl or bcr probe was mixed in hybridisation buffer containing 50 % formamide, 10 % Dextran sulphate in 20xSSC. With this mixture, different concentrations of probes abl (50-200 ng) and bcr (50-200 ng per hybridisation) were tested. However, in order to suppress cross hybridisation, 5 μg to 15 μg of human placental DNA and salmon sperm DNA each in 10 μl hybridization buffer were tested.

b) Denaturation of probe mixture and target DNA:

Denaturations of probe and target DNA were performed separately and simultaneously. In simultaneous denaturation, the hybridisation mixture was applied to the slides which after immersed in acetone for 10 minutes and air dried. Hybridisation mixture was covered with a coverslip, sailed the edges of it with cow gum. This was then placed in a 80-90 °C oven for 7-10 minutes to denature simultaneously hybridisation mix and chromosomal DNA. After denaturation, the slides were immediately transferred to waterbath at 37-43 °C and incubated overnight for hybridisation.

In separate denaturation, hybridisation mix was placed in a waterbath at 60-75 °C for 5-15 minutes for denaturation of probe and competitor DNA in hybridisation buffer. After this period the tube was transferred to waterbath at 36-38 and prehybridisation was performed at the same temperature for 30-120 minutes for preanneling. During the preanneling time, DNA on the slide was denatured separately by immersing the slide in 70 % formamide in 2xSSC at 60-70 °C for 2-5 minutes. After denaturation the slides were immediately dehydrated in 70 % cold ethanol and then a ascending series of alcohol (70 %, 90 % and 100 %). Then the slides were air-dried.

10-13 µl of prehybridised mixture was placed on the slide and covered with a coverslip avoiding the formation of air bubbles. The coverslip was sealed with cow gum. The slides were incubated overnight as described above (simultaneous procedure).

c) Washing

The next day, the cow gum was gently removed and slides were soaked in 2xSSC and 2-3 changes of 50 % formamide in 2xSSC at 37-50 °C for 3-5 minutes each. With these washings, the coverslips, unbinding probe and excess of hybridisation mix will be automatically removed. The slides were then incubated with the washing solution containing 5 % nfdm and 0.005% Tween-20 in 4xSSC at 10-30 minutes at 36-38 °C.

d) Immunochemical detection:

Two types of immunochemical detection system were used. The first was fluorescein isothiocynate (FITC) avidin (vector) -biotinylated anti avidin (vector) system for biotinylated probes. Alternatively, for two layer amplification, fluorescein isothiocyanate (FITC) avidin-biotinylated anti

avidin FITC system was used. The second was antidigoxigenin fluorescein (Boehringer) - FITC anti-sheep IgG system for digoxigenin labelled probe.

In the first detection system, 150 µl of fluorescein isothiocyanate (FITC) avidin (2: 1000 dilution) (2 mg/ml; Vector) was applied on the slides and covered with a parafilm to ovoid evaporation of detection reagent. Incubation was performed at room temperature or at 37 °C for 15-30 minutes. Then the slides was washed three times in washing solution for 3-5 minutes at 37-50 °C. The washing solutions were 4xSSC containing 0.05 % Tween-20 or 4xSSC containing 5 % nfdm and 0.05 % Tween-20 and phosphate saline buffer (PBS) containing 0.05 % Tween-20 . After last washing, the surrounding part of marked area of the slides were wiped with Kimwipes tissue.

For second layer, 150 µl of biotinylated anti avidin diluent (2 mg/ml; vector) was applied on the slides. Incubation was similar duration of first layer at room temperature or at 37 °C. After same washes as above, another 150 µl of fluoresceinated Avidin diluent was applied on the slides for the last layer. Incubation and washing of the slides were performed as above. After the last washing, the slides were soaked in 4xSSC containing 0.005 % Tween-20. The slides were then immediately dehydrated in a ascending ethanol series (70 %, 90 % and 100%) and air-dried at room temperature.

Alternatively, for the second layer (for short procedure) biotinylated anti avidin FITC (Vector) was used in the same condition as the first layer mentioned above when biotinylated antiavidin FITC became commercially available.

The second detection system was used for the probe labelled with digoxigenin-dUTP. In this detection, two layers were used by applying sheep antidigoxigenin fluoresceinated (FITC) FAB fragment (200 µl / ml) (Boehringer) for the first layer of amplification and FITC anti-sheep IgG (1.5 mg/ml) (Vector) for the second layer of amplification of signals. For the first layer was performed by applying 150 µl of sheep antidigoxigenin fluoresceinated (FITC) FAB fragment diluent (2.5:1000 dilution). For the second layer, 150 µl of FITC anti-sheep IgG diluent (2.5:1000 dilution) was applied. All the rest of steps, such as incubation of slides and washing conditions were the same, as described in the detection of biotinylated probe. Dilutions of detection reagents were done in 4xSSC containning nfdm and 0.005 % Tween-20. This detection system was mainly used dual-colour FISH study.

e) Counterstaining:

The slides treated with fluorescein isothiocynate (FITC) were stained with 10 µl of counterstain medium which contains propidium Iodide (PI) (5-10 µg/µl) and DAPI (5-10 µg/µl) in antifade medium. The slides were then covered with a glass coverslip. Nail vanish was applied around the edges of the coverslip to seal.

2.2.6.2. Optimisation of dual-colour fluorescence *in situ* hybridisations

a. Simultaneous Hybridisation:

First, the simultaneous hybridisation was accomplished using PJM 128 and D12Z1 centromeric probes for chromosomes 8 and 12 respectively, because these probes give intense signal with uni-colour FISH study. Next, simultaneous hybridisation was optimised for locus specific cosmid DNA probes (abl-18, bcr-19 and bcr-51), following the optimisation of procedure using the probes for uni-colour FISH.

Prior to the denaturation, the slides were immersed in acetone for 10 mins and air-dried. On the one hand the target DNA was denatured in 70 % formamide, 2xSSC for 2-3 minutes at 65-70 ^{o}C and dehydrated in an alcohol series (cold 70%, 90% and 100%) according to the method described for repetitive probe. To optimise the dual colour, one centromeric probe was labelled with digoxigenin while the other one was labelled with biotin. The labelled probes in hybridisation buffer without competitor DNA were then denatured at 65 -70 °C for 7-10 minutes. This mixture was applied to the slides containing denatured DNA area. Hybridisation reaction was sealed under a coverslip and incubated in waterbath (moist chamber) at 37 - 42 ^{o}C overnight (16 hours).

With locus specific DNA probe, simultaneous hybridisation was carried out by modifications of the procedure described by Lebo et al (1991) and Trask et al (1991). First of all hybridisation conditions such as concentration of probe and competitor DNA, and prehybridisation step was adopted from section 2.2.5.4.1. for each locus specific cosmid probes. They were combined and tested for simultaneous hybridisation. For denaturation of the probe mixture, 1 µl (100-140 ng) quantities digoxigenin

labelled bcr and 1 µl (100 ng) quantities of biotin labelled abl probe, 0.5 - 0.7 µl salmon sperm DNA (20 mg / ml) and 1.1 µl of human placental DNA (10 mg/ml) were added to 8-10 µl hybridisation buffer, mixed very well. However, as an alternative 1.0 µl of Cot-1 DNA (10 mg/ml) instead of human placental DNA was used for the same aim. The probes were denatured at 60 -70 ^{o}C for 7 - 10 minutes and the repetitive sequences allowed to preanneal for 30 minutes to one hours at 37 ^{o}C before being applied to denatured targets. The preannealed probe solution was applied to the slides, covered with a 22x22 mm coverslip and the edges of coverslip sealed with cow gum. The hybridisation reaction took place at 37 - 42 ^{o}C overnight (16 hours).

When using a combination of repetitive probe (centromeric probe) and locus specific probe, or whole chromosome specific library probe in a simultaneous hybridization, the pre hybridization step must be carried out for each probe independently. For example, for locus specific abl and bcr probes, prehybridisation was carried out as same described for uni-colour hybridization system. Each individual probe was used at optimised concentrations and pre hybridisation conditions and then combined together just before hybridisation. The rest of the procedure was as described above. Because the prehybridisation condition for each probe is different that is simultaneous hybridisation of one probe could affect the other one. For example locus specific probe requires competitor DNA while centromeric probe does not require. And also this has to be mentioned that the combinative hybridisation of commercial probe and other probe which was prepared in the department was carried out in the same way as above.

b. Detection of Dual Colour :

Next day slides hybridised to both biotinylated and digoxigenin labelled probes were soaked in 2xSSC at 38 - 44 °C for 3 -5 minutes to remove the coverslip . The slides were then washed twice or three times with 50 % formamide for 3-5 minutes at 38-44 °C followed by three minutes washing in 2xSSC at same temperature. The slides were then blocked with 4xSSC containing 5% non-fat dried milk (Marvel) and 0.05% Tween-20 for 10 - 30 minutes at 37 °C.

Probe detection was achieved in two ways: The first method was adopted from Johnson et (1991) with some modifications. In accordance with this protocol simultaneous detection was carried out in only one step. After blocking, the slides were incubated with a mixture of anti digoxigenin Rhodamine (Boehringer) (2 : 1000 dilution) and fluoresceinated avidin (2.5 ; 1000 dilution) at room temperature for 30 minutes or at 37 °C for 15-30 minutes. After incubation, the slides were washed twice in 4xSSC containing nfdm and Tween-20 at 37-44 °C for 3-5 minutes, followed by one wash in 4xSSC at same temperature, and then dehydrated in an alcohol series (70%, 90% and 100%) and air-dried.

Because the one step detection method is sometimes insufficient to get signals, further amplification of signal was required and a second detection method was tested as described by Reid et al (1992) and Trask et al (1991) with some modifications: After blocking with 4xSSC containing 5 % nfdm and 0.005 % Tween-20, the slides were sequentially incubated with a mixture of Rhodamine avidin (Vector) (2:1000 dilution) and sheep antidigoxigenin fluorescein FAB fragment (Boehringer) (2.5: 1000 dilution); with a mixture biotinylated anti avidin (Vector) (2:1000 dilution) and fluoresceinated anti sheep IgG (H+L) (Boehringer) (2.5:1000 dilution);

and finally with Rhodamine -avidin DCS (Vector) (2:1000 dilution) for 15 -30 minutes at 37 °C, or at room temperature. After each incubation slides were washed with washing solution (4xSSC containing 5% nfdm and Tween -20). Detection reagents used were diluted in washing solution (4xSSC containing 5% nfdm and Tween -20).

c. Counterstaining :

The slides prepared for bicolour FISH were stained with 10 µl counterstain medium containing DAPI (6 µg /µl) in antifade medium, while the slides treated with fluorescein isothiocynate (FITC) were stained with 10 µl of counterstain medium which contains propidium Iodide (PI) (7 µg/µl) and DAPI (6 µg/µl) in antifade medium. The slides were then covered with a glass coverslip and the edges of coverslips were sealed by nail vanish.

d. Visualisation:

Observation of signals was achieved by either epifluorescence microscope (Zeiss Axioplan) equipped with specific filter sets BP450-490 FT510), or epifluorescence microscope (Zeiss Axioplan) equipped with a digital imaging camera and computer software system.

Using conventional epifluorescence microscope, the results were photographed on Ektrachrome 400 ASA colour slide film (Kodak). For unicolour one exposure was required while for dual colour a triple exposure including one through filter for Rhodamine (excitation: 540-560), filter for FITC (Excitation: 490) and UV filter for DAPI was necessary.

Using the digital image system, the results were obtained with Zeiss Axioplan epifluorescence microscope fitted with photometric camera Dr3

and digital image acquisition (16 bit colour). The computer used is an Apple Macintosh Quadra 950 (4 RAM and 230 HD). The signals were selectively imaged with a filter set specifically prepared by Omega (Digital Scientific), for FITC, Rhodamine and DAPI to minimise image offsets. The photographs were taken with an sublimation full colour printer (Mitsubishi) using sublimation printing paper (A4-SPW).

2.3. Application of uni- and dual-colour FISH on leukaemia samples

With optimised uni- and dual-colour FISH, some leukaemia cases were analysed using locus specific DNA probes, whole chromosome specific DNA probe and chromosome specific centromeric probes. During this research, normal cases were used as control and to optimise the technique. Leukaemia groups studied are Chronic Myeloid Leukaemia (CML), Acute Promyelocytic Leukaemia (AML-M3) and Chronic Lymphocytic Leukaemia (CLL)

a. Chronic Myelogenous Leukaemia (CML):

i) Uni-colour FISH was applied to the Ph positive and negative cases,

ii) Dual-colour FISH was applied to the Ph positive, negative cases and variant Ph cases.

b) Acute Promyelocytic Leukaemia (AML-M3): Dual colour FISH was applied to AML-M3 and AML-M3v cases.

c) Chronic Lymphocytic Leukaemia (CLL): Uni-colour FISH using α-satellite centromeric probe for chromosome 12 was applied to CLL.

CHAPTER THREE

RESULTS

3. Results

3.1. Detection of a unique sequence probe, GMGY10, using alkaline phosphatase and fluorescence *in situ* hybridisation

To become familiar with the techniques involved, an initial experiment was carried out with the highly repetitive unique sequence probe GMGY10 (DYS59) using first the alkaline phosphatase technique, and then fluorescence in situ hybridization technique.

Before hybridisation with the probe labelled with biotin-11-dUTP, karyotytpically normal male metaphase chromosomes were pre-banded to identify individual chromosomes with lipsol and trypsin banding. The lipsol and the trypsin bandings were performed according to the methods described by Garson et al (1987).

The results showed the lipsol method to be more successful than the trypsin method. However, lipsol banding required fresh slides (1-2 days old) for the best results, whereas the age of slides was not so important for trypsin banding. After banding, in order to destain and clean the slide, pretreatments with Xylol/Ethanol mix and methanol/acetic acid mix were carried out. However, this did not improve the hybridisation signal.

For *in situ* hybridisation, chromosomal and probe DNAs were denatured either in the oven at 75-85 °C simultaneously or separately with chromosomal DNA being denatured in 70 % formamide in 2xSSC and the probe at 65-70 °C prior to the hybridisation. No difference was found between the two denaturation methods. However, simultaneous

denaturation in the oven is shorter and easier. The hybridisation was performed in the water bath overnight at 38-42 °C. After the detection with alkaline phosphatase, the signal of the probe GMGY10 was visualised on the short arm of chromosome Y **(Figure 5)** under the phase contrast microscope.

In addition, the same probe GMGY10 was detected by using the fluorescein avidin and biotinylated goat anti-avidin system. For fluorescence *in situ* hybridisation (FISH), the denaturation and hybridisation were basically similar to the alkaline phosphatase procedure. On the other hand, the stringency of the detection conditions such as temperature and salt content in post hybridisation washing solution, was found to be effective on the background. The optimal washing temperature and salt concentration were found to be between 40 to 45 °C, and 3xSSC and/or 4xSSC. Even under this post-hybridisation condition, for visualisation of the repetitive unique sequence probe GMGY10 (4.5 kb), three layers of amplification with fluoresceinated avidin was sufficient. More than three layers of amplification of fluoresceinated avidin resulted in increased non-specific background signals. The probe GMGY10 was visualised on the short arm of the Y chromosome **(Figure 6)** under the epi-fluorescent microscope.

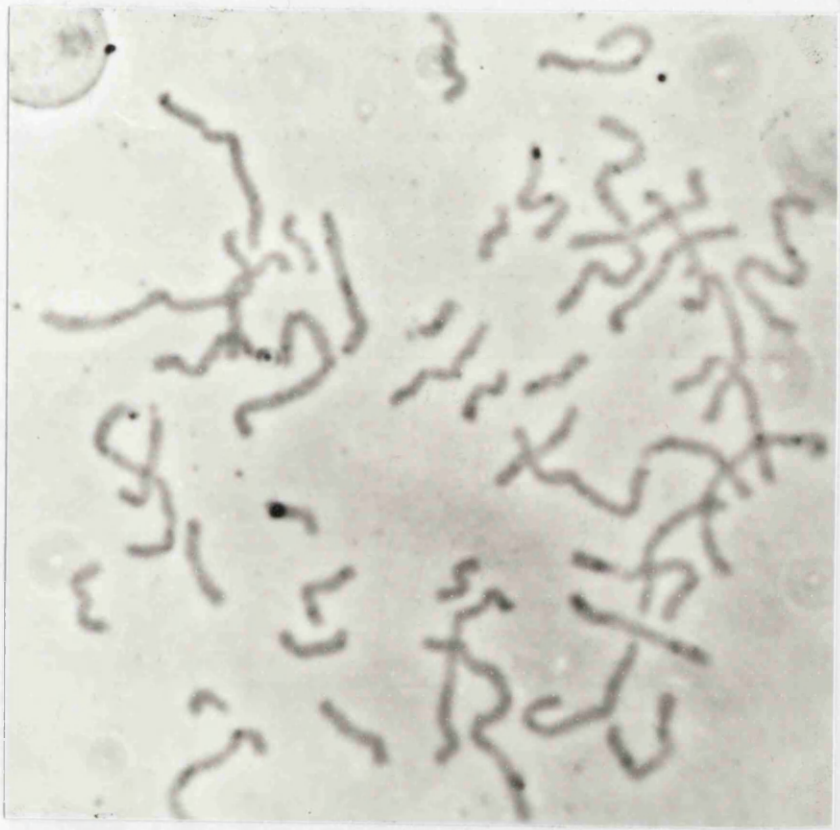

Figure 5. Photomicrograph of non-isotopic in situ hybridisation with GMGY10 probe using alkaline phosphatase detection.

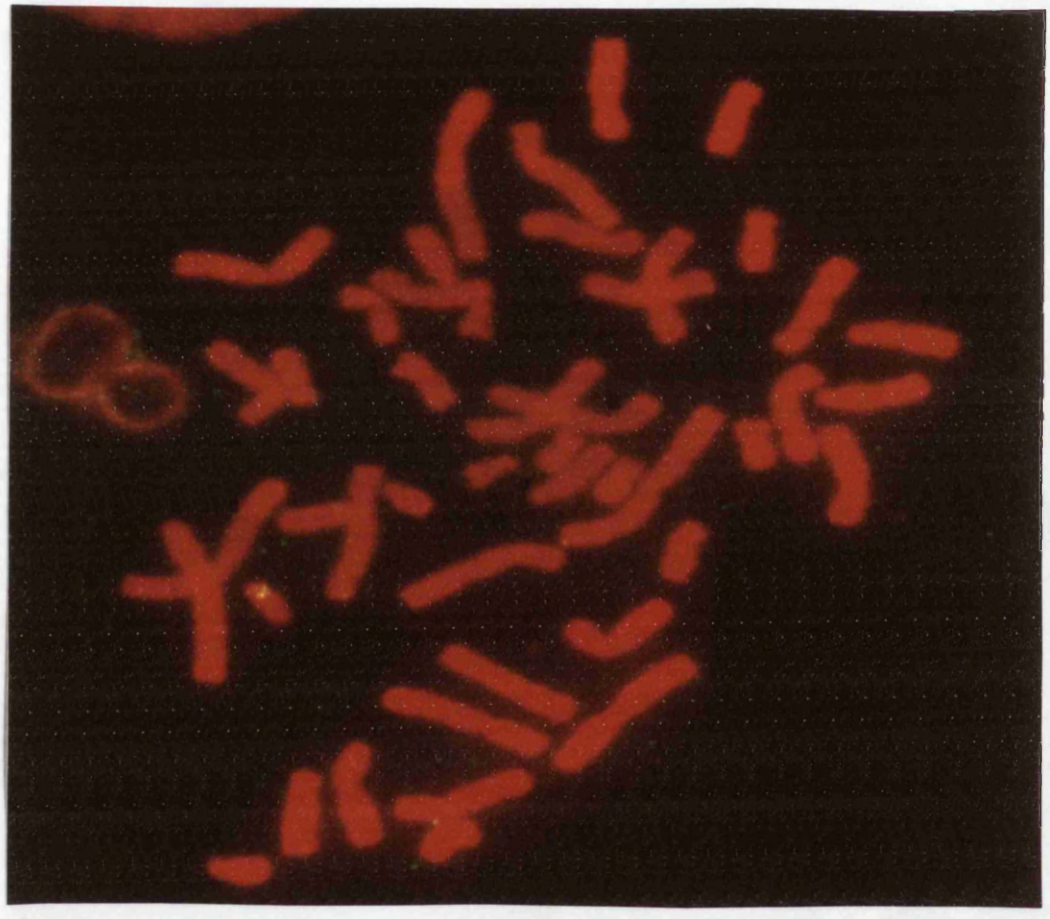

Figure 6. Photomicrograph of non-isotopic in situ hybridisation with GMGY10 probe using FISH.

3.2. Chromosome suppression *in situ* hybridisation using whole chromosome specific pBS-18 and pBS-21 libraries

The optimisation of the probe preparation, hybridisation and detection for the chromosome *in situ* suppression (CISS) hybridisation was evaluated with the pBS-18 and the pBS-21 library probes, since it was initially hoped to use these in the leukaemia studies. Conditions for *in situ* hybridisation with the pBS-18 and the pBS-21 library probes in these experiments were found to deviate from standard conditions, under which the concentration of probes and competitor DNA were determined by Lichter et al (1988), Cremer et al (1988) and Goldman et al (1992).

3.2.1. Restriction enzyme digestion of the plasmid DNA

The probes pBS18 and pBS-21 are specific libraries for chromosomes 18 and 21 respectively. Each probe insert had been cloned into the vector pBS. In order to check whether the recombinant molecule contained the insert of interest (i. e. probe specific chromosome library fragments), the DNA from both libraries was digested with enzyme *Hind III.* No smear or bands were seen on the agarose gel indicating small number of clones had inserts.

3.2.2. Preparation of chromosome specific library probes

A probe for *in situ* hybridisation should have DNA fragments with a length smaller than 500 nucleotides, since it is necessary for the probe molecules to diffuse into the dense matrix of cells and chromosomes. For this purpose, first, the plasmid DNAs containing inserted chromosome specific library DNAs, was digested with DNase by

manipulating the DNase concentration before probe labelling, and was run on a 0.8% agarose gel (i.e. from both chromosome libraries for 18 and 21). Most of the DNA fragments obtained had a length of 500 -700 base pairs.

3.2.3. Chromosome *in situ* suppression hybridisation and detection

The undigested 21 and digested whole chromosomes 18 and 21 specific DNA libraries were individually labelled with Biotin-11-dUTP by using nick translation as described in the Materials and Methods chapter. Chromosome *in situ* suppression hybridisation (chromosome painting) with undigested and digested chromosomes 18 and 21 library DNAs was tested on normal male specimens. The concentration of the probe used was between 200 ng and 700 ng per hybridisation area (22x22 mm).

The result showed that 200 to 600 ng of the undigested pBS-21 library exhibited non-specific specked hybridisation signals over the other chromosomes. Using over 600 ng of probe caused an increase in the background, even when competitor DNAs were used. The use of competitor DNAs (i.e. salmon sperm and human placental DNA) did not improve the specific signals of chromosome 21 and did not reduce the background. In addition, different hybridisation times and temperatures were tested, however, the result was not improved.

The following experiments were performed in order to determine the optimum probe concentration, reassociation time and temperature for ISH using the 21 library probe, pBS-21. 200 ng to 400 ng (per hybridisation reaction) of digested pBS-21 library probe were hybridised to karyotypically normal male metaphases and nuclei for chromosome *in situ* suppression hybridisation. Using 200 to 400 ng digested probe

without and with competitor DNA showed that the signals did not completely cover the target chromosomes (incomplete coverage), and were observed as speckles. Optimum coverage with digested 21 library probe (600 to 700 ng per hybridisation area) was obtained using simultaneous salmon sperm DNA (1500 ng/hybridisation) and human placental DNA (1500 ng / hybridisation). The use of individual competitor DNA failed to suppress the cross-hybridisation. Clearly higher concentration of human placental DNA caused an apparent suppression of signal intensity over the targets. With digested chromosome 21 library probe, the centromeric regions of other acrocentric chromosomes were not effectively suppressed. The prehybridisation was performed at 37 to 42 °C for 30 mins and 1.5 hours. The optimum prehybridisation and hybridisation temperatures were for at least one hour at 37 °C, and overnight (about 16 hrs.) at between 39 and 41 °C, respectively.

In addition to chromosome suppression *in situ* hybridisation in the chromosome spreads, interphase nuclei were also evaluated on the same slides. The results obtained from the optimised CISS hybridisation with digested pBS 21 library probe were not interpretable because the hybridisation signals present as large domains which are fuzzy and tend to overlap.

Detection of hybridisation with the digested pBS-21 probe was carried out using the fluoresceinated avidin and biotinylated anti-avidin system. Before the detection stages, the slides were washed using different washing stringencies. The slides were first washed in 2xSSC and in 50 % formamide three times at 38 to 45 °C for 5 minutes each. The optimum wash temperature was found to be at between 42 and 45

°C for an average of 5 mins. Higher temperatures caused an apparent decrease of signal intensity, especially above 50 °C under these washing conditions. Low temperature (less than 39 °C) resulted in excess background. After these stages, the slides were preincubated in wash solution for 15 to 30 minutes, in order to suppress the protein binding region on the slides. All these stages reduced the probability of background.

After incubating with wash solution, the slides were incubated in immunofluorescent reagent to produce a fluorescent signal at the sites of probe hybridisation. For first layer, fluoresceinated avidin was applied onto the slides, and incubated at either room temperature or at 37 °C for at least 15 to 30 mins and then for second layer biotinylated anti-avidin was applied. For last layer, first layer application with fluoresceinated avidin was repeated as first layer. A range of temperatures and incubation times were tested but no significant improvement in quality of the signals or reduction in the background was observed. However, fifteen minutes of incubation was a critical point to permit amplification of signals. Two amplifications of signals with fluoresceinated avidin were sufficient to enhance the decoration of chromosome 21 **(Figure 7)**. Between incubations with detection reagents the optimum wash was obtained at 44 °C for 5 minutes. Lower temperature increased the background.

After optimising the hybridisation and detection conditions of chromosome *in situ* suppression hybridisation with digested pBS-21 library, two cases were evaluated.

The first was clinically Down's syndrome, which was detected to have a 47, XY, +21 karyotype, by Giemsa banding. Following chromosome suppression hybridisation, three fluorescently labelled chromosome 21s were demonstrated as shown in **Figure 8.**

The second case was an individual suspected a clinical grounds of having trisomy 21, but found on two occasion to have a normal karyotype by G-banding. Application of CISS hybridisation using digested whole chromosome 21 library (pBS-21) showed that both chromosome 21s were normal and no other area of hybridization was observed. It was concluded that chromosome 21 was not involved in any translocation.

