CHROMOSOME PAINTING USING MICRODISSECTION TECHNIQUES

EUNICE-GEORGIA G. STEFANOU

Thesis submitted to
The Faculty of Medicine, University of Glasgow
For the degree of
Doctor of Philosophy (Ph.D)

The Duncan Guthrie Institute of Medical Genetics
University of Glasgow

© August, 1997

ProQuest Number: 13832093

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 13832093

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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.

EUNICE-GEORGIA G. STEFANOU

To my parents

George and Vivian

and my brother

Demetrios-Constantinos

Σε κάθε προσπάθειά σου, βάλε αρχή το Θεό
που είναι η αρχή κάθε αγαθού, για να γίνει
κατά Θεόν εκείνο που αποφάσισες να κάνεις.

Αββάς Μάρκος
(Πατέρας της ερήμου, αρχές πέμπτου αιώνος μ.Χ.)

In all your endeavours place God first, He who
is the beginning of all goodness, so that it will
be done by God that which you have decided to
do yourself.

Abba Marcus
(Father of the desert, early fifth century A.D)

ACKNOWLEDGEMENTS

I would like to express my utmost gratitude and appreciation to:

Professor J. M. Connor for offering me the opportunity to undertake this course and for his effortless encouragement throughout my period of study;
Dr. Elizabeth Boyd for her supervision, constant support, guidance and understanding;
Dr. George Lanyon for his guidance and helpful discussions on all molecular aspects of this project;
Dr. Mair Crouch for teaching me the first steps in chromosome microdissection;

Dr. Jeffrey M. Trent and his group, especially Dr. X.-Y. Guan , Colin Cargile and Yuan at the NIH, Laboratory of Cancer Genetics, Bethesda, Maryland, USA, for their outstanding generosity, immense help and wonderful hospitality during my visit in July 1996;

Ms. Debra Lillington at the Department of Medical Oncology, ICRF, St. Bartholomews Hospital, London, for all her help and advice during my visit in July and August 1995; Dr. Andrew Shelling at the Institute of Molecular Medicine, ICRF, John Radcliffe Hospital,Oxford, for all his advice and help with the DOP-PCR;

Mr. Fen Tang Yang at the Department of Pathology, University of Cambridge for kindly providing the isolated FACS chromosome 4 samples.

Dr. Rosemary Akhurst and her group, especially Dr. Sarah Johnson and Dr. Alison Trainer, for allowing the use of their technical facilities;

Ms. Anne Theriault for being such a wonderful friend; Ms. Betty O'Hare for her friendship and technical help and Ms. Ann Ross for requesting my 'urgent' orders which always arrived promptly;

Dr. Mojtaba. Mohaddes, Dr. Saeed Ghaffari, Dr. Mee Hye Kim and Dr. Patricia Ong for their invaluable friendship;

All the staff members of the DGI, especially the cytogenetics group, for their help, support and for making my stay here an everlasting memory;

My dear friends Dr. Angeliki Malliri, Aris and Yiouli Bitzeni, Nikos Georgosopoulos, Achilleas Anagnostopoulos, Alexis Alexiou and Maria Giannakopoulou for being there when needed;

This project would not have been completed without the patience, trust and continuous support of my beloved parents and brother. THANK YOU AND MAY GOD BLESS YOU.

TABLE OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: RESULTS

CHAPTER 4: DISCUSSION

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMCA	:	Amino-methyl coumarin-acetic acid
AML	:	Acute myeloblastic leukaemia
ANLL	:	Acute nonlymphocytic leukaemia
~	:	Approximately
Biotin-11-dUTP	:	Biotinylated deoxyuracil 5'-triphosphate
cen	:	centromere
CGH	:	Comparative Genomic Hybridisation
CISS	:	Chromosomal in situ supression hybridisation
Contig	:	Contiguous sequence
COP	:	Cruachem Oligonucleotide Purification
Cot	:	Concentration over time
DAPI	:	4',6-diamino-2-phenyl-indole
dATP	:	Deoxyadenosine 5'-triphosphate
dCTP	:	Deoxycytidine 5'-triphosphate
dGTP	:	Deoxyguanine 5'-triphosphate
dNTP	:	Deoxynucleotide 5'-triphosphate
dTTP	:	Deoxythymidine 5'-triphosphate
dH_2O	:	Distilled water
Dig	:	Digoxigenin
dms	:	Double Minutes
DNA	:	Deoxyribonucleic acid
DNA pol	:	DNA polymerase
DNP	:	Dinitrophenol
DOP	:	Degenerate Oligonucleotide Primer
e.g.	:	exempla gratia (for example)
et al.	:	et alia
FACS	:	Fluorescent Activated Cell Sorting
fg or fm	:	femtogram
FISH	:	Fluorescence In Situ Hybridisation

FITC	:	Fluorescein isothiocyanate
G-banding	:	Giemsa banding
HSRs	:	Homogeneously stained regions
inv	:	inversion
gm (or gr)	:	gram
ISH	:	In Situ Hybridisation
Kb	:	Kilobase
LINEs	:	Long Interspersed Nuclear Elements
Mb	:	Megabase
mg	:	milligram
ml	:	milliliter
µg	:	microgram
µl	:	microliter
ng	:	nanogram
OD	:	Optical density
p	:	short arm of a chromosome
PBS	:	phosphate-buffered Saline
PCR	:	Polymerase Chain Reaction
pg	:	picogram
PHA	:	Phytohaemagglutinin
PRINS	:	Primed in situ nucleic acid synthesis
q	:	long arm of a chromosome
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
SINEs	:	Short Interspersed Nuclear Elements
SDS	:	Sodium dodecyl sulfate
SSC	:	Saline sodium citrate
TE	:	Tris-HCL/EDTA
tel	:	telomere
Tris	:	Tris (hydroxymethyl) aminomethane
Tm	:	Melting temperature
UV	:	Ultra Violet

v/v	:	volume per volume
w/v	:	weight per volume
YAC	:	Yeast Artificial Chromosome

SUMMARY

Chromosome microdissection is a technique that was first described in 1981 by Scalenge et al. Since then, several modifications have been made and it was recently applied to dissect abnormal parts of aberrant chromosomes, the origin of which was unknown, with the ultimate aim of identifying the origin of the dissected chromosomal material (Meltzer et al. 1992). The aim of this project was to develop the technique, so that it could be further used as a molecular cytogenetic tool in the delineation of chromosome aberrations encountered in the diagnostic workload of this department which remain unidentifiable by using standard cytogenetic techniques.

The steps associated with the technique involved the microdissection of several copies of a specific chromosomal region, PCR amplification using a degenerate oligonucleotide primer (DOP primer with six 'N' degenerate bases), labelling of the amplified product with biotin-11-dUTP and finally, reverse chromosome painting by hybridising the labelled product on normal metaphase cells. The location of the hybridisation signal would then reveal the chromosomal origin of the microdissected DNA of interest.

All parameters that could affect the efficiency of the DOP-PCR amplification, such as the primer concentration and annealing temperatures were evaluated by using low concentrations of at first, human genomic DNA and finally FACS isolated copies of chromosome 4 as a positive control. Satisfactory results meant that the values of the parameters tested were kept constant for all further attempts.

Contamination was the main drawback in interpreting the PCR results. The nature of the DOP primer that could amplify literally any type of DNA template, in conjunction with the fact that the starting microdissected material did not exceed in total a few hundred femptograms (fg) (an average size band equals to 15-50fg, $1fg=10^{-9}$ μg) made the amplification extremely sensitive to extraneous contaminations. It was therefore necessary to sequentially introduce multiple specific

precautions (i.e. barrier-pipette tips, autoclaving all solutions, preparing small aliquots of each of the PCR reagents to allow only a single use per experiment and performing all steps involved with the PCR inside a sterile safety cabinet) in order to prevent contamination.

Several approaches to chromosome microdissection were investigated. Successful results were obtained by dissecting the fragment of interest and transferring it into a collection tube by gently touching the tip of the needle inside the collection drop, so as to release the DNA fragment without having to break the tip of the needle inside the tube. For this, it was very important to obtain the correct size of needles. Successful micro-FISH probes were produced by less than ten fragments, proving the efficiency of the technique. Successful results were obtained from microdissections of the chromosome regions 7p21-31, 21q21-22, 16cen, 16cen→q22 as well as double minutes present in an abnormal Acute Myeloid Leukaemia case (AML). Signals produced by FISH were very bright , covering fully the corresponding region. Using competitor DNA totally eliminated cross-reactivity with the other chromosomes of the cells. The successful micro-FISH results indicated that the conditions of all steps involved were appropriate for further generation of micro-FISH probes, providing that contamination was either absent or present at very low levels at the first critical round of amplification.

The micro-FISH probe for 16cen→q22 was used on three ANLL cases to reconfirm the numerical and structural rearrangements of chromosome 16 previously identified by conventional cytogenetic techniques and to search for involvement of the chromosome 16 in other rearrangements unidentifiable by previous cytogentic studies. In one case where multiple abnormalities such as monosomy 16 and marker chromosomes were present in the cell population, the micro-FISH 16cen→q22 probe revealed two extra chromosomes (one chromosome of an E size and another one of a C size group) to contain material fron the 16q region. In another AML case the origin of double minutes (dms) present in the cell culture was successfully deliniated by microdissection of seven dms and using them as a probe for reverse chromosome painting on normal metaphase cells. The hybridisation revealed the dms

to derive from the chromosomal region 8q24 where the c-myc gene (being often amplified in several types of cancer) is localised.

These results suggest that chromosome microdissection should have the potential for introduction into routine clinical practice. The next stage is to use the optimised approach described here in an unselected series of cytogenetic cases to see if its potential as a diagnostic tool can be realised.

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 UNIQUE AND REPETITIVE SEQUENCE DNA

1.1.1 Genes: unique sequence DNA

Genes that encode for enzymes and other proteins are called structural genes. About 50,000-100,000 structural genes (with an average length size of 20-50 kb) have been estimated to encode in the DNA of the human genome. Each structural gene is usually represented only once in the haploid genome and is therefore unique sequence DNA. Approximately four thousand such genes have been identified so far, many of which have been associated with human inherited diseases which result from mutations occuring in the nucleotide sequence of these genes. However, most of the human DNA is made up of introns (noncoding part of a gene) and repetitive sequence DNA.

1.1.2 Repetitive sequence DNA

Repetitive sequence DNA involves multiple copies of DNA base sequences that are identical or nearly identical to each other and are generally found outside of coding regions. They vary in length from a few to thousands of bases and in copy number from a few hundred to several million. Two types exist: the highly repetitive sequences that include all sequence families with more than 10^6 copies in the haploid genome and the moderately repetitive ones with 10^2-10^5 copies present in the haploid genome.

Moderately repetitive DNA consists of a variety of interspersed repetitive sequence families present in varying degree of repetition. These families often are interspersed with longer stretches of non-repetitive DNA. They are found in both euchromatin and heterochromatin and include genes which code for ribosomal RNAs and histones. The members of interspersed repeats can be in the form of a short unit (SINES) less

than 500 basepairs (bp) repeated up to one million times per genome. The majority of SINES in humans belong to a single family called Alu family with a consensus sequence of 300 bp. With approximately 300,000-900,000 copies in the genome and an average distance between copies of ~4kb, they are usually found in intergenic DNA, introns and untranslated 3'regions of genes, thus comprising up to 6 percent of the total DNA. Long interspersed sequences (LINES) are of several kilobases in length with the most frequent the L1 or KpnI sequences being up to 6.4 kb long with a reiteration frequency of 20,000-100,000 copies in the human genome. Sequences belonging to the LINES family show variation among individual members. However, the members of the family within a species are relatively homogeneous compared to the variation shown between species. Several mechanisms have been described for generating SINES and LINES sequences such as DNA transposition events (retroposition), gene dublications and chromosomal rearrangements, with the process of 'retroposition' being the most dominant one. For this event to occur, RNA molecules are copied into DNA with subsequent integration of those copies into new genomic sites. The newly integrated sequence is called a 'retroposon' to show that it has been transposed through an RNA intermediate molecule which can favour retroposition because of its poly(A) rich region. Studies have revealed that LINES are derived from transcripts of RNA polymerase II whereas SINES diverge from transcripts of RNA polymerase III which terminate in an oligo(U)-rich sequence capable of self-priming reverse transcription at an internal oligo(A) region (Deininger et al. 1986).

Highly repetitive DNA is not transcribed into RNA. It consists of tandemly repetitive sequences which are highly conserved and are found in the chromosome centromere or telomere regions as well as on the arms of some chromosomes. Based on the rate of reassociation during denaturation-renaturation experiments, they are classified into two family groups: family (I) which consists mainly of 'satellite' DNA sequences (uninterrupted arrays of tandemly arranged units of 6-12 bp) and family (II) consisting of 'minisatellite' DNA sequences of a much shorter length (e.g. TA and GC repeats and CpG islands). Four major satellites have been described: I, II, III and IV, with satellites II and III (2% and 5% of the human genome, respectively) being

much more abundant than satellite I (0.5% of the genome). Satellite II is present mainly on the heterochromatic regions of the long arm of chromosomes 1 and 16, whereas satellite III is located predominantly on chromosome 9 and the acrocentric chromosomes (groups D and G). Each of the satellite DNAs (I to III) is composed of a mixture of different repeated sequences. Satellite I consists of two repeated units, a 17 bp and a 25 bp unit arranged in alternating arrays throughout the genome. Satellites II and III consist of a 42 bp and 45 bp consensus repeated unit respectively, with a 5 bp repeat (5'-ATTCC-3') present in it (Vogt et al. 1990, Tagaro et al. 1994). The human α satellite DNA family is composed of tandemly arranged monomer units of ~171 bp (Choo et al. 1991). Chromosomes have been differentiated into three major groups of chromosome-specific alphoid repeated families. Alphoid families located on chromosomes belonging to one group are capable of cross hybridising with each other but not to chromosomes from different groups. Thirty alphoid sequence subfamilies have been described according to whether they are specific for a single chromosome or are shared by more than one chromosome. Another class of highly repetitive tandem DNA has been described. Monomer repeat units of ~68 bp compose the β satellite DNA which is located in tandem arrays on chromosome 9 and the acrocentric chromosomes (Waye et al. 1989).

1.2 CYTOGENETICS: THE STUDY OF CHROMOSOME ABERRATIONS

1.2.1 Constitutional and aquired chromosome aberrations

A variety of human diseases have been associated with chromosome modifications where essential genetic information is missing or extra DNA is present. It has been shown over the years that chromosome aberrations contribute significantly to birth defects, fetal loss and genetic disorders. Structural aberrations such as deletions, translocations, inversions, isochromosomes and ring chromosomes may result in mental and physical dissabilities and may even be lethal due to genetic imbalances. Numerical aberrations of the autosomal chromosomes can cause severe congenital abnormalities (e.g. Down's syndrome; 47,+21), whereas sex chromosome

aberrations can severely affect the phenotype of the individual (e.g. Kleinefelter's syndrome; 47,XXY).

Chromosome changes in cancer and leukaemias have revealed important information as to the diagnosis, classification and prognosis of the disease. Specific aberrant chromosomes are consistently present in some specific cancers. Oncogenes found to be located at or near breakpoints, when relocated , may trigger the initiation of a particular cancer. Over 90% of the patients suffering from chronic myelogenous leukaemia (CML) carry a distinct marker, the Philadelphia chromosome (Ph^1), a product of the t(9;22)(q34;q11) translocation, which results in the fusion of the bcr (breakage cluster region) gene together with the abl (Abelson) gene. As a result of that a new transcription unit (mRNA) is formed which is then translated into an abnormal protein. Because of the variable breakpoints on chromosome 22, two different mRNAs may be produced depending on whether one of the small exons of the bcr genes is included in the Philadelphia chromosome. Speculations regarding the involvement of the abnormal fusion protein in the progression of the disease state that it might play a role in the abnormal proliferation of white cell progenitors in the chronic form of the illness. Disruption at the gene level is also the main cause of Burkitt's lymphoma (BL) with the consistent translocation t(8;14)(q24.1;q32.3) which involves the deregulation of the c-myc protooncogene. Numerical aberrations are often encountered in acute non-lymphocytic leukaemia (ANLL) such as trisomy 8, and monosomy 5 which are considered as primary karyotypic changes for the particular group of leukaemias. Primary karyotypic events may be the key event in the cause of the disease and establishing such aberrations can provide us with valuable information as to the possible genes involved and the consequences of these aberrations. Identifying secondary chromosome changes such as isochromosome 1, trisomy 8, and trisomy 19 is critical as to indicating worsening of the disease. Chromosome structural aberrations such as homogeneously staining regions (HSRs) and double minutes (dms), which can result in gene amplification (and consequently in overexpression of their products), have been identified only in malignant cell lines. Finally, marker chromosomes are often encountered in the karyotype of leukaemic patients. These may be very short (of the G group size) and with a very obscure

morphology, thus making their recognition very difficult by standard cytogenetic techniques.

The wide variety of chromosome aberrations reported and their involvement in the pathogenesis of genetic disorders make necessary the development of diagnostic tools that could open the way for detecting obscure aberrations and consequently improving the diagnosis.

1.2.2 Technical improvements in cytogenetics

With the technical improvements over the last few decades, cytogenetics has led to fundamental insights into the study of chromosome aberrations and related disorders. First, Tjio and Levan in 1956 reported the correct number of chromosomes in a human cell as forty six. In 1959 Lejeune and colleagues reported that an extra chromosome 21 was associated with Down's syndrome. Major advances were made in human tissue culture and chromosome preparation methodology in 1950's and 1960's (Hsu, 1952: Hypotonic treatment; Moorhead et al. 1960: Phytoheamaglutinin treatment). During the late 1960's techniques for the conventional staining of mitotic chromosomes were developed allowing the recognition of each human chromosome individually (Casperson et al. 1968). In the early 1970's, new staining methods added an important dimension to the analysis of chromosome morphology. In 1976, the first of several techniques to obtain elongated chromosomes with 500 to 2,000 bands per haploid set was reported by Yunis (Yunis et al. 1976). This led to the discovery of several very small chromosome deletion syndromes (Allanson et al. 1985, Miller-Dicker syndrome del(17)p13.3; Buehler et al. 1984, the Tricho-Rhino Phalangeal syndrome del(8)q24.1; Curry et al. 1982, X-linked chondrodysplasia punctata with ichthyosis del(X)p32-22) as well as the del(15)q13 responsible for the Prader-Willi and Angelman syndromes (Ledbetter et al. 1981). With high resolution banding the exact nature and breakpoints of other chromosome aberrations were defined. In addition, new chromosome abnormalities in leukaemic bone marrow specimens and other human neoplasms were revealed (Hagemeijer et al. 1979; Yunis et al. 1978).