Based on the 21 library probe optimisation, whole chromosome 18 library probe was evaluated under the defined conditions for digested pBS-21 library. For digested pBS-18 library the optimum concentration for chromosome *in situ* suppression hybridisation was found to be 600 ng per hybridisation reaction. However, the results showed consistently that the probe hybridised to chromosome 2 centromeric region in addition to chromosome 18, as shown in **Figure 9.** Even with low concentration of the 18 library (i.e. between 200 and 400 ng per hybridisation reaction), the centromeric regions of chromosome 2 lit up although chromosome 18 was not itself painted. Further investigation revealed that when CISS was performed with higher concentration of competitor DNA, hybridisation to chromosome 18 was again suppressed.

Work with these probes was discontinued because pBS-21 library for chromosome painting required too much probe DNA and the pBS-18 library constantly revealed the chromosome 2 centromeric regions.

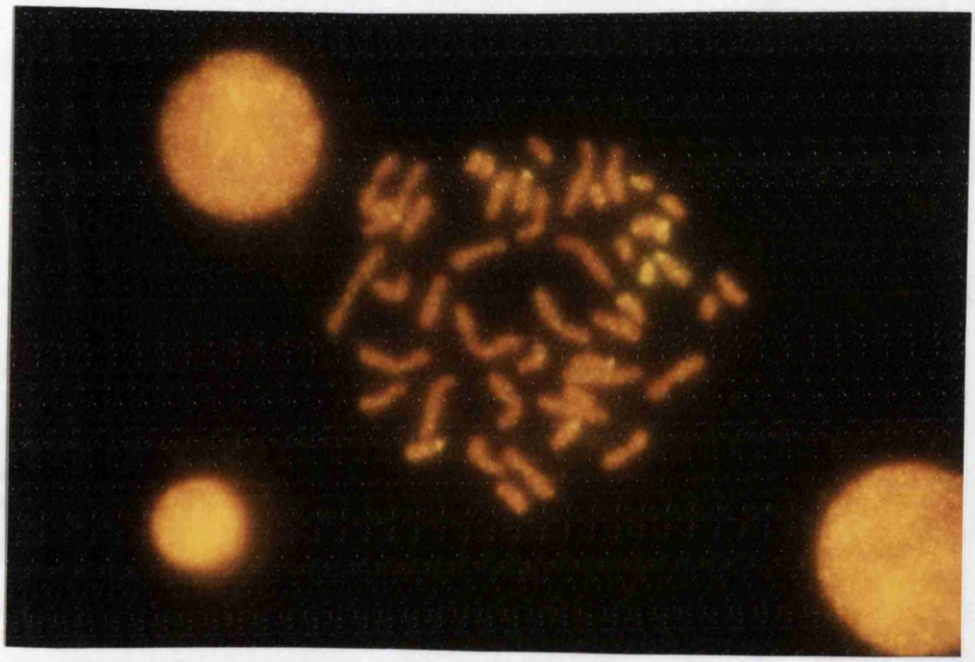

Figure 7. Photomicrograph of CISS hybridisation of metaphase chromosome of a karyotypically normal blood sample using whole chromosome 21 specific library.

In the following illustration denotes (a) conventional epifluorescence microscopy and (b) enhanced by digital imagining.

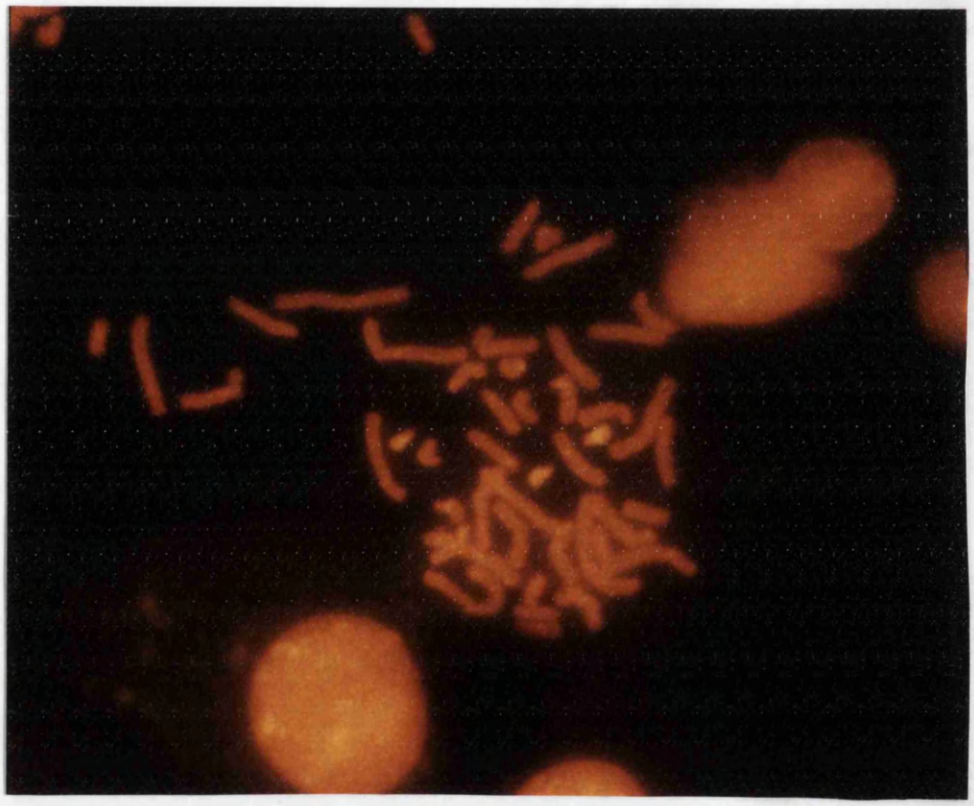

Figure 8. Photomicrograph of CISS hybridisation on metaphase chromosomes of case no 1 karyotype 47, XY, +21. Three chromosomes 21 observed.
a)

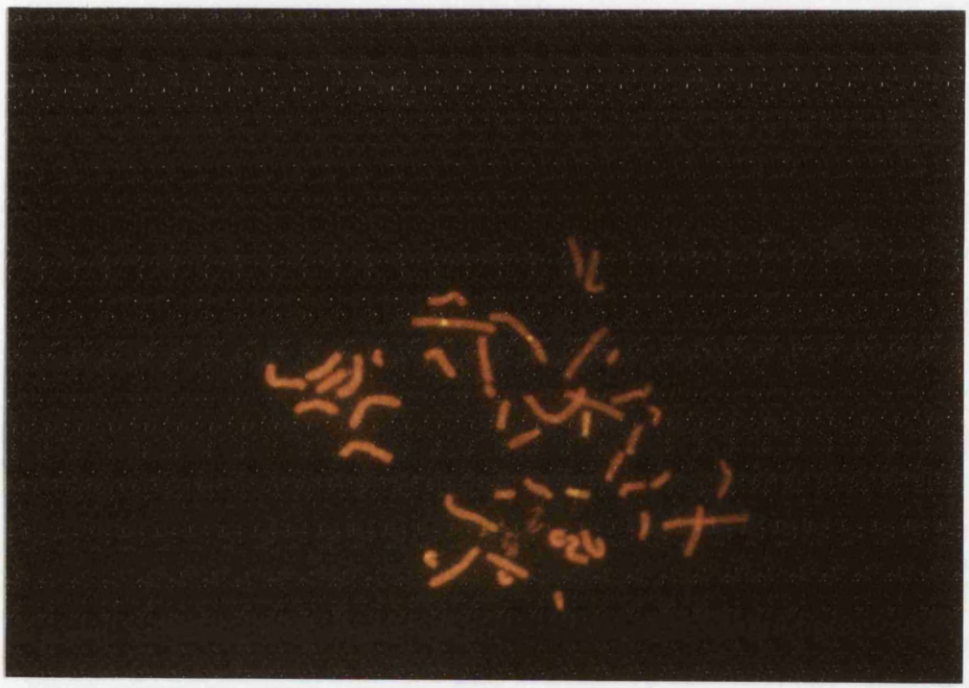

Figure 9. Photomicrograph of metaphase chromosomes from a karyotypically normal blood sample after CISS hybridisation with chromosome 18 specific library. Two chromosomes 18. Signals are also observed at the centromeric area of chromosomes 2.

3.3. Uni- and dual-colour FISH with chromosome specific centromeric probe, locus specific probe, and library probe and in combination

During the optimisation of FISH protocol for uni-colour and dual-colour, several procedures described in the recent literature were evaluated using the centromeric probes, the locus specific probes and the library probes. In this study, first of all α-satellite centromeric probes for chromosome 8 (PJM 128) and for chromosome 12 (D12Z1) were used and uni- and dual-colour FISH were optimised successfully. Thereafter, first locus specific cosmid probes (abl and bcr) were optimised for uni-colour FISH. Based on the uni-colour FISH procedure, dual-colour FISH for cosmid probes abl and bcr was optimised. However, some steps in the protocol did not noticeably improve the results, and thus they were omitted. However, some of the steps in the protocol are remarkably resistant to many parameters. They will be presented at each step.

3.3.1. Preparation of probes and slides for uni-colour and dual-colour FISH

Both locus specific probes (abl-18, bcr-19 and bcr-51) and chromosome specific centromeric probes (PJM 128, D12Z1 and D22Z2) DNA were successfully isolated from their host cells by using CIRCLEPREP KIT.

For probe labelling, the nick translation kit (BRL) and random prime kit (Boehringer) were successfully used. However the combination of the nick translation kit reagents (BRL) and the random prime digoxigenin label reagent (Boehringer) resulted in a digoxigenin labelled probe which

yielded as good a result as that from the random prime labelling system and was less expensive to produce.

One of the most important factors in *in situ* hybridisation is to have clean and well spread metaphase chromosomes or interphase nuclei. For instance, cytoplasmic artefacts can prevent probe penetration into the DNA matrix and increase the signal-background ratio. Slides were prepared from leukaemic cases, and from the control group. Fixed samples were dropped on to clean wet slides, and were then aged overnight at 42 °C. This preparation was found to be optimum for uni- and dual-colour FISH experiments with bone marrow preparations from leukaemia cases. Various pretreatments of the slides before hybridisation were investigated but did not improve the results. These included post fixation of slides in methanol : acetic acid (3:1), dehydration of slides and also treatment with *RNase*. In this study, the result showed that proteinase K treatment was not needed, since non-penetration of the probe did not appear to be a problem.

On the other hand, in the chronic lymphocytic leukaemia study which was carried out on interphase nuclei, the best results were obtained from slides containing lymphocytes that had been separated by using histopaque, rather than using whole blood cells.

3.3.2. Probe mixture

In the hybridisation buffer, several factors influenced the intensity and specificity of the hybridisation signal for both unicolour and dual-colour FISH; in particular, the purity and stability of the DNA probe, the size of the insert DNA into vector, the size of the probe fragments and competitor DNA used in the hybridisation reaction. While repetitive probe

hybridisation does not need competitor DNA, for locus specific probe and whole chromosome specific DNA library, unlabelled competitor DNA in the hybridisation reaction is one of the most important factors. By carefully controlling these parameters, strong and specific fluorescent hybridisation signals can be obtained in the absence of appreciable fluorescence background.

Biotinylated abl-18 and bcr-19 and bcr-51 were first used for uni-colour FISH. Optimum signal intensity was obtained with 100 ng of ABL-18 in 10µl (10 ng/µl) hybridisation buffer containing 1.1 µl of human placental DNA (10 mg/ml) and 0.7 µl of salmon sperm DNA (20 mg/ml). Lower concentration of the probe and higher concentration of competitor DNAs resulted in a very small signal, however higher concentration of probe resulted in higher cross-hybridisation despite the adjustment of competitor DNA.

On the other hand, bcr-19 and bcr-51 were tested in different concentrations using human placental DNA and salmon sperm DNAs. Neither of these probes was completely satisfactory for FISH, because they gave cross hybridisation and signal was found to be weak. So the optimal concentration of bcr-19 probe in hybridisation buffer was in the range of 100 ng to 140 ng with 1.1 µl of human placental DNA (10 mg/ml) and 0.7 µl of salmon sperm DNA (20 mg / ml).

Dual-colour FISH optimisation was first investigated using the centromeric probes PJM 128 (chromosome 8) and D12Z1 (chromosome 12) first singly and then simultaneously. For single hybridisation and simultaneous hybridisation, the optimum each probe concentration was found to be 50 ng in 10 ul hybridisation without competitor DNA.

Second, for dual hybridisation of abl and bcr, the best result was obtained from the combination of 100 ng labelled abl-18 probe and 100 -140 ng of one of the labelled bcr probes in the hybridisation reaction. This contained competitor DNA in the same concentrations as previously used for uni-probe hybridisation.

3.3.3. Denaturation and hybridisation

Both separate and simultaneous denaturation of probe and target DNA were tested. Simultaneous denaturation of locus specific DNA and target DNA affected hybridisation in several ways such as chromosome morphology, cross hybridisation and signal intensity. For this reason, further denaturations were carried out separately. Thus, target DNA was denatured in 70 % formamide in 2xSSC at 65-70 °C for 2 to 3 minutes and then transferred through an ascending series of ethanol concentrations. Longer denaturation of target DNA and an increase in temperature both resulted in poor chromosome morphology.

Denaturation of the centromeric probes or the locus specific probes in the hybridisation mixture for single or simultaneous hybridisation was performed at 65 °C for 10 minutes.

In order to perform prehybridisation, 60 minutes incubation at 37 °C was found to be sufficient to suppress cross-hybridisation for both the ABL-18 and the BCR-19 and the BCR-51 probes for single probe hybridisation and simultaneous hybridisation.

Hybridisation temperatures of the abl and bcr probes were found to be very flexible. Hybridisation was carried out at 37 to 42 °C for 15 hours or longer.

When the abl and bcr probes or whole chromosome specific library probes were used in combination with centromeric probes for simultaneous hybridisation, the locus specific or whole chromosome specific library probe in the hybridisation mixture was denatured as above and prehybridised in the hybridisation buffer containing competitor DNA (which had already optimised for unicolour hybridisation) at 37 °C for 60 minutes. Just before adding this mixture, the already denatured centromeric probe was mixed to the prehybridised hybridisation mixture, and subsequently the mixture was applied on the denatured target DNA. Simultaneous prehybridisation of the centromeric probe with either the locus specific probes or the whole chromosome specific probe resulted in a decreased intensity of the centromeric probe signals. The combinatorial hybridisation was carried out in accordance with the requirements of the locus specific probe or the whole chromosome specific probes, because these needed a longer hybridisation time (16 hours) than the centromeric probes.

3.3.4. Washing and detection

Post hybridisation washing was performed first in 2xSSC, two changes of 50 % formamide washing solution and then two changes of 2xSSC at 42 - 44 °C for 3-5 minutes. These conditions were found to be optimal for the abl and bcr probes while the centromeric probes (such as PJM 128, D12Z1 and D22Z2) did not need a specific washing temperature, the temperature range of 37 to 45 °C for 3-5 minutes being adequate. After washing with 2xSSC to remove the coverslips and 50% formamide to remove the unhybridised sequences, the slides were incubated in a 4xSSC containing nfdm and Tween-20 in order to suppress the unspecific binding of the antibody. However, this step is not necessary for the centromeric probes

(PJM128 and D12Z1), because they can give very sharp and specific signals. Between the detection layers or after detection (for one step detection), washing with only 4xSSC including Tween- 20 and phosphate saline buffer containing Tween-20 were tried, but they did not alter the quality of the results.

The detection was carried out for unicolour and simultaneous detection by using two different detection reagents which were diluted in wash solution. The concentration of the detection reagent is very important since higher concentrations increased the background and suppressed the signal intensity.

For uni-colour FISH detection, 150 µl of diluted Avidin FITC was placed on each marked area of the slide and covered with parafilm to spread homogeneously, and to avoid evaporation of detection reagent during the incubation. Incubation of the slide at 37 °C for 15 minutes produced the best results. A second layer with biotinylated anti avidin and a third layer with Avidin-FITC detections were performed under the same condition. Since biotinylated goat anti avidin-FITC became commercially available two layers of amplification were used. These amplification gave sufficient intensity of signal. One layer of detection was sufficient to achieve good results for the centromeric probes, the library probes and even with the abl and bcr probes when these were applied to well-prepared slides.

In dual-colour FISH, the detection layer was found to be as important as the dilution of the detection reagent. This is because high concentrations and more than two layer amplifications with detection reagents conjugated with Rhodamine or Texas red stained the nuclei and metaphase chromosomes with a reddish colour. Because of this

restriction, especially for the locus specific abl and bcr probes, dual colour FISH always resulted in weaker hybridisation signals than those of the individual hybridisations. However, the centromeric and library probes were found to be tolerated this restriction.

3.3.5. Counterstaining and visualisation

Other critical steps in FISH procedures are counterstaining and visualisation. For uni-colour FISH, the combination of propidium iodide and DAPI was used as a counterstain and the optimum concentration was found to be 7 μg/μl propidium iodide and 6 μg /μl DAPI in the antifade medium. Higher concentrations suppressed the probe signal whereas lower concentrations failed to visualise the chromosomes. Also freshness of counterstain reagents was found to be important.

For the dual-colour FISH study, only DAPI counter stain was used with a concentration of 6 μg/μl DAPI in antifade medium. Propidium iodide was not suitable for counterstain for red fluorescence signals because it suppressed the dual-colour signal and especially the red signals.

In order to visualise the hybridised probe (s), initial results with uni-colour and dual-colour FISH studies were analysed using a Zeiss Axioplan epi-fluorescence microscope coupled with the fluorescence filters of excitation 345 for DAPI, 540-560 for Rhodamine and 490 for FITC fluorochromes.

Slides counterstained with the combination of propidium iodide and DAPI for unicolour showed the green - yellow coloration of the hybridised probe abl-18, bcr-19, bcr-51, and the centromeric probes such as chromosomes 8 and 12. However, the bcr-19 and bcr-51 probes showed some small signal

due to cross hybridisation on the other chromosomes, and in the uni-colour FISH study, these were found to be suppressed by increased propidium iodide concentration. As a result of this fact the bcr probes have been successfully used for uni-colour FISH study.

In contrast to unicolour FISH, both bcr-19 and bcr-51 probes in the dual colour FISH study resulted mostly in unsuccessful hybridisation signals. Nevertheless some cases exhibited acceptable hybridisation signals by paying good attention. To improve the signal intensity, the two bcr probes (bcr-19 and -51) were combined together to amplify the same region and this application resulted in slightly better hybridisation signals.

For photography, exposure times for both the uni-colour and the dual-colour FISH depended completely on the intensity of the hybridisation signals. Of course, the centromeric and library probes need shorter exposure time than the locus specific probes for abl and bcr because the centromeric and library probes give brighter and sharper signals. The initial results were obtained by using Kodak Extrachrome 400 ASA colour slide film. According to the optimisations of the locus specific and centromeric probes, localisation of those probes can be visualised.

The dual-colour FISH signals needed two or three exposures in order to transfer the image to the 400 ASA colour slide film. The results showed that the signals could not be picked up on the film, even though each signal separately could be seen under the microscope. With this system, even the centromeric probes showed a low intensity signal when compared with the individual exposure. The reason is that multiple exposure of the colour film can not adequately display and resolve images from samples being detected with dual-colour FISH.

This restriction was overcome when the digital imaging system was purchased by the department. This system allowed the collection of a separate image of each signal and these images could be combined and then stored on computer software. Thus, even signal invisible to the eye could be observed. All results have subsequently been visualised by using the digital image system coupled with the computer system. The optimised use of centromeric probes for chromosome 8 and 12, and locus specific abl and bcr cosmid probes localisation can be seen as in **Figures 10 and 11.**

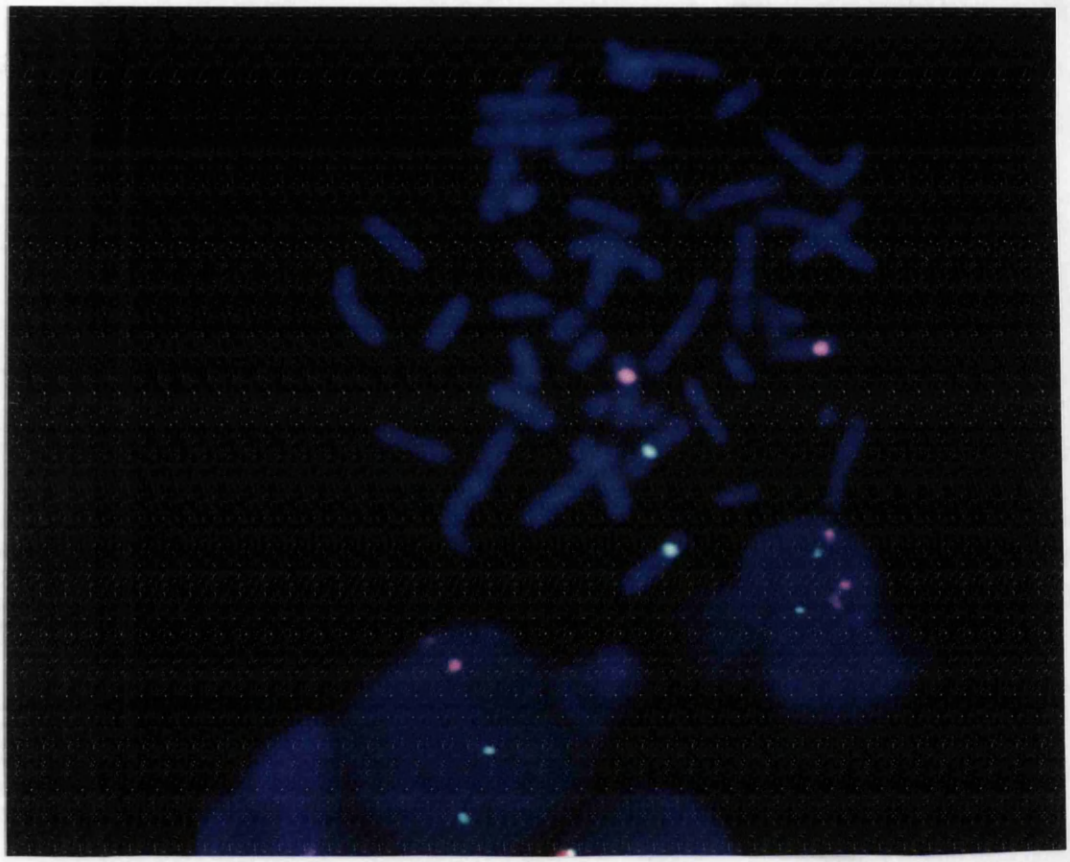

Figure 10. Simultaneous in situ hybridisation of α-satellite repetitive sequence probes for chromosome 8 (green) and chromosome 12 (red) on both metaphase and interphase nucleus.
b)

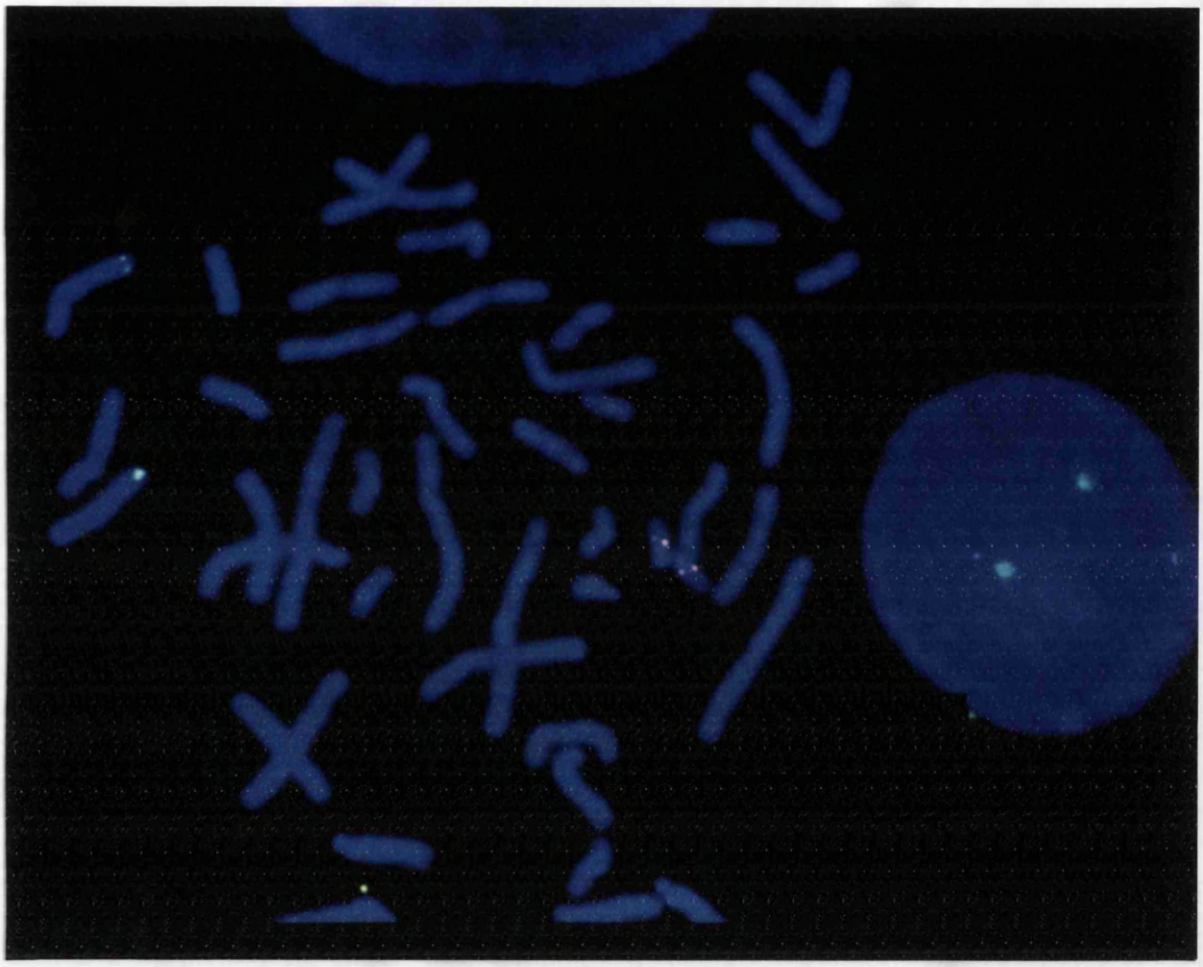

Figure 11. Simultaneous in situ hybridisation of locus specific cosmid probes for abl (green) and bcr (red) on both metaphase chromosome and interphase nucleus.

b)

3.4. Application of uni- and dual-colour FISH in selected haematological disorders

Following the optimisation of the several parameters involved in FISH, these objectives in leukaemia studies were investigated:

A. In CML -

i) investigate the use of the FISH abl/bcr probes in interphase nuclei as a diagnostic test for, or confirmation of, the Ph chromosome.

ii) study of Ph negative and variant Ph cases;

B. In AML-M3 -

i) improved detection of the incidence of t(15;17) using FISH.

ii) reliability / comparability of interphase studies,

C. In CLL -

i) detection of trisomy 12 in interphase and its reliability as an aid to diagnosis.