Banding techniques became the central theme of every cytogenetic laboratory. However, in many cases, chromosomal abnormalities could not be detected even with all of the available technology. More specifically, conventional cytogenetic study limits analysis to the few cells arrested in metaphase and excludes from analysis the vast majority of cells remaining in interphase. Moreover, conventional cytogenetic analysis is entirely dependent on the production of high-quality metaphase preparations which are not always available, particularly in leukaemia and tumour cytogenetics. It is significantly restricted for cells that cannot easily be grown in culture and finally, it is limited in that it is labour intensive, requires highly trained technologists for interpretation and is tremendously time consuming. Therefore, it became necessary to develop new strategies in order to overcome the above limitations.

1.3 MOLECULAR CYTOGENETICS: A new era in diagnosis and research

Combining molecular biology techniques with cytogenetics has led to the establishment of molecular cytogenetics as the field for analysing the human karyotype by localising nucleic acid sequences on the human genome. The concepts involved are based on two principles (Marmur et al. 1960):

1. The DNA double strands are held together by hydrogen bonds which are weaker than the covalent sugar-phosphate bonds.
Hence, when using high temperature or certain chemicals (formamide and alkali) the complementary strands separate leaving the sugar-phosphate backbone intact.

2. The two strands can reanneal to each other once the conditions return to normal (i.e. with the temperature falling below the melting temperature).
Reannealing of two DNA molecules depends on collision of the complementary strands and follows second order kinetics. The rate of the reaction is governed by the equation:

$$\frac{dC}{dt} = -kC^2 \qquad (1)$$

where C is the concentration of single stranded DNA at time t and k is a reassociation rate constant. By intergrating this equation between the limits of the initial DNA concentration C_0 at time t= 0 and the concentration C of the DNA that remains single stranded after time t, the progress of the reaction can be described as

$$\frac{C}{C_0} = \frac{1}{1 + k \cdot C_0 t} \quad (2).$$

C_0t is considered as the parameter controlling the reassociation reaction and under conditions where the reaction is half completed at time t , the parameter is described as :

$$C_0 t_{\frac{1}{2}} = \frac{1}{k}$$

The $C_0t_{\frac{1}{2}}$ is directly related to the amount of DNA in the genome and therefore the complexity of the genomic DNA can be assessed by the kinetics with which denatured DNA reassociates.

Hence, the annealing of a defined molecular probe to complementary target DNA sequences which are usually fixed to a microscope slide in metaphase or interphase conformation (*in situ*) can be performed. This molecular cytogenetic approach is called *in situ* hybridisation and can be used to form DNA-DNA, DNA-RNA and RNA-RNA hybrids with the purpose of localising and identifying specific nucleic acid sequences.

Direct visualisation of the chromosomal localisation of a specific probe was first reported by Gall and Pardue, and by John et al. in 1969. They used radioactively labelled ribosomal RNA as a probe to detect amplified ribosomal genes in oocytes of the toad Xenopus. In this study the RNA probe was labelled with tritium (^3H) and the hybrids were detected with autoradiography. This resulted in silver grains covering specific chromosomal regions that contained the target DNA sequences. Routine isotopic *in situ* hybridisation protocols were established in many laboratories during the 1970's . Human DNA sequences of few hundred base pairs in length could be detected by autoradiography. Single copy genes such as the insulin gene and, β-

globin genes were localised on metaphase chromosomes (Harper and Saunders 1981; Malcolm et al. 1981).

1.3.1 FISH: Developments and applications

Although autoradiography is a sensitive means of visualisation, it has several drawbacks which limit its use in the laboratory such as difficulty and expense of use and disposal of radioactively labelled probes, long exposure time, limited shelf life and decreased banding resolution.

The above drawbacks made the development of non-isotopic *in situ* hybridisation using non-radioactive hapten labels necessary. With the use of fluorochromes (fluorescent molecules) conjugated to the labelled probes, fluorescent *in situ* hybridisation (FISH) in biological studies was first introduced in the late '70s. Indirect immunofluorescence detection of RNA probes was demonstrated by Rudkin et al. (1977) followed by Bauman et al. (1980). Its advantages over hybridisation with isotopically labelled probes have established FISH as a powerful diagnostic and research tool in cytogenetics, prenatal and postnatal diagnosis and tumour biology. (i) Non-isotopic detection methods are considerably faster than autoradiographic procedures. (ii) Non-isotopic probes are chemically stable and are not subject to special disposal requirements. (iii) The combination of different coloured probe-labelling schemes can make possible the simultaneous detection of two or more target DNA sequences. (iv) Hybridisation signals can be more precisely localised than isotopic signals, thus allowing an analysis at much higher spatial resolution. (v) Improved fluorescent microscopy with digital imaging systems can enhance the sensitivity. (vi) A variety of amplification procedures can greatly enhance the signal detection. (vii) Hybridisation of repetitive sequences commonly found throughout the genome can be suppressed by prehybridisation of probes with unlabelled genomic DNA.

Numerical and structural aberrations involving greater than approximately 5 Mb of DNA can be detected by karyotyping. However, fluorescence detection of the probes provides the highest resolution possible at the light microscopy level. Since nick

translated labelled probes containing a biotinylated nucleotide analogue were first described in the early '80s (Langer et al. 1981; Langer-Safer 1982), several laboratories made refinements so that sequences as small as 5 kb could be seen (Bhatt et al. 1988; Lawerence et al. 1988). Further developments led to the detection of sequences as small as 1kb on banded chromosomes (Fan et al. 1990) and resolution of distances within a gene such as the human dystrophin gene was achieved (Lawrence et al. 1990). More recent studies have dramatically increased the resolution within the DNA chromatin by a technical development known as fibre FISH (Raap et al. 1996). FISH is applied onto fibre DNA that has previously been released from DNA proteins and has been stretched on glass slides to produce maximum chromatin resolution.

Recombinant DNA technology has made possible the use of a variety of probes according to the DNA region of interest. Chromosome library probes are used for chromosome painting when structural and numerical abnormalities must be investigated both in prenatal diagnosis and cancer cytogenetics (Smit et al. 1991; Gray and Pinkel 1992). Single gene disorders such as Duchenne/Becker muscular dystrophy are investigated by using locus specific probes (Mahler et al. 1990) and probes revealing the chromosome number in interphase cells have been of great value in prenatal diagnosis (Zheng et al. 1992).

For single gene disorders, the signal intensity was enhanced by improving the already existing fluorescent detection systems after applying additional layers of immunochemicals (Landegent et al. 1985; Kosma et al. 1987; Ried et al. 1990). The need to interpret complex chromosomal abnormalities led to the development of multicolour FISH and improved microscope systems (i.e. digital imaging camera systems controlled by appropriate computer hardware and software), thus making feasible the simultaneous detection of multiple probes each labelled with a distinct hapten (Ried et al. 1992a; Dawerse et al. 1992). Further developments by introducing combinatorial labelling schemes (i.e. ratio mixing FISH) increased the number of unique sequences that could be enumerated in a single hybridisation experiment, while minimizing the number of different probe haptenisation and detection systems required (Reid et al. 1992b; Nederlof et al. 1990; Mohaddes et al. 1996).

Successful efforts to visualise chromosomes of the first and second polar body for accurate cytogenetic analysis of oocytes have made it possible to perform pre-implantation diagnosis of common aneuploidies by FISH using probes specific for chromosomes X, 18 and 13/21 (Verlinsky Y. et al. 1996).

The recent developments of multiplex FISH (M-FISH) and spectral karyotyping (SKY) where all 46 chromosomes could be identified with uniquely distinctive colours, using just five fluorochromes, CCD imaging microscopy and special computer software, has made it possible to identify both simple and complex anomalies present in pre- and postnatal diagnosis and cancer cytogenetics, thus expanding greatly the capability of molecular cytogenetics in reliably detecting chromosome aneuploidy (Speicher et al. 1996, Schrock et al. 1996)

1.3.2 FISH: Critical stages

The sensitivity and consequently the success of FISH depends on a number of factors such as (1) quality and preparation of the biological material that could affect its permeability to probe and detection reagents (2) type of probe construct (3) denaturation of probe and target DNA (4) hybridisation conditions (5) sensitivity of the method for signal detection and visualisation (Figure 1.1).

1.3.3 FISH: Preparation of biological samples

Fluorescent *in situ* hybridisation can be performed on a variety of tissues and cell types such as lymphocytes and fibroblasts, bone marrow and tumour cells , amnion and chorion samples.

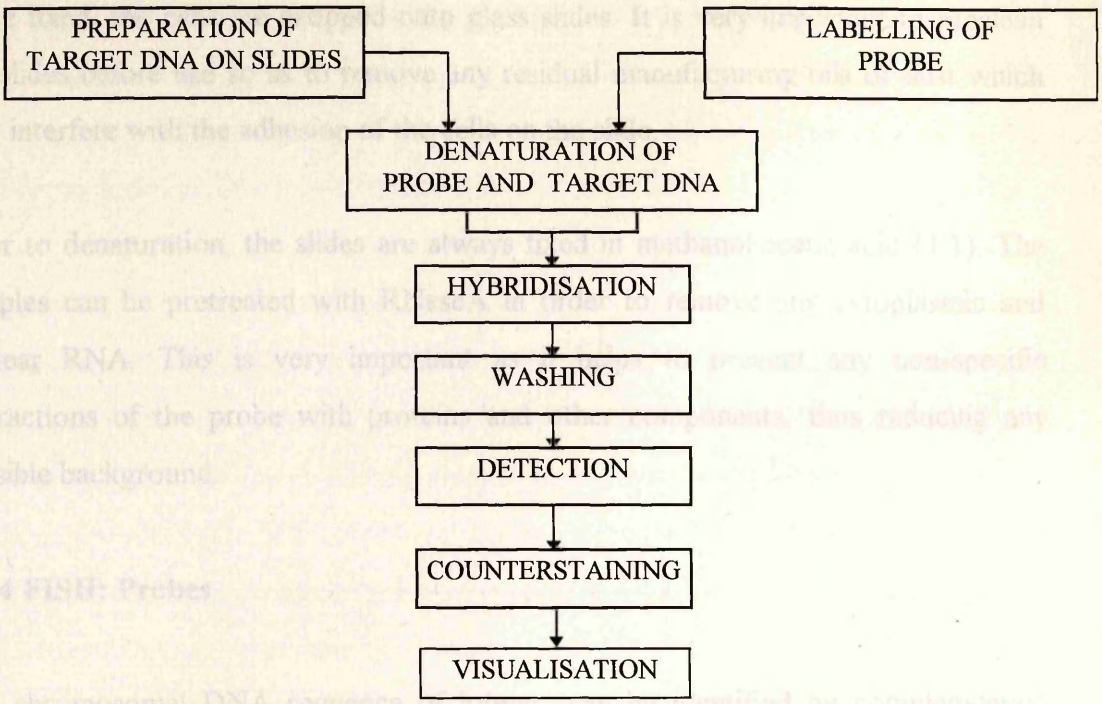


Figure 1.1 An outline of the fluorescence *in situ* hybridisation procedure.

Treatment with hypotonic solution (KCl) will swell the metaphase and interphase cells prior to fixation. The length of exposure and concentration of the hypotonic solution may have a remarkable effect upon chromosome morphology since less than optimum hypotonic treatment may result in tightly packed, non dissectable metaphase chromosomes. Good quality metaphase spreads are necessary when assessing the fluorescent signals.

Fixation with methanol:acetic acid (3:1) will preserve the cells, stop all their function and prevent further swelling. It is very important for the cells to be well fixed so that we prevent diffusion and loss of cellular DNA during post hybridisation and detection washes of FISH. Endogenous nuclease activity and consequent loss of DNA is also prevented. However, if overexposed to fixative, although well preserved the material will be less accessible for *in situ* hybridisation.

Once fixed, the cells are dropped onto glass slides. It is very important to preclean the slides before use so as to remove any residual manufacturing oils or dust which may interfere with the adhesion of the cells on the slide.

Prior to denaturation, the slides are always fixed in methanol:acetic acid (3:1). The samples can be pretreated with RNaseA in order to remove any cytoplasmic and nuclear RNA. This is very important as it helps to prevent any non-specific interactions of the probe with proteins and other components, thus reducing any possible background.

1.3.4 FISH: Probes

The chromosomal DNA sequence of interest can be identified by complementary hybridisation to certain probes. Probes may be defined on the basis of their origin and on the basis of their target. Recombinant DNA technology has produced a pool of probes cloned into specific vectors. Once inserted into the vector, the recombinant DNA can be propagated into the host bacteria. Then the probe is isolated, labelled and used for FISH. The average size of a probe should be 100-300 bp. Probes of a shorter length may not form enough stable hybrids, whereas probes longer than 1kb may have tissue penetration problems and may also increase the chance of repetitive signals.

The following types of DNA can be used as cloning vectors for probe DNA:

- Plasmids are widely used as cloning vehicles. They are extrachromosomal circular pieces of bacterial DNA (ranging in size from 1-200 kb) that can carry up to 5 kb inserted foreign DNA.
- Bacteriophages are linear DNA bacterial viruses that can propagate fragments of probe DNA from 5-10 kb in size.
- Cosmid vectors are modified plasmids that can integrate sequences of probe DNA from 25-40 kb in length. Such probes have been used to localise breakpoints of chromosomal translocations involved in a variety of cancers (Selleri et al. 1991).

• Finally, YACS (Yeast artificial chromosomes) have been developed to clone very large fragments of probe DNA, ranging in size from 100-700 kb and to study chromosomal aberrations (i.e. cryptic translocations, deletions) involved in syndromes such as Miller-Dieker and DiGeorge (Kuwano et al. 1991).

Probes can also be defined according to their targets in the following general classes:

• Locus specific DNA probes are stretches of unique DNA sequences which occur once or only a few times in the genome. They contain all or part of a particular gene and are important for the molecular analysis and diagnosis of genetic disorders. The hybridisation efficiency of the probe to the single copy target depends on the type of the cloning vector used. Moreover, the larger the insert size is, the larger the target and consequently the larger the hybridisation signal is. Probes of less than 2 kb single copy sequences can detect 40% to 50% of all target sites (Lawrence et al. 1985; Landegent et al. 1987; Fuscoe et al. 1989; Mahler et al. 1990).

• Repetitive DNA probes are short sequences of DNA which are repeated many times and are present around the centromere of each chromosome as well as in other locations such as the long arm of the Y chromosome (e.g. α-satellite centromeric probes, pericentromeric heterochromatic probes, rRNA gene probes, telomeric probes). They can be used to identify marker chromosomes and detect numerical abnormalities. The high number of target sequences improves the quality of signal obtained.

• In 1989, Koch et al. described a new ISH method for identifying chromosomal regions on metaphase and interphase cells called PRimed *IN Situ* labelling (PRINS). According to this technique, synthetic oligonucleotides 10 to 50 bp long are used as probes to detect high and low copy repeats and single copy genes (Cinti et al. 1993). The oligo which serves as a specific primer anneals to the denatured target DNA material (which serves as the template for the primer-probe), thus, inducing *in situ* synthesis of labelled DNA by either DNA polymerase or reverse transcriptase and nucleotide triphosphates as precursors.

• Whole chromosome specific DNA probes consist of DNA sequences that are distributed densely or more or less continuously over a particular chromosome so that the chromosome which is targeted by this probe appears almost completely

painted in both metaphase and interphase nuclei. This method of identifying the target chromosome is known as *forward chromosome painting*. Several approaches have been used to construct whole chromosome library probes (i.e. paints).

In one approach, chromosome paints are constructed from somatic cell hybrids - the products of fusing human and rodent tissue cells- which have the tendency to lose human chromosomes and retaining the rodent genome as they grow in culture. In another approach, chromosomes of one type are purified by fluorescence activated cell sorting (FACS). Metaphase cells are disrupted to release the chromosomes into suspension and these are stained with a mixture of two different fluorescent dyes. The suspension is fed into a fluorescence-activated sorter, where single chromosomes pass in a stream through a pair of laser beams. A double fluorescent signal - characteristic for nearly every chromosome type- is emitted by each chromosome. This signal is used to produce a flow karyotype. The chromosomes of interest are sorted by adding a small electric charge to the droplet created at the end of the flow stream, allowing the droplet to be deflected out of the stream into a collection tube.

Chromosomes isolated by either of the approaches mentioned above are cloned into a vector by digesting with restriction enzymes and ligation (Van Dilla et al. 1986; Fuscoe et al. 1986). The recombinant molecule can then be propagated into a host and finally be used as a probe for FISH (Collins et al. 1991). Experiments with such libraries did not always result in fully painted chromosomes. Certain libraries failed to hybridise to the centromeric regions of the target chromosomes whereas others hybridised weakly to the short arms of acrocentric chromosomes. This could be explained by the fact that certain repetitive sequence regions of the library construct did not contain the restriction enzyme sites that are compatible with the cloning vector, hence leading to insufficient cloning and consequently, to poor hybridisation efficiency.