3.4.1. Studies in Chronic Myeloid Leukaemia

3.4.1.1. Studies on the Philadelphia chromosome with uni-colour FISH

The locus specific cosmid probes abl-18 and bcr-19 and -51 were applied to chromosome preparations from fourteen CML cases at diagnosis and at follow up which were known to be Philadelphia positive in at least a proportion of cells. The FISH results using abl probe are given in **Table 4.** Transfer of abl-18 from the long arm of chromosome 9 to the long arm of the Ph chromosome (chromosome 22) was detected even in poor quality preparations unless the chromosomes were very badly spread **(Figure 12).** In case 4, three out of six metaphases exhibited an extra abl signal on an

extra Ph chromosome which confirmed the cytogenetic result. As a unexpected result, in one metaphase from the same case two abl signals appeared on both the long and short arms of the one homologue of chromosome 9. This chromosome was identified by staining of DAPI which identifies the heterochromatin region of chromosome 9. The significance of this finding is not clear and could represent a translocation or isochromosome, or might be artifact.

When used singly bcr cosmid probes gave normal localisation on chromosome 22 or on derived chromosome 22 (Ph chromosome) in cases where this had been identified cytogenetically **(Figure 13)**. In one metaphase out of four in case 9 one bcr signal appeared to be clearly located on the long arm of a D group chromosome.

Table 4: Uni-colour fluorescence *in situ* hybridisation on metaphase spreads: application to 14 cases of Ph positive Chronic Myeloid Leukaemia using the abl cosmid probe as confirmation of cytogenetic results.

CASE NO:	METAPHASE WITH RE-ARRANGEMENT OF abl PROBE	CYTOGENETIC RESULTS
1.	2 / 2	46, XX, t(9;22) [19]
2.	2 /2	46,XY, t(9;22) [10] / 46, XY [6]
3.	2 / 3	45, XY, -21, t(9;22) [4] / 46, XY, t(9;22) [43] / 46, XY [4]
4.	6/ 6*	46, XY, t(9;22) [3] / 47, XY, t(9;22),+Ph [17]
5.	failed	46, XY, t(9;22) [18]
6.	2 / 2	46, XY, t(9;22) [18]
7.	5 / 5	46, XY, t(9;22) [19]
8.	non informative	46, XY, t(9;22) [10]
9.	2 / 6	46, XY, t(9;22) [9] / 46, XY [11]
10.	4 / 4	46, XY, t(9;22) [15] / 46, XY [5]
11.	8 / 8	46, XY, t(9;22) [14]/ 46, XY [1]
12.	17 / 17	46, XY, t(9;22) [9]
13.	no mitosis	no mitosis
14.	2 / 2	46, XX, t(9;22) [20]

*: 3 out of 6 metaphases with duplication of abl re-arrangement.

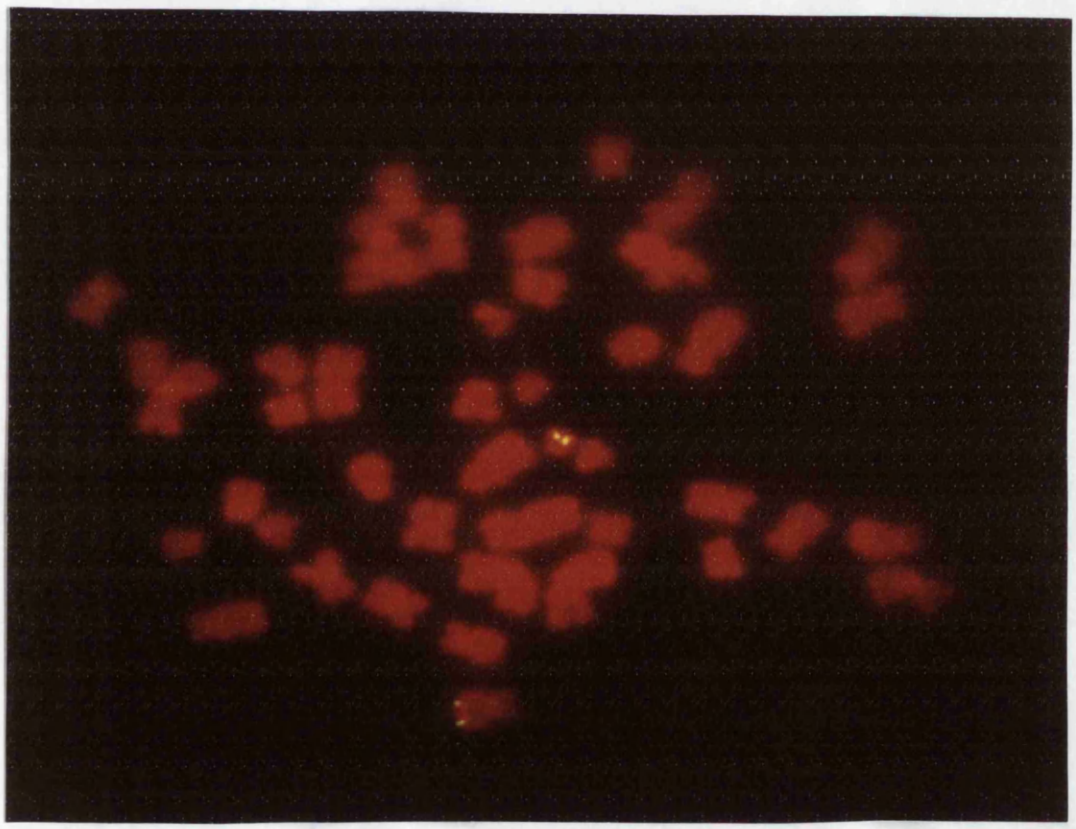

Figure 12. Hybridisation results on the metaphase spreads of bone marrow cells of a Ph positive CML patient. DAPI and Propodium iodide counterstaining: one abl signal (FITC) on the long arm of chromosome 9, one abl signal (FITC) on the long arm of Ph chromosome representing the fusion of bcr-abl genes on the derived chromosome 22.

b)

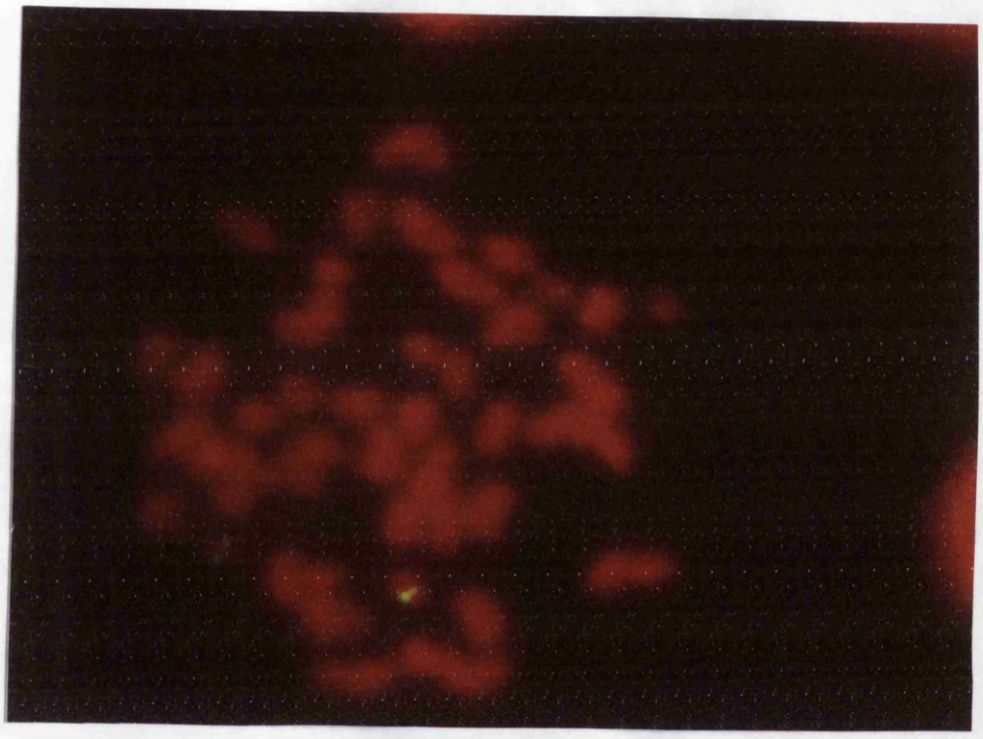

Figure 13. Hybridisation results on the metaphase spreads of bone marrow cells of a Ph positive CML patient. DAPI and Propodium iodide counterstaining: one of bcr signals (FITC) on the long arm of chromosome 22 and other (FITC) on the long arm of Ph chromosome (derived chromosome 22).

b)

3.4.1.2. Uni-colour FISH applied to metaphase spreads in case of Philadelphia negative

Between 1991 and the beginning of 1994, twelve cytogenetically Philadelphia chromosome negative patients with a provisional diagnosis of chronic myeloid leukaemia (CML) were monitored by using uni-colour FISH with the abl and bcr cosmid probes. In addition, by using α-satellite repetitive probe (PJM 128) for chromosome 8 and a specific cosmid contig probe for chromosome 21, extra numerical chromosome abnormalities which were detected by cytogenetics in these patients were confirmed **(Table 5).**

Uni-colour FISH was applied to metaphase spreads from these twelve cytogenetically Ph negative patients. Interestingly, one out of the twelve cases (case 12) showed the standard bcr-abl fusion on the long arm of chromosome 22. This patient had a 47, XYY karyotype without a cytogenetically recognisable translocation. (This result was confirmed by applying dual-colour FISH in section 3.4.1.3, case No 12). The remaining cases (Nos. 1-3, 5, and 8-11) were found to have normal abl signal localisation. In case No 7 no metaphases were found. In all these cases, the localisation of the bcr signals was on the long arm of chromosome 22.

In cases 4 and 6, trisomy 9 was confirmed using abl cosmid probe which produced three signals detected with abl cosmid probe by using the FISH technique. In case 4, one out of sixteen metaphase spreads showed an extra abl signal on an extra chromosome 9. In case no 6, seven out of

fifteen metaphases were detected with abl signal on the long arm of three C group chromosomes (**Figure 14**). From DAPI staining this result would equate with trisomy for chromosome 9.

Case no 2 confirmed trisomy 8 by using chromosome specific α-satellite probe for chromosome 8 whereas the abl and bcr probes resulted in normal localisation of the abl and bcr signals.

By applying the specific cosmid contig probe for chromosome 21 on case 6, the FISH results revealed that eleven out of eighteen metaphase spreads had an extra copy of cosmid contig signals on chromosome 21 indicating trisomy 21 (**Figure 15**).

As an unexpected result, case 10 had one metaphase (1 out of 12 metaphases) with the abl signals lighting up on the long arm of one G group chromosome which could not be identified. In the same case, one metaphase was found to be tetraploid with normal abl signal location.

Table 5: Uni-colour fluorescence *in situ* hybridisation on metaphase spreads: application to 12 cases of Philadelphia negative Chronic Myeloid Leukaemia using abl and bcr probes.

CASE NO	PROBES	NUMBER OF METAPHASE AND LOCATION OF SIGNAL	CYTOGENETIC RESULTS
1.	abl	9 normal	46, XX [20]
	bcr	5 normal	
2.	abl	15 normal	46, XX [8] /
	bcr	5 normal	47, XX, +8 [12]
	PJM 128	7three signals	
3.	abl	10 normal	46, XY [17]
	bcr	4 normal	
4.	abl	16 normal	46, XX [20]
	bcr	17 normal	
5.	abl	11 normal	46, XY
	bcr	9 normal	
6.	abl	8 normal 7 three signals	46, XY [4] / 47, XY, +21 [1] / 47, XY, +9 [3] /
	bcr	5 normal	48, XY, +9, +21[7]
	PJM128	5 normal	
	Chr.21 cos. contig	11 three signals 7 normal	
7.	abl	no metaphase	46, XY [21]
	bcr	no metaphase	
8.	abl	8 normal	46, XY [50]
	bcr	4 normal	
9.	abl	2 normal	46, XY [20]
	bcr	8 normal	
10.	abl	12 normal	46, XY [20]
	bcr	6 normal	
11.	abl	7 normal	46, XY [20]
	bcr	4 normal	
12.	abl	4 translocated on chromosome 22	47, XYY [20]
	bcr	3 normal	

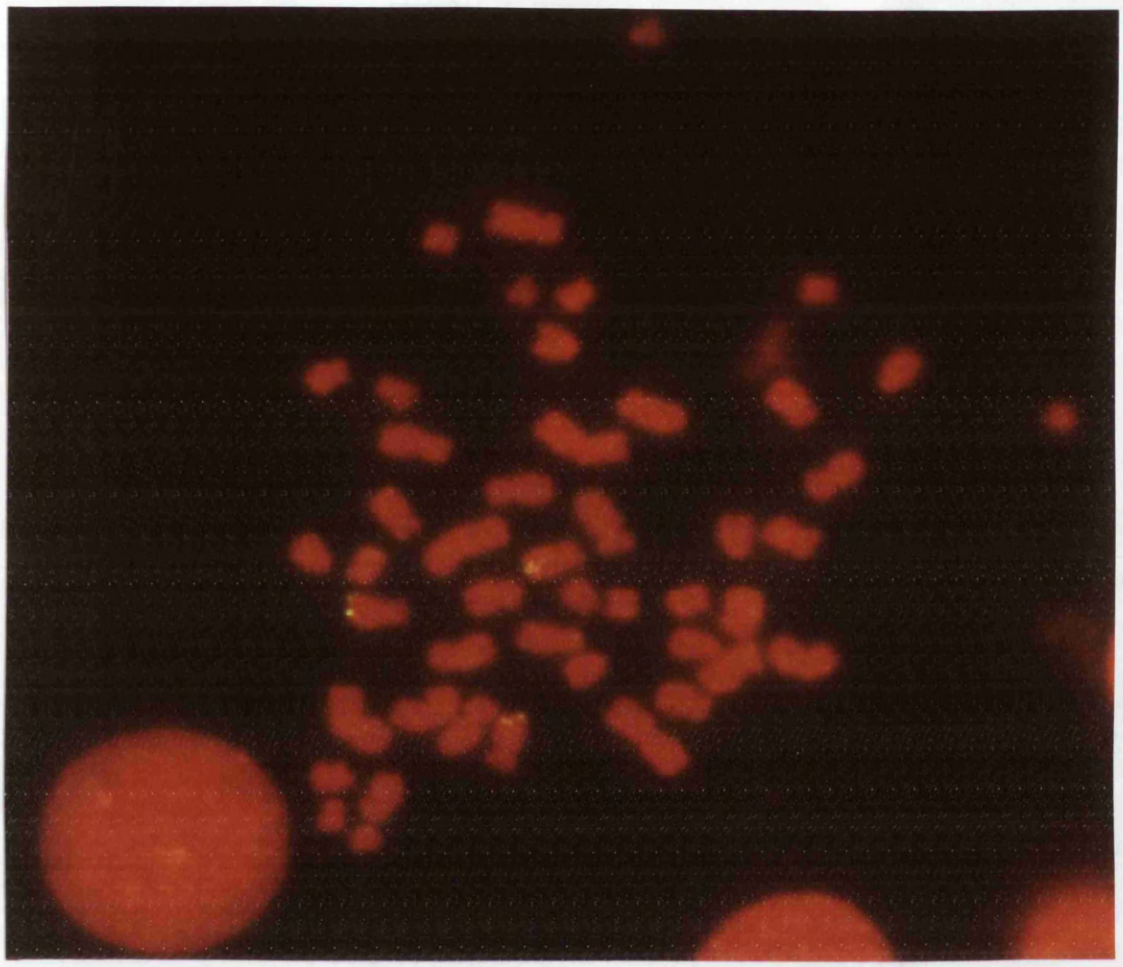

Figure 14. Hybridisation with the abl cosmid probe on metaphase spread of bone marrow cells of a Ph negative CML patient who was trisomic for chromosome 9 showed abl signals on the long arms of three copies of chromosome 9.

b)

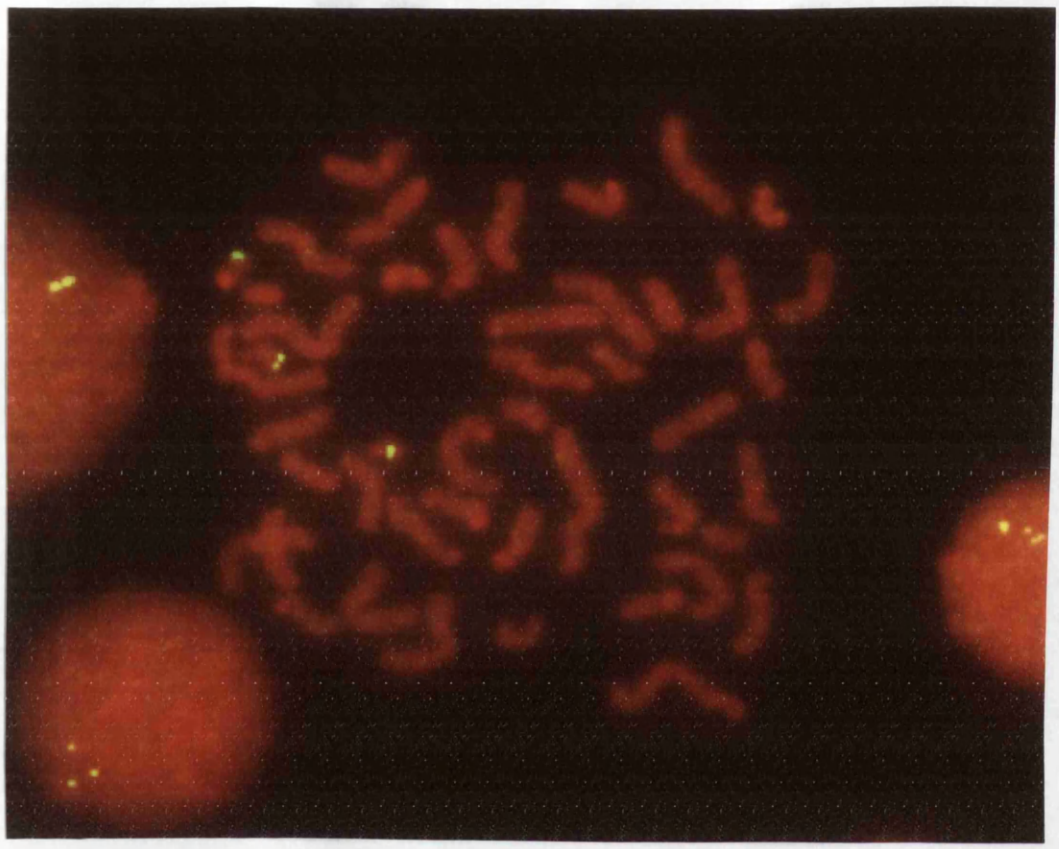

Figure 15. Hybridisation with the cosmid contig probe for chromosome 21 on metaphase spread of bone marrow cells of a Ph negative CML patient who was trisomic for chromosome 21 showed cosmid contig signals on the long arms of three copies of chromosome 21.

b)

3.4.1.3. Application of dual-colour FISH on interphase nuclei and metaphases from chronic myeloid leukaemia cases

Optimised dual-colour FISH using the abl and bcr cosmid probes was applied to interphase nuclei and metaphase spreads by visualising with the digital image system coupled with the computer system. Three control cases with normal karyotype and thirteen CML cases including five cases (cases 1, 5, 7, 11 and 12), which had also been analysed by uni-colour FISH (see sections 3.4.1.1 and 3.4.1.2), were studied and results are shown in **Table 6.**

In general, 50 nuclei were analysed from each case in the interphase study, but some cases (nos.1, 2, 3, 9 and 11) did not have an adequate number of good quality nuclei. In this instance as many as possible were studied.

In the control group, the incidence of a red-green fusion in interphase nuclei as a false positive was observed to be 6 % for control case 1 (mean, 4 %) and the rest of the nuclei had two individual red and two individual green signals. However, no metaphases bearing the red-green fusion on either chromosome 9 or chromosome 22 from those controls were observed **(Table 6).**

Cytogenetically, eight out of the thirteen cases (nos. 1-6, 9 and 11) were Philadelphia positive and three of the cases (nos. 8, 12 and 13) were Philadelphia negative. In case 7 cytogenetic analysis had failed and case 10 was a blood sample from a patient previously analysed cytogenetically and shown to be Ph positive in all mitoses from bone marrow specimen.

The results are given in **Table 6**, and showed that a fusion signal was detected in at least a proportion of nuclei from all cases known to be positive in a least a proportion of cells **(Figure 16)**, and in the case where cytogenetic analysis had failed. The fusion signal was also observed in one of the three Ph negative cases **(Figure 17)**. The other Ph negative cases showed results in the control range.

In the metaphase study, metaphase spreads were obtained only from cases 3, 5, 6, 12 and 13, and only a few metaphases were obtained, however the bcr-abl fusion could be seen very easily on the derivative chromosome 22 in the Philadelphia positive cases. However, on some of the metaphase spreads from case 1, the red-green fusion representing the bcr-abl fusion signal appeared only as a green colour. It is assumed that there is fusion of red-green signals on the der(22) with suppression of the red signals **(Figure 18)**. Case 12 exhibited nine out of nine metaphases with the red-green fusion of the bcr-abl on the long arm of chromosome 22. The suppression of the red signal, which was mentioned above, can also be seen on one chromatid of chromosome 22 in case 12. **(Figure 19).**

Table 6: Dual-colour fluorescence *in situ* hybridisation on metaphase spreads and interphase nuclei: application to control group (n=3) and 13 cases of Chronic Myeloid Leukaemia using the abl and bcr probes.

CASE NO	INTERPHASE FISH RESULTS	METAPHASE FISH RESULTS	CYTOGENETIC RESULTS
A. Control Group			
1.	3 apparent fusion 47 normal	15 normal	46, XY
2.	1 apparent fusion 49 normal	15 normal	46, XX
3.	2 apparent fusion 48 normal	15 normal	46, XY
B. Patient Group			
1.	15 fusion	4 fusion	46, XY, t(9;22)
2.	20 fusion 5 normal	no metaphase	46,XY, t(9;22) [30]
3.	14 fusion 1 normal	3 fusion	46, XX, t(9;22)
4.	non-informative	non informative	46, XY, t(9;22)
5.	46 fusion 1 double fusion 3 normal	3 fusion 1 non-informative	46, XX,t(9;22) [20]
6.	32 fusion 2 double fusion 16 normal	2 fusion	46, XY, t(9;22) [1]
7.	16 fusion 34 normal	no metaphase	Failed
8.	1 fusion 49 normal	10 normal	46, XY

9.	15 fusion 9 normal 1 three abl and two bcr signals	no metaphase	46,XX,t(9;22) [3] / 46, XX [17]
10.	44 fusion 6 normal	no metaphase	not analysed
11.	14 fusion 1 normal	no metaphase	46,XY,t(9;22)[14] / 46, XY [1]
12.	41 fusion 1 double fusion 8 normal	9 fusion	47, XYY
13.	48 normal 1 three abl and two bcr signal 1 two abl and three bcr signal	8 normal	46, XY

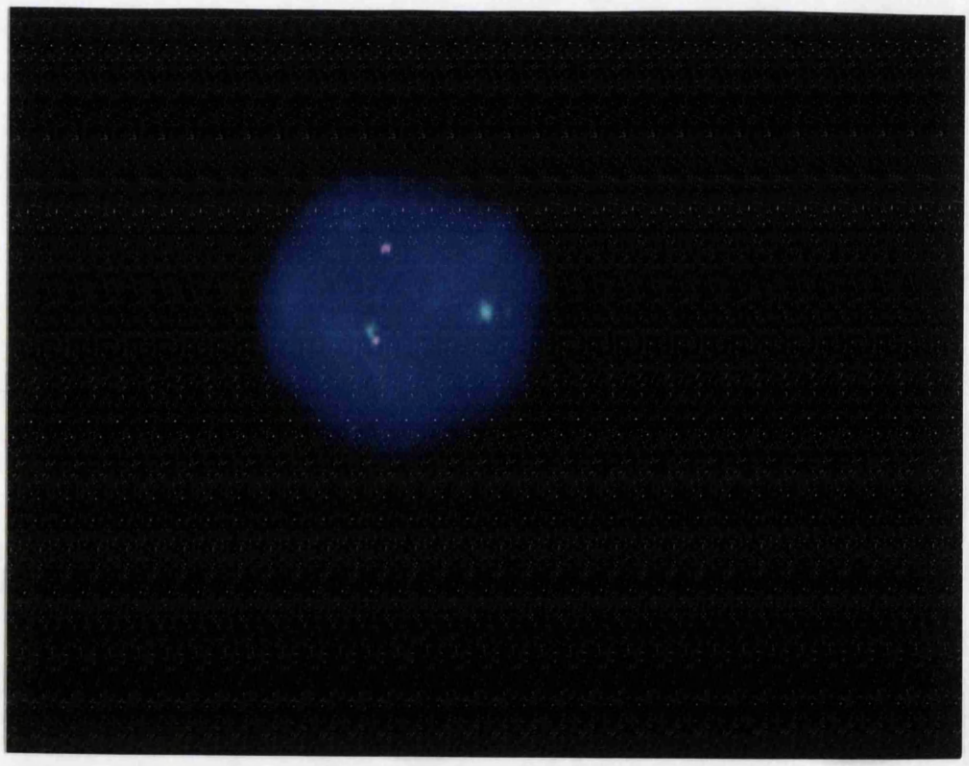

Figure 16. Double hybridisation with the cosmid probes for abl and bcr on bone marrow specimen of cytogenetically Ph positive CML patient. Dual hybridisation on interphase nuclei with DAPI counterstaining, abl (FITC) signal, bcr (Rhodamine) signal and the bcr-abl (red-green) signals representing the fusion of the bcr-abl gene.

b)

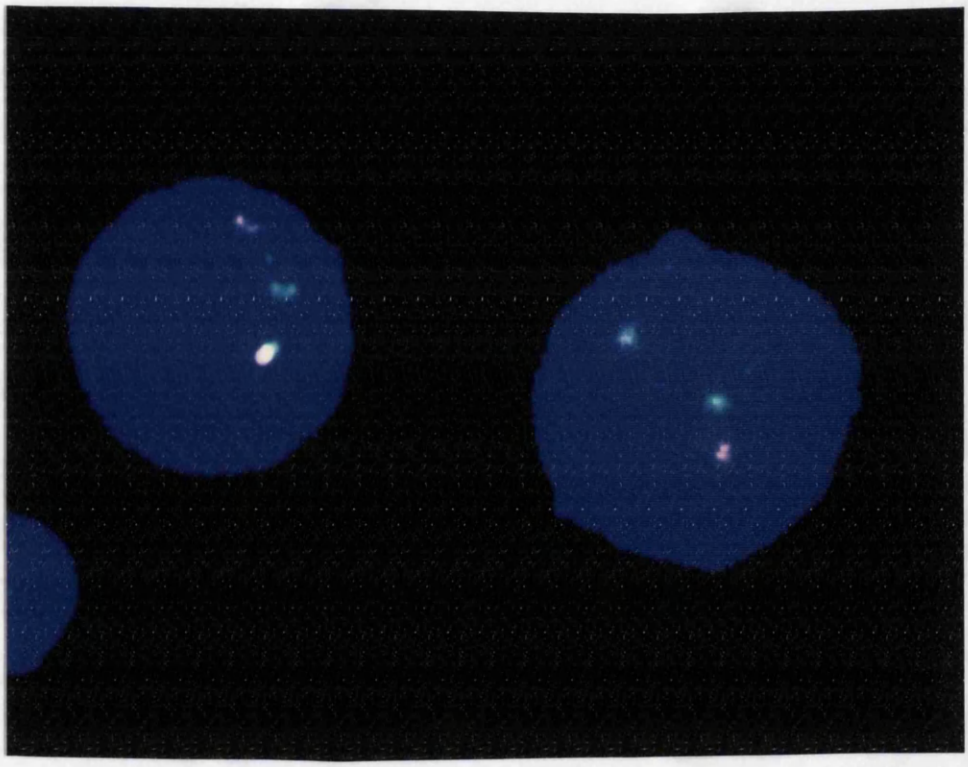

Figure 17. Combined hybridisation with the cosmid probes for abl and bcr on bone marrow specimen of cytogenetically Ph negative CML patient (case no 12) dual hybridisation in interphase nuclei with DAPI counterstaining, abl (green) signal, bcr (red) signal and the bcr-abl (the red-green) signal.

b)

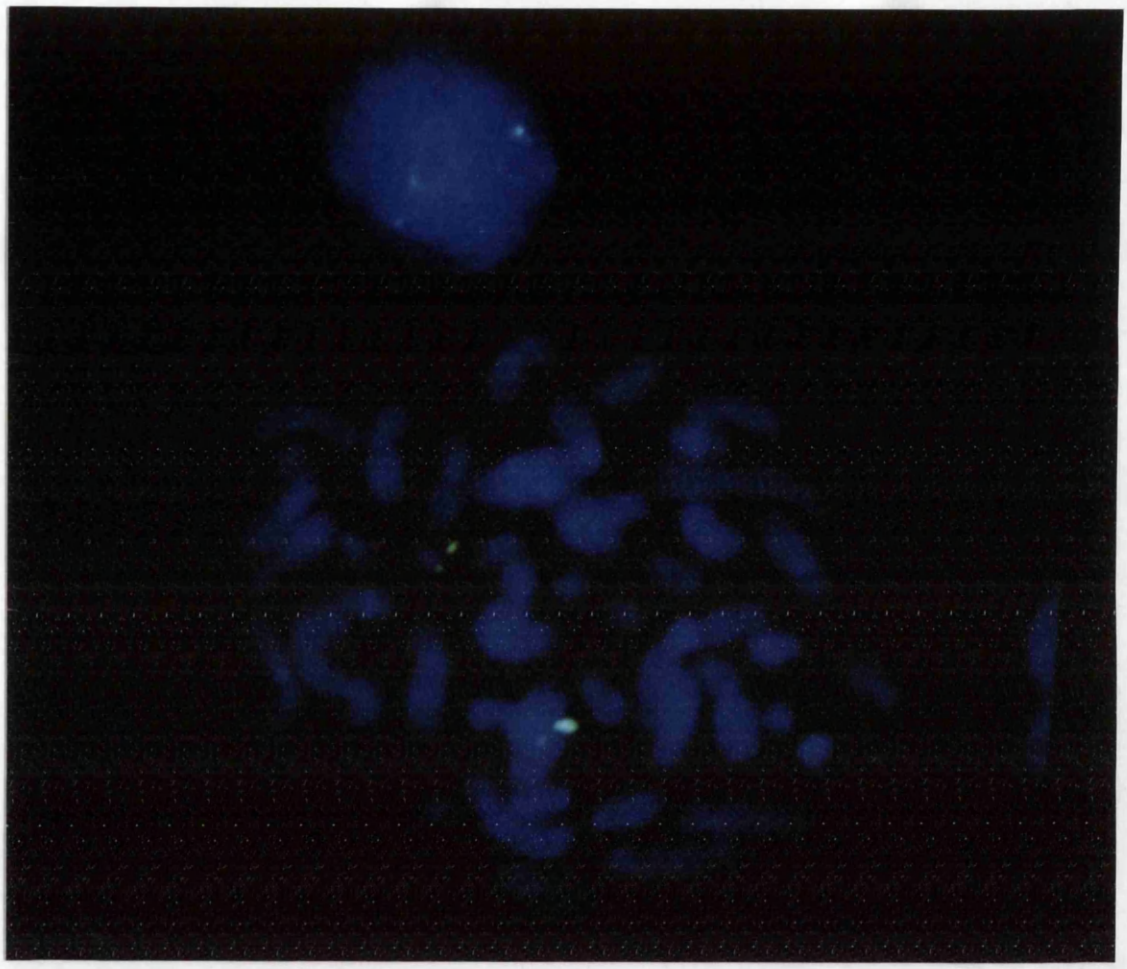

Figure 18. Dual hybridisation with the cosmid probes for abl and bcr on bone marrow specimen of cytogenetically Ph positive CML patient. Dual hybridisation on metaphase spread with DAPI counterstaining. abl (FITC) signal on long arm of chromosome 9 and bcr (red) signal on the long arm of chromosome 22 and the bcr-abl (green) signal on the long arm of Ph chromosome. It is assumed that there is fusion of red-green signals on the der(22) with suppression of the red signals.

b)

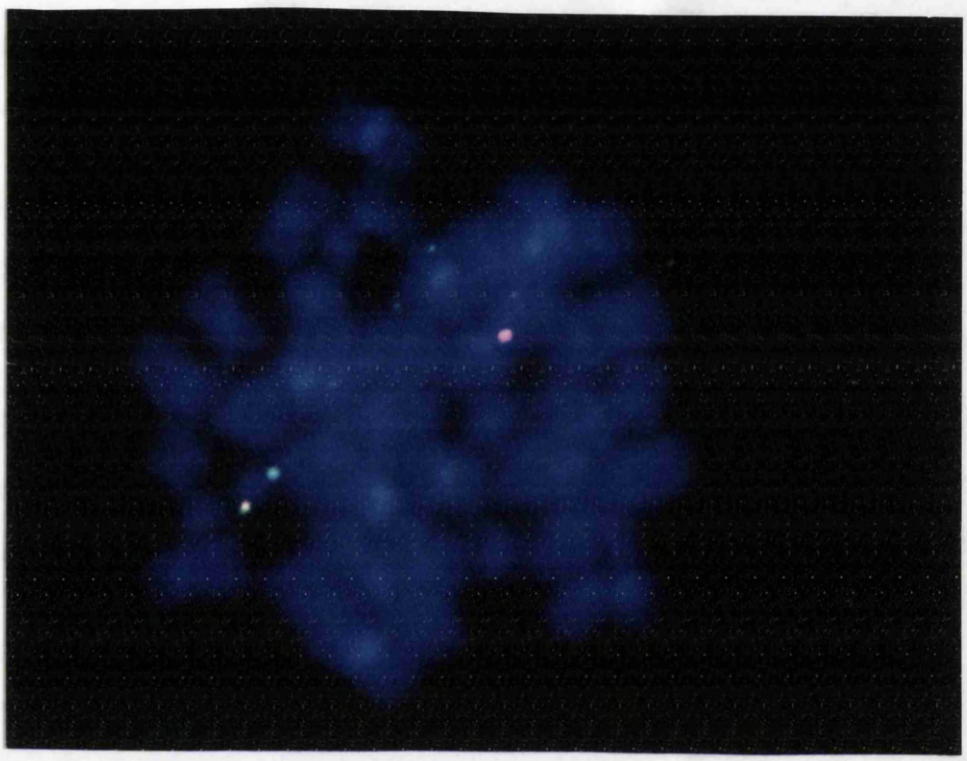

Figure 19. Double hybridisation with the cosmid probes for abl and bcr on bone marrow specimen of cytogenetically Ph negative CML patient (case no 12). Dual hybridisation on metaphase spread with DAPI counterstaining. abl (FITC) signal on long arm of chromosome 9 and bcr (red) signal on the long arm of chromosome 22 and the bcr-abl (red-green) signal on the long arm of chromosome 22.

b)

3.4.1.4. Investigation of variant Philadelphia chromosome translocations in CML

In this part of the study, two CML cases were analysed using uni- and dual-colour FISH in order to evaluate the nature of the complex translocation. For this purpose, abl and bcr cosmid probes, whole chromosome specific library probes for chromosomes 3, 6, 8, 9 and 22, and alpha satellite repetitive probes for chromosomes 3, 8 and 22 were used individually or in combination.

Cytogenetic analysis of bone marrow cell from the first case showed that the complex translocation involves chromosomes 6, 8, 9 and 22 with break points at 6q25, 8q22, 9q34 and 22q11 (46, XX, t(6; 8; 9; 22))**(Figure 20).**

Dual colour FISH with the abl and the bcr cosmid probes revealed the interphase nuclei exhibiting the red-green fusion signals (21 out of 25 nuclei) indicating the bcr-abl gene fusion **(Figure 21).**

Dual colour FISH was also applied on metaphase spreads by using the combination of an 8 centromeric probe detected with Rhodamine and chromosome 6 library probe detected with FITC. The result with chromosome 6 painting showed a normal chromosome 6 being painted with a green colour whereas the other homologue was re-arranged, because the long arm of one homologue chromosome 8 showed to be partially painted with FITC (which had a red centromeric region) indicating the existence of chromosome 6 material.

For further investigation, a combination of the 9 library probe detected with Rhodamine and the chromosome 8 library probe detected with FITC was applied on the same case. The results showed one chromosome 9

painted red, the other homologue was shown to be re-arranged as although most of the chromosome painted red, part of the long arm was painted green. The results also showed one chromosome 8 painted green, the other homologue was shown to be re-arranged as although the whole of the short arm and part of the long arm were painted green. There was a small area of unpainted material on the distal end of the long arm. These results would be consistent with transfer of material from 8q to 9q and the attachment of additional material from another chromosome to the breakpoint on 8q **(Figure 22).** In addition, chromosome 22 library probe was applied but the results showed that chromosome 22 gave cross-hybridisation with the centromeric area of the other acrocentric chromosomes. Therefore, this probe was uninformative.

In the second case, all cells were Ph chromosome positive with a complex translocation between chromosomes 9, 17 and 22 with break points at 9q34, 17p13, 22q11. Most cells had an additional translocation between the long arm of chromosome 3 and the long arm of the homologue of chromosome 22 not involved in the Ph chromosome complex translocation resulting in a karyotype 46, XX, t(3;22)(q13;q12), t(9;17;22)(q34;p13;q11) **(Figure 23).** Some of these cells also had a structural alteration of the long arm of chromosome 20. Thus, FISH analysis was performed using the abl and bcr cosmid probes, α-satellite 3 and 22 centromeric probes, and whole chromosome specific 3 and 22 library probes.

By applying dual-colour FISH, the combination of the abl and the bcr probes showed the presence of bcr-abl fusion in the majority of interphase nuclei. For further analysis of these translocations, combined probe hybridisation was applied. Using dual-colour FISH with whole chromosome specific 3 library probe (FITC) and a 22 centromeric probe

(Rhodamine), the 3 library probe painted the normal chromosome 3, the derivative chromosome 3 and part of the derivative chromosome 22 (identified by a red centromeric region) not involved in the Ph chromosome complex translocation **(Figure 24)**.

However, dual application of whole chromosome specific 22 library (detected with FITC) and α-satellite centromeric probe for chromosome 3 (detected with FITC) with uni-colour FISH showed painting of chromosome 22 material on the der(22) t(9;17;22)(q34;p13;q11) (Philadelphia chromosome) on the short arm of the der(17) t(9;17;22) (q34;p13;q11) and also on the distal part of the long arm der(22) t(3;22)(q13; q12) and on part of the long arm of the der(3) t(3;22)(q13;q12) **(Figure 25)**. This revealed the nature of the involvement of chromosome 17 in the complex Ph chromosome translocation as well as the reciprocal translocation between the other homologue of chromosome 22 and chromosome 3.

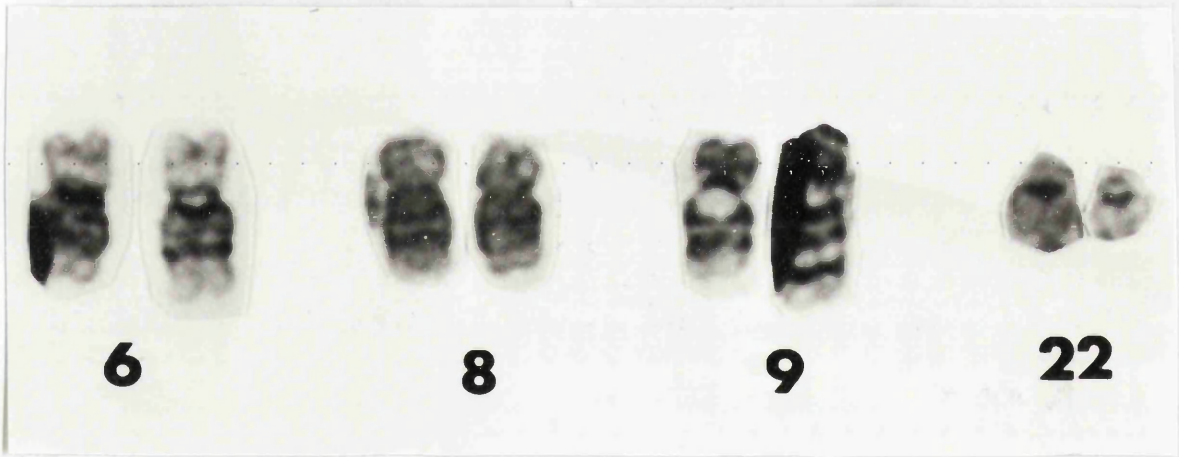

Figure 20. Partial karyotype of chromosomes involved in t(6;8;9;22)(q25; q22;q34;q11).

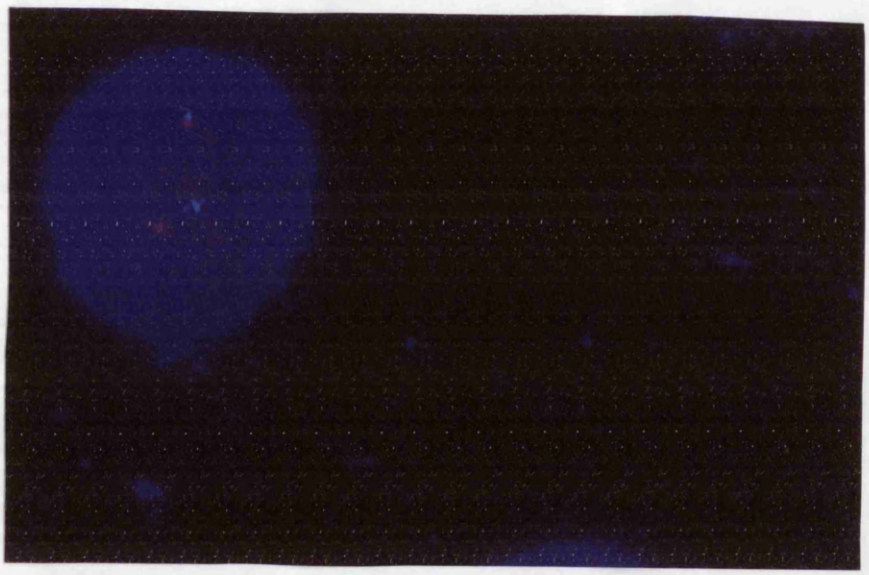

Figure 21. Double hybridisation with the cosmid probes for abl and bcr on bone marrow specimen of cytogenetically complex translocated CML patient. Dual hybridisation to interphase nucleus with DAPI counterstaining. abl (FITC) signal, bcr (Rhodamine) signal and the bcr-abl (red-green) signals representing the fusion of the bcr-abl gene.

b)

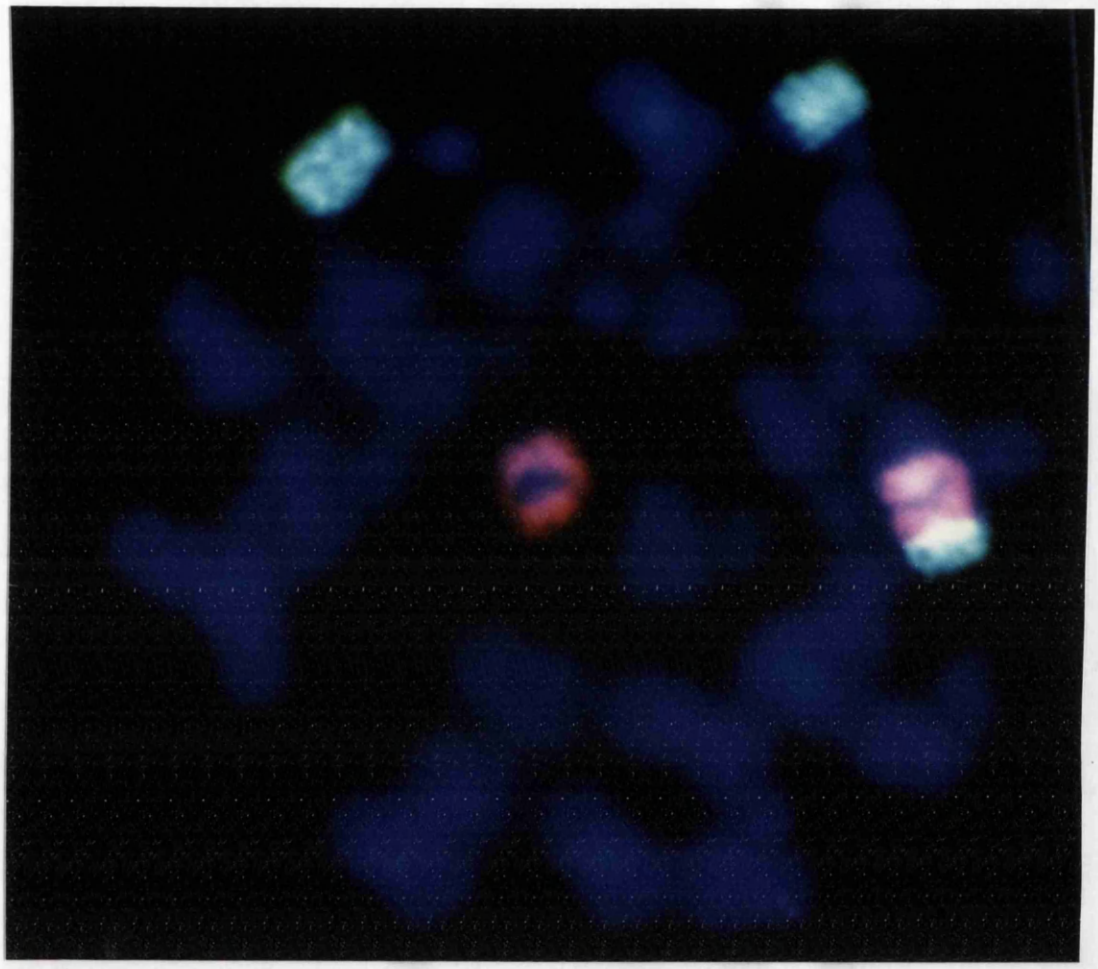

Figure 22. Dual-colour FISH on bone marrow metaphase from a patient with CML and 46, XX, t(6;8;9;22). The biotin-labelled chromosome 8 library is detected by FITC (green), the Rhodamine labelled chromosome 9 library.

b)

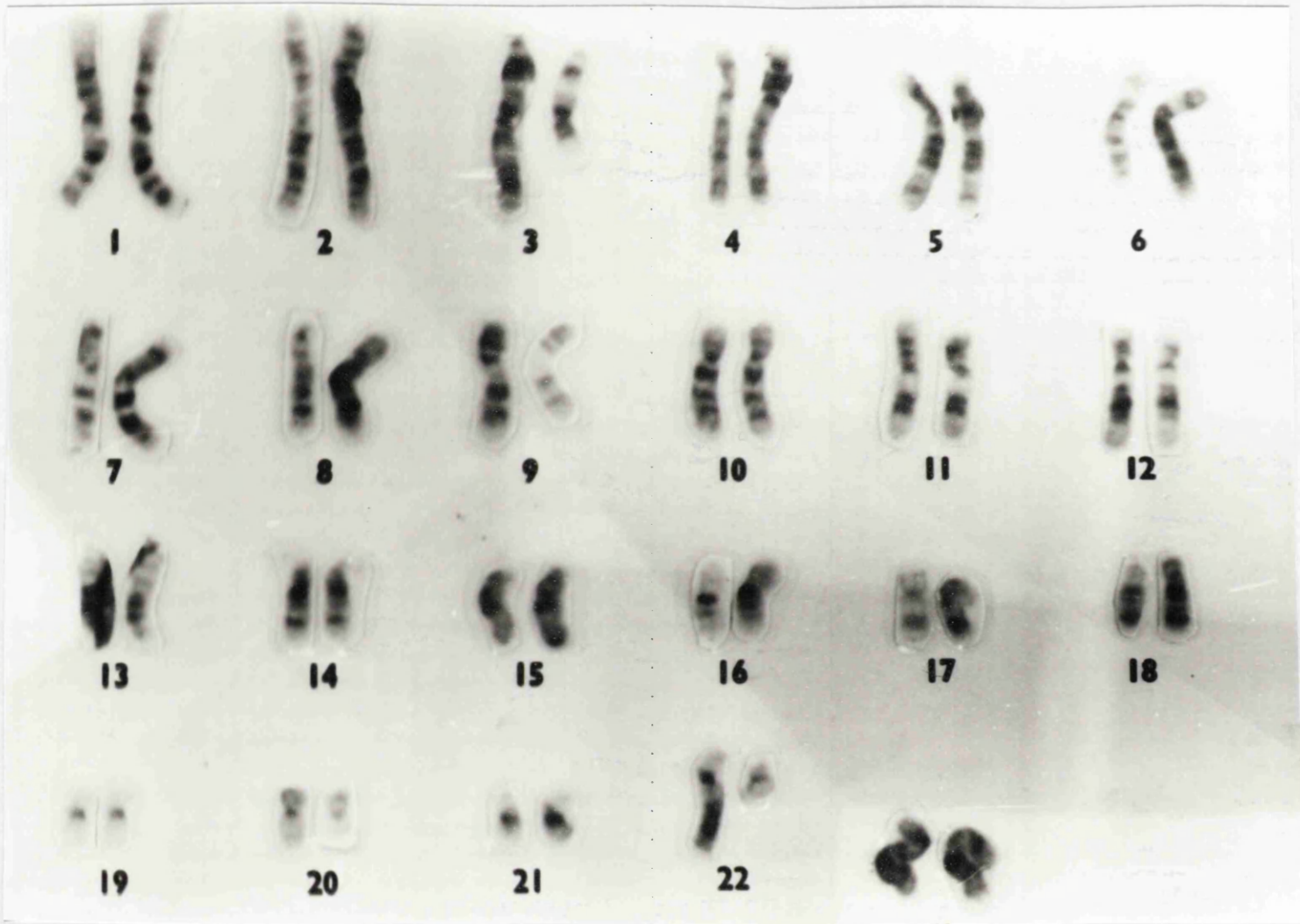

Figure 23 : Chromosome analysis with G-banding which showed 46, XX, t(3;22)(q13;q12), t(9;17;22)(q34;p13;q11).