Cloning procedures were improved by the introduction of PCR technology. Human DNA constructs isolated by somatic cell hybrids, were amplified by IRS-PCR (interspersed repetitive sequence-polymerase chain reaction), when oligonucleotides

homologous to Alu- and or L1 repeats were used as primers to successfully amplify human DNA regions, providing that these blocks of repetitive DNA were located within a distance that can be bridged by PCR (Nelson et al. 1989; Ledbetter et al. 1990; Lichter et al. 1990). Generation of such libraries from hybrids by Alu- and or L1 PCR proved to be far simpler than cloning DNA fragments from sorter-purified chromosomes (Lengauer et al. 1990). However, these libraries contained only fragments that are flanked by repeated sequences spaced less than few kilobases apart, thus limiting their application only to DNA sources that contain frequently repeated sequences. This restriction was overcome by using once again sorted-purified DNA, this time amplified by a linker-adaptor oligonucleotide primer (Vooijs et al. 1993). More specifically, the sorted chromosomes are digested with a restriction enzyme recognizing a four-base pair DNA sequence and ligated on both ends to an adaptor oligonucleotide. Then, the entire collection of DNA fragments is amplified by PCR using a primer homologous to the adaptor oligonucleotide. Finally, flow sorted chromosomes can be used directly for FISH once they are amplified by PCR. The application of amplified FACS isolated probes has been of great importance in characterising chromosome constituents in aberrations involved in leukaemias (Suijkerbuijk et al. 1992) and clinical cytogenetic cases associated with dysmorphic features, malformations and mental retardation (Rack et al. 1993).

In general, chromosome painting has been extensively used in analysing and identifying structural rearrangements whether constitutional or in neoplastic cells, and in identifying the origin of chromosomal segments unidentified by G-banding (Cremer et al. 1988; Pinkel et al. 1988; Hulten et al. 1991). However, the success of library probes depends on suppression of hybridisation signals from common repetitive sequences shared by all chromosomes (i.e. Alu, KpnI and LINE segments). This is accomplished by a procedure known as Chromosomal *In Situ* Supression hybridisation (CISS) which involves the addition of unlabelled genomic DNA (total human DNA) in the hybridisation mixture (Lichter et al. 1988). This will act as a competitor against the target DNA by prehybridising to the library probe and consequently suppressing the repetitive sequences present in it, by a reannealing procedure that is based on rapid reassociation kinetics (Figure 1.2).

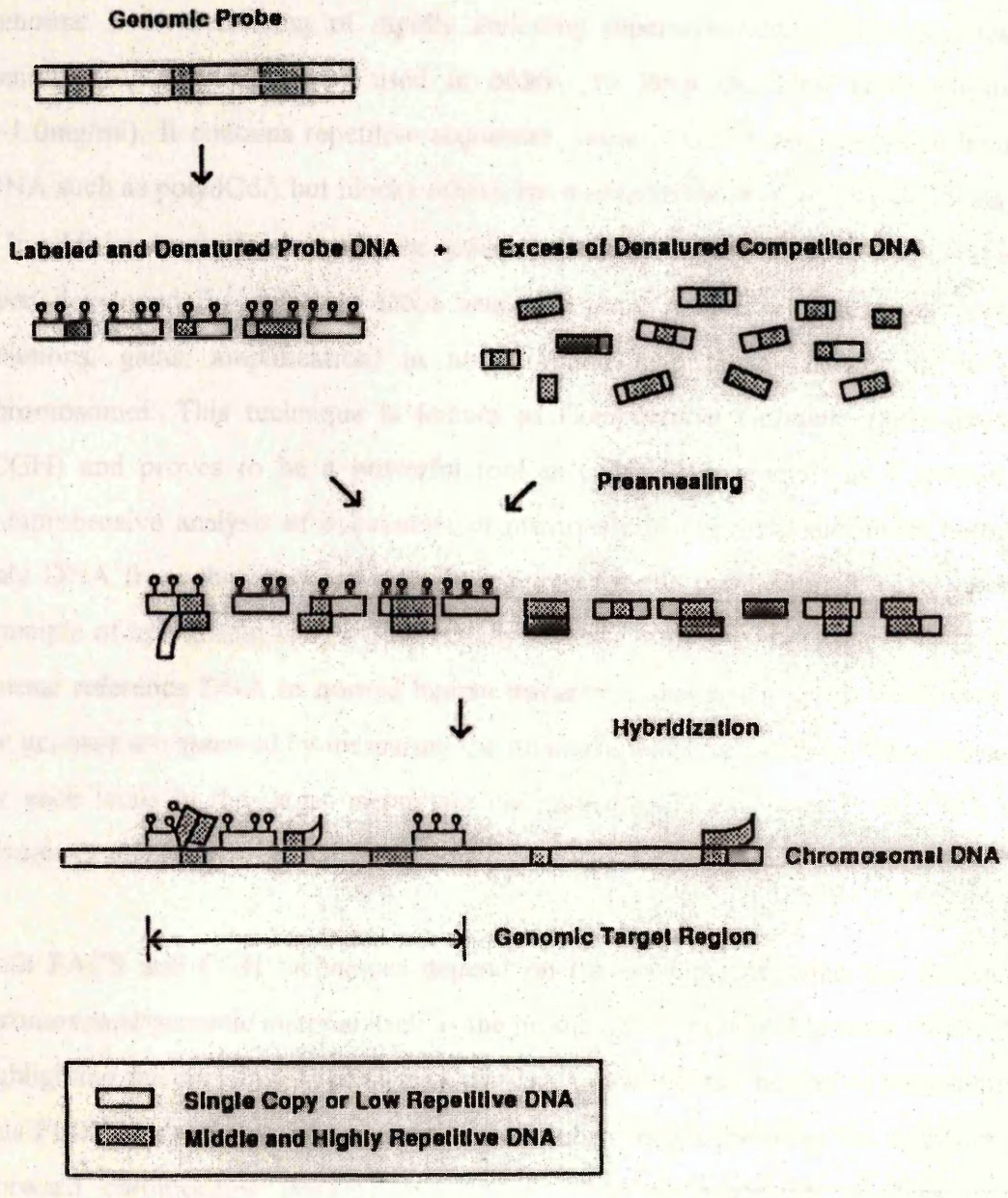


Figure 1.2 Schematic representation of chromosomal *in situ* suppression hybridisation (CISS). (Taken from Lichter et al. 'Chromosome analysis by non-isotopic *in situ* hybridisation', Rooney D.E. and Czepulkowski; 1992).

Any type of human DNA source containing a sufficiently high concentration of repetitive elements may serve as competitor DNA such as total human genomic DNA, human placental DNA and Cot-1 DNA. The latter is a fraction of human genomic DNA consisting of rapidly annealing repetitive elements. Salmon testis genomic DNA can also be used in order to keep the total DNA constant (~1.0mg/ml). It contains repetitive sequences, some of which are present in human DNA such as polydCdA but blocks others, most notable the Alu- and KpnI repeats.

• In addition to single chromosome specific paints, total genomic DNA probes have been developed for detecting DNA sequence copy number changes (i.e.. losses, deletions, gains, amplification) in tumours and map these changes in normal chromosomes. This technique is known as *Comparative Genomic Hybridisation* (CGH) and proves to be a powerful tool in cancer cytogenetics as it permits a comprehensive analysis of imbalances in previously inaccessible specimens because only DNA from the abnormal case is required for the procedure. It relies on the principle of hybridising *in situ* differentially labelled total genomic tumour DNA and normal reference DNA to normal human metaphase spreads. Genetic imbalances in the genome are assessed by measuring the tumour/control fluorescence intensity ratio for each locus in the target metaphase chromosomes (Kallioniemi et al. 1992; du Manoir et al. 1993).

Both FACS and CGH techniques depend on the principle of using the abnormal chromosomal/genomic material itself as the probe onto normal metaphases, therefore, highlighting the constituents of the aberrant DNA directly onto normal chromosomes. This FISH approach has been termed "reverse chromosome painting" as opposed to "forward chromosome painting" which as previously described, involves using normal chromosomes as probes onto metaphases containing the chromosome of interest (Carter et al. 1992).

The accuracy of the "reverse" over "forward chromosome painting" has been well documented by observing the limitations of the latter. Chromosomes with small rearrangements or chromosomes containing additional DNA material of unknown origin can only be analysed by trying several chromosome libraries in turn until

hybridisation of the abnormal chromosome is observed. When a deletion is involved, this can be missed as it cannot be visualised when "painting" the region of interest with a library probe. However, both, "reverse" and "forward" chromosome painting approaches can complement each other especially when studying large marker chromosomes (chromosomes of unknown origin) where more than one chromosome is involved in its composition. By using the "reverse" marker-paint its origin can be identified whereas the "forward" paints can help to establish the precise location of the different chromosome regions on the marker chromosome itself (Blennow et al. 1992). The accuracy of this combined approach has been reconfirmed by several studies on both constitutional abnormalities (De Albuquerque Coelho et al. 1996) and aberrations present in cancer (Meltzer et al. 1992).

1.3.4.1 Chromosome microdissection

Another approach of isolating abnormal chromosome DNA and using it as a probe for "reverse chromosome painting" has been recently described. This method known as *chromosome microdissection* involves the physical dissection of the actual chromosomal region of interest and its subsequent labelling by in vitro amplification-labelling, for use as a probe for FISH .

(A). Microdissection for microcloning

Chromosome microdissection was initially applied in the construction of region specific genomic libraries in order to facilitate physical mapping and cloning of disease-related genes since linkage studies using DNA polymorphisms were difficult to accomplish due to the lack of narrowly spaced markers. The first chromosome microdissection was performed in 1981 by Scalenge et al. on fragments from section 3 of the salivary gland X chromosome of Drosophila Melanogaster. These were digested with *Eco*RI, ligated to a lambda phage vector and *in vitro* packaged , finally producing recombinant phage clones which hybridised *in situ* to the chromosomal region of interest. Using this microtechnique, Bates et al. (1986) successfully cloned DNA sequences from the short arm of human chromosome 2 thus providing a rapid method to generate linked markers to genetic diseases for

which the chromosomal location was known. However, working with unstained preparations made it difficult to identify most human chromosomes. For this, experiments were performed by using somatic cell hybrids where the chromosome of interest was present as the only one, or one of the very few human chromosomes in the hybrid cell line. Alternatively, chromosomes flow-sorted directly onto coverslips could be used for dissection (Kao 1987).

The wide use of microdissection and microcloning for physical mapping of the human genome was initially limited by the poor cloning efficiency (i.e. the low number of isolated clones and the inadequate size of cloned fragments). Dissecting unstained chromosomes and adjusting the fixation (by minimising acetic acid treatment so as to avoid DNA depurination) and cloning protocols (by pre-ligation and phosphorylation of the vector so as to decrease the number of nonrecombinant clones and facilitate the formation of lambda DNA concatamers thus increasing the packaging efficiency) improved significantly the yield of microcloning (Weber et al. 1990).

The large number of DNA fragments (50-200) which needed to be dissected and the unbanded chromosome preparations were still the big drawbacks in the applicability of the technique. These limitations were circumvented by the introduction of the PCR technology. In this method, 20-40 fragments from banded chromosomes were dissected, treated with proteinase K, digested with a frequently cutting restriction enzyme (e.g. *Rsa*I), ligated to a plasmid vector (e.g. pUC plasmid) and amplified by using the plasmid vector sequences as targets for PCR primers. Finally, the inserts were subcloned into a second plasmid vector in order to generate a library (Ludecke et al. 1989; Ludecke et al. 1990).

Another method employed the ligation of dissected DNA to a linker-adaptor (a 20-24 nucleotide molecule) rather than to a plasmid before PCR amplification (Saunders et al. 1989; Johnson et al. 1990; Kao et al. 1991). In addition, improvements in sample preparation made possible the dissection of GTG-banded chromosomes, hence making feasible the dissection of any stained visible region of the human genome (Senger et al. 1990). Such developments on the microdissection-microcloning technique led to numerous studies of genetic disorders.

(B). Microdissection for micro-FISH

The strategy of reverse chromosome painting by using microdissected chromosomal fragments of DNA as the starting material which were then *in vitro* amplified, was first described in 1992 by Meltzer et al. The technique, termed *Micro-FISH,* was used in order to identify a chromosomal translocation involved in a malignant melanoma cell line previously unrecognisable by conventional cytogenetic techniques. Since then, several groups have applied this technique both in clinical and cancer cytogenetics, suggesting that it might be applied to diagnostic problems. However, several precautions must be taken in order to eliminate any factors that might possibly affect the quality of the micro-FISH probes produced.

I. Chromosome preparations

Synchronising agents used in order to obtain elongated chromosomes can potentially damage the DNA (e.g. methotrexate). Thus, it is better to use unsynchronised methods. However, when high resolution chromosomes are required, thymidine is preferable as the agent that will block the cells at a specific stage of the cell cycle. After release, cells reach the end of their cycle in synchrony.

In order to accumulate cells at metaphase, a mitotic inhibitor, usually colchicine (Colcemide) is used. An average of 15 minutes incubation should yield chromosomes of an appropriate length for microdissection. Shorter incubation will give longer chromosomes but allow fewer cells to reach metaphase thus decreasing the possible copy number of dissected fragments. Longer incubations (up to one hour) will give a much higher number of cells available for microdissection but with chromosomes much shorter in length.

Fixation with acetic acid can cause depurination of the dissected DNA. Therefore, a new protocol is adopted, which uses 70% ethanol as a prefix, followed by a short step of fixation in methanol/acetic acid (10-20sec). However, this method is not

suitable for whole blood samples as adequate fixation is required to remove the red cells and debris. Therefore, it is preferable to separate the red from the white cells in advance (by a lymphocyte separation protocol). Otherwise, cells are harvested according to the conventional cytogenetic protocols, however, trying to expose them to fixative for a shorter time.

Spreading of the chromosomes is crucial as it can greatly facilitate the microdissection procedure itself, prevent contamination caused by touching regions other than the target one (if chromosomes are lying very close to each other) and minimize the time of the dissection procedure (average timing 2 hrs. for twenty copies). However, chromosomes should not be overspread to the extend that some are lost from the cell. This would make karyotyping more problematic and identification of the target chromosomes difficult. This applies particularly where the chromosome morphohology is poor (i.e. bone marrows).

To prevent degradation of the DNA during ageing in dry air once dropped on the glass coverslips, chromosome preparations are stored at -20°C for several weeks up to months either in a dry chamber or in 70% ethanol. Banding of the chromosomes used to be thought to cause damage to the DNA, especially when microcloning was performed, nevertheless, conventional cytogenetic protocols are followed by the majority of groups involved in micro-FISH.

II. Microscopy and microdissection

Initial studies on microdissection for the purpose of microcloning were performed by using the oil chamber method (Scalenghe et al. 1981). According to this method, the coverslip with the chromosomal material is placed (with the chromosomes side down) on top of a glass chamber with a rectangular groove in the middle of it, filled with paraffin oil. Dissection is performed on a conventional microscope under a high power dry objective (at least 40X). Glass needles with a very fine end are driven with precise movement by a micromanipulator attached to the microscope. The dissected fragment is then transfered immediately to the parafin oil. All subsequent steps involved in microcloning are carried out in the oil chamber containing a nanoliter microdrop and by using nanolitre volumetric pipettes (Bates et al. 1986).

A second approach towards microdissection and collection of the subsequent fragments called laser microdissection was first described in 1986 by Monajembashi et al. According to this method, microdissection is performed by using a UV laser attached to the microscope. The laser beam is focused and directed on to the glass slide with the cells. All cells and chromosomes surrounding the target chromosomal region, as well as the unwanted segments within the desired chromosome are destroyed by the laser beam, leaving the chromosome fragment intact and ready to be recovered (Ponelies et al. 1989). The approach was adopted by a number of groups and successfully applied in constructing libraries from the fragile X region and the distal half of the short arm of human chromosome 4 (Djabali et al. 1991; Hadano et al. 1991; Lengauer et al. 1991).

Recent studies using microdissection for the purpose of chromosome painting have been carried out by using glass microneedles and a coverslip placed upright on an inverted microscope. The inverted microscope should consist of a rotatory stage that can rotate around the centre of the field and also move on X and Y axes, so that the chromosomal region of interest can be oriented appropriately prior to cutting. The microscope should have a short and efficient light path and a long working distance condenser to give maximum clearance for microdissection equipment. The condenser could have in addition to phase contrast a Nomarski differential interference contrast (DIC) if required for a different purpose (i.e. microinjection). Low power objectives (x4 up to x40) and high power dry lenses (x60, x100) are also necessary for fast scanning of the slide for metaphase cells and identification as well as precise dissection of very small chromosomal regions. A three dimentional micromanipulator with a mechanical or hydraulic drive is needed for the controlled-fine movement of the needle. A fine drive joystick is used to reduce hand movement during dissection, separation and collection of the fragment.

It is absolutely essential for the whole microscope system to be placed on a vibration free table to avoid any possible vibrations that could interfere with the very sensitive movement of the microneedle towards the region of interest at dissection.

Finally, the glass microneedles should have a very fine tip with a diameter of ~0.5μm capable of dissecting DNA fragments such as chromosome bands with an average width of 0.2-0.5μm. Needles initially come as glass capillaries which are then pulled under a microelectrode puller. The capillary is mounted into holders. Half way through the distance between the two holders, there is a metal filament. When particular values of the four parameters (i.e. heat, velocity, pulling force and time) are selected , the 'pulling' procedure will commence, with the filament producing heat which will heat up the capillary and eventually separate it into two pieces by pulling over the ends of the capillary towards different directions. The shape and the length of the tip of the needle produced, depends on several factors such as the type of rod glass used, the thickness of the wall and the values of the parameters with which the puller is set up. For a tip of a certain diameter, the needle can be ground on a micropipette grinder, which will bevel the pipette to increase opening diameter while retaining the small size tip. The coupling of a video camera to the microscope is not necessary, but could allow monitoring of the microdissection procedure if desired.

The first step in microdissection involves the scanning of the coverslip under low power magnification for appropriate cells. Then, once the cell has been selected and the chromosome of interest orientated so that it is perpendicular to the axis of the needle, the high magnification is used and the needle is placed carefully on a holder attached to the microscope. The needle is then lowered towards the chromosome at a suitable angle of ~ 45°, until it touches its surface. Using a micromanipulator, the needle is moved across the selected chromosome region and cut through. It can then be picked up immediately once it has been excised and adhered to the needle and can then been transferred to the PCR tube. Alternatively, once dissected it can be pushed aside on the slide and then, by approaching it with the same needle, it can be lifted up by electrostatic attraction produced by forces between the chromatin DNA and the glass. The material is then transferred into a small Eppendorf tube to which the PCR components will be added (Figure 1. 3 a-c).