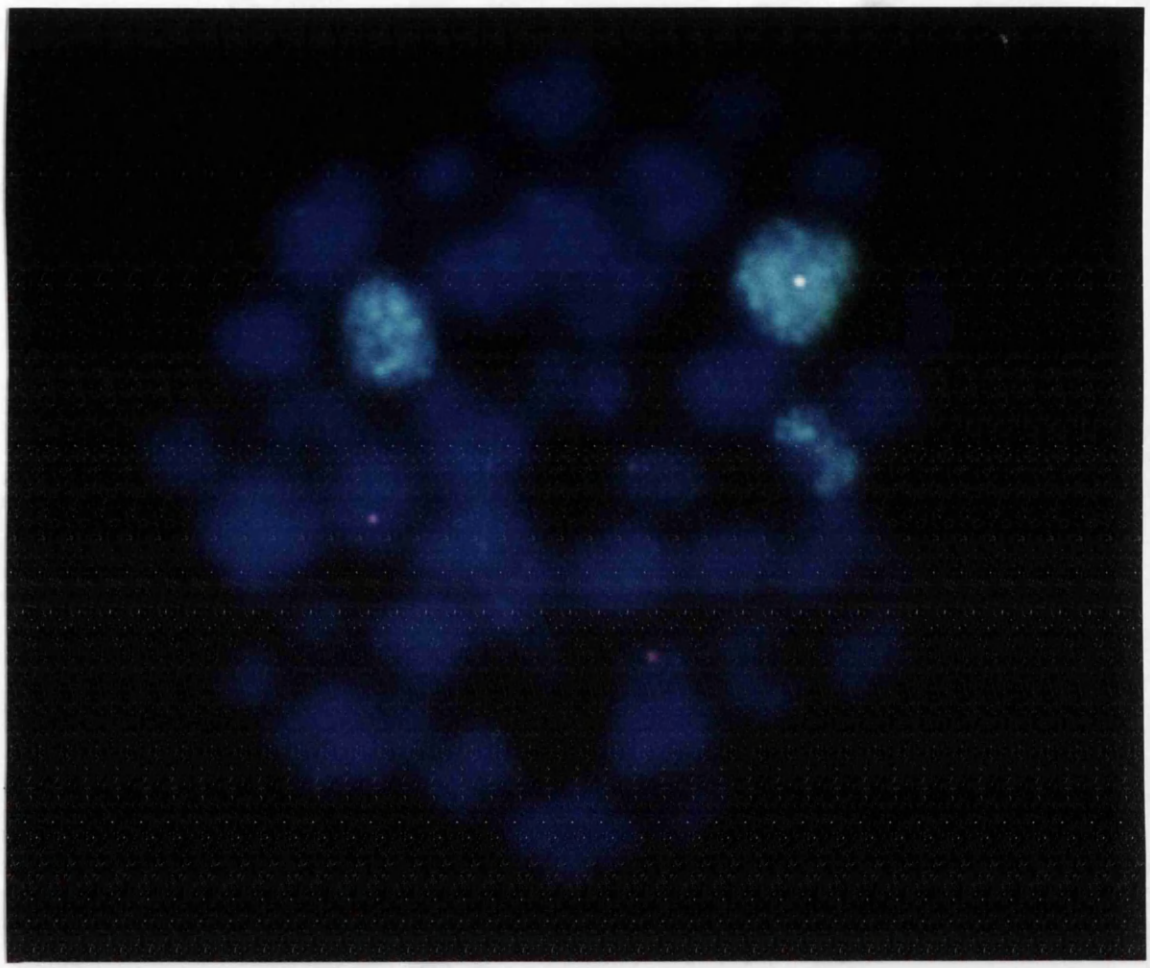

Figure 24. Dual-colour FISH on bone marrow metaphase from a patient with CML and (46, XX, t(3;22); t(9;17;22)). The digoxigenin-labelled chromosome 22 centromeric probe is detected by Rhodamine (red), the biotin labelled chromosome 3 library is detected by FITC (green).
The position of 22 centromeric probe is not clear from the photograph but these regions could be demonstrated by microscopy. The exposure needed to bring out red signals intensified cross hybridization which produced unspecific signals.

b)

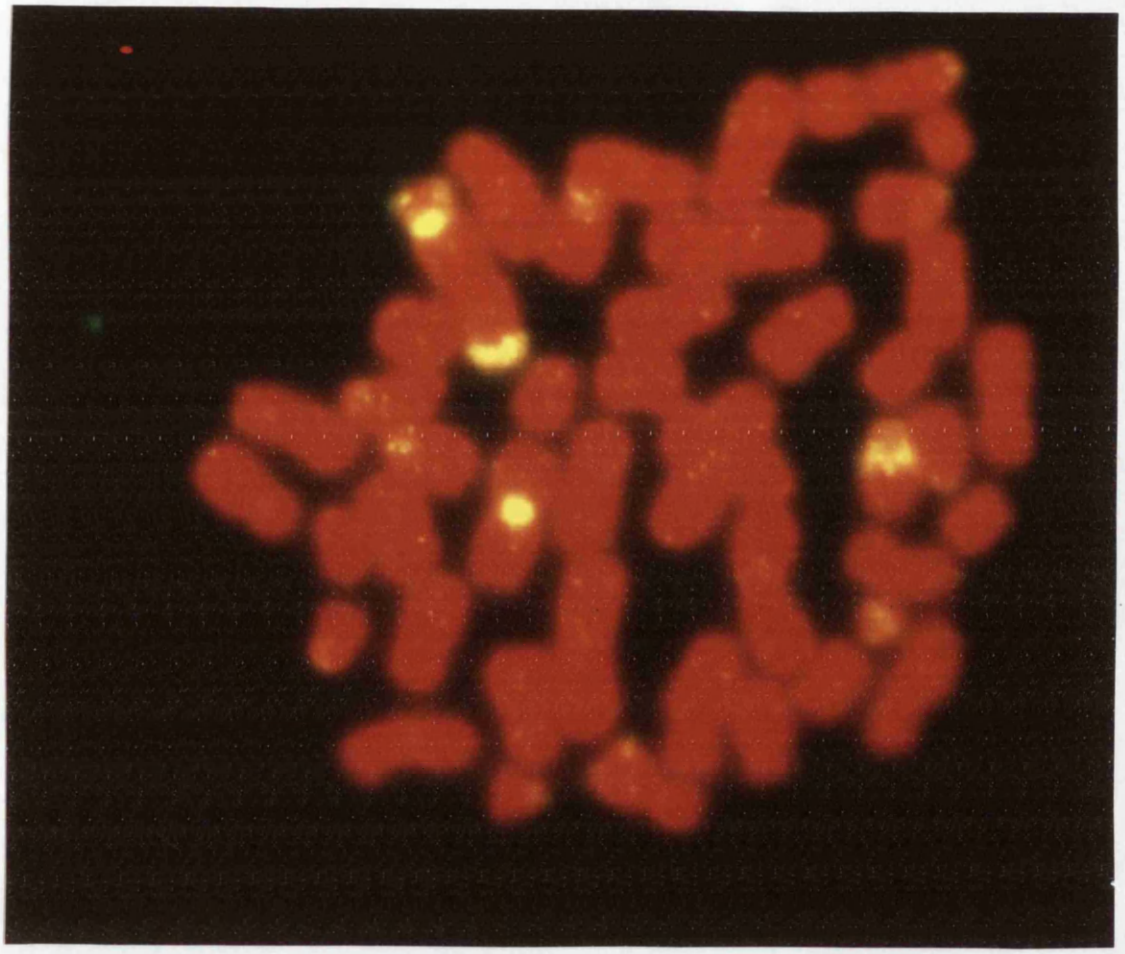

Figure 25. Uni-colour FISH on bone marrow metaphase from a patient with CML and (46,XX, t(3;22); t(9;17;22)). The biotin-labelled chromosome 3 centromeric probe is detected by FITC (green), the biotin labelled chromosome 22 library is detected by FITC (green).

b)

3.4.2.1. Application of dual-colour FISH in case of Acute Promyelocytic Leukaemia (AML-M3)

The translocation t(15;17)(q22;q12 or 21) is specific for AML-M3 but may be difficult to detect in chromosome preparations of sub optimal quality. Hence the probe set (P5119-D/B; ONCOR) was used to study preparations from 3 female and 4 male patients with AML-M3 and AML-M3v by dual-colour FISH. Two karyotypically normal females (AML) and three karyotypically normal males (one MDS and two AML) were used as controls.

Cytogenetic analysis of both the AML-M3 cases at different stages of investigation and the cytogenetic analysis of the control cases had been carried out by staff in the bone marrow section of the department **(Table 7).**

In this study normal localisation of red and green signals is on chromosomes 17 and 15 respectively which would normally occupy separate domains within the nucleus **(Figure 26).** Fusion of red and green signals would equate with re-arrangement between chromosomes 15 and 17 in particular t(15;17)(q22; q21).

In the control group (cases 1 to 5) at least 50 interphase nuclei were examined. Normal localisation of red and green signals was seen in 84.6% to 96% of interphase nuclei from all control specimens. A few cells (from 1 to 4 cells out of 50) from all controls showed apparent fusion of red green signals which may be due to translocation or isochromosome, or might be artifact. One or two cells from all controls showed duplication of red or green signals, presumably tetraploidy.
In addition, metaphase spreads from the same specimens were examined by dual colour FISH. Results showed that both red and green signals

individually located on the long arms of chromosomes 15 and 17, respectively **(Figure 27) (Table 7)**. No metaphase from the control material showed the translocation chromosome or the presence of any unexpected signal.

Table 7: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads from bone marrow preparations hybridised with the t(15;17) cosmid probe mixture (Case no 1 (925357), 2 (921141), 3 (940907), 4 (913958) and 5 (925054). All these control cases had a normal karyotype.

CONTROL CASES

INTERPHASE

		FISH RESULTS				
Case Nos	N	1R1G1F	1R1G2F	3R2G	3G2R	Total
1.	47	3				50
2.	47	2	1			50
3.	48	2				50
4.	44	4		2	2	52
5.	48	1		1		50
METAPHASE						
1.	4					4
2.	4					4
3.	6					6
4.	5					5
5.	15					15

N: represents normal localisation- two red signals for chromosome 17 and two green signals for chromosome 15.

R: represents chromosome 17 break point region as a red signal,

G: represents chromosome 15 break point region as a green signal,

F: represents the fusion of chromosomes 15 and 17 break points as a red-green signal (F).

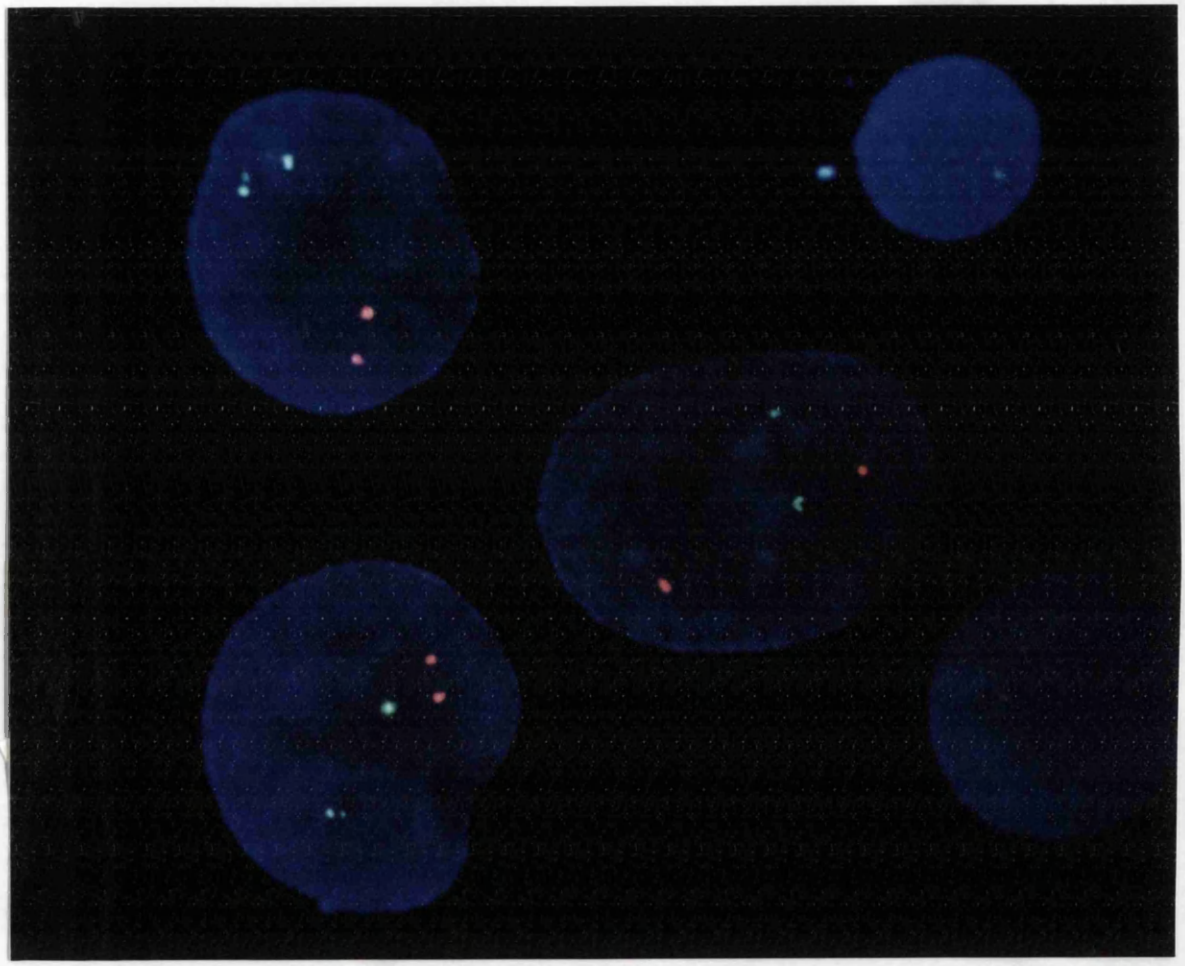

Figure 26. Dual-colour FISH on bone marrow: Normal interphase from a patient with AML (as a control). The digoxigenin-labelled and biotin labelled t(15;17) translocation probe signals on chromosome 17 (red) and chromosome 15 (green). DNA is stained with DAPI.

b)

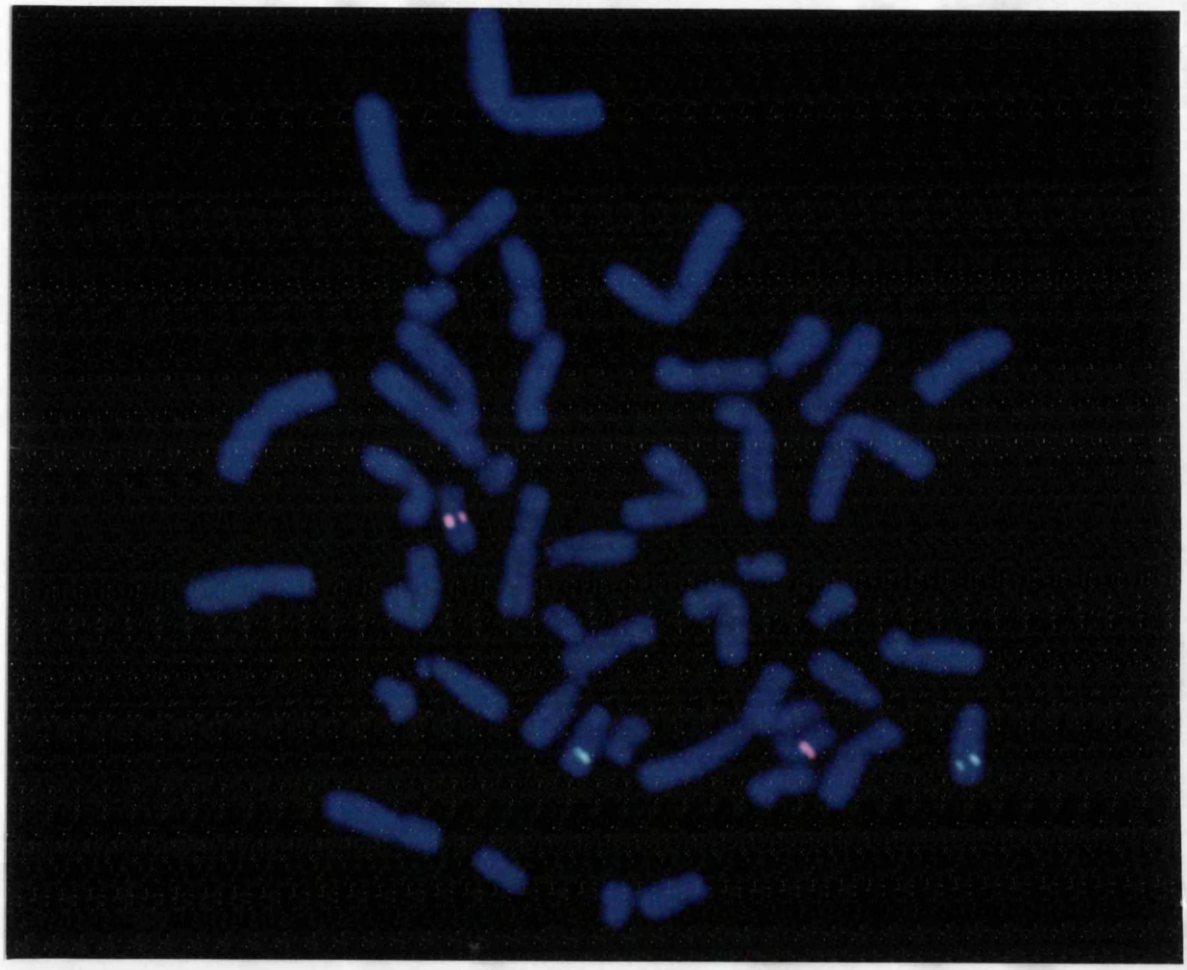

Figure 27. Dual-colour FISH on bone marrow: Normal metaphase from a patient with AML (control). The digoxigenin-labelled and biotin labelled t(15;17) translocation probe signals on chromosome 17 (red) and chromosome 15 (green). DNA is stained with DAPI.

b)

In the patient group, in order to determine the t(15;17) on interphase nuclei and metaphase spreads, specimens were obtained from bone marrow and peripheral blood from patients with AML-M3 at different stages of investigation. Direct and twenty-four hour unstimulated cultures were examined from these patients (cases 6 to 12). In the metaphase study, as many metaphase spreads as possible were examined. This was dependent upon the quality of the preparations.

CASE 6:

Serial specimens from a 25 years old male with AML-M3 (case no 6) were received in the department. Four of these bone marrow specimens were available for FISH analysis. Cytogenetic results at different stages of the disease are listed in **Table 8.**

SAMPLE 1) A diagnostic marrow sample taken on 20 / 1 / 93 revealed 90 % of interphase nuclei from both direct and 24 hour preparations to have fusion of red-green signals **(Figure 28)**, confirming the presence of t(15;17). 6 % of nuclei from direct preparations and 8 % of nuclei from 24 hour cultures showed normal localisation of signals indicating that a normal cell line may also have been present. **(Table 8).**

SAMPLE 2) A subsequent marrow sample taken 8 / 2/ 93 revealed 92 % and 94 % of interphase cells from direct and 24 hour cultures respectively to have fusion of red-green signals **(Table 8).**

SAMPLE 3) A pre-allograft sample taken on 16 / 7 / 93 when the patient was in clinical remission revealed 88 % of interphase nuclei to have normal localisation of signals. 12 % of nuclei, however clearly showed red-

green fusion signals suggesting the presence of t(15;17) as a minor clone conforming the continued presence of the abnormal clone **(Table 8)**.

SAMPLE 4) A subsequent pre allograft sample taken on 28 / 8 / 93 showed only 2 % of interphase nuclei with red-green fusion signals. The remaining 98 % of nuclei showed normal localisation of signals. These results were from direct cultures only. This result was indistinguishable from the control values.

In the metaphase study, the main drawback was the lack of the metaphase spreads. As can be seen on **Table 8**, a very small number of metaphase spreads was obtained. Dual- colour FISH studies showed red-green signal fusion on the long arm of chromosome 17 in metaphases from the diagnostic (24 hour cultures only) and in the subsequent specimen (both direct and 24 hour cultures) taken three weeks later **(Figure 29)**. No red-green signal fusion was observed in any of the two subsequent remission samples. These results are in direct agreement with the cytogenetic results.

Table 8: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads hybridised with the t(15;17) cosmid probe mixture (Case 6; serial bone marrow specimens nos: 1(930276), 2(930574), 3 (933137), 4 (933735)).

A.INTERPHASE

SPECIMEN		FISH RESULTS					
Specimen Taken on	Culture Type	N	1R1G1F	1R1G2F	Other	Total Cells	Cytogenetics Results
1. 20.1 93	Direct	3	45	2		50	46, XY [12]
20.1.93	24 hour	4	45	1		50	46,XY, t(15;17) [6] / 46, XY [2]
2. 8.2.93	Direct	2	46		2*	50	
8.2.93	24 hour	3	47			50	46, XY, t(15;17)
3. 16.7.93	Direct	44	6			50	46, XY [24]
4. 23.8.93	Direct	49	1			50	46, XY [12]

B.METAPHASE

1. 20.1 93	Direct	1				1	
20.1.93	24 hour	1	2			3	
2. 8.2.93	Direct		1			1	
8.2.93	24 hour		4			4	
3. 16.7.93	Direct	3				3	
4. 23.8.93	Direct	10				10	
23.8.93	24 hour	3				3	

*: More than three signals each of colour in one nucleus.

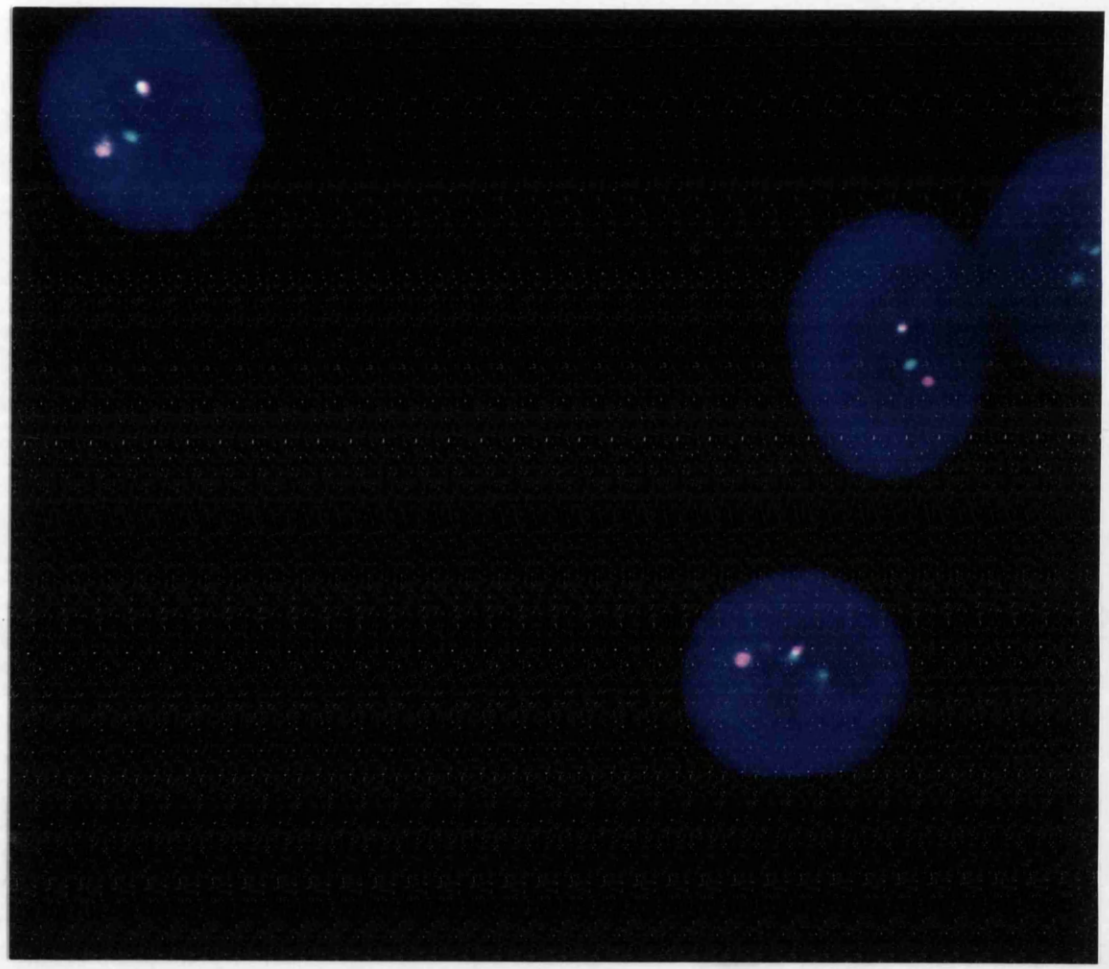

Figure 28. Dual-colour FISH with the t(15;17) cosmid probe mixture on bone marrow interphase from a patient with AML-M3 (Case no 6) showed the red-green fusion, representative of the translocation between chromosomes 17 and 15 on chromosome 17, and single red and green hybridisation signals, representative of break points on chromosomes 17 and 15.

b)

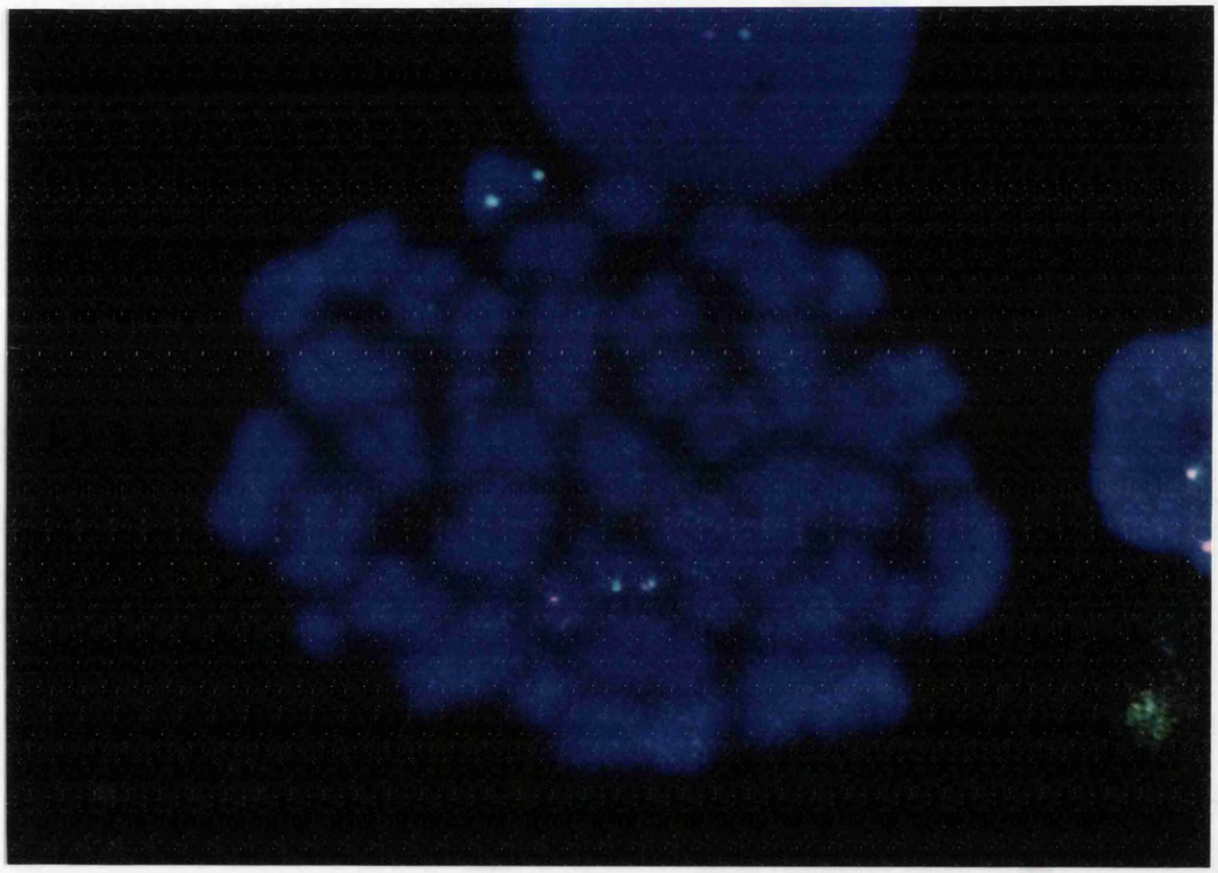

Figure 29. Dual-colour FISH with the t(15;17) cosmid probe mixture on bone marrow metaphase from a patient with AML-M3 (Case no 6) showed the red-green fusion, representative of the translocation between chromosomes 17 and 15 on chromosome 17, and single red and green hybridisation signals, representative of break points on chromosomes 17 and 15.

b)

CASE 7:

Serial marrow specimens from an 8 year old male with AML-M3 were obtained. Cytogenetic results as different stages of the disease are listed in **Table 9.**

SAMPLE 1) A diagnostic marrow sample taken on 22 / 7 / 93 revealed 60 % of interphase nuclei from direct preparation to have the fusion of red-green signals. 36 % of nuclei from direct preparation showed normal localisation of signals.

SAMPLE 2) A subsequent marrow sample - post chemotherapy taken on 19 / 8 / 93 revealed 38 % of nuclei from direct preparation to have the fusion of red-green signals. 56 % of nuclei from direct preparation showed normal localisation of signals. The preparation of 24 hours culture yielded insufficient material for scoring.