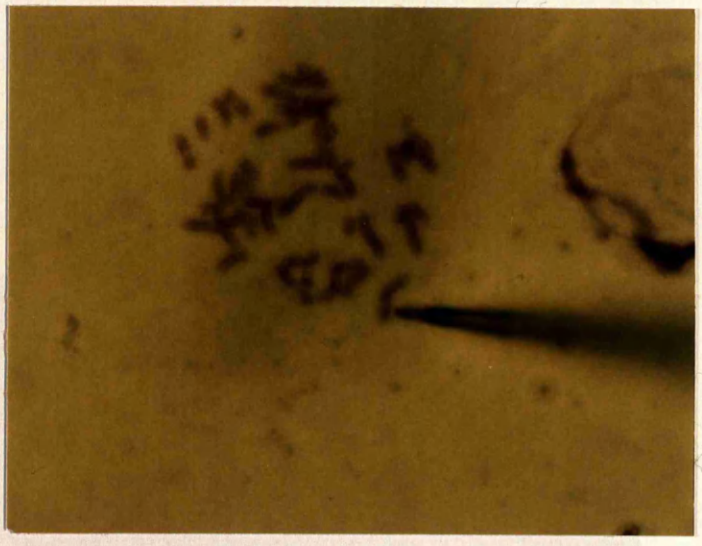


Figure 1.3a Microdissection of the 3q25-26 chromosome region. The glass microneedle is aimed at the region of interest.

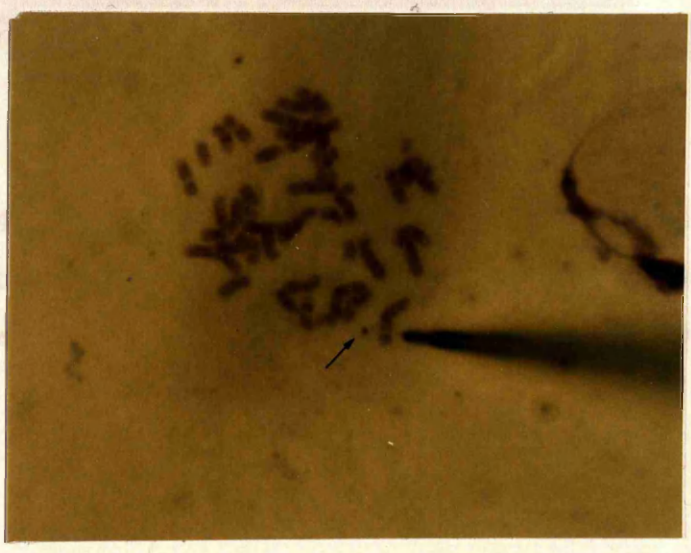


Figure 1.3b Microdissection of the 3q25-26 chromosome region. The needle has cut through the region of interest. The dissected fragment is visible (indicated by an arrow) lying by the side of the long arm of chromosome 3.

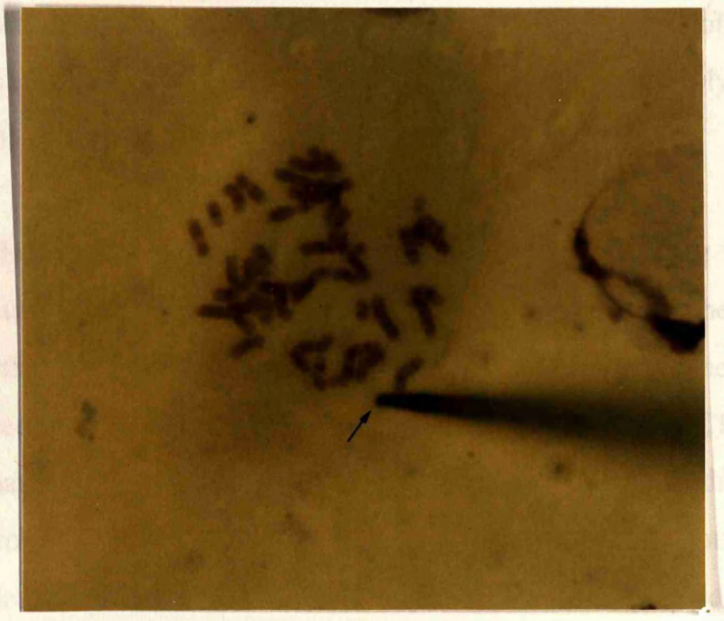


Figure 1.3c Microdissection of the 3q25-26 chromosome region. The needle has lifted up the dissected fragment (seen adhered on the very end of the tip, indicated by an arrow) which is then transferred into a collection tube.

III. In vitro amplification by DOP-PCR

Since twenty to forty fragments of dissected material can yield only a few hundred femtograms to a few picograms of DNA (an average size G band in metaphase contains ~15-50 fg DNA), it is necessary to amplify this starting material in vitro so that it can be used later as a probe for FISH. The polymerase chain reaction developed by Mullis et al. (1986) can produce, enzymatically, multiple copies of a specific DNA sequence by using forward and reverse oligonucleotide primers flanking the region of interest and hybridising to the opposite DNA strands. Repeated cycles of template DNA denaturation, primer annealing and extension of the annealed primers, under the influence of the *Thermus aquaticus* (Taq) DNA polymerase leads to an exponential accumulation of template DNA.

Until recently, the most commonly used PCR technique for amplifying whole human genomic DNA was the ISR-PCR (interspersed repetitive sequence) where sequences homologous to the Alu family of repetitive DNA were utilised as primers to amplify the region between adjacent Alu sequences. A drawback of such a PCR was shown to be the fact that because Alu repeats were not evenly distributed within the human genome, the probe produced was not painting the target region uniformly. In 1990 Wesley et al. described the use of a 20-mer primer with complete degeneracy at positions 4, 5, 6 and 7 from the 3'end.

5'- T T G C G G C C G C A T T N N N N T T C -3'

This nonspecific primer could anneal to any type of target DNA under low stringency due to its specific 3'end, the complementary sequence of which could be found ~every 64 bases. Successful matching of the primer could then be facilitated by the annealing of at least one of the degenerate 'N' bases to the target DNA.

In 1992, Telenius et al. (1992a) described a novel polymerase chain reaction where a primer consisting of a partially degenerate oligonucleotide sequence could result in a rapid, species-independent, efficient amplification. This Degenerate Ologonucleotide Primer (DOP), (or else called 'universal primer', UN-1), was successfully used to

analyse a chromosomal rearrangement in FACS sorted chromosomes derived from a lymphoblastoid and a thyroid carcinoma cell line. Designed by Telenius et al. (1992b), the degenerate oligonucleotide (22-mer) primer contains six specific bases at its 3' end. These short sequences occur frequently throughout the genome, thus allowing amplification at multiple loci. Furthermore, they tend to separate initiation sites, consequently increasing the product size. Once annealing of the 3' end has taken place, the priming is further stabilised by simultaneous annealing at one or more of the degenerate nucleotides. At the first few cycles, the annealing temperature does not exceed 30°C, since this facilitates annealing of the degenerated sites of the primer at short target sequences, hence allowing sufficient priming to initiate DNA synthesis at frequent intervals along the template.

5'- C C G A C T C G A G N N N N N N A T G T G G -3'

(where N=A,C,G or T)

These six random bases can yield 4^6 primers of different sequence as opposed to the single sequence of a nondegenerate primer, thus making its applicability universal. The 5' specified restriction site sequence (CTCGAG) for *Xho*I is necessary for microcloning purposes. Moreover, it allows efficient annealing of the primer, especially to previously amplified DNA, thus, enabling a higher annealing temperature (56°C-62°C) to be used in further cycles (Figure 1.4).

The products of amplification are within a range of 200 to 600 bp, hence, yielding a product smear when tested under gel electrophoresis. Some specific bands may be due to primer related products such as primer dimers and concatamers because of annealing of the primers to each other, however, this is not expected to affect the efficacy of the PCR, since the presence of DNA template can compete and inhibit formation of such products.

This new application of DOP-PCR simplifies the procedure for amplifying microdissected material. It is independent of the presence of repetitive sequences on the target region and increases the specificity and quality of the micro-FISH probes

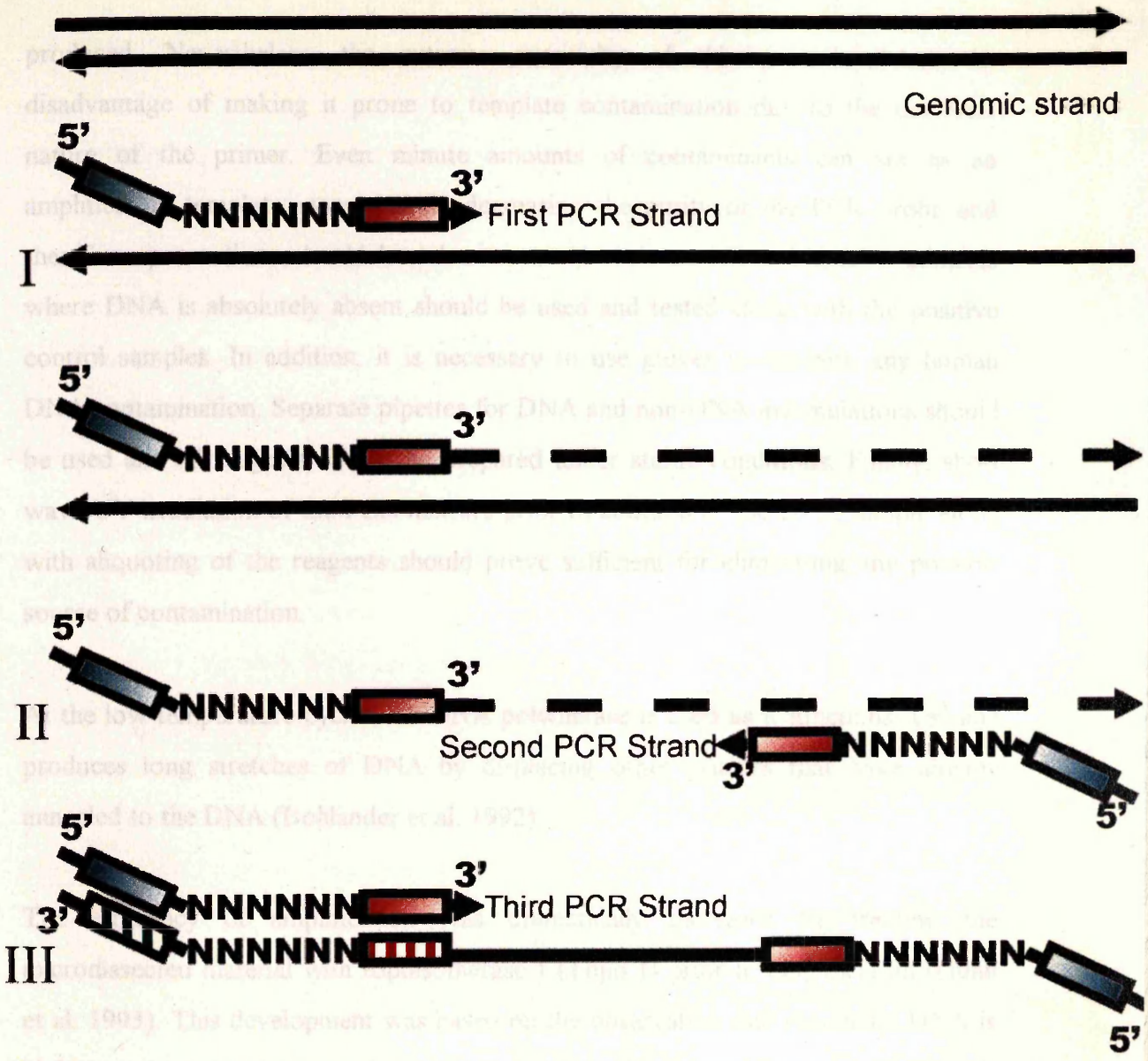


Figure 1.4 Diagrammatic representation of the DOP-PCR amplification.

The first low annealing temperature cycles will produce DNA strands that will comprise the full length of the primer at one end (I) and its complementary at the other (II), hence, enabling increase of the annealing temperature at the following cycles to a degree requiring the full length of the primer to anneal (III). (Adapted from Bohlander et al. 1992 and Telenius et al. 1992a).

produced. Nevertheless, the extreme sensitivity of this procedure has the disadvantage of making it prone to template contamination due to the universal nature of the primer. Even minute amounts of contaminants can act as an amplification template, consequently decreasing the purity of the PCR probe and therefore, precautions should be taken to avoid such an effect. Negative controls where DNA is absolutely absent should be used and tested along with the positive control samples. In addition, it is necessary to use gloves to diminish any human DNA contamination. Separate pipettes for DNA and non-DNA manipulations should be used and all reagents should be prepared under sterile conditions. Finally, short wave UV irradiation of the PCR mixture prior to addition of the DNA sample along with aliquoting of the reagents should prove sufficient for eliminating any possible source of contamination.

At the low temperature cycles, T7 DNA polymerase is used as it functions well and produces long stretches of DNA by displacing other primers that have already annealed to the DNA (Bohlander et al. 1992).

The efficiency of amplification was dramatically increased by treating the microdissected material with topoisomerase I (Topo I) prior to amplification (Guan et al. 1993). This development was based on the observation that chromatin DNA is highly condensed and supercoiled when in the metaphase stage. Since this might limit the access of both the primer and the polymerase to the DNA of the chromatin structure, Topo I (product of the topA gene of Escherichia coli) was used as the enzyme which can relax negative supercoils from duplex DNA. When binding to DNA, it makes a transient break in one of the DNA strands by nicking at the 5'-phosphate end and covalently linking it to a tyrosine residue in the enzyme, resulting in the transfer of a phosphodiester bond from the DNA to the protein and hence, ensuring input of energy for its function.

1.3.5 Labelling of probes

Non radioactive nucleic acid labelling can be performed in two different ways: direct and indirect. In the direct method, the probe is either labelled with a reporter molecule (hapten) chemically or enzymatically, or with a fluorescent molecule and can then be detected and visualised microscopically immediately, once *in situ* hybridisation has been completed. In contrast, for the indirect method, the label cannot be visualised directly until a second molecule, a reporter binding molecule, binds to the probe label, hence, enabling detection of the probe hybridisation sites.

A number of reporter molecules have been introduced, the most commonly used being biotin and digoxigenin (others include: dinitrophenyl, aminoacetylfluorene, mercury and sulfonate). Biotin is a member of the vitamin B complex (found in egg white). Acting as a analogue of dUTP, it attaches to the C-5 position of the pyrimidine ring through a linker arm of at least 11 carbon atoms to ensure access of the detection reagents, without interfering with the hydrogen bonding between the probe and the target nucleic acid (Figure 1.5). Digoxigenin is isolated as a steroid from digitalis plants (*Digitalis purpurea* and *Digitalis lanata*). When incorporated at the probe, it is linked to uridine nucleotides at the number 5 position of the pyrimidine ring through an 11 carbon atom linker arm (Figure 1.6)

Both biotin and digoxigenin are enzymatically incorporated into the probe by nick translation, random-primed labelling and PCR. Other haptens such as 2-acetylaminofluorene (AAF), dinitrophenyl (DNP), mercury and sulfonate are labelled enzymatically. However, enzymatic labelling is preferred to chemical labelling since it results in highly labelled nucleic acid probes.

1.3.5.1 Nick translation

Nick translation was first introduced by Rigby et al. (1977) for the labelling of *in situ* hybridisation probes with radioisotopes. The incorporation of biotin and digoxigenin (Langer et al. 1981; Holtke et al. 1990 respectively) as nonradioactive labels into

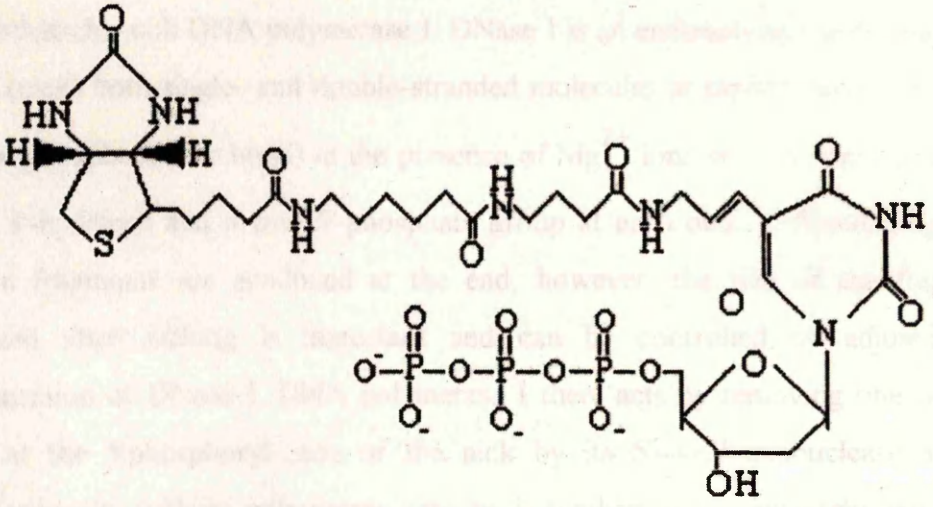


Figure 1.5 Schematic representation of the biotin molecule.

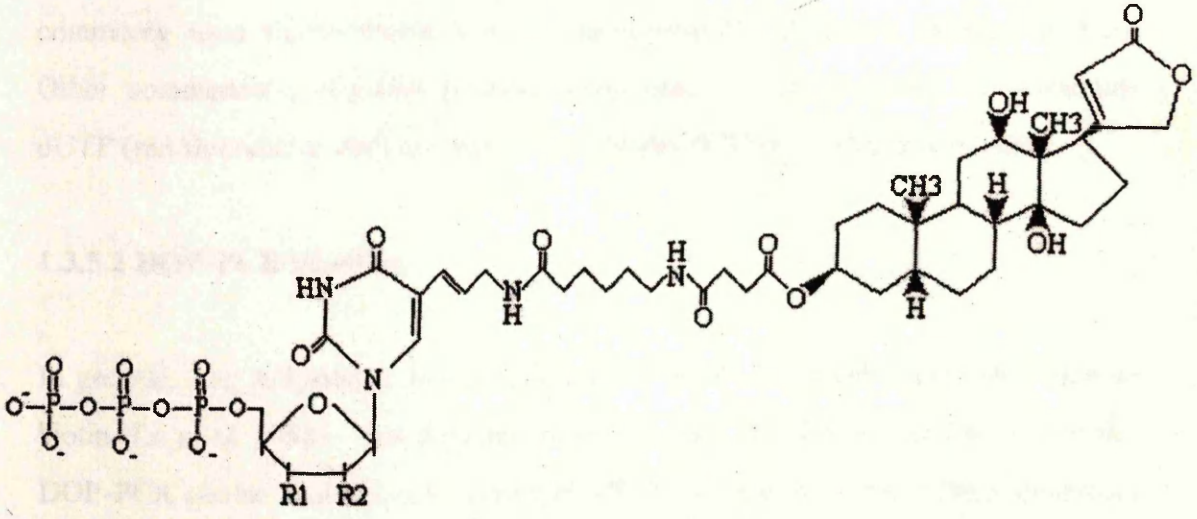


Figure 1.6 Schematic representation of the digoxigenin molecule.

DNA soon made it probably the most popular method for DNA labelling.

The technique employs two enzymes: bovine pancreatic deoxyribonuclease (DNase I) and Escherichia coli DNA polymerase I. DNase I is an endonuclease with the ability to cut (nick) both single- and double-stranded molecules at random sites (i.e. at any internal phosphodiester bond) at the presence of Mg^{2+} ions in vitro, thus, producing a free 3'-hydroxyl and a free 5'-phosphate group at each nick. Different lengths of labelled fragments are produced at the end, however, the size of the fragments produced after nicking is important and can be controlled by adjusting the concentration of DNase I. DNA polymerase I then, acts by removing one or more bases at the 5'phosphoryl side of the nick by its 5'--->3' exonuclease activity. Simultaneously, with its polymerase activity, it synthesises a completely new strand by incorporating sequentially all four deoxyribonucleotides and their analogues (i.e. labelled ones) to the 3'-hydroxyl termini, while degrading the existing strand as it proceeds, thus, filling the gap and resulting in movement of the nick along the DNA.

As mentioned previously, by nick translation, fluorescein nucleotide analogues can also be incorporated for the purpose of direct labelling (Dirks et al. 1991). The most commonly used fluorochrome used is fluorescein-dUTP (green fluorescein dye). Other commercially available fluorochromes used for this purpose are resorufin-dUTP (red fluorescent dye) and hydroxycoumarin-dUTP (blue fluorescent dye).

1.3.5.2 DOP-PCR labelling

In general, Taq polymerase has been shown to accept modified nucleotides such as biotin (Lo et al. 1988) and digoxigenin (Seibl et al. 1990) as substrates. Therefore, DOP-PCR can be used not only to amplify DNA, but also to produce large quantities of labelled probe DNA suitable for *in situ* hybridisation. The procedure involves exactly the same steps followed in an ordinary PCR reaction, with the exception that a hapten label (i.e. biotin) is also added to the PCR mixture so that it will be incorporated with the added nucleotides while the extension of the new strand takes

place. Both labelling procedures can be used equally, but incorporation of the label by DOP-PCR is less time consuming and hence, more preferable.

1.3.6 Purification of the labelled probe

The procedure of labelling results in labelled probe along with unincorporated labelled nucleotides. These have to be removed prior to hybridisation of the probe in order to avoid any backround. A number of methods have been designed (BioSpin , Sephadex G-50, and Amicon centricon columns) to purify the probes by removing such nucleotides. These are based on the principle of passing the probe mixture through a filter membrane/matrix inside the column. Unincorporated labelled nucleotides are trapped in the matrix thus allowing the labelled probe to pass through and be collected in its purified form after spinning the column. The probe is then precipitated and the successful incorporation of the reporter molecule to it can be evaluated by a dot-blot analysis.

1.3.7 Denaturation, hybridisation, washes and detection

In order for successful in situ hybridisation to take place between the probe and the target DNA, both molecules should first be converted to single stranded DNA (Figure 1.7). Therefore, denaturation is a prerequisite that will allow thereafter the single strand sequences to reanneal to each other in a complementary fashion. Acid, alkaline and heat treatments can be used for this denaturation purpose. However, such treatments may affect the morphology of the target DNA and thus, a balance between *in situ* hybridisation signal and morphology must be found experimentally. In order to avoid deterioration of the target DNA morphology, organic solvents can be used that reduce the thermal stability of double stranded polynucleotides. Formamide is widely used for this purpose. This is a destabilising molecule which inhibits the formation of nucleic acid complexes while at the same time it lowers the melting temperature *Tm* of DNA (temperature at which half of the DNA is present in a single stranded form) by 0.72°C for each 1% formamide added, thus, preserving the DNA morphology. Consequently, by using formamide, prolonged heating of the

target DNA or high temperatures (90°C-100°C) are avoided. Treatment of only a few minutes at 65°C-70°C proves to be sufficient for denaturation. For the labelled probe heat denaturation is popular because of its experimental simplicity and great effectiveness.

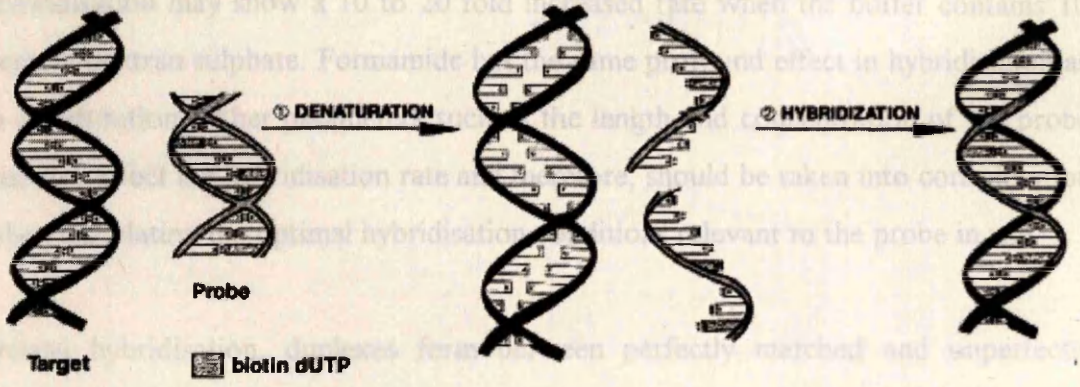


Figure 1.7 Denaturation and hybridisation of the DNA molecules. (Adapted from Dyer et al. 1991; Barch M.J. The ACT Cytogenetics Laboratory Manual)

Simultaneous denaturation of both the probe and the target DNA is possible and can be achieved by heat when a coverslip is placed on top of the slides. Despite the fact that this approach is more simple and safe due to the absence of the hazardous formamide solvent, it may damage the chromosomal morphology as high temperature is required for the denaturation to take place. For better denaturation results, factors such as the probe length and the GCpair content of the target DNA should be also be taken in consideration. For competition *in situ* hybridisation (CISS), where the labeled probe is allowed to reanneal with unlabelled competitor DNA prior to hybridisation, so as to suppress any repetitive sequences present that might create non-specific signals, the chromosonal DNA and the probe are denatured separately.

Once denatured the single stranded probe is applied on the slide with the target DNA and are then left to hybridise (reanneal) at an optimal temperature of 37°C. Both, the rate of renaturation and the thermal stability of the DNA hybrids are dependant on specific parametres. Using the correct temperatures at which denaturation and

renaturation of the DNA molecules occur, the hybridisation efficiency can increase dramatically. Inclusion of certain solutions into the hybridisation mixture can improve the quality of hybridisation. Most importantly, high salt concentrations (eg. SSC) increase the stability of the nucleic acid duplexes. Dextran sulphate is a polymer that, by forming a matrix in the hybridisation mixture, concentrates the probe DNA. Hybridisation may show a 10 to 20 fold increased rate when the buffer contains 10 percent dextran sulphate. Formamide has the same profound effect in hybridisation as in denaturation. Other parameters such as the length and concentration of the probe can also affect the hybridisation rate and therefore, should be taken into consideration when calculating the optimal hybridisation conditions relevant to the probe in use.

During hybridisation, duplexes form between perfectly matched and unperfectly matched sequences. It is important to remove the latter as well as any excess of unhybridised probe, so as to eliminate non-specific binding. For this, it is necessary to perform post-hybridisation washes under stringent conditions that will permit retention of actual hybrids only. Varying the salt and formamide concentrations present in the washing solution as well as the wash temperature can increase the specificity of hybridisation. However, better results are obtained when hybridisation is performed at a high stringency and washing at similar or lower stringency.

Following the stringency washing steps, a blocking step is used to remove high background. The blocking reagent (human serum, non fat milk or any other blocking reagent) contains antibodies which can bind to cellular and nuclear proteins and supress them from binding to the antibodies conjugated to the fluorescent dyes during detection steps.

Detection of the hybridised probe depends on the type of label used. For probes labelled directly with fluorescent nucleotides there is no requirement to perform any blocking antibody incubation. But when a reporter molecule (hapten) such as biotin or digoxigenin is used as a label, more steps may be required for detection to take place. A fluorochrome (signal-generating system) can be conjugated to a primary antibody which is raised against the hapten-label (Figure 1.8).

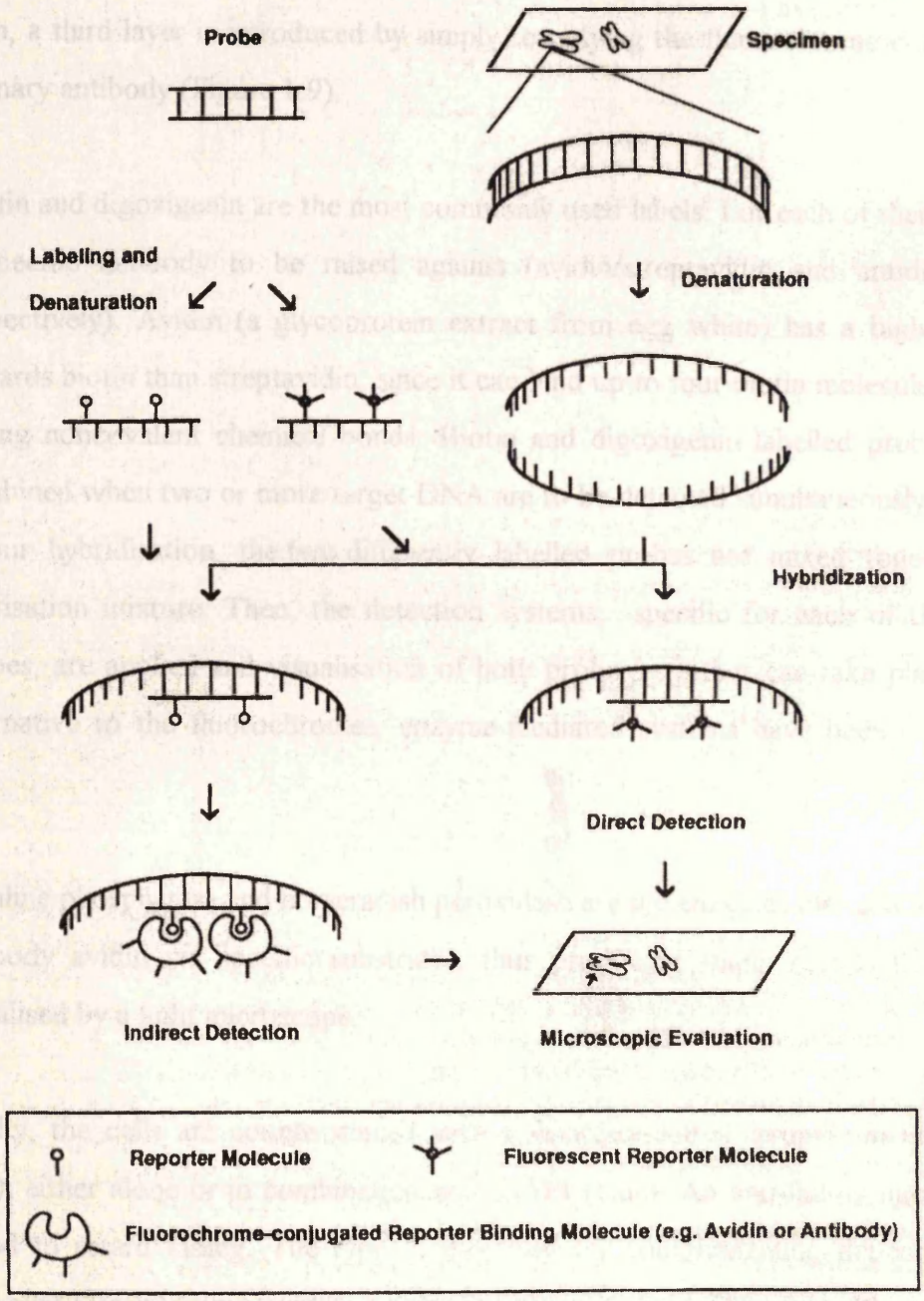


Figure 1.8 Schematic representation of FISH using direct and indirect signal detection systems. (Taken from Rooney and Czepulkowski, 1992)

Alternatively, a second antibody (raised against the species which produced the primary antibody) is used with the particular fluorochrome being attached to it. The signal intensity can be further enhanced, if after adding the second antibody on its own, a third layer is introduced by simply reapplying the fluorochrome conjugated - primary antibody (Figure 1.9).

Biotin and digoxigenin are the most commonly used labels. For each of them, there is a specific antibody to be raised against (avidin/streptavidin and antidigoxigenin respectively). Avidin (a glycoprotein extract from egg white) has a higher affinity towards biotin than streptavidin, since it can bind up to four biotin molecules through strong noncovalent chemical bonds. Biotin and digoxigenin labelled probes can be combined when two or more target DNA are to be detected simultaneously. For dual colour hybridisation, the two differently labelled probes are mixed together in the hybrisation mixture. Then, the detection systems, specific for each of the labeled probes, are applied and visualisation of both probes together can take place. As an alternative to the fluorochromes, enzyme-mediated systems have been used in the past.

Alkaline phosphatase and horseradish peroxidase are the enzymes that can bind to the antibody avidin via specific substrates, thus producing stable signals that can be visualised by a light microscope.

Finally, the cells are counterstained with a fluorescent dye, propidium iodide (PI) (red), either alone or in combination with DAPI (blue). An anti-fading agent is also added to retard fading. The type of dye used for counterstaining depends on the particular fluorochrome used as a signal detection system. Therefore, PI can be used in conjunction with fluoro-green (FITC) and fluoro-blue (AMCA), but not with fluoro-red (Texas/rhodamine) as the signals of the latter will be masked by the red PI stain. Similarly, DAPI can be used in conjunction with fluoro-green (FITC) and fluoro-red, but not with fluoro-blue, as both of them give blue fluorescence.

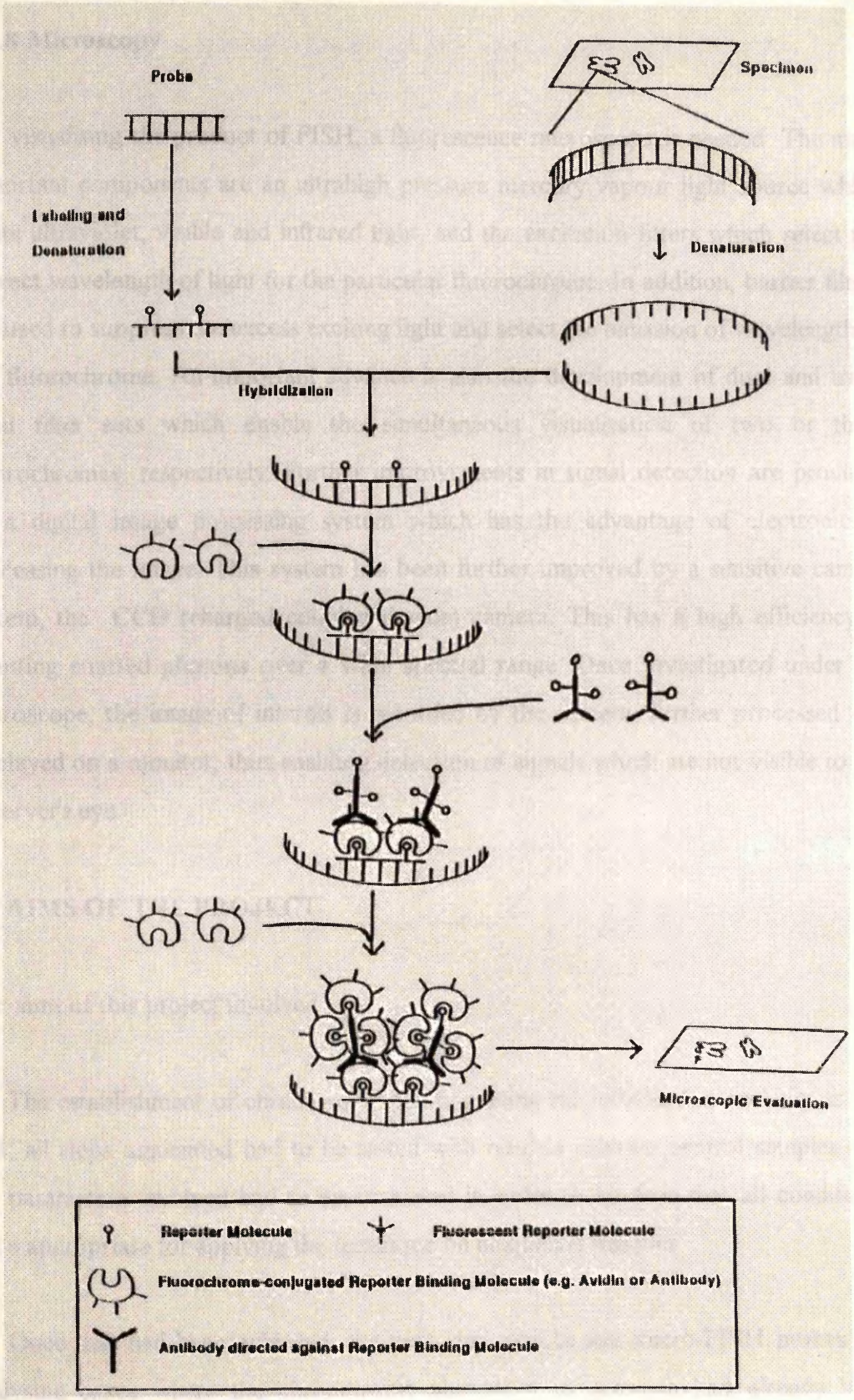


Figure 1.9 Schematic representation of FISH using a two step signal detection system. (Taken from Rooney and Czepulkowski, 1992)

1.3.8 Microscopy

For visualising the product of FISH, a fluorescence microscope is needed. The most important components are an ultrahigh pressure mercury vapour light source which emits ultraviolet, visible and infrared light, and the excitation filters which select the correct wavelength of light for the particular fluorochrome. In addition, barrier filters are used to suppress the excess exciting light and select the emission of wavelength of the fluorochrome. An important advance is also the development of dual and triple band filter sets which enable the simultaneous visualisation of two or three fluorochromes, respectively. Further improvements in signal detection are provided by a digital image processing system which has the advantage of electronically processing the image. This system has been further improved by a sensitive camera system, the CCD (charged coupled device) camera. This has a high efficiency in counting emitted photons over a wide spectral range. Once investigated under the microscope, the image of interest is recorded by the camera, further processed and displayed on a monitor, thus enabling detection of signals which are not visible to the observer's eye.