SAMPLE 3) A subsequent marrow sample taken on 16 / 11 / 93, when the patient was in clinical remission, revealed 10 % of nuclei from direct preparation to have the fusion of red-green signals. The remaining 90 % of nuclei showed normal localisation of signals. The preparation of 24 hours culture yielded insufficient material for scoring.

SAMPLE 4) A subsequent marrow sample taken on 22 / 2 / 94 revealed only 4 % and 2 % of interphase nuclei from direct and 24 hour preparation to have the fusion of red-green signals. The remaining of 96 % of nuclei from direct preparation and 98 % of nuclei from 24 hours culture showed normal localisation of signals.

Application of dual-colour FISH to metaphase spreads from the diagnostic specimen revealed one out of four metaphases (from the direct

preparation) to have red-green fusion (on the long arm of chromosome 17) together with one extra red signal on what appeared to be the long arm of a D group chromosome. Red-green signal fusion was noted on the long arm of chromosome 17 in three out of eight metaphases in the direct preparations from the subsequent specimen taken three weeks later. Confirming the continuing presence of t(15;17) **(Table 9).**

Table 9: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads from bone marrow preparation hybridised with the t(15;17) cosmid probes mixture (Case 7; Serial bone marrow specimens nos: 1 (933219), 2 (933685), 3 (935147), 4 (940846)).

A. INTERPHASE

SPECIMEN		FISH RESULTS					
Specimen Taken on	Culture Type	N	1R1G1F	1R1G2F	Other	Total Cells	Cytogenetics Results
1. 22.7. 93	24 hour	18	30		2*	50	46,XY,t(15;17)[10] / 46, XY [2]
2. 19.8.93	Direct	28	19	3		50	46,XY,t(15;17)[15]
19.8.93	24 hour	Preparation inadequate					
3. 16.11.93	Direct	45	5			50	46, XY [21]
16.11.93	24 hour	Preparation inadequate					
4. 30.12.93	Direct	48	2			50	No metaphase
30.12.93	24 hour	49	1			50	46, XY [20]

B. METAPHASE

1. 22.7. 93	24 hour		3		1	4
2. 19.8.93	Direct	5	3			8
19.8.93	24 hour	No metaphase spreads found				
3. 16.11.93	Direct	7				7
16.11.93	24 hour	3				3
4. 30.12.93	Direct	6				6
30.12.93	24 hour	16				17

*: one red-green fusion, two red and one green signal in two nuclei.

CASE 8

Serial blood specimen from an 8 year old male with AML-M3 were taken. Diagnostic cytogenetics was performed 4 / 5 / 93 at diagnosis at another centre, where the karyotype was reported as: 46, XY, t(15;17)(q22;q12) [2] / 46, idem, der(3) t(3;12)(p12;q12) [14] / 46, XY [5] **(Table 10).**

SAMPLE 1) A post allograft (male donor) marrow sample taken on 7 / 12 / 93 revealed 26 % of interphase nuclei from 24 hours culture to have the fusion of red-green signals. The remaining 72 % of nuclei showed normal localisation of signals. Cytogenetic analysis was not performed on this sample.

SAMPLE 2) A subsequent post allograft marrow samples taken 14 / 12 / 93 when the patient was clinically completed first remission, revealed 8 % of nuclei from direct preparation to have fusion of red-green signals. The remaining 92 % of nuclei showed normal localisation of signals.

The karyotype from 24 hour culture was reported as 46, XY with no evidence of the t(15;17), although the origin of the cells could not be confirmed. Metaphase analysis of the direct and 24 hours cultures from both specimen showed no fusion of red-green signal on the long arm of chromosome 17 **(Table 10).**

Table 10: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads from blood samples hybridised with the t(15;17) cosmid probe mixture (Case 8: Specimens nos: 1(935486), 2(935619)).

A.INTERPHASE:

SPECIMEN		FISH RESULTS				
Specimen Taken on	Culture Type	N	1R1G1F	1R1G2F	Other	Total Cells
1. 7.12.93	Direct	Non informative				
7.12.93	24 hour	36	13		1	50
2. 14.12.93	Direct	46	4			50

B.METAPHASE

1. 7.12.93	Direct	20				20
7.12.93	24 hour	33				33
2. 14.12.93	Direct	10				10

CASE 9:

Serial blood specimens from a 47 year old male with AML-M3 were examined. Cytogenetic analysis of the direct preparation from the first specimen showed the karyotype to be 46, XY, inv(9)(p11q13)c [2] / 46, XY, inv(9)(p11q13)c, t(15;17) (q22;q21) [3]. The second specimen was not set up for cytogenetics. The inverted chromosome 9 was considered to be a normal constitutional chromosome polymorphism. 1 % of the population have this inv(9). It is considered to be of no clinical significance.

SAMPLE 1) A diagnostic blood sample taken on 7 / 2 / 94 revealed 80 % of nuclei from direct preparation to have the fusion of red-green signals.

However, 8 % of nuclei had a regular fusion of red-green signal, a normal green signal, a normal red signal and an extra red signal in the same nuclei. The remaining nuclei (12 %) had normal hybridisation signals.

SAMPLE 2) A diagnostic blood specimen taken on 8 / 2 /94 revealed 76 % of nuclei from the direct preparation to have the fusion of red-green signals. However, 14 % of nuclei had a regular red - green signal fusion, one normal green signal, one normal red and an extra red signal in the same nuclei. The remaining nuclei (10 %) showed normal hybridisation signals **(Table 11)**.

Both of these results are in broad agreement and would confirm the cytogenetic findings, although the finding of one extra red signal in a small number of cells from both samples is unclear. Unfortunately FISH examination of metaphase preparations was not possible as no metaphases were found.

Table 11: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads from blood samples hybridised with the t(15;17) cosmid probe mixture (Case no 9: Blood specimen 1(940628)).

INTERPHASE:

SPECIMEN		FISH RESULTS				
Specimen Taken on	Culture Type	N	1R1G1F	1R1G2F	Other	Total Cells
1. 7.2.94	Direct	6	40		4*	50
2. 8.2.94	Direct	5	38		7*	50

*: Nuclei have a regular fusion of red-green signal and one green and two red signals on the same nuclei.

CASE 10:

A diagnostic marrow specimen from a 27 years old female with AML-M3 was received in the department. Cytogenetic analysis of bone marrow cells revealed the karyotype as 46, XX, t(15;17)(q22;q11-12) [1], 46, XX der(15) t(15;17)(q22;q11-12), ider (17) t(15;17)(q22;q11-12) [5], 46, XX [11] **(Figure 30).**

SPECIMEN) A diagnostic marrow sample taken on 4 / 3 / 91 revealed 60 % of nuclei to have the fusion of red-green signals. 18 % of nuclei showed normal localisation of hybridisation signals. 22 % of nuclei examined showed the double fusion of red - green signals with one individual red and one green signal on the same nuclei **(Figure 31) (Table 12).**

In metaphase studies with FISH, no metaphase spreads were obtained from the direct preparations.

Table 12: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads hybridised with the t(15;17) cosmid probes mixture (Case 10: Bone marrow specimen no 1 (910958)).

INTERPHASE:

SPECIMEN			FISH RESULTS			
Specimen Taken on	Culture Type	N	1R1G1F	1R1G2F	Other	Total Cells
1. 4.3.91	Direct	9	30	11		50

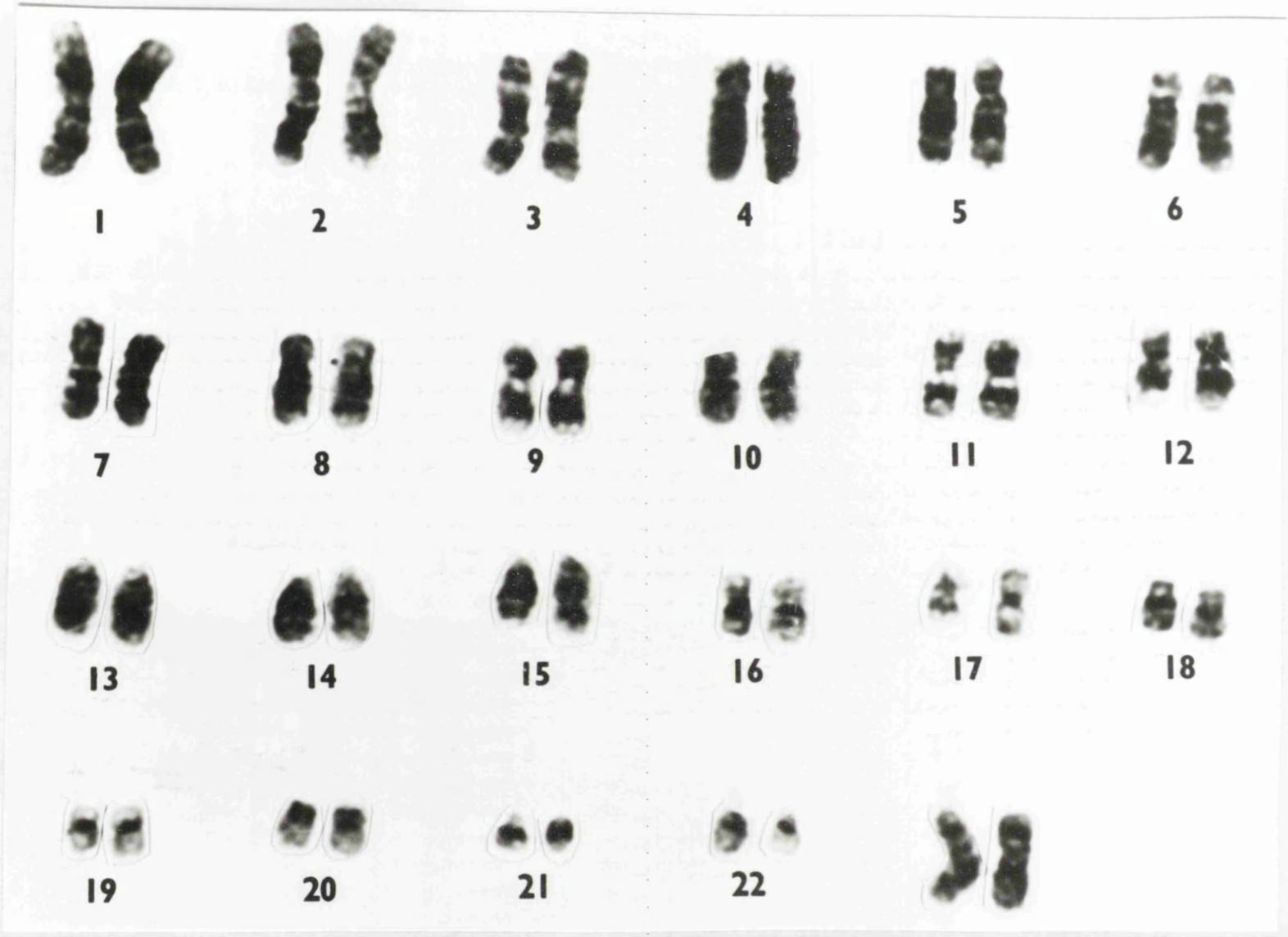

Figure 30. Chromosome analysis with G-banding, which showed 46, XX der(15) t(15;17)(q22;q11-12), ider(17) t(15;17)(q22;q11-12) in five out of seventeen metaphases.

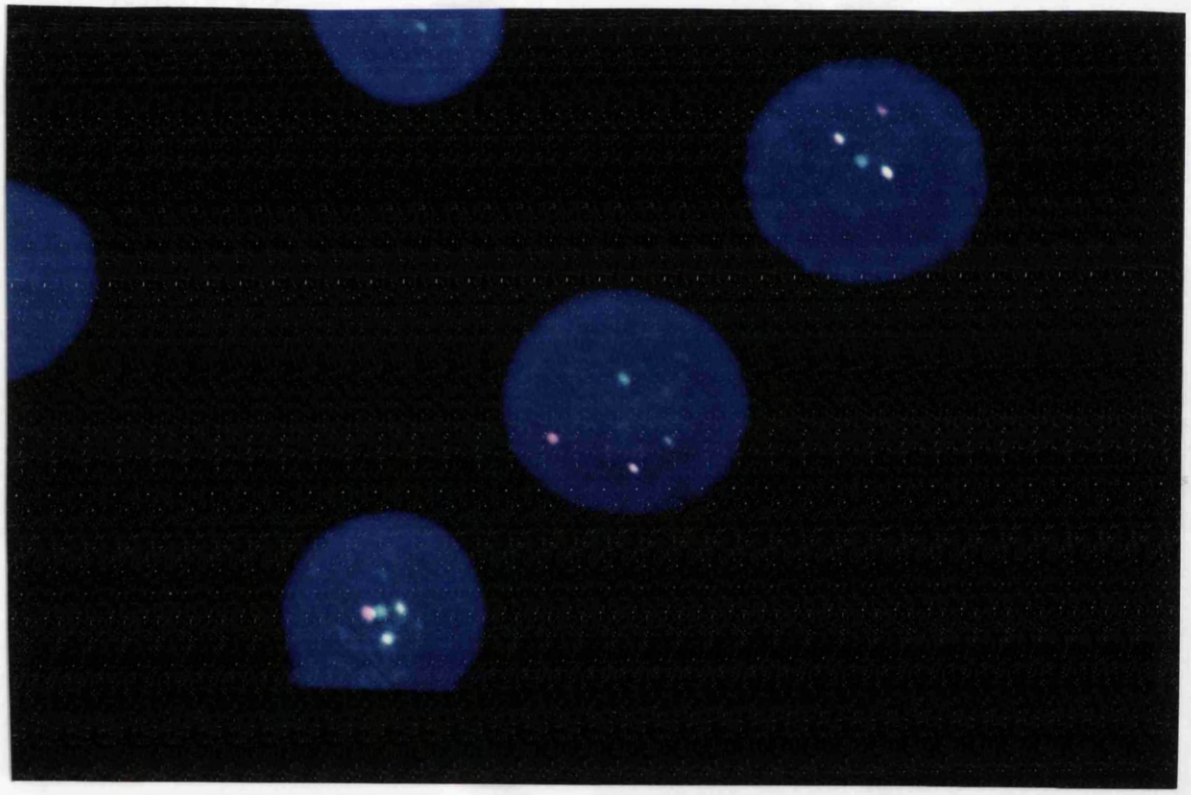

Figure 31. Dual-colour FISH with the t(15;17) cosmid probe mixture on bone marrow interphase from a patient with AML-M3 (Case no 10) showed the double red-green fusion, representative of the translocation between 17 and 15 on chromosome 17 in nuclei. Two individual green, one red-green fusion and one red signals in another interphase nuclei.

b)

CASE 11:

A bone marrow specimen from a female who is 29 years old was obtained. Chromosome analysis revealed 46, XX, i(8)(q10) t(15;17)(q22;q11-12) [2], 46, XX, dic(15) t(15;17;8), der (17) t(15;17)(q22;q11-12) [8] **(Figure 32).**

SPECIMEN) A diagnostic marrow specimen taken on 10 / 3 / 92 revealed 70 % of nuclei from direct preparation to have the fusion of red-green signals. 24 % of nuclei showed normal localisation of signals **(Table 13).**

In this case, despite the large number of nuclei, no metaphase spreads were available to examine the nature of the translocation.

Table 13: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads hybridised with the t(15;17) cosmid probe mixture (Case 11: Bone marrow specimen no: 921078).

INTERPHASE:

SPECIMEN		FISH RESULTS				
Specimen Taken on	CultureType	N	1R1G1F	1R1G2F	Other	Total Cells
1. 10.3.93	Direct	12	35	2	1*	50

*: One nucleus bore 1R+2G+1F.

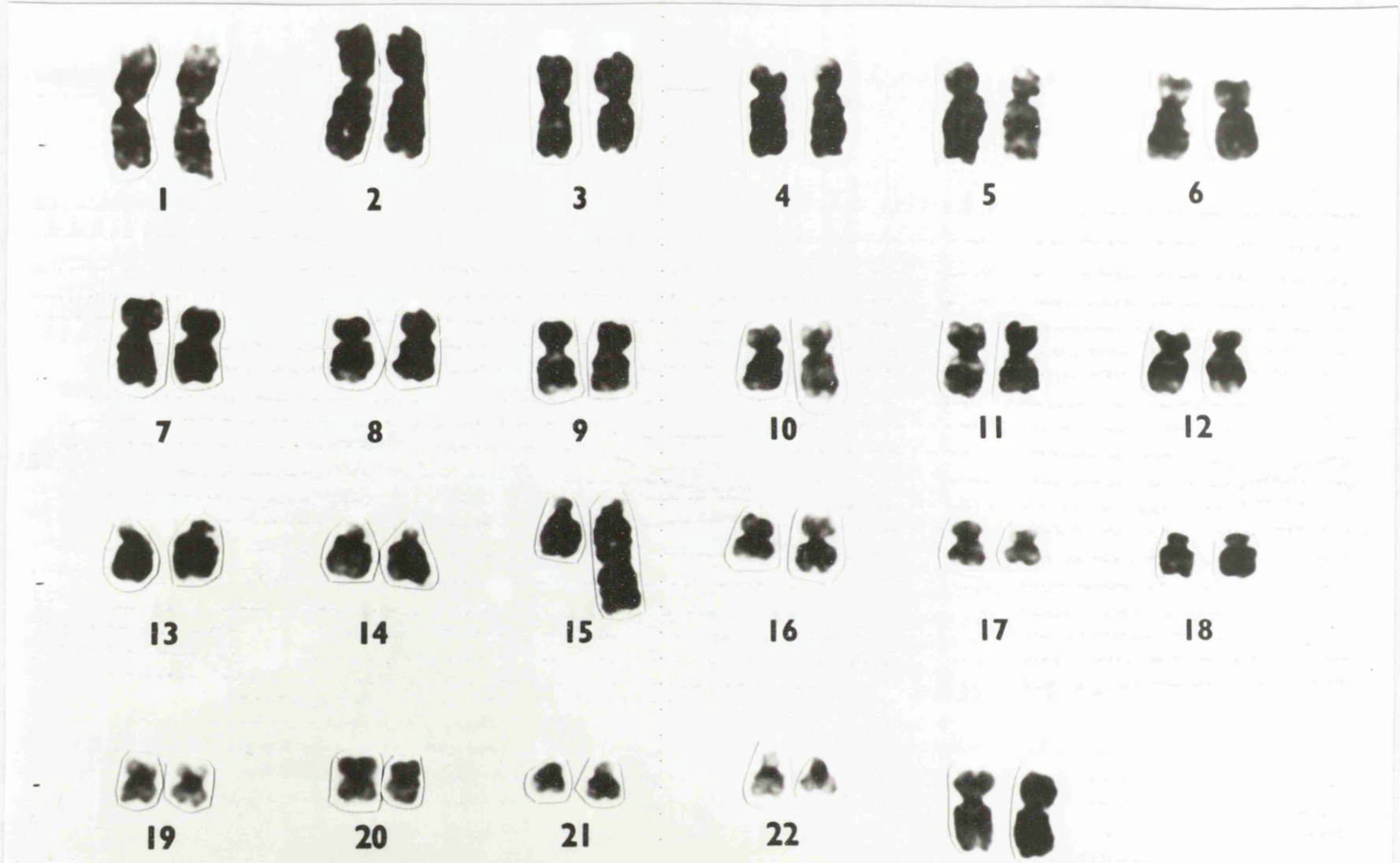

Figure 32. Chromosome analysis with G-banding, which showed 46, XX, dic(15) t(15;17;8), der(17) t(15;17)(q22;q11-12) in eight out of ten metaphases.

CASE 12:

A marrow specimen from an 18 year old female with AML-M3 was obtained. Chromosome analysis revealed a 46, XX karyotype from a direct preparation (analysis of one cell only) and 46, XX, t(15;17) [19] karyotype form a 24 culture.

SPECIMEN A diagnostic marrow sample taken on 5 / 10 / 93 revealed 86 % of nuclei from direct preparation and 82 % of nuclei from 24 hour culture preparation to have the fusion of red-green signals. The remaining 10 % of nuclei from direct preparation and 14 % of nuclei from 24 hours culture showed a normal localisation of signals **(Table 14).**

In metaphase study using dual-colour FISH, only two metaphase spreads were obtained and both showed red-green fusion indicating the t(15;17) on the long arm of chromosome 17.

Table 14: Distribution of dual-colour hybridisation signals in interphase nuclei and metaphase spreads hybridised with the t(15;17) cosmid probe mixture (Case 12: Marrow specimen 1 (934444)).

INTERPHASE:

SPECIMEN			FISH RESULTS			
Specimen Taken on	CultureType	N	1R1G1F	1R1G2F	Other	Total Cells
1. 5.10.93	Direct	5	43	1	1*	50
5.10.93	24 hour	7	41	2		50

*: One nucleus has 1R+2G+1F.

3.4.3. Application of uni-colour FISH in cases of Chronic Lymphocytic Leukaemia

In this section of the study, uni-colour FISH with an α-satellite repetitive centromeric probe (D12Z1) for chromosome 12 was applied to interphase nuclei and metaphase spreads obtained from cases of CLL.

26 patients with CLL (7 females and 19 males, aged between 61 to 83) and 26 normal control cases were evaluated for chromosome 12 copy number with the α-satellite repetitive probe (D12Z1). The first fifteen of control group (7 female and 8 male) were age matched with the patient group (more than 50 years of age). 100 nuclei were scored from each sample. The results of the control group are shown in **Table 15 A** and the patient data is given in **Table 15 B.**

Two methods of sample preparation were investigated. In cases 6-26 whole blood preparations were used, while in cases 1-5 the mononuclear cells were separated from the remainder of the sample and used to produce preparation. The latter method showed essentially similar results although it was found to give better quality slides and less background hybridisation.

Analysis of freshly obtained peripheral blood specimens from 26 control cases demonstrated 0, 1 and 2 hybridisation signals in 3 % (in two cases), 6 % (in two cases) and 98 % of nuclei, respectively. Three signals were visualised in 4 % of nuclei in one case. The remaining cases showed three hybridisation signals in less than 4 % of nuclei **(Table 15 A).** None of the control cases showed different sized signals in the nuclei.

15 metaphases were analysed from each control case. Neither trisomy 12 nor different size of chromosome 12 centromeric signals were observed using FISH with D12Z1.

In the CLL group, uni-colour fluorescence *in situ* hybridisation was performed on specimens from 26 patients. Analysis of the distribution of signals in nuclei prepared from patients with CLL showed that 16 individuals had values within the control range. (Patients 2, 4, 7, 9, 10, 12, 13, 14, 17, 18, 19, 21, 23, 24, 25 and 26) **(Table 15 B)**

Case 1 and 8 had respectively 65 % and 68 % of nuclei with three signals. This was interpreted as indicating trisomy 12 **(Figure 33)**. In case 1, this was confirmed by the presence of three cells with trisomy 12 among 50 metaphases scored-a result taken to indicate the presence of a trisomic line in PHA stimulated culture in CLL **(Figure 34) (Table 16).** Case 8 was also believed to be a trisomy 12 although this was not present in mitoses from the PHA stimulated culture. Cases 20 and 22 showed three signals in 10 % and 14 % of nuclei respectively, and both were interpreted as indicating the presence of a proportion of abnormal cells. From a metaphase study of case 6, 18 and 20, three copies of chromosome 12 centromere appeared to be present in one or two cells **(Figure 35) (Table 16)**. From DAPI staining, one of these chromosomes did not have the morphology of chromosome 12. The explanation of this finding is unclear. In all cases this chromosome appeared to be smaller than a C-group chromosome.

In cases 3, 11 and 15 the majority of nuclei (85 %, 95 % and 60 %, respectively) exhibited two signals. However, it was observed that there was a noticeable difference in the size of the two signals. One signal

appeared to be extremely small **(Figure 36).** This was confirmed in a metaphase study **(Figure 37).** A similar result was obtained from a subsequent specimen from two of these patients. Two other cases 5 and 23 showed a size difference between the two signals in a smaller percentage of nuclei (9 % and 5 %, respectively) **(Table 15 B).**

In cases 3, 5, 6, 9, 12 and 17, the analysis was repeated after certain time **(Table 15 B).** In cases 3, 5, 9,12 and 17, results were similar. In case 6, from the first specimen fewer than 100 cells were examined because a large number of nuclei exhibited a shape and size difference.

Table 15 A: Distribution of hybridisation signals with α-satellite chromosome 12 centromeric probe in interphase nuclei from control cases.

INTERPHASE PREPARATION, FISH RESULTS						
CASE NO:	INTERPHASE EXAMINED	NUMBER OF INTERPHASE FISH SIGNALS				
		0	1	2	3	4
1.	100	--	1	98	1	-
2.	100	-	4	96	-	-
3.	100	2	3	93	2	-
4.	100	1	2	97	-	-
5*.	32	-	2	29	1	-
6.	100	2	4	94	-	-
7.	100	3	3	93	1	-
8*.	62	-	2	60	-	-
9.	100	-	5	91	4	-
10.	100	-		98	2	-
11.	100	1	2	97	-	-
12.	100	3	2	94	1	-
13*.	41	-	6	35	-	-

14.	100	-	4	96	-	-
15.	100	-	3	97	-	-
16.	100	1	6	91	2	-
17.	100	-	5	94	1	-
18*.	60	1	3	55	1	-
19.	100	-	4	95	1	-
20.	100	1	3	95	1	-
21.	100	2	2	96	-	-
22.	100	-	1	98	1	-
23.	100	2	2	95	1	-
24.	100	-	2	98	-	-
25.	100	-	3	97	-	-
26.	100	-	3	96	-	1

*: Fewer than 100 cells were examined from these cases because a large number of nuclei exhibited a shape and size difference making interpretation difficult in these nuclei.

Table 15 B: Distribution of hybridisation signals with α-satellite chromosome 12 centromeric probe in interphase nuclei from CLL cases.

CASE		NUMBER OF INTERPHASE FISH SIGNALS					
NO & CELL SOURCE	Date Specimen Received	Total Cell	0	1	2	3	4
1. PB	1.10.93	100	1		34	65	
2. PB	1.10.93	100		2	96	1	1
3. PB	1.10.93	100		7	91*	2	
3. PB	15.4.94	100		1	99*		
4. PB	15.10.94	100		1	98	1	
5. PB	8.10.93	100	2	5	93*		
5. PB	28.1.94	100		3	95*	2	
6**. PB	8.10.93	66	5	15	45	1	
6. PB	17.2.94	100		1	97	2	
7. PB	21.10.93	100		3	97		
8. PB	28.10.94	100	1		30	68	1
9. PB	29.10.93	100		2	98		
9. PB	14.1.94	100		2	97	1	
10. PB	29.10.93	100	1	3	93	3	
11. PB	15.11.93	100	1	2	96*	1	
12. PB	18.11.93	100		3	95	2	
12. PB	18.3.94	100		5	93	2	
13. PB	23.11.93	100		4	95	1	

14. PB	3.12.93	100		7	92	1	
15. PB	3.12.93	100	1	7	88*	4	
16**. PB	10.12.93	70	1	2	66	1	
17. PB	10.1.94	100		2	96	2	
17. PB	18.3.94	100		3	95	2	
17. PB	15.4.94	100		1	99		
18. PB	14.1.94	100	1	2	96	1	
19.PB	10.2.94	100		6	94		
20. PB	24.2.94	100		2	87	10	1
21. PB	11.3.94	100		1	97	2	
22. PB	8.3.94	100			86	14	
23. PB	15.4.94	100		2	98*		
24. PB	22.5.94	100		1	98	1	
25. PB	12.5.94	100		2	96	2	
26.PB	12.5.94	100		5	94	1	

PB: Peripheral Blood

*: certain percentage of nuclei has different size of signals
**: Fewer than 100 cells were examined from these cases because a large number of nuclei exhibited a shape and size difference making interpretation difficult in these nuclei.