1.4 AIMS OF THE PROJECT

The aims of this project involved:

(1) The establishment of chromosome painting using microdissection techniques. At first, all steps associated had to be tested with reliable positive control samples and the parameters involved had to be evaluated in order to confirm that all conditions were appropriate for applying the technique on diagnostic samples.

(2) Once that had been achieved, the next step was to use micro-FISH probes for analysing cases where the chromosome aberration in question had already been identified, in order to test the reliability of the technique by reconfirming the aberration. The final aim was to further utilise the technique as a

routine technique to define aberrations that remained unresolved by standard cytogenetic methods.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Safety

Culturing and harvesting of human peripheral blood cells was carried out in a biological safety cabinet. Laboratory coat and gloves were worn at all times during the experiments. All Eppendorf tubes, plastic universals and tips used were sterile. Dangerous chemicals, such as toxic and carcinogenic reagents (i.e. formamide) were always handled with care in a fume hood.

2.1.2 Solutions and reagents

For culturing human cells:

1. Iscoves culture medium

   Into 100 ml of Iscoves medium, the following components were added asceptically:

   Heparin (5,000 units/ml) 1ml

   L-glutamine (100x) 1ml (1%)

   Penicillin/Streptomycin 1ml (1%)

   The medium was mixed and stored at 4°C until further use.

2. Complete medium

   In 100 ml Gibco RPMI 1640 medium, containing L- glutamine, the following components were added:

   Fetal calf serum (FSC) 20 ml (20%)

   Penicillin/Streptomycin 1 ml (1%)

3. Wash medium

   Serum free Gibco RPMI 1640 medium containing 1% P/S

Phytohaemaglutinin (PHA)

Into one vial of PHA powder, add 10 ml of sterile distilled water, mix well and keep at 4°C.

For harvesting cells:

4. Hypotonic solution- KCl

In 1000 ml of distilled water disolve 5.592 gm of Potasium chloride (0.075M)

In 1000 ml of distilled water dissolve 2.8gm of Potasium chloride (0.0375M)

For banding metaphase cells:

5. Sorenson's buffer

To 1000 ml of distilled water add

9.48 gm Disodium dihydrogen phosphate and

9.08 gm Potassium dihydrogen phosphate

6. Trypsin solution

To 1000 ml of Sorenson's buffer dissolve 1.2 gm of Difco Trypsin.

7. Lipsol solution

1 ml of Lipsol detergent solution was added to 100 ml saline.

8. Saline

In 1000 ml of distilled water dissolve two saline-tablets.

9. Staining solution

In 2000 ml of methanol dissolve 3 gms of Leishmanns powder.

10. Buffer-pH 6.8

In 1000 ml of distilled water dissolve one buffer tablet-pH 6.8

For COP (Cruachem Oligonucleotide Purification) column primer purification:

11. Solutions

Solutions	Amount used
Acetonitrile	2 ml
2 M Trethylamine Acetate (TEAA, pH 7.0)	2 ml
Deionised water	3 ml
Ammonium Hydroxide /water (1.10)	3 ml
2% (v/v) Trifluoroacetic Acid (TFA) Water	2 ml
20% (v/v) Acetonitril/water	2 ml

All solutions were commercially available and stored at 4°C.

For PCR purposes:

12. Collection drop buffer

Components	Stock solution	50 ml stock of 10X	1X
Tris-HCl (pH 7.5)	1M	(400mM) 20 ml	40mM
NaCl	5M	(500mM) 5 ml	50mM
$MgCl_2$	1M	(200mM) 10 ml	20mM

Add up to 50 ml with distilled water, make aliquots and store at -20°C. Add 1µl of it into 5µl collection drop (containing dNTPs, DOP primers and distiled water).

13. PCR buffer

Components	Stock solution	50 ml stock of 10X	1X
Tris-HCl(pH 8.4)	1M	(100mM)	10mM
KCl	2M	(500mM)	50mM
$MgCl_2$	1M	(20mM)	2mM

Make up to 50 ml with distilled water, aliquot and store in -20°C. Add 5 µl into 50 µl of PCR mixture (containing the rest of the components).

14. dNTPs

100mM of stock solution (25mM each) in water (pH 7.5) was used to prepare the working solution with a final concentration of 0.2mM for each dNTP. This was made by taking 5µl of each dNTP and adding dH_2O up to 250µl (2mM). Five microliter of this dilution was used in each 50 µl PCR reaction.

15. T. B. E. buffer (5X)

In 1000 ml of distilled water disolve 108 gm Tris base
55 gm Boric acid
9.3gm EDTA

Keep at room temperature.

16. T. E. buffer

10mM Tris-HCl was mixed with 1mM EDTA, pH 8.6. After filter-sterilising the solution, it is kept at room tempetature.

17. 1 Kb ladder

100µl of stock ladder solution are mixed with 200µl of loading mix and 800µl of distilled water.

18. Loading mix

Mix 30% glycerol with 0.25% BPB (Bromophenol blue) in dH_2O.

For Dot-Blot assay:

19. AP 7.5 buffer

0.1 M Tris-HCl pH 7.5 mixed with 0.1M NaCl and 2mM $MgCl_2$

20. AP 9.5 buffer

0.1M Tris-HCl pH 9.5 mixed with 0.1M NaCl and 50mM $MgCl_2$

21. Blocking buffer

3% BSA in AP 7.5 buffer

Dilution buffer

0.1 mg/ml sheared salmon sperm DNA in 6X SSC pH 7.0

22. Developing solutions

75 mg/ml nitroblue tetrazolium (NTB) in 70% dimethyl formamide and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylforma-mide.

For FISH purposes:

23. 20X SSC, pH. 5.3

In 1000 ml distilled water disolve 88.2 gm Sodium citrate

175 gm Sodium chloride

by stiring. Adjust the pH with conc. HCl and then autoclave at room temperature. (SSC solutions could be kept at room temperature for up to a year)

24. 4X SSC, pH. 7.0

Dilute 20X SSC (stock) into 1/5 (i.e. 200 ml 20X SSC in 800 ml distilled water), adjust the pH, autoclave and store as above.

25. 2X SSC, pH. 7.0

Dilute 20X SSC (stock) into 1/10 (i.e. 100ml 20X SSC in 900 ml distilled water), adjust pH, autoclave and store as above.

26. 4X SSC, 0.05% Tween-20 (4X SSCT)

In 100 ml of 20X SSC, add 250 µl of Tween-20 and make up to 500 ml with distilled water by stiring, store as above.

27. Denaturation buffer (70% Formamide)

To 15 ml of 2X SSC (pH 7.0) add 35 ml formamide (99%)-Fluka and store at 4°C till use.

Hybridisation buffer

Mix 5 ml of 50% formamide with 2 ml of 50% dextran sulphate, 0.5 ml of 20X SSC and 2.5 ml sterile water. Once aliquoted, store at -20°C.

28. Washing solution (50% Formamide)

To 25 ml of 2X SSC (pH 7.0) add 25 ml formamide and store 4°C till use.

29. Wash A solution

20X SSC	100	ml
Tween-20	250	µl
Dried skimmed milk	25	gm

All three components were added to 500 ml solution adjusted with dH_2O, mixed on low heat while stiring and then, spun at 2000rpm in 50 mls aliquots.

30. FITC-avidin solution (5µg/ml)

From a stock solution (2 mg/ml), 0.25 µl were added in 99.5 µl of wash A for every 100 µl required per hybridisation area.

31. Biotinylated anti-avidin solution (5 µg/ml)

From a stock solution (2 mg/ml), 0.25µl were added in to 99.75 µl of wash A for every 100 µl required.

32. Phosphate bufferred- saline (PBS), pH. 7.0

In 1000 ml of distilled water dissolve 10 Dulbecco PBS tablets, adjust pH., autoclave and store at room temperature.

33. DAPI (4,6-Diamidino-2-Phenonyl-Indole) (40µg/ml)

Dilute 1µl of DAPI (stock:1mg/ml) in 24µl of distilled water, mix well and store in dark at 4°C.

Propidium iodide (20 μg/ml)

Dilute 1μl of PI (stock:10mg/ml) in 499 μl of distilled water, mix well and store in dark at 4°C.

For Southern blotting:

34. 6X agarose gel loading buffer

Mix together 0.25% bromophenol, 40% (w/v) sucrose and 1X TBE buffer and store at 4°C.

35. 10% SDS

Dissolve 100gm of electrophoresis-grade SDS in 900 ml of dH_2O. Heat at 68°C to assist dissolution. Adjust the pH by adding few drops of concent-rated HCl. Then adjust volume to 1000 ml with dH_2O and dispense into aliquots.

36. 0.1% SDS/2X SSC

From the stock solutions, 10% SDS and 20X SSC, add 10 ml and 50 ml respectively into 940 ml dH_2O.

37. 0.5% SDS/2X SSC

Add 50 ml of 10% SDS (stock) and 50 ml of 20X SSC(stock) into 900 ml of dH_2O.

38. 0.25% SDS/2X SSC

Add 25 ml of 10% SDS and 50 ml of 20X SSC into 925 ml of dH_2O.

2.2 METHODS

2.2.1 Sources of DNA material

For optimising the several steps involved in micro-FISH, two types of DNA material were used: (a) extracted human genomic DNA and (b) fluoresence activated sorted chromosomes kindly provided by the Department of Pathology, University of Cambridge,U.K. Microdissection was performed on chromosomes from normal peripheral blood samples and from cell cultures of abnormal bone marrow samples.

2.2.2 Preparation of coverslips for microdissection

Glass coverslips, 22mm x 50mm in size were exposed to UV light for 10-20min. They were then immersed into 80% ultra-pure ethanol, air dried in a sterile hood and kept inside sterile containers at room temperature until further use. A few hours prior to dropping of the cell suspension, slides were placed at -20°C to facilitate adequate spreading of the cells.

2.2.3 Lymphocyte separation of peripheral blood samples for the use of microdissection

1. Approximately, 10 ml of fresh heparinised peripheral blood is needed for setting up two cultures.
2. Fresh peripheral blood was diluted with an equal volume of serum free RPMI 1640 medium.
3. 3 ml of lymphocyte separation medium were placed into a centrifuge tube and 7 ml of diluted blood was layered on top by carefully running it down the side of the tube from a plastic pipette.
4. The sample was centrifuged at 1500 rpm for 20 min. The red cells formed a pellet at the base of the tube whereas the white cells formed a buffy coloured layer at the interface between the separation medium and the diluted serum.

5. The buffy coat layer was harvested using a 1 ml syringe whilst avoiding drawing up the separation medium and the blood serum.
6. It was then placed into a fresh centrifuge tube, topped up to the 10 ml mark with serum free medium, mixed well by inverting the tube and centrifuged at 1000rpm for 10 min.
7. The supernatant was removed, cells were resuspended in a further 10 ml serum free medium and centrifuged again at 1000rpm for 10 min.
8. Finally, once the supernatant had been removed, the cell pellet was resuspended into 5 ml of cultured RPMI 1640 medium.
9. 0.1 ml of PHA was added to the cell suspension and the cells were incubated at 37°C for 48 hrs.

2.2.4 Harvesting synchronised cultures for microdissection, using thymidine block

1. 0.1 ml of thymidine (0.3 mg/ml) was added to the 5 ml culture and incubated at 37°C for a further 16 hrs.
2. The thymidine block was released by centrifuging at 1000rpm for 10 min.
3. Removing the supernatant cells were resuspended in 5 mls of wash medium.
4. After being centrifuged for another 10 min at 1000rpm., the supernatant was removed, cells were resuspended in 10 ml of complete medium and incubated at 37°C for 4hrs and 55min.
5. 0.1 ml of colcemide (0.1 μg/ml) was added to the culture for a further 10 to 20 min.
6. Cells were centrifuged at 1000rpm for 10 min, the supernatant was removed and cells were resuspended in 0.075M KCl (prewarmed at 37°C).
7. After incubating for 10min at 37°C, cells were centrifuged at 1000rpm for 10 min., the supernatant was removed and 5 to 10 ml of ice-cold prefix (70% ethanol) were added. The culture was placed at -20°C for 30min (at this stage, samples could be stored for months prior to further use).
8. After centrifuging for 10 min at 1000rpm, the supernatant was removed, and cells were resuspended in 1 to 2 ml of ice-cold fixative (3 parts methanol /one part glacial acetic acid) depending on the size of the pellet, by constantly agitating the tube.

9. Cells were immediately dropped onto clean, ice-cold coverslips and dried in a sterile hood.
10. They were then placed into a sterile container and stored at -20°C until further use.

2.2.5 G- banding

All solutions used were filtered into sterile universal containers.

Staining of coverslips was carried out in a safety fume hood cabinet. Coverslips were either left at room temperature prior to staining, or were incubated for 48 to 72 hrs at 37°C, in order to achieve better banding results.

2.2.5.1 Trypsin

1. Coverslips were immersed in trypsin solution for 8 to 15 sec.
2. They were then rinsed in saline solution and stained was applied (1 part of Leishmanns stain to three parts of buffer pH 6.8) for 2 min. and 30 seconds.
3. Coverslips were rinsed in buffer and then in distilled water to remove any excess of stain.
4. After air drying, they were ready to be used for microdissection

2.2.5.2 Lipsol

Alternatively, chromosomes could be banded by treating in 1% Lipsol solution.

1. Coverslips were immersed in Lipsol for 7 to 15 seconds.
2. They were then rinsed with saline and stained by using Leishmanns stain diluted in pH 6.8 buffer (1:3) for ~2 min. and 30 seconds.
3. Slides were then washed with pH 6.8 buffer.

2.2.6 Preparation of needles and microdissection

Borosilicated glass capillaries (containing a filament) with a size of 1.2mm-outer diameter and 0.68mm-inner diameter were used. Wearing gloves and handling with sterile forceps the capillaries were mounted on a Model P-87, Flaming/Brown micropipette puller (Sutter Instruments). By applying heat in the middle of the capillary and pulling forces at its opposite ends, two needles were produced, both with a very fine tip at the end. Careful adjustment of the four parameters available, was necessary to obtain optimal results (heat, pull, velocity and time). The needles were then rinsesd with 80% ethanol and were air dried by placing them in a sterile cabinet. Prior to microdissection, they were exposed to UV light and then, were used accordingly.

An OLYMPUS (IMT2-NIC) inverted microscope, with a Narishige hydraulic micromanipulator were used for microdissection. For each dissection, a separate needle was used. Ten to fifteen fragments from a specified region were dissected each time. The fragments were transfered inside a small Eppendorf tube (0.2 ml), by simply touching the tip of the needle to the 5μl of PCR reaction mix contained in the tube.

2.2.7 Synthesis and preparation of primers

2.2.7.1. DOP primer

A degenerate oligonucleotide primer (Telenius Universal primer) of 22 nucleotides in length with the following sequence was synthesised :

5'- C C G A C T C G A G N N N N N N A T G T G G - 3'

The primer was prepared by a '391 Synthesizer PCR-MATE' (Applied Biosystems) and was extracted from the columns as follows:

I. Oligonucleotide deprotection

1. 1 ml of ammonium hydroxide (40mM) was drawn into a polypropylene (blue) syringe and and was connected to one end of the column.
2. An empty syringe was attached to the other end.
3. By gently pushing the NH_3OH through the column, the barrel of the second syringe was displaced.
4. The NH_3OH was pushed back and forth through the column, 2 to 3 times, periodically for 2-3 hrs, ensuring that the column was always filled with liquid. The column should decolourise from yellow to white.
5. The NH_3OH was withdrawn and expelled into small Nunc tubes.
6. The volume was made up to approx. 2.5 mls by adding NH_3OH the cap was put on tightly and the primer was incubated in a 55°C waterbath, overnight.
7. Afterwards, the primer could be stored at -70°C.

II. Ethanol precipitation of oligonucleotides

1. 450 µl of the deprotected primer were aliquoted into a large (1.5 ml) Eppendorf.
2. 50 µl of 3M Sodium Acetate and 2 volumes of 95% ethanol were added and contents were agitated gently for 30 sec to 1 min.
3. The primer was then incubated at -70°C for 1hour and 30 min. and afterwards centrifuged for 20 min at 12000rpm
4. The supernatant was carefully removed and the pellet was washed with 85% ethanol (by spining for 2 min at 12000rpm). The ethanol was then discarded and the pellet air-dried.
5. The pellet was then resuspended in 200 µl of TE buffer and was stored at -20°C.

III. Estimation of DOP-primer concentration

Five microlitre of DOP-primer was added to 995 µl of dH_2O in a 1.5 ml Eppendorf and mixed well. The suspension was transferred into a quartz tube, the optical density was

measured at A_{260} in a UV/VB spectrophotometer and the concentration was calculated as follows:

As a useful approximation, 1 OD unit of a single stranded oligonucleotide consists of about 33 mg, by mass. One molar (1 M) of oligonucleotide has a number of OD units equal to10X the number of bases i.e. 220 for the 22oligomer DOP-primer.

Hence, the gram molecular weight = 220 X 33 = 7260 gr/L

1 M =7260 gm/L

i.e. 100 pmol correspond to 726 ng

Since, the final concentration of the primer should be 2μM = 100pmol per 50 μl PCR reaction, aliquots of 100 pmol are prepared after diluting accordingly the stock solution (following estimation of the concentration after the OD reading).

IV. COP (Cruachem Oligonucleotide Purification) column DOP-primer

After synthesis, the trityl-ON oligomer was deprotected following the procedure previously mentioned.

1. A syringe from which the plunger had been removed, was connected to the main body of a COP cartridge.
2. By holding it in a vertical position above a disposal beaker, 2 ml of acetonitrile solution were poured inside, the plunger was reinserted and the solution was gently pushed through the cartridge at a rate of 1-2 drops per second.
3. The cartridge was first removed from the main syringe body and then, the plunger was taken off.
4. By repeating the procedure of step 2, the cartridge was flushed with 2 ml of TEAA solution, and 2 ml of de- ionised water. (The amount of water added could affect the recovery of the purified primer, hence for 15-50 oligomers the recomended amount should be added, i.e. 2 ml).
5. The oligo primer solution was loaded to the cartridge as described in step 2.
6. The eluate was collected and loaded onto the cartridge for a second time.

7. The cartridge was then flushed sequencially by 3 ml of dilute ammonium hydroxide, 2 ml of de-ionised water and 2 ml of 2% TFA to detritylate the support-bound primer.
8. The purified primer was recovered by flushing the cartridge with 1 ml of 20% (v/v) acetonitrile-water.
9. The purified sample was evaporated to dryness and resuspending dH_2O inside a NUNC tube. Finally, the OD and the concentration were calculated accordingly.

2.2.7.2 Chromosome 3 centromere specific primers

Amplification of the centromere of chromosome 3 was performed using a set of primers, specific for the repetitive alphoid sequences present at that region. The sequence of the primers used was described as follows:

D3Z1(+):5'-TCT GCA AGT GGA TAT TTA AA-3' and
D3Z1(-): 5'-TGA GTT GAA CAC ACA CGT AC-3'

Primer concentrations were kept standard at 200 ng/μl each, per reaction. Amplification took place under the following conditions:

Cycle Temperature	Time	Cycles
95°C	3 minutes	1X
95°C	1 minute	30X
54°C	1 minute	
70°C	3 minutes	
70°C	10 minutes	1X

2.2.8 DOP-PCR amplification

2.2.8.1 Precautions

All procedures that involved PCR and all PCR reactions were performed in a containment hood in order to avoid any contamination. Gloves were worn all the time. A set of Gilsen pippettes were used strictly for the PCR reactions. Tips with a plaque filter in them were used to avoid any particles from the barrel of the pipette contaminating the PCR solutions. All solutions were prepared in deionised water (Millipore, "Milli RQ" water purification system). All glassware was autoclaved. Only sterile plasticware were used. All reagent solutions used were aliquoted into small quantities (i.e. one per experiment!) to ensure no contamination from other sources.

2.2.8.2 First DOP-PCR amplification

An MJ, PTC 100 Programmable thermal controller-cycler (Peltier-Effect cycling) was used for this amplification.

A collection drop of 5 μl PCR mix was added into a 0.2ml Eppendorf tube. The components of the mix were as follows:
A 1X collection buffer consisting of 40 mM Tris-HCl (7.5), 50 mM NaCl and 20mM $MgCl_2$, along with 7 pmol of DOP-primer (100μM stock concentration) and 0.2mM each dNTP, adjusted with dH2O.

Once the dissected fragments were collected inside the collection drop of the tube, 0.1 μl of Topoisomerase I enzyme (1 unit) was added to allow relaxation of the coiled DNA. Following an incubation at 37°C for 30 min. the reaction was terminated by heating at 96°C for 10 min. Amplification was initiated with 7 cycles at low annealing and extention temperatures in order to produce primer- terminated DNA copies. For this, ~0.3 units of T7 DNA polymerase (Sequenase Version 2.0, USB) were added to the 5μl reaction mixture. More specifically, the enzyme was diluted 1 to 8 in enzyme

dilution buffer (Sequenase, 13units/μl) and 0.2μl were added after the denaturation step of each cycle.The thermal cycle program was carried out as follows:

Cycle Temperature	Time	Cycles
94°C	1minute	7X
30°C	1 mins 30 sec	
37°C	2 minutes	

Once the last cycle was completed, 45 μl of PCR reaction mixture were added on top, containing PCR buffer (10mM Tris-HCl of pH 8.4, 50mM KCl, 2mM $MgCl_2$) along with 75 pmol of DOP-primer and 0.2mM dNTP, adjusted with dH2O. After a denaturation step of 95°C for 3 min was performed, 0.8 μl of Taq polymerase LD (high purity Perkin Elmer product) were added (Hot Start PCR to avoid non-specific annealing of the primer, before the correct annealing temperature is reached) to the reaction mixture and the following cycles were performed:

Cycle Temperature	Time	Cycles
94°C	1minute	35X
56°C	1 minute	
72°C	3 minutes	

An extention of 72°C for 10 min completed the reaction.

2.2.8.3 Second DOP-PCR amplification

For this amplification a Perkin-Elmer/Cetus DNA thermal cycler 480 was used. An aliquot of 5 μl template DNA from the previous amplification was added to a new Eppendorf (0.5 ml), in a reaction mixture containing the following components:

5 μl of 10X PCR buffer (Perkin-Elmer/Cetus), 5 μl dNTPs (0.2mM each) and 150 pmol of DOP-primer, adjusted to 50 μl with dH_2O. After a denaturation step at 95°C for 3 min, 0.5 μl of Taq polymerase (5 units/μl-Perkin Elmer) were added to the reaction mix. This was then overlayed with an equal volume of parafin oil and the high annealing temperature cycles were repeated 30 times.

Cycle Temperature	Time	Cycles
94°C	1 minute	30 X
56°C	1 minute	
72°C	1 minute	

The reaction was completed after an extention step at 72°C for 10 min.
Fewer cycles (15-25) could give the same yield of PCR product, thus minimising the duration of the experiment. In addition, the time of the 72°C extension step could be decreased to just 5 minutes.

2.2.8.4 Gel electrophoresis of the DOP-PCR products

Visualisation of the PCR products took place in the following way:
1.2 gm of agarose was dissolved in 60 ml of 1X TBE buffer at a microwave oven for 3 minutes. When the temperature reached less than 60°C, 2 μl of 1% ethidium bromide were added to the mixture to enable visualisation of the DNAs. The gel (2%)was poured onto the electrophoresis gel casting unit. When the agarose solidified, the DNA samples were loaded. 4 μl aliquots of amplified DNA and 4μl of 1 kb ladder (in order to

determine the size of the products) were individually mixed with 1 µl of loading mix and loaded on the gel. They were run at 100mA for one hour. Products were visualised using a UV light transilluminator (257 nm) coupled with a black and white Sony monitor(SSM-920CE). The images were printed by a Sony video graphic printer (UP-860 CE).

2.2.9 Probe labelling

Two ways of labelling the amplified product could be followed:

I. Directly by DOP-PCR and II. by nick translation.

2.2.9.1 DOP-PCR labelling

Labelling took place during the second amplification reaction (i.e. high annealing temperature cycles) by adding 20µM biotin -11 -dUTP as the reporter molecule to the reaction. Accordingly, the concentration of dNTPs was slightly altered, by decreasing the concentration of dTTP in the mix to 160µM (instead of 200µM).

Once the thermal cycle program was completed (30 cycles and a 72°C extension step), a purification step was carried out to remove any unlabelled nucleotides from the mix.

I. Purification of the DOP-PCR labelled probe

The entire amplified product was then purified on BioSpin P6 columns (BioRad) according to the manufacturer's instructions:

1. The column was left at room temperature, for the gel to melt and settle (after removing it from the fridge, where it was kept stored)
2. Its top cap was removed and the snap-off tip was snapped off to allow excess buffer to drain by gravity.
3. The column was placed in a collection tube and centrifuged for 2 min at 2500rpm.

The collection buffer was discarded and the sample (~45 µl) was applied directly to the center of the column allowing the liquid to drain into the gel between successive drops of the sample.

4. The column was placed in a clean collection tube and was centrifuged at 12000rpm (9500g) for 4 minutes.

II. Precipitation of the purified probe

The purified product was ethanol precipitated by adding 1/10 volume of 3M NaAc, 1 µl glycogen and 2 volumes of ice-cold 85% ethanol. After placing at-70°C for 30 min, the sample was centrifuged at 12000rpm for 20 min, the supernatant was discarded and the pellet was dried in a vacuum dessicator. At the end, the pellet was suspended in 25 µl of 1X TE buffer and the purified-labeled probe was stored at -20°C until further use.

III. Measurementof probe concentration

The concentration of the DNA probe was estimated by measuring the optical density (O.D.) at 260 nm in a spectrophotometer. 5 µl of aliquot probe DNA was added to 995 µl of dH_2O, vortexed and then measured. The concentration of DNA in the suspension was calculated with the following formula:

Cons. = ODA_{260} x DF x 50 where Cons.= Concentration of the probe at µg/ml or ng/µl, A_{260} = Absorbance at 260nm wavelength, DF= Dilution factor (X200) and 50 is a constant corresponding to 50 µg DNA per ml at 260nm per 1 unit OD.

2.2.9.2 Nick translation

1. Following the DOP-PCR, the amplification products were precipitated by ethanol. To the 50µl of product, 1/10 volume of 3M sodium acetate (NaAc) along with 1 µl of glycerol (20 mg/ml) were added. After adding 2X volume of ice cold 85% ethanol, the contents of the tube were mixed by tapping the tube. It was then placed

at -70°C for 30 min (or at -30°C for few hours or overnight) to allow DNA precipitation. The tube was then spun for 20-30 min at 14000rpm. The supernatant was carefully discarded and the pellet was air dried for one hour. Alternatively, the tube was placed for 15 min in a vacuum dessicator to speed up the process.

2. Afterwards, the pellet was dissolved in 20μl of 1X TE buffer and the OD reading of the PCR product was measured at A_{260} nm.
3. A nick translation kit was used and its components were added sequentially to a 1.5 ml Eppendorf tube kept in ice as follows:

Solution A4 (dATP, dCTP, dGTP)	5 μl
DNA to be labeled	1 μg
Biotin-11-dUTP	3 μl

The volume was adjusted to 45 μl with distilled water provided in the kit (solution E). 5 μl of DNA polymerase I enzyme (solution C) were added, the contents were mixed well and incubated at 15°C for 90 min. The reaction was stopped by adding 5 μl of stop buffer (solution D).

4. The labelled product was purified by ethanol precipitation in order to remove any unincorporated nucleotides. 4.6 μl of NaAc and 1 μl glycogen along with 122 μl of ice-cold 85% ethanol were added to the probe. The contents were mixed well, placed at -70°C for 30 min and spun at 12000rpm for 30min. Once the supernatant was removed, the pellet was dried and dissolved in 20 μl of 1X TE buffer. It was then stored at -20°C until further use.

2.3 DOT-BLOT ASSAY TO TEST LABELLING

In order to test the biotin-labelling of the probe, the following assay was performed by using at the same time as a control commercially available standard or previously used, well-labelled probe:

1. 1 μl aliquots of the different dilutions of standard-control DNA and, in parallel, 1 μl aliquots of the same concentrations of test DNA were applied on nitrocellulose filter (Hybond N+, Amersham).
2. The filter was baked for 1 hour at 80°C.

4. The filter was sealed in a plastic bag, together with 10 ml of blocking solution and was incubated at 37°C for 30 min.
5. The bag was then opened at one end, the blocking solution was removed and a freshly prepared solution of streptavidin-conjugated alkaline phosphatase (diluted to a concentration of 1μg/ml in AP 7.5) was added. The bag was sealed again and incubated at 37°C for 30 min.
6. The filter was removed from the plastic bag, washed in a dish with AP 7.5 buffer (twice for 5 min at room temperature), followed by AP 9.5 buffer (10 min at room temperature).
7. The filter was sealed again in a plastic bag, together with the developing solution: 33 μl of NBT were added to 5 ml of AP 9.5 buffer. After careful mixing (no vortex!), 25 μl of BCIP was added; the resulting solution was again mixed gently. It was then incubated at 37°C until colour development was appropriate. Any time between 15-60 min was concidered as sufficient.
8. The filter was taken out of the plastic bag and washed in a dish with TE buffer in order to stop the colour reaction.
9. After air drying, the assay was evaluated: the colour intensities of the test and control DNA should be comparable.

2.4 PRE-TREATMENT OF SLIDES FOR FISH

Glass microscope slides were placed in 5% Decon solution overnight. They were then rinsed thoroughly under running tap water, for at least two hours and stored in 70% ethanol till used. After rinsing them again thoroughly under tap water, cell suspension obtained by conventional cytogenetic techniques was dropped on them. Slides were examined under the microscope (phase contrast) and areas carrying an adequate number of metaphase cells were marked. They were then fixed for 1 hour in 3:1 methanol/acetic acid and then air dried. Afterwards, they were de-hydrated in an ethanol series (50%, 70%, 90%, 100%) and placed in a 42°C oven overnight. Following this, 100 μl of RNase (100μg/ml) were added on each slide. Coverslips were placed on top and slides were then incubated at 37°C for one hour. This step was necesssary to remove any endogenous hybridisable RNA. Slides were then washed

twice in 2X SSC with agitation (3 min each). Prior to denaturation they were passed through a series of alcohols and air dried.

2.5 DENATURATION AND HYBRIDISATION

2.5.1 Probe preparation and denaturation

Three types of DNA probes were used:

A. Human genomic DNA as a positive control. This was mainly used at a concentration of 100-150 ng for FISH. Cot-1 DNA (0.5-1.0 mg/μl) was also added to the hybridisation mixture in order to test the efficiency of the chromosome painting under suppression conditions.

B. Whole chromosome four paint. This was obtained by the FACS technique, where the starting material (25-30μl), contained approximately 300 copies of the specific chromosome. Cot-1 DNA was added in the hybridisation mixture in order to suppress any cross-hybridisation with commonly shared sequences of other chromosomes. Two hybridisation areas were tested on each slide : one with and the other one without Cot-1 DNA.

C. Several regions from different chromosomes were dissected for the purpose of micro-FISH. These included band regions, centromeric regions, half arms, whole chromosome arms and whole chromosomes. In most of the cases, ~150 ng of the micro-FISH probe was added together with Cot-1 DNA (0.5-1.0 mg/μl) in the hybridisation mixture.

1. Approximately 100-150 ng of the labelled probe were mixed with 0.5-1.0 μg of Cot-1 DNA in 10 μl of hybridisation buffer.
2. The mix was denatured for 10 minutes in a 70°C water bath and was then placed in a 37°C waterbath for one hour.

2.5.2 Denaturation of fixed chromosome preparations

1. Slides were denatured by placing them in a 70% prewarmed formamide solution, at 70°C for 3 minutes.
2. They were then dehydrated through a series of ice-cold ethanols (70%, 90%, 100%), three minutes each, and were finally air-dried for 10-15 minutes.

2.5.3 Hybridisation

1. The probe mix was removed from the 37°C waterbath, briefly spun and added directly on the marked area of the slide.
2. When Cot-1 DNA was not added to the mix, the probe was resuspended in hybridisation buffer and denatured at 70°C for 10 miutes (while air-drying the denatured slides). The mix was very briefly spun and was immediately added to the slide.
3. A coverslip was placed on top of the slide, sealed with rubber cement and the slide was hybridised overnight at 39 to 42°C, in a water bath.

2.6 POST-HYBRIDISATION WASHES

1. Washing solutions, inside glass coplin jars, were prewarmed in a 42°C waterbath.
2. Slides were first placed in 2X SSC (room temperature) for 5 minutes to loosen the rubber cement.
3. After carefully removing the sealant with forceps, slides were placed in 2X SSC at 42°C for 5 minutes. By briefly agitating, coverslips would come off.
4. Slides were then washed at 42°C, in three 50% formamide washes, 5 minutes each.
5. This was followed by two washes in 2X SSC, 5 minutes each, at 42°C.
6. Slides were then blocked in wash A solution (250 µl of Tween-20 detergent; 25gm of non-fat dried milk made up to 500 ml with 4X SSC), for 30 minutes at 37°C.