Table 16: Distribution of hybridisation signals with α-satellite chromosome 12 centromeric probe on metaphase spreads from the CLL cases.

METAPHASE PREPARATION, FISH RESULTS

CASE NO:	METAPHASE EXAMINED	NORMAL POSITIONING OF 12 CENTROMERE PROBE	TRISOMIC FOR 12 CENTROMERE	DIFFERENT SIZE SIGNALS
1.	50	47	3	-
2.	10	10	-	-
3.	16	-	-	16
4.	7	7	-	-
5.	12	12	-	-
6.	26	25	1•	-
7.	10	10	-	-
8.	3	3	-	-
9.	30	30	-	-
10.	6	6	-	-
11.	20	-	-	20
12.	29	29	-	-
13.	no metaphase	-	-	-
14.	6	6	-	-
15.	7	-	-	7
16.	4	4	-	-
17.	21	21	-	-
18.	18	16	2•	-
19.	20	20	-	-
20.	11	10	1•	-
21.	2	2	-	-
22.	18	18	-	-
23.	6	6	-	-
24.	5	5	-	-
25.	14	14	-	-
26.	9	9	-	-

•: Marker chromosome

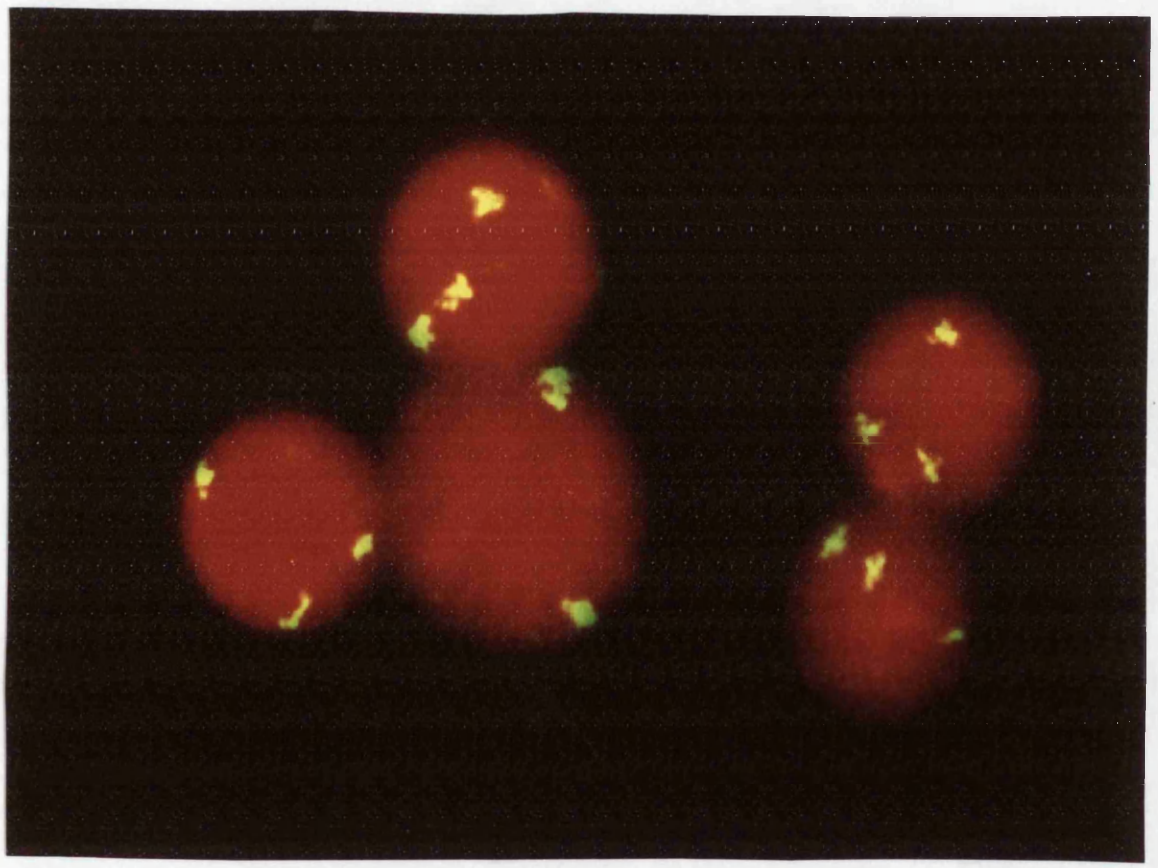

Figure 33. Uni-colour FISH with α-satellite centromere specific probe 12 in interphase nuclei from a patient with CLL (case no 1). Three distinct signals indicate trisomy 12. Case no 1

b)

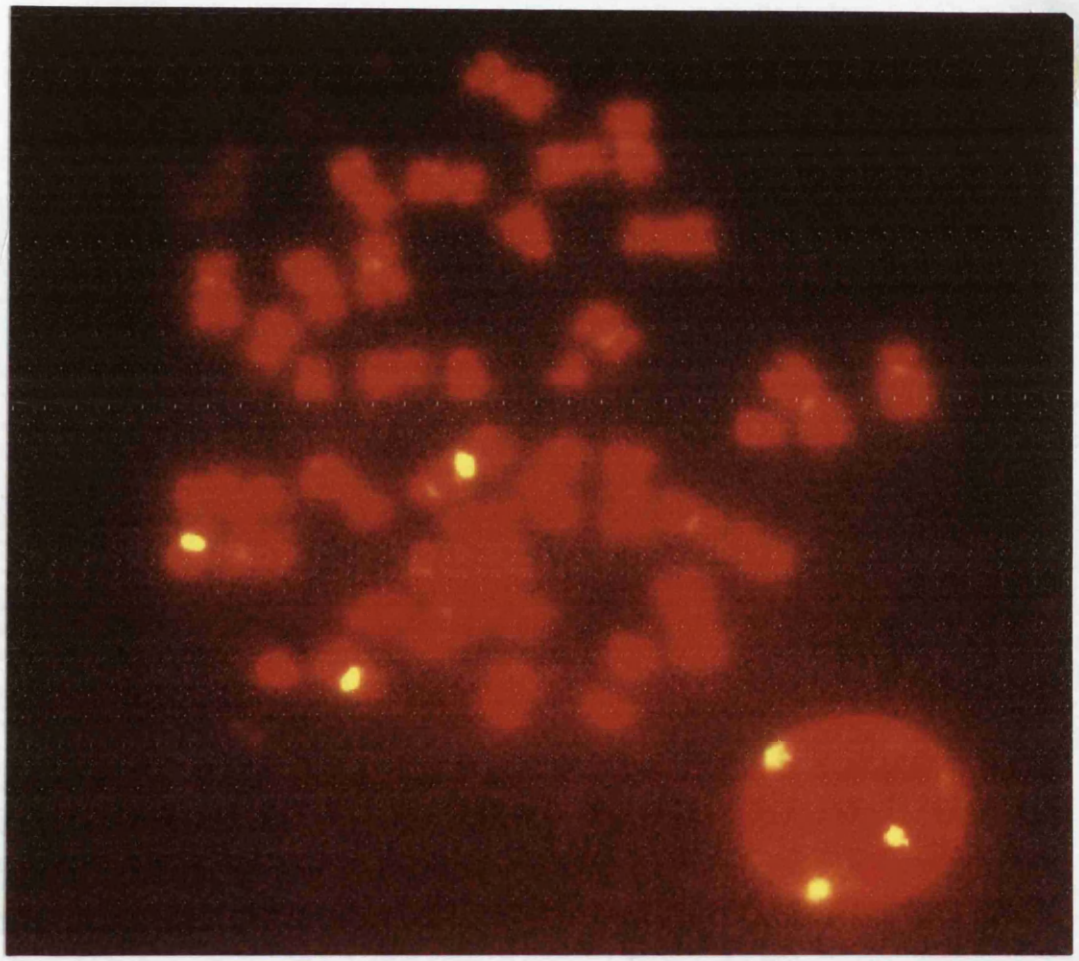

Figure 34. Uni-colour FISH with an α-satellite DNA probe for chromosome 12 showed trisomy 12 on metaphase spread.

b)

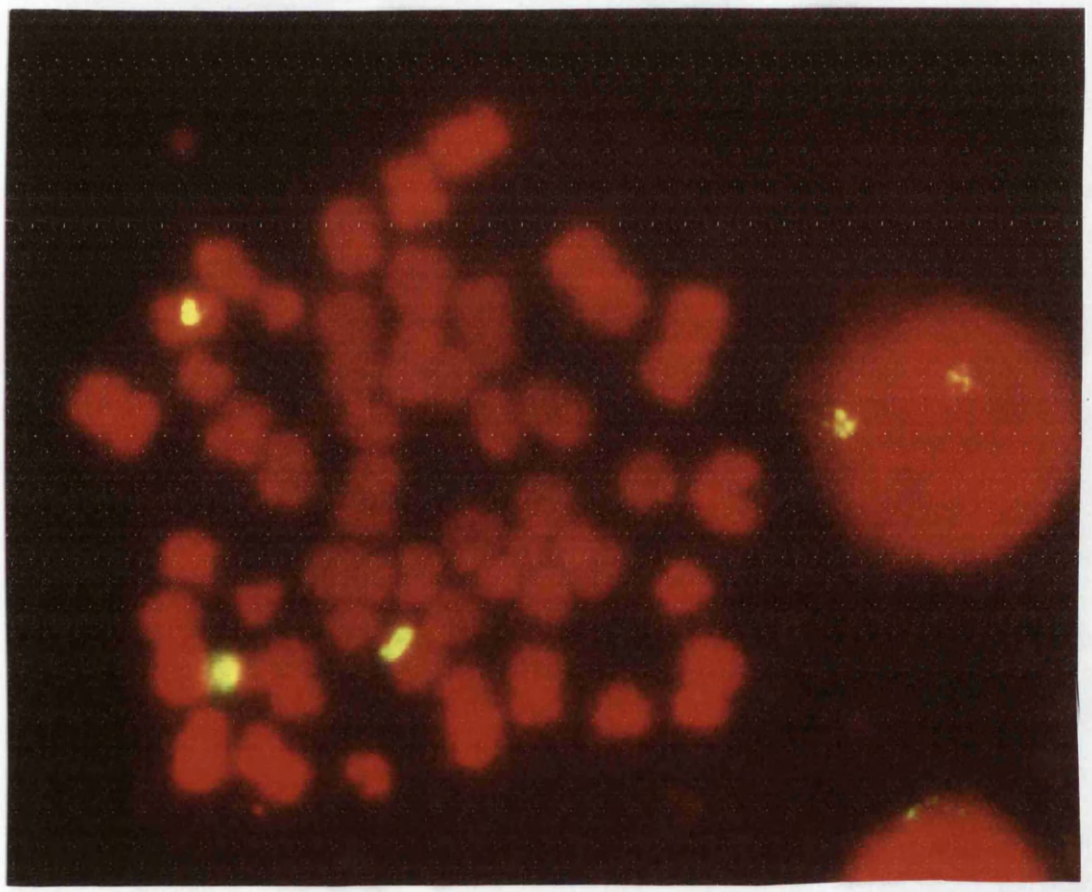

Figure 35. Metaphase spread with labelling of centromers of two chromosomes 12 and the marker using uni-colour FISH with an α-satellite DNA probe for chromosome 12 (PI staining).

b)

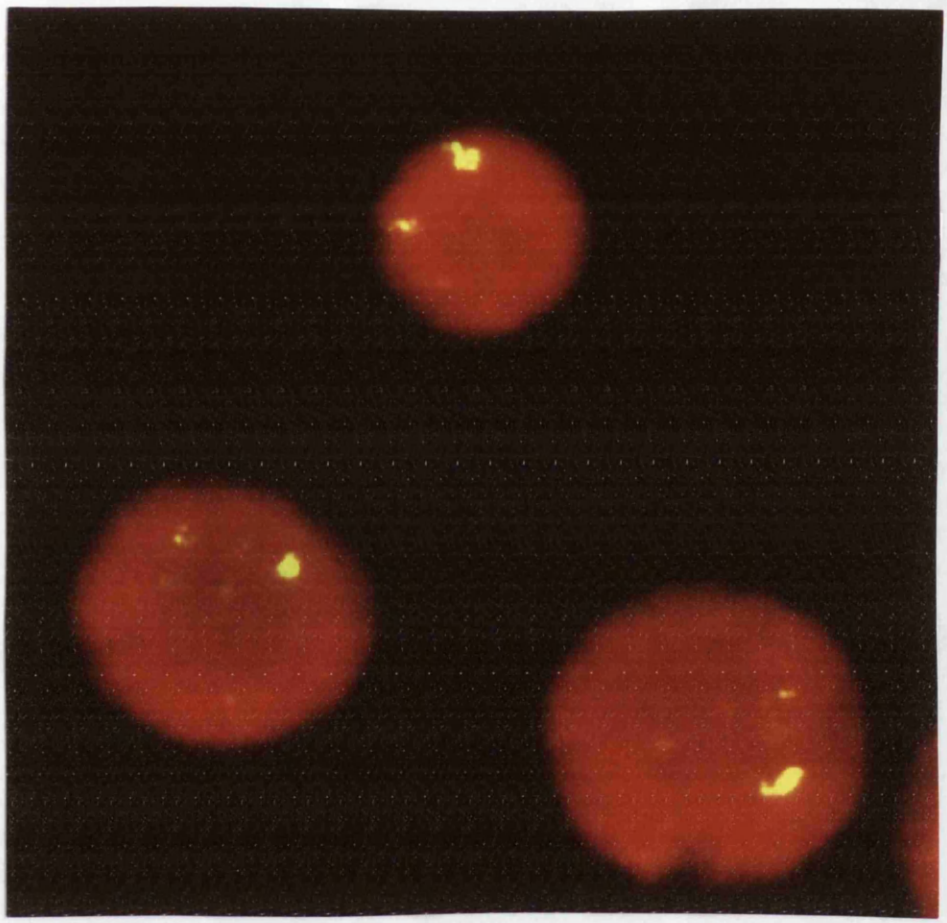

Figure 36. Uni-colour FISH with α-satellite centromere specific probe 12 in interphase nuclei from a patient with CLL. Two distinct signals in nuclei with clear difference in size between the two signals.Case No 3.

b)

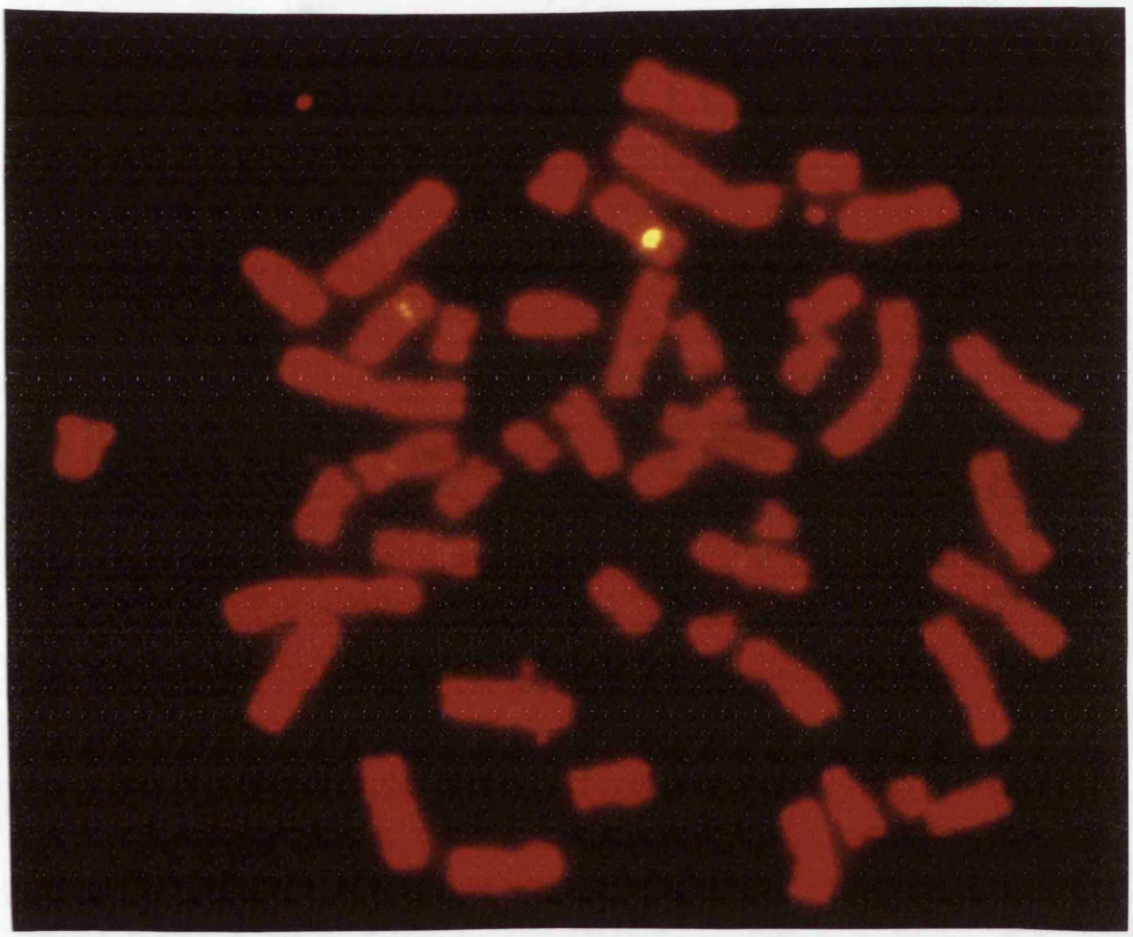

Figure 37. Uni-colour FISH with an α-satellite DNA probe for chromosome 12 showed one signal to be smaller than other signal. Case No 3.

b)

CHAPTER FOUR

DISCUSSION

4. Discussion

4.1. Optimisation of chromosome *in situ* suppression (CISS) hybridisation

Specific delineation of individual chromosomes by CISS hybridisation (chromosome painting) has been very useful for the cytogenetic analysis of diagnostic and research cases alike. Several factors in the CISS hybridisation procedure had to be optimised for whole chromosome specific library probes 21 (pBS-21) and 18 (pBS-18) before being applied to clinical cytogenetics. The initial experiments were carried out according to the methods described by Lichter et al. (1988) and Cremer et al. (1988), but these were unsuccessful. The probes were therefore tested to confirm the presence of the DNA insert of interest. This was accomplished by digesting the plasmid DNA with restriction enzyme (*Hin dIII*). Running the digested plasmid DNA on agarose gel showed a very low yield of the required probe. This could explain the failure of the initial optimisation technique with these probes.

In spite of the low yield of chromosome 21 library probe, the optimisation of probe concentration was extensively focused on controlling other parameters in the CISS hybridisation technique. The probe concentration was not the same as in the protocols described by Pinkel et al. (1988), Lichter et al. (1988) and Goldman et al. (1992). Undigested labelled chromosome 21 library did not give any reasonable coverage of chromosome 21s even with a high concentration (>600 ng per hybridisation area). To improve the coverage of the signals on the target, the probe was digested into 500 to 700 bp with *DNase* . The digested chromosome 21 library probe gave reasonable coverage with 600 ng to 700 ng per hybridisation area (22x22 mm). Unfortunately this library probe also gave very high cross hybridisation with other acrocentric

chromosomes. Similar results were reported by a number of authors (Lichter et al. 1988; Isa, 1991; Rauch et al. 1992). Although other parameters in the CISS procedure such as concentration of competitor DNA, denaturation, hybridisation condition and detection, were subsequently optimised, optimal CISS was not achieved with the probe. As a result, the experiments with the whole chromosome specific library probe (pBS-21) for chromosome 21 were not continued, because for each hybridisation area , more than half of the nick translation labelled product was required in order to get a reasonable coverage.

The second library probe to be optimised was whole chromosome specific library probe for chromosome 18 (pBS-18) and this was optimised under the procedure described for digested whole chromosome specific library probe for chromosome 21. Using 600 ng of whole chromosome specific probe for chromosome 18 in 10-13 μl of hybridisation buffer, the result showed sufficient coverage, but the centromeric area of chromosome 2 constantly lit up brighter than the chromosomes 18. The most likely explanations for cross hybridisation with the centromeric region of chromosome 2 are : (1) the contamination of the stock with chromosome 2 centromeric sequence; (2) the propagation of whole chromosome specific library inserts of the vector might not be pure; (3) there may be sequence homology between chromosome 18 and the centromeric region sequence of chromosome 2. That could explain why a higher concentration of whole chromosome specific library was required to obtain complete coverage. Consequently, this group of experiments was stopped for the same reasons as for the chromosome 21 library probe.

4.2. Optimisation of uni- and dual-colour FISH using chromosome specific centromeric probes and locus specific probes.

Both α-satellite repetitive centromeric probes for chromosomes 8 and 12 and locus specific probes for abl and bcr were successfully isolated from their host cells using commercial CIRCLEPREP KIT. These probes were also successfully labelled with digoxigenin using random prime labelling and with biotin and digoxigenin using nick translation system.

Each probe was individually optimised for uni-colour FISH and then optimised again in different combinations of the probes for dual-colour FISH. Some factors in the procedure were found to be helpful to the optimisation of uni- and dual-colour FISH in clinical applications. However, some of the stages in the procedure did not improve the results, and some parameters were not conducive to fast, easy and reliable results.

The first critical step in the procedure is slide quality. Good chromosome and interphase nucleus morphology are very important. Chromosome preparations from bone marrow cells from patients with leukaemia are generally of a very poor quality and so the hybridisation result quality was not as good as for the other specimens. It is for this reason that the slide quality should be as good as possible. However, pre-treatments of slides with fixative (3:1; Methanol: Acidic acid), ascending alcohol series (70 % to 100 % ethanol) and RNase were not found to improve the results noticeably and thus were omitted. However, the slides should be fresh especially for the locus specific cosmid probes for abl and bcr. In order to get a fresh slide, specimen storage in fixative (3:1; methanol : acetic acid) for a long period was preferable to the prepared slide being kept in different conditions such as under refrigeration (Pinkel et al. 1988) or at room temperature for a long period.

The second factor which plays a very important role in the sensitivity of the FISH technique is the purity and stability of the probe. The sensitivity of FISH technique depends on the purity and stability of the probe. The α-satellite centromeric probes for chromosomes 8 and 12 were found to be very stable and pure, while the α-satellite repetitive probe for chromosome 22 was slightly weaker. In the case of the locus specific probes, the bcr cosmid probes (bcr-19 and bcr-51) were found not to be stable and pure. When these probes were used for dual-colour FISH they were not very successful. The combination of bcr probes increased the hybridisation signal quality, but it was critical that each step was taken very carefully. Otherwise, the application of these probes is not informative in clinical cytogenetics with dual-colour FISH. The abl cosmid probe was pure and stable, and gave a strong signal. It can be used successfully for both uni- and dual-colour FISH. Similar results were obtained with these probes by Grosveld (Leiden University, Holland) and Boavida (Lisboa, Portugal) (Personal communication).

The third critical factor is the concentration of the probe and competitor DNA. Large probes contain repetitive sequences which are shared by all other genomic DNA sequences. Therefore, these repetitive sequences are blocked by unspecific hybridisation to unlabelled competitor DNA. In this project, clearly the high concentration of probe (bcr or abl probes) or low concentration of competitor DNA resulted in an increase of the background-target signal ratio. When simultaneous or individual use of 100 ng abl and 130 ng bcr cosmid probes with 1.1 µl of whole human DNA (10 mg / ml) (or with the same concentration of human Cot-1 DNA) plus 0.7 µl of salmon sperm DNA (20 mg/ml) were used, the best results were achieved. The use of the above concentration of competitor DNA apparently improved the hybridisation signal, but no difference was found

between human whole DNA (human placental DNA) and human Cot-1 DNA for suppression of cross hybridisation. On the other hand, this step was not necessary for chromosome specific centromeric probes, since the probe sequence is specific and consists of short repetitive sequences. Without using competitor DNAs and pre-incubation, the appropriate concentration of centromeric probes consequently gives a specific and sharp signal with both uni- and dual-colour FISH.

The fourth factor is the denaturation of the probe and target DNAs. Denaturation is recommended to be performed separately for uni- and dual-colour FISH, whereas α-satellite repetitive centromeric probes did not need to be denatured separately. Separate denaturation is essential for successful uni- and dual-colour FISH with locus specific abl and bcr probes. Simultaneous denaturation of the probe and target DNAs might lead to a loss of the signal intensity and poor morphology of chromosomes and nuclei, due to the high temperatures used in the procedure. The presence of formamide in the hybridisation buffer and denaturation solution reduces the temperature required for denaturation and hybridisation of the probe and target DNA. The target DNA was denatured in 70 % formamide in 2xSSC at 65-70 °C for $2^{1/2}$ - 3 minutes. This time is critical for leukaemic specimens because of the very poor quality of the targets. An increase in the temperature or denaturation time had an adverse effect on the cell and chromosome morphology. Probe DNA (in the hybridisation mix) was denatured at 65-70 °C for 6-10 minutes, followed by the pre-hybridization of the probe mixture including competitor DNA. After denaturation of the probe mixture, a pre-hybridization is often necessary to prevent background staining because recombinant DNA isolated from eucaryotic DNA often contains genomic repetitive sequences (e.g. the Alu sequence in human DNA). Therefore, for the abl and bcr

cosmid probes using the above concentration and competitor DNA, the optimum time for pre-hybridization was found to be 1 hour. A shorter incubation (less than one hour) resulted in an increase of cross hybridisation. Therefore proper suppression of cross hybridisation requires at least one hour for the cosmid abl and bcr probes.

The next step is the hybridisation. The locus specific abl and bcr cosmid probes required a longer hybridisation time (13-16 hours) to allow proper annealing. In contrast, hybridisation of the α-satellite repetitive sequence probes was performed overnight at between 37 and 42 °C for uni- and dual-colour FISH. This time was not critical and even 8 hours hybridisation was sufficient for re-annealing of centromeric probes. Furthermore, in the literature, shorter hybridisation times (1 hour) for α-satellite centromeric probes has been reported (Bienz et al., 1993).

Another critical step in FISH is detection. For the library probes and the α-satellite repetitive centromeric probes, one layer of amplification of target sequences was found to be sufficient for uni-colour FISH detection. As for simultaneous detection of hybridised probes (α-satellite, chromosome specific library probes and cosmid probes), the targets in nuclei or metaphase spreads can be visualised by only one application of the combination of detection reagents conjugated with FITC, and with Rhodamine. Even good and pure locus specific cosmid probes could be detected with one layer detection. In contrast, when the probes yield a small signal and are not pure and stable, more than one layer amplification is needed. This step is crucial, especially for dual-colour FISH study with cosmid probes (the abl and bcr probes).

Another step is counterstaining. The final concentration of counterstain reagents has a dramatic impact on the signal intensity. In the uni-colour

FISH study, the slides were counterstained with antifade medium containing propidium iodide (PI) and DAPI. The signals were apparent on interphase nuclei and metaphase spreads for both centromeric and locus specific cosmid probes for abl and bcr. In dual colour FISH study, either a higher or normal optimised concentration of propidium iodide suppressed the hybridisation signal whereas centromeric probes tolerated this concentration in the counterstain. That is why, in the dual-colour FISH study, the use of PI solution was not recommended, thus the best dual-colour resolution was obtained with antifade medium containing only DAPI. In addition, higher concentrations of DAPI also had the same effect as PI. So, for uni- colour FISH, the combination of 7 μg/μl propidium iodide and 6 μg /μl DAPI in the antifade medium resolved the optimal dual-colour FISH for the abl and bcr cosmid probes.

In the FISH procedure, the final step is microscopy for interpretation of the results. Initial results were visualised with conventional epi-fluorescence microscopy. In this system, uni-colour FISH results can be visualised easily for especially strong signals, but the dual-colour results (green and red) are routinely visualised on either a separate filter set with more than one exposure, or by one exposure with a dual band pass filter set. With separate filter sets, the difference in optics between the filters may result in a shift in placement of the signals with respect to each other. This poses a problem for applications where precise placement is essential for detailed studies such as gene rearrangement, and cellular organisation. Multiple exposure of colour film cannot adequately display and resolve images from multi labelled small size probes. To overcome these problems, double-band pass filter sets have been developed and allow the simultaneous recording of fluorochromes such as FITC and Rhodamine. Such filter sets may become very important in dual-colour

FISH. However, because a double-band pass filter represents a compromise between optimal wavelengths for red and green signals, small hybridisation signals such as those for bcr were noted to be weaker although double band pass filter gave a very good resolution with strong centromeric and library probes. With digital imaging devices, like the system used in this study, the image quality of hybridised cells is improved and they can then be collected and stored into computer memory or software. In contrast, although digital images are easier to get result and can be subjected to powerful image processing, the registration problem is still present. Therefore, very good computer knowledge is necessary to optimise the system for each type of experiment. The development of this (computerised) system has made FISH a faster and more reliable technique.

4.3. Application of uni- and dual-colour FISH in selected haematological disorders

Uni- and dual-colour FISH techniques using α-satellite repetitive sequence probes for chromosomes 8, 12 and 22 were optimised successfully, but these techniques had to be optimised by refining each step of the FISH procedure for the locus specific cosmid abl and bcr probes. The optimised techniques were applied to cases of leukaemia including CML, AML-M3 and CLL in order to detect the appropriate structural or numerical abnormalities.

4.3.1.1. Application of uni- and dual-colour FISH in Chronic Myeloid Leukaemia (CML)

Rapid detection and reliable assessment of structural and numerical abnormalities are very important in cancer genetics, since certain chromosome abnormalities have been shown to be significant in terms of the diagnosis and prognosis of the disease. The development of the FISH technique has allowed the use of chromosome specific DNA probes to detect chromosome abnormalities and rearrangements of genes on metaphase spreads and interphase nuclei. This technique has enough accuracy to detect the bcr-abl fusion, although hybridisation signals for the bcr and abl cosmid probes are small and nonspecific hybridisation signals might obscure the true signal. In fact, a relatively large hybridisation signal for the bcr-abl fusion is detectable by metaphase- and interphase-dual-colour FISH and can be easily distinguished from a background of nonspecifichybridisation signals.

In this part of the study, it was demonstrated that; (1) the bcr-abl fusion could be detected on metaphase spreads by the uni-colour FISH technique using the cosmid abl and bcr probes and (2) the bcr-abl fusion could be

detected on both metaphase and interphase nuclei by refining the dual-colour FISH technique using the abl and bcr cosmid probes. In addition, dual-colour FISH could have great potential for the detection of complex rearrangement by dual-colour FISH with combinatorial hybridisation (i.e., centromeric probe-library probe, library -library probes or other combinations)

In the literature, very few studies have been published regarding the application of FISH on CML cases. Tkachuck et al. (1990) and Arnoldus et al. (1990) successfully used the bcr and abl probes to detect the bcr-abl fusion in interphase nuclei and metaphase spreads by dual-colour FISH. In addition, Arnoldus et al. (1990) applied the library probes for chromosomes 9 and 22 to detect the Ph chromosome. Zhao et al., 1993 successfully applied FISH using library probes to CML patients in residual proliferating leukaemic cells in complete remission.

4.3.1.2. Application of uni-colour FISH in Chronic Myeloid Leukaemia (CML)

Uni-colour FISH was applied to fourteen CML cases which were cytogenetically Philadelphia positive, and twelve patients with a provisional diagnosis of CML who were cytogenetically Philadelphia negative, using direct and cultured bone marrow and peripheral blood cells in order to detect the bcr-abl fusion.

More than 90 % of CML patients are genetically characterised by the fusion of the bcr-abl genes as a consequence of translocation on chromosome 22 known as Philadelphia chromosome (Zhang et al., 1993). The application of uni-colour FISH revealed the bcr-abl fusion accurately on metaphase spreads and even on very poor quality metaphases. However, in case no 8, the FISH results could not be interpreted due to

high background and unclear signals. In some cases (case 13), however, no metaphase spreads were found. In case no 4, cytogenetic analysis showed the presence of two Philadelphia chromosomes. This was confirmed on metaphase spreads by applying uni-colour FISH. Despite very limited metaphases obtained from some cases (3 and 9), uni-colour FISH revealed the frequency of the leukaemic cells having the bcr-abl fusion. This approach provides potential for determining the frequency of the abnormalities indifferent cell lineages and is fast and sensitive.

The remaining 5% to 10% of CML cases are Philadelphia negative (Heim and Mitelman 1987). Many cases previously thought to be Ph-negative have now been shown by molecular techniques to contain the rearrangement between the bcr and abl genes. Nacheva et al.(1993) and Hagemeijer et al. (1993) reported the molecular heterogeneity in Ph-negative CML cases using molecular techniques and FISH. The accurate detection of this marker is important because patients who are Ph-negative generally show reduced survival rates.

Twelve Ph negative cases with a provisional diagnosis of CML were analysed in total, and in eleven out of these FISH with the abl and bcr cosmid probes confirmed that no fusion between bcr and abl had occurred. Interestingly, one case (no 12) showed a sub-microscopic fusion of the bcr-abl genes on the long arm of one homologue of chromosome 22 (this result was also confirmed with dual-colour FISH on both interphase nuclei and metaphases spreads), whereas the cytogenetic study on this case gave a Philadelphia negative result. This result demonstrates the molecular heterogeneity of Philadelphia negative CML.

Comparison of the results from FISH and conventional cytogenetic analysis in this study suggests that FISH is more sensitive in detecting

sub-microscopic rearrangements. The other advantage of using FISH over conventional cytogenetic techniques is that when insufficient numbers of good-quality metaphases are available for analysis by conventional cytogenetic techniques, an adequate number of metaphases may be analysed by FISH to determine the bcr-abl fusion. Additionally, easy identification of the presence or absence of the bcr-abl fusion by uni-colour FISH allows rapid examination of a large number of metaphases on cytogenetically Philadelphia negative samples. FISH therefore provides a rapid, simple and quantitative way of monitoring patients with Ph-negative CML and can also aid conventional cytogenetic analysis in the identification bcr-abl fusion.

The application of FISH also confirmed additional numerical abnormalities in this group of cases. The most common abnormality, which is trisomy 8, (Heim and Mitelman, 1987) was detected in one case. After applying FISH, the cytogenetic finding of one CML patient (case no 6) was evaluated and the cytogenetic detection of trisomy 9 and trisomy 21 cells was successfully confirmed.

4.3.1.3. Dual-colour FISH on interphase nuclei and metaphase spreads from Chronic Myeloid Leukaemia cases

The simultaneous detection of dual-hybridization has several advantages; including increased efficiency, smaller sample requirement, and potential for analysis of a larger number of chromosomal abnormalities (Reid et al. 1992). Recently, dual-colour FISH using locus specific cosmid probes for the abl and bcr genes has been used for the detection of the bcr-abl fusion in interphase nuclei and metaphase spreads of CML patients. The first application of this technique for detection of the bcr-abl fusion was described by two independent groups (Tkachuk et al. 1990 and

Arnoldus et al. 1990). Later, Nacheva et al (1993) and Chen et al (1993) successfully applied the bcr and abl probes using dual colour FISH to both Philadelphia negative and positive cases. These approaches were based on the simultaneous hybridisation of the abl and bcr probes that localise to the translocation breakpoint. Application of this technique is fast and sensitive, and provides potential for determining the frequency of abnormality in different cell lineages.

In the present part of study, dual-colour FISH using abl and bcr cosmid probes was applied to detect the bcr-abl fusion on both metaphase spreads and interphase nuclei.

On the metaphase spreads of the bcr-abl positive CML cases, the bcr cosmid probe signals were consistently located on the long arm of chromosomes 22 and one of the abl probe signals was located on the long arm of the normal chromosome 9 whereas the other abl signal was located on the abl part of the bcr-abl fusion gene on chromosome 22. These signals could be distinguished very easily in metaphase spreads. In some cases, the fusion appeared greenish yellow in colour. A similar result was reported by Tkachuk et al. (1990). This may be attributed to the following factors: (1) the abl cosmid probe sequence is larger than the bcr probes sequence so the abl signals dominate the bcr signal, (2) the bcr cosmid probes are less stable and strong, as reported by other groups, Grosveld, and Boavida (personal communication), and (3) the bcr probe normally covers the break point cluster region of chromosome 22. If the break point occurs further downstream on the bcr gene, the hybridisation with the bcr cosmid probe could give a weaker signal because the bcr probe is not able to hybridise to enough sequences on the bcr gene on chromosome 22. In some cases (3, 7 and 9-10) metaphases spreads were not obtained. However, despite the small number of metaphases obtained from the

remaining cases, the metaphase results indicate a good correlation with cytogenetic results.

Some false positive fusion signals were found in interphase nuclei at a frequency of 4 % (mean of 3 controls) of the nuclei in the control group, while no false positive was observed on metaphase spreads. Other studies have shown false-positive fusion signals in nuclei at a frequency of about 2 % (Tkachuk et al., 1990) and 4 % (Chen et al. 1993). The difference in these frequencies may be attributed to the different reagents, procedures, and scoring criteria used. False positive results may be due to random co-localisation of the abl and bcr signals. Therefore, less than 4 % red-green fusion signals in the patient group was not considered to be significant.

Tkachuk et al (1990) and Chen et al., (1993) reported that application of dual-colour FISH on interphase nuclei is fast and sensitive, and also provides potential for identifying classic and variant Philadelphia chromosomes. In case 7, although both metaphase FISH study and conventional cytogenetic analysis failed, the interphase FISH study revealed the red-green fusion signals in a significant number of nuclei. In addition, in cases 2 and 9-11 no metaphase for FISH was obtained but interphase dual-colour FISH result showed the red-green signal fusion in correlation with cytogenetic results. In cases 9 and 11 the observed frequency of cells with the fusion signal indicates that some population of cells are normal, indicating a good correlation between cytogenetic analysis and FISH result. In particular, in case 12, FISH showed 82 % of nuclei bearing the red-green signal fusion, whereas G-banding showed this case to have normal 22 and 9 chromosomes. Despite the obvious advantages of the interphase study (e.g. obviating the need for metaphase cell population), there are still problems to be overcome. Although it was possible to score some cells in all cases, in the majority it was not possible

to analyse 50 nuclei. The reasons include the instability of the bcr probe, the quality of the specimen (bone marrow quality is generally very low) and the technical difficulties of dual-colour FISH. Another factor is that, during the analysis of the signal, three exposures were applied to pick up each signal and then to combine them in one nucleus with the digital imaging system, this procedure had to be carried out swiftly before the signals were allowed to fade. Therefore, from some cases a very limited number of nuclei (less than 50) were examined.

Consequently, based on these findings and those of previous studies, this approach can be used to identify the bcr-abl fusion in bone marrow and blood sample from all Ph-negative and -positive CML cases. This approach significantly expands the capabilities of the clinical cytogenetic laboratory. This approach is sensitive. This technique is also faster than other diagnostic technique, because it is performed on interphase nuclei.

4.3.1.4. Variant Philadelphia translocations in CML

Two CML cases with variant translocations were analysed by using dual-colour and uni-colour FISH techniques. The experiments were based on simultaneous hybridisation of two probes (i.e. a combination of abl and bcr cosmid probes, and centromeric and library probes). The quality of the specimens was not good.

The first experiments were carried out using dual-colour FISH with abl and bcr cosmid probes. Based on the interphase dual-colour FISH results, there is no doubt that the bcr and abl probes can be used to identify the bcr-abl fusion, as reported by Arnoldus et al. (1990), Tkachuk et al. (1990) and Chen et al. (1993), by finely optimising each step of the procedure, and using more stable and stronger probes. Chen et al., (1993) also applied these locus specific probes (abl and bcr) on variant translocation, the bcr-

abl fusion event has been detected in cells from CML patients with variant translocation successfully. Of course, this would not be expected to be useful in defining all of the derivative products of the variant translocation, even though the application of these probes (abl and bcr cosmid probes) has the obvious advantage in delineating complex rearrangement in metaphase cells by showing abl and bcr signal localisations, and in analysing the bcr-abl fusion on interphase nuclei when there is a the lack of metaphase spreads.

Therefore, the combinations of centromeric and library probes and/or library-library probe combination were applied on metaphase spreads in order to define the nature of variant translocation. In spite of the non-informative nature of the experiment with 22 library probe, where there was a very high level of cross-hybridisation with other chromosomes, translocations involving 6, 8, 9 and 22 were successfully demonstrated by dual-colour FISH. In the second case, the nature of the translocations [t(3;22); t(9;17;22) occurring in combination] was successfully revealed by using dual- and uni-colour FISH with simultaneous hybridisation of the probes. In particular, this demonstrated the presence of material from 22q on the short arm of chromosome 17, which was not certain from cytogenetic analysis.

Comparison of the results from conventional cytogenetic analysis and FISH in the present study suggest that FISH is a more sensitive method in detecting the variant translocations in CML, because when insufficient number of good- quality metaphases are available for analysis by conventional cytogenetic methods, adequate number of metaphases may be analysed by FISH to identify of variant translocation. In addition to this advantage, easy identification of variant and classic Phs cells by FISH allows rapid monitoring of a large number of metaphases. However,

another advantage of FISH over conventional cytogenetic analysis is that FISH does not need cytogenetic qualification.

4.3.2. Application of dual-colour FISH in cases of Acute Promyelocytic Leukaemia (AML-M3)

Acute Promyelocytic Leukaemia (AML-M3 and -M3v) has a unique balanced reciprocal translocation between chromosomes 15 and 17.

In the literature, a few studies have been so far published regarding the application of FISH on AML-M3 and -M3v cases (Glasser et al., 1994 and Borrow J., et al 1994). Borrow et al (1993) used whole chromosome specific library probes for chromosomes 15 and 17, and also cosmid probes for RARA and PML loci in order to confirm the molecular results. These probes (RARA and PML) were also applied to the patients who had a leukaemic relapse after therapy (Glasser et al., 1994).

Cytogenetic analysis of specimens taken at different stages of AML-M3 can be shown to have a translocation between chromosomes 15 and 17. In general as treatment is administered, the incidence of the translocation decreases until eventually a normal karyotype is observed. This translocation is more likely to be picked up cytogenetically in cultured preparations rather than direct preparations.

In an attempt to determine the presence of the translocation between chromosomes 15 and 17 in cases of AML-M3 and -M3v at diagnosis or at follow up, chromosome specific cosmid probes were applied. In this study, application of dual-colour FISH on interphase nuclei confirmed the translocation to be present in cytogenetically positive cases. In addition, some remission (clinically), pre-allograft and post-allograft specimens where the translocation had not been detected cytogenetically, exhibited some nuclei with evidence of the translocation between chromosome 15 and 17.

In one case with a normal karyotype (case 8), a level of fusion signals (26 %) (from 24 hours culture specimen) was found although a direct preparation from a subsequent specimen (direct preparation) showed a level of red-green fusion of signals within the control range. This might suggest that the t(15;17) was present as a minor clone at diagnosis which was not detected cytogenetically.

An AML-M3v case (case 10) was shown to have a variant translocation of t(15;17) with the karyotype 46, XX, t(15;17) [1], 46, XX der(15) t(15;17)(q22;q11-12), ider (17) t(15;17)(q22;q11-12) [5], 46, XX [11]. Application of dual colour FISH on interphase nuclei showed double red-green fusion of signals in a significant proportion. From the karyotype it would be expected that double fusion signals would be seen on both arms of the iso derivative chromosome 17. However, the position of these signals was not always as close together as would be expected if they were located on the same chromosome.

An identification of the t(15;17) was not always made by cytogenetics on direct preparations, since the translocation is more usually demonstrated cytogenetically in cultured cells. In these cases, interphase analysis with FISH confirmed that the translocation was present. In particular, from direct culture, where no metaphase were found for cytogenetic analysis, the interphase FISH results showed the fusion of red-green signals. Identification of the presence of the translocation in these cases illustrates an important advantage of interphase analysis over conventional chromosome analysis; i.e., the interphase method is not dependent on the proliferative activity or the presence of metaphase spreads. The detection of the rearrangement in remission or pre-allograft specimens which are considered to be cytogenetically normal by cytogenetic studies reveals a second advantage; i.e. the application of dual-colour FISH may be able to

detect a low frequency of the abnormality on interphase nuclei where the number of fusion signals is higher than that found in the control cases.

Interphase analysis indicated that in some cases (cases 6, 7 and 8) a small percentage 10 to 12 % of nuclei exhibited the red-green fusion. In the control group, the incidence of the red-green fusion on interphase nuclei was found to be less than or equal to 8 % of nuclei. From this study of a limited number of control cases, the finding of red-green fusion in more than 8 % of cells in specimens is considered to be significant in indicating the presence of translocation between chromosomes 15 and 17.

The sensitivity of interphase analysis in the detection of the target chromosomal abnormality in cases 6, 7, and 8 raised some questions. In some cases it is not readily apparent whether a relatively low level of fusion signals is indicative of the presence of the t(15;17) at a low level or simply accords with the level of fusion within the control group range. As many cells as possible should be analysed both from interphase and metaphase preparations. To determine at what level the number of fusion signals becomes significant a much larger control group is needed. For AML-M3 cases with a borderline result, PCR could be used to detect whether or not the re-arrangement was present.

As a conclusion and recommendation, dual-colour FISH appears to have a potential in the detection of re-arrangements in interphase nuclei, the detection of sub-microscopic abnormalities in interphase and metaphase cells, and aiding in the definition of complex rearrangements on metaphase spreads. By using other disease-locus specific probes, dual-colour (multi-colour) FISH can aid the identification of abnormalities associated with other malignancies in metaphase spreads and interphase cells. It is recommended that chromosome analysis should be done

concurrently to help clarify any uninformative or unexpected results including variant translocations which might appear on FISH analysis.

4.3.3. Application of uni-colour FISH in cases of Chronic Lymphocytic Leukaemia

Trisomy 12 has been reported to be the most common cytogenetic abnormality in patients with chronic lymphocytic leukaemia. Among patients with chronic lymphocytic leukaemia, trisomy 12 is found primarily in those with excess B-lymphocytes by using the combined cytogenetic analysis and immunotyping (Knuutila et al. 1986) and the combined FISH and fluorescence activated cell sorter (FACS)-separated technique (Escudier et al. 1993).

CLL patients are generally middle aged or elderly. The percentage of trisomy 12 cells does not appear to correlate with age, but the majority (57.7 %) of patients were men. Catovsky et al. (1989) reported that the incidence of male patients with CLL was almost twice that of women.

The results of the present study demonstrated that uni-colour FISH with an α-satellite repetitive DNA probe for chromosome 12 is a valuable supplement to conventional cytogenetics for numerical and centromeric aberrations (in term of signal intensity). The use of FISH allowed the detection of as many cases of trisomy 12 as possible in a shorter time than conventional cytogenetic analysis. Of course, other abnormalities such as duplications, deletions and translocations can be detected by using new specific probe products.

The sensitivity of detecting trisomy 12 was increased over that of conventional cytogenetic techniques because, in this study and previous studies, the application of FISH on interphase nuclei showed a more accurate percentage of trisomy 12 in cases of CLL in which conventional cytogenetic analysis was unable to detect the abnormality without the aid of B-cell mitogens.

Previous studies reported different incidence of trisomy 12 in CLL: 18 %, 20 %, 30 %, 35 % , 54 % (Dohner et al. 1993; Cuneo et al. 1992; Anastasia et al. 1992; Escudier et al. 1993 and Beinz et al. 1993, respectively). However, Raghoebier et al. (1992), Que et al. (1993) and Losada et al. (1991) reported lower incidence of trisomy 12 in CLL cases with 11 %, 11.5 % and 13.3 %, respectively. In this study, a total of 26 patients with CLL were studied for the incidence of trisomy 12. Only 4 of the 26 cases (15.3 %) exhibited trisomy 12. This may reflect different methods of patient selection and inclusion criteria.

In two of the four patients with trisomy 12, the percentage of interphase nuclei with three signals was about 65 %. Similar frequencies of interphase nuclei with three hybridisation signal (24-72 %) in patients with trisomy 12 were reported in the study by Anastasia et al. (1992). The other two patients (cases 20 and 22) with trisomy 12 were found to have 10 to 14 % of interphase nuclei with three signals. Similar results were reported by Escudier et al. (1993). This frequency might indicate the presence of mosaic trisomy 12 (Raghoebier et al. 1992).

In the control group, the highest percentage of trisomic cells was found to be 3 % of nuclei as a false positive (in one case), thus in the CLL group the finding of three signals in more than 3 % of cells was considered significant. However the proportion of the control cells with two signals was 93 to 98 %, but never 100 %. The reason might be attributed to the poor penetration of the probe, DNA damage, or incomplete denaturation of target.

Uni-colour FISH was also performed on metaphase spreads obtained from cultured cells from each case. In only one case (no 1), by analysing 50 metaphases, trisomy 12 was seen in 6 % of metaphases while 67 % of the

interphase nuclei reflected trisomy 12 by exhibiting three hybridisation signals. In the other CLL cases with trisomy 12 (cases 8, 20 and 22), FISH was not able to demonstrate trisomy 12 in the metaphase spreads. The cultures were stimulated with Phytohemagglutinin which stimulates mostly T-cells, only 5-6 % B-cells are stimulated (Knuutila et al, 1986). Losaada et al. (1991) and Cuneo et al. (1992) reported similar results that patients with B-cell CLL with a normal karyotype were found to have trisomy 12 by interphase FISH analysis.

In CLL, a correct interpretation of the pathogenic implications of a chromosome abnormality in cultured cells stimulated with a mitogen or uncultured cells is very important. In cases of trisomy 12 identified with FISH which had a normal karyotype or had insufficient metaphase spreads, dividing normal T cells may have led to a misleading result (Knuutila et al. 1986; Gahrton et al. 1987). As a consequence, in this study the result demonstrated that trisomic abnormalities originated in the B cells and not in the T cells.

An interesting finding in this study is that in cases 3, 5, 11 and 15, FISH results on both interphase nuclei and metaphase spreads exhibited one of the hybridisation signals to be smaller than the other. This phenomenon was reported by Rodriguez et al. (1992) in human male germ cell tumours, but the smaller size of hybridisation signal was due to an iso-chromosome 12. In fact, in this study no metaphases from any of the cases showed metacentric chromosomes indicating iso-chromosomes. However, this type of FISH result has not so far been reported in CLL cases. One assumption may be that the nucleus contains two normal chromosome 12s with one being deeper inside and, due to insufficient probe penetration, produces a weaker signal. The second assumption may be that there is a centromeric abnormality of chromosome 12, and thus

one signal appears smaller than the other signal in the same nucleus. Metaphase FISH result showed the same abnormality as interphase FISH, except for case no 5. Thus the first assumption is most likely for case no 5. Sized difference signals may be due to deletion or alteration of α-satellite repetitive sequences in centromeric region, one of the homologue chromosome 12.

FISH is also useful in the detection of marker chromosome origin (Rauch et al. 1992 and Liehr et al. 1992). The application of FISH on metaphase spreads showed aberrant results in a small number of cells. Cases 6, 18 and 20, in addition to two normal signals on both homologues of chromosome 12, had one extra signal appearing on the centromeric region of a small metacentric chromosome which otherwise remains unidentified. In most of these cells this chromosome appeared as a super numerical marker chromosome which appeared to be derived in part from chromosome 12.

As demonstrated in this part of the study, the FISH technique with an α-satellite repetitive centromeric probe for chromosome 12 constitutes a rapid and precise method for the detection of trisomy 12.

FISH is thus a very useful additional cytogenetic tool but will not replace conventional analysis since information can only be obtained in relation to the specific probe used.

4.4. Recommendations for future research

Dual-colour FISH has been shown both in this study and by other authors to be a reliable, rapid and relatively simple technique for detecting microscopic and submicroscopic abnormalities in interphase nuclei and metaphase spreads.

A number of recent developments have led to improvements in FISH technology. These include (1) new techniques for the construction of probes, such as microdissection and YAC cloning, (2) development of multi-colour FISH and (3) the development of image enhancement systems.

Recently, the development of new techniques for the construction of probes has overcome major limitation of diagnostic and experimental techniques. In addition, multi-colour FISH (more than two colour) using different labelling and detection systems is now available. This technique is based on hybridisation of probe sets each labelled with different reporter molecule ratios. Technically, the development of scanning systems has also improved the resolution of hybridization signals.

With the combination of these improvements, the use of the FISH technique promises to be of value in the detection of genetic alterations of importance in the diagnosis of cancer and solid tumours, because cytogenetic analysis of human tumours is often technically difficult due to the presence of multiple abnormal cell lines and complexity of the chromosomal pattern. Finally, the technique will become a powerful research and routine diagnostic tool with the capability to detect genetic alterations in several areas.

CHAPTER FIVE

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