2.7 DETECTION

1. 100 µl of 5 µg/ml FITC conjugated avidin (diluted in filtered wash A solution) were applied onto each hybridisation area. The slide was covered with parafilm and incubated at 37°C for 20 minutes.
2. After removing the parafilm, slides were washed three times in wash A (5 minutes each), at 42°C.
3. After carefully draining the reagent off the slides, 100 µl of 5 µg/ml biotinylated anti-avidin (diluted in filtered wash A) were applied on each hybridisation area and the slides were incubated again, at 37°C for 20 minutes.
4. Slides were washed three times in wash A (5 minutes each) at 42°C.
5. Finally, a second layer of 100 µl (5 µg/ml) FITC conjugated avidin was added to each area of hybridisation, slides were covered with parafilm and were left at room temperature for 20 minutes.
6. Slides were then washed in wash A (three times, 5 minutes each) at 42°C, and in 4X SSCT (0.5% Tween 20), twice (3 minutes each) at room temperature by agitation.
7. Afterwards, they were washed in PBS solution for 5 minutes and finally dehydrated through an ethanol series (50%, 70%, 90% and 100%) and were air dried.
8. Since the cells displayed FITC (green) signals, they were counterstained using both DAPI and Propidium Iodide as follows:

0.75 µl of DAPI (40 µg/ml) and 1.2 µl of Propidium Iodide (20 µl/ml) were resuspended in 85 µl of mounting medium AF1 (Citifluor Ltd.). Twelve microlitre of the suspension were applied on each hybridisation area and a 22x22mm coverslip was placed on top and sealed with nail polish. Slides were stored at 4°C till examination under the microscope. Alternatively, slides were counterstained only with DAPI. 4 µl of DAPI (stock: 1 mg/ml) were added into a coplin jar containing 50 ml of 2XSSC. Slides were immersed for 5 min and then left to air dry. Ten microliter of antifade solution were added to each hybridisation area and a coverslip was placed on top.

2.7.1 Alternative reagents in blocking step and detection

Instead of Wash A (4X SSCT, nonfat dry milk), Human Serum type AB (17%) could be used as a blocking reagent. This was prepared by simply adding 300 µl of AB Human serum into 1700 µl of 4X SSCT.

1. After processing the slides through a series of two washes in 50% formamide (7 minutes each, at 42°C), one wash in 2X SSC (5 minutes at 42°C) and one wash in 4X SSCT (5 minutes at room temperature), 100 µl of the prepared human serum blocking agent were applied to each slide and incubated at 37°C for 15 minutes.
2. 100 µl of 5 µg/ml FITC conjugated avidin (diluted in filtered human serum/4X SSCT blocking agent) were applied onto each slide. The slides were covered with parafilm and incubated at 37°C for 30 minutes.
3. They were then washed three times in 4X SSC (3 minutes each at 42°C).
4. A second layer of 100 µl Anti-avidin FITC was applied onto each slide and incubated at 37°C for 30 minutes.
5. Following three washes in 4X SSC (3 minutes each) at 42°C, slides were dehydrated in a series of alcohols as previously mentioned.
6. They were then stained by immersing in 50 ml of 2X SSC containing 4 µl of DAPI stock solution (1mg/ml), at room temperature for 5 minutes.
7. After being airdried, 10 µl of citifluor were added per hybridisation area, coverslips were mounted with nail polish and slides were stored at 4°C.

2.8 VISUALISATION

A Zeiss Axioskop epifluorescence microscope equipped with a cooled CCD camera (Photometrics, KAF 1400) was used in order to analyse the results obtained by FISH. The CCD camera was controlled by an Apple Macintosh computer (Quadra 950) and a Smartcapture software (Digital Scientific). The images were first visualised under the microscope using a motorised excitation filter set and an appropriate emission filter block. They were then captured and processed by using the Smartcapture software which allowed automated colour image capturing. The captured images were printed on sublimation printing papers (A4-SPW), using a Mitsubishi colour printer.

2.9 SOUTHERN BLOTTING

2.9.1 Digestion of genomic DNA

Approximately 10 μg of human genomic DNA was digested using the appropriate restriction endonuclease in a total volume of 40 μl containing 1X appropriate enzyme buffer and 40 units of the specific enzyme (Not 1). The digest was incubated overnight in a water bath at 37°C.

2.9.2 Agarose gel electrophoresis

The digest was resolved in 20 cm 0.8% agarose gel prepared in 1X TAE buffer and containing 0.5μg/ml ethidium bromide (10mg/ml). Samples were prepared by adding 4 μl of 6X agarose gel loading buffer (0.25% bromophenol blue, 40% w/v sucrose in 1X TBE buffer) to the 40 μl digest. Samples were then loaded alongside 1 kb ladder (Gibco BRL) in the gel and electrophoreses at 52 volts/cm for 4 hours. After electrophoresis the gel was visualised and photographed under UV light (254nm) and the wells were then cut off. The gel was then transferred to a plastic box, upside down and rinsed in water. It was then treated in a depurination solution (0.2M HCl) for 15-20 min, then rinsed in water. The gel was transfered to a denaturation (0.5M NaOH, 1.5M NaCl) solution for 30 min, then rinsed again in water and neutralised in two changes of neutralisation solution (3M NaCl, 0.5M Tris pH 7.5) for 15 min each.

2.9.3 Setting up the transfer apparatus and Southern transfer of digested DNA

The transfer apparatus comprised a tray with a raised platform for the gel to sit on. The tray was half filled with 1.5 liters of 10X SSC. The platform was covered by double layer of 3MM Whatman paper wick with its ends dipped into the SSC. The 3MM paper was left to soak the SSC solution and became completely wet. Any air bubbles between the paper and the platform were smoothed out. After the neutralisation step the gel was placed on the wick on the platform and again any air bubbles between the gel and the 3MM wick paper were smoothed out. A gel-size Hybond N membrane was cut,marked

using a permanent pen, wet in 2X SSC and layered on to the gel taking care to get rid of any air bubbles that were trapped between the membrane and the gel. A gel-size double sheet of 3MM paper was wet in 2X SSC and layered on to the membrane followed by a stack of absorbent paper (paper towels).

A glassplate and a weight of approx. 500 gr were laid on top of the absorbing paper stack. The blotting was carried out for 12-16 hours at room temperature. The Hybond N membrane was rinsed in 5X SSC to remove residual agarose , placed between two sheets of 3MM paper and was exposed in UV light for 4 min to crosslink the DNA fragments to the Hybond N membrane. The filter was then wrapped in a Saran wrap and stored in the cold room (4°C) until hybridisation.

2.9.4 Labelling of the human genomic DNA

The human genomic DNA probe was removed from 37°C and was radioactively labelled using the random primed DNA labelling kit (Boehringer Mannheim) according to the manufacturers protocol. 23 µl of the probe (100ng) was boiled for 6 min, briefly spun and incubated at 37°C for one minute. The probe was then added to a mix of 2 µl each of dATP, dGTP and dTTP, 1.5µl of Klenow enzyme, 4 µl of enzyme buffer and 6 µl of ^{32}P dCTP (Amersham) and the contents of the tube were mixed by repeated pipetting. The tube was then incubated at 37°C for two hours and the reaction was then stopped by the addition of 2 µl of 0.5M EDTA. The labelled probe was then separated from the unincorporated ^{32}P dCTPusing NICK^{TM} columns (Pharmacia Biotech.) which were prepacked columns containing sephadex G-50 DNA grade. The column was rinsed once with 3 ml 1X SSC by allowing it to drip through by gravity. The labelled probe (~40 µl) was added to the top of the suspended column and then 400 µl 1X SSC was added to the column and allowed to drip through. A Sarstedt tube was then placed under the column and a further 400 µl 1X SSC was added to the top of the column and collected. This contained the labelled probe after its separation from the unincorporated ^{32}P dCTP. Two microlitre of the collected probe were added to ascintillation vial along with 1.5 ml ecosint and put into a scintillation counter to count.

2.9.5 Prehybridisation of filter

About 10 ml of QuikHyb solution (hybridisation solution; Stratagene) were added into a plastic universal and placed in a 65°C oven for 10 min for the solution to become less viscous. Approximately 50 ml of tap water were added to a hybridisation cylinder bottle. The filter was placed into a flat plastic tray containing tap water. It was rolled up and put into the hybridisation bottle. The bottle was rotated until the filter had attached itself to the sides. The water was then poured off and the bottle was turned upside down to allow any excess to run off. About 1 ml of the QuickHyb solution was left in the universal and the rest was added to the bottle containing the filter. Both, the bottle with the filter and the universal were placed into the oven for 15 minutes. Following that, 100 µl of salmon sperm DNA (10 mg/ml) were added to the already labelled probe and were boiled for 2 min (denaturation step).

2.9.6 Hybridisation of filter

While the probe was boiling, the universal containing the 1 ml of QuickHyb solution was removed from the oven. The bottle containing the filter was also taken out. After the required 2 minutes denaturation, the probe/salmon sperm DNA was added to the universal and mixed well. It was then added to the bottle and placed in the oven for one hour.

2.9.7 Washing the filter after hybridisation

After hybridisation, the Quik Hyb solution was discarded and the filter was rinsed in 0.1% SDS/2X SSC while inside the bottle. The filter was washed once by adding 10 ml of 0.1% SDS/2X SSC to the bottle and returning it to the 65°C hybridisation oven for 10min. The filter was then taken out from the bottle and placed in a plastic tray with 0.1% SDS/0.5X SSC and washed by shaking at 65°C for a further 10 min. The filter was monitored using a series 900 minimonitor and if a signal greater than 10 cpm (count per minute) was detected, it was then washed at increasing stringency (65°C,

01% SDS/0.25X SSC then 0.1% SDS/0.1X SSC) taking care not to over wash it (filter was monitored every 3 min).

2.9.8 Autoradiography

After washing the filter, it was briefly dried and covered by a plastic wrap. It was then placed in an autoradiography cassette with intensifying screens and exposed to Kodak Diagnostic AR Imaging film with the DNA side of the filter facing the film. The position of the upper border of the filter was marked on the film surface so that a ruler could be used to estimate the size of the bands detected. The cassette was stored in a -40°C freezer for 2 to 7 days before developing the film.

CHAPTER 3

RESULTS

CHAPTER 3: RESULTS

3.1 OPTIMISATION OF DOP-PCR USING HUMAN GENOMIC DNA

3.1.1 Selecting human genome DNA samples

The first step in optimising the conditions for DOP-PCR was to test the several parameters involved, by using as a control target material, human genomic DNA. For this, good quality samples are necessary. Several extracted DNA samples were tested. The OD value for each of them was measured and the concentration was calculated. After appropriate dilutions, 10 ng per µl was chosen as the concentration of each target DNA per PCR reaction. Low OD reading samples were excluded from this experiment.

A 50 µl PCR reaction (table 3.1.1) was carried out by first denaturing the contents at 95°C for 5 minutes. Two and a half units of Taq polymerase were added to the mix and eight cycles of amplification at a low annealing temperature (30°C) were performed. The annealing temperature was then increased to 62°C (as recommended by Telenius et al. 1992) and samples were further amplified through a series of 30 cycles. Finally, a 10 minute extension step at 72°C was performed.

	Buffer	dNTP's	DOP primer	H_2O	Taq polymerase	DNA
Final concentration	1X	0.2 mM each	2µM		2.5 units	10 ng
volume	5 µl (10X)	5 µl	1 µl	34.5 µl	0.5 µl	1 µl

Table 3.1.1 Final concentrations of the DOP-PCR components in a 50 µl reaction mix.

PCR reagents were commercially available. The buffer consisted of 100mM Tris-HCl pH 8.3, 500mM KCl and 15mM $MgCl_2$. Five samples were amplified. A tube containing all PCR reagents, but no target DNA, was used as the negative control for testing for any contamination present in the reaction mix. The results were analysed on a 2% agarose gel in 1X TBE buffer (Figure 3.1.1).

A strong smear of numerous multiple bands was produced from the amplification of each sample. The absence of smear/bands from the negative control lane indicated that the PCR reaction was free of contamination. The size range of the smears varied among the five samples, with sample 3 (~150 bp to 290 bp) showing the smallest DNA fragments, in contrast to sample 4 which had a smear with a great intensity from ~270 bp to 1.0 kb. The rest of the samples showed the expected smear of multiple bands from 220 bp to ~600 bp. Sample 4, along with samples 1 and 2 were used as controls in further experiments.

3.1.2 Testing the efficiency of DOP-PCR on low concentration DNA samples

To ensure successful amplification of the minute amounts of microdissected material (600 fg = $600x10^{-6}$ ng = $6x10^{-13}$ gm), the efficiency of DOP-PCR on low concentration DNA samples had to be tested. A series of dilutions (10 ng, 5 ng, 1 ng, 0.9 ng and 0.8 ng) on human genomic DNA were carried out and the diluted samples were successfully amplified by one round of DOP-PCR which included the low annealing temperature cycles (Figure 3.1.2). A smear of 220 bp to 1.4 kb was visualised on all samples tested, with the greatest intensity between 400 bp and 1000 bp. No contamination was present in this reaction (lane 1).

A series of further dilutions (550 pg, 400 pg, 250 pg and 100 pg) were carried out on the scale of pg/µl . Following the first round of amplification, samples were subjected to a second round of DOP-PCR, though, by omitting the eight cycles of low annealing temperature. Aliquots of 5 µl from the first PCR were used as a DNA template. Because of the low DNA concentration, PCR products were not visible after the first amplification. However, visualisation of the second round PCR products revealed a

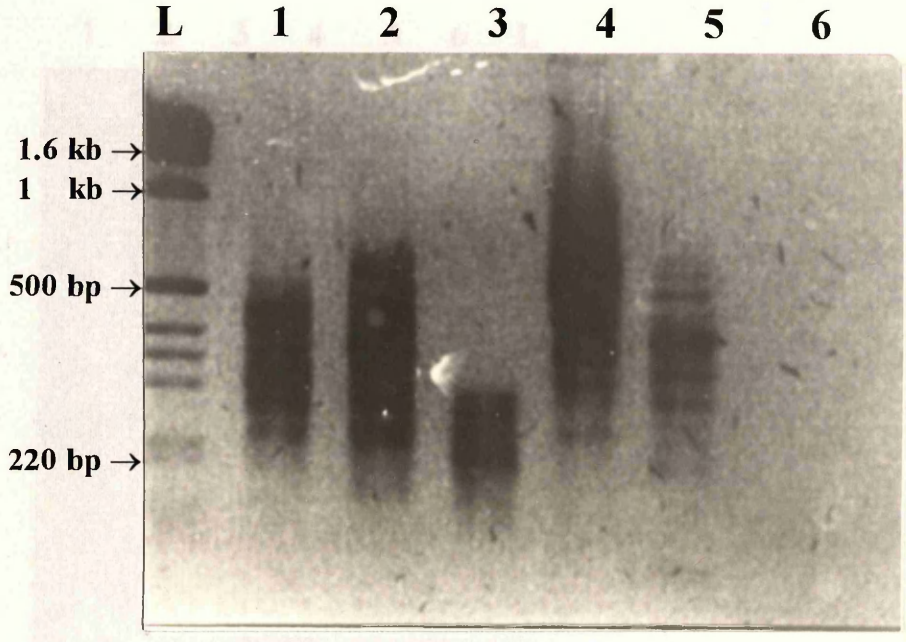


Figure 3.1.1. Agarose gel electrophoresis of DOP-PCR amplified human genomic DNA samples

L, 1kb ladder.

Lanes **1-5,** 10 ng/μl human genomic DNA samples.

Lane **6,** PCR reaction with no template DNA.

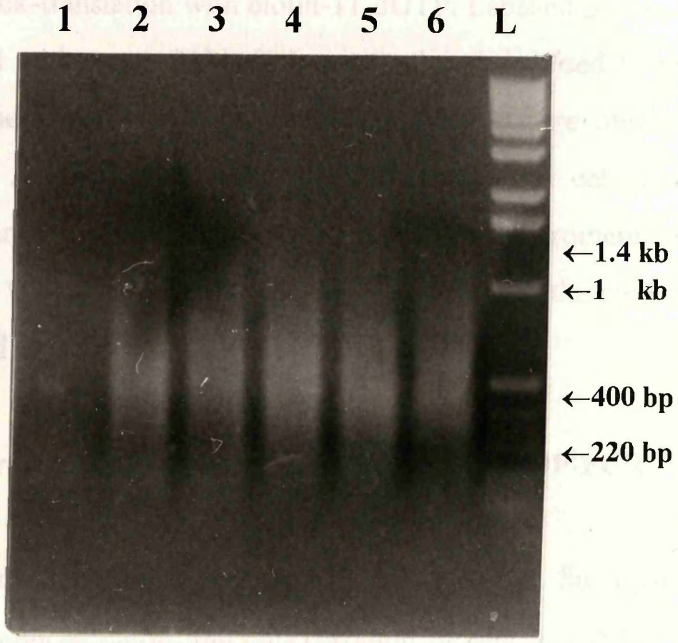


Figure 3.1.2 DOP-PCR products of human genomic DNA dilutions

Lane **1,** negative control.

Lanes **2-6,** different concentrations (ng/μl) of human template genomic DNA:

Lane **2,** 10 ng. Lane **3,** 5 ng. Lane **4,** 1 ng. Lane **5,** 0.9 ng. Lane **6,** 0.8 ng.

L, 1 kb ladder.

smear of 200 bp to 800 bp with a strong intensity around the 430 bp region in all PCR samples (Figure 3.1.3). Absence of smear from the negative control (lane 5) showed that no contamination was present in this reaction.

Four of the amplified products were randomly selected for further experiments (5 ng, 800 pg, 400 pg, 100 pg). These were ethanol precipitated, resuspended in 1X TE buffer and labelled by nick-translation with biotin-11-dUTP. Labelled probes (100 ng/μl) were first pre-annealed with cot-1 DNA (0.5 μg/μl), then hybridised to normal metaphase cells from peripheral blood samples. Results by FISH were obtained from all four amplified probes. All chromosomes present in the metaphase cells were evenly painted by the total genomic DNA probes apart of the centromere regions of some chromosomes (1, 9 and 16) that were left unpainted due to the competitor DNA, Cot-1(Figure 3.1.4 a-d).

3.1.3 Testing parameters involved in the efficiency of DOP-PCR

Several parameters could affect the efficiency of DOP-PCR. Such parameters involved the DOP primer concentration, the concentration of $MgCl_2$ and the primer annealing temperature. It was necessary to either reconfirm or adjust accordingly the values of these parameters established from the previous studies, by investigating the working conditions under which low concentrations of target DNA could be amplified successfully.

3.1.3.1 DOP primer concentration

Four different DOP primer concentrations were tested, each of them on two target DNA samples of 1 ng/μl and 500pg/μl respectively (Table 3.1.2). The rest of the PCR reagents (200μM each dNTPs, 1X buffer and 2.5-3.5 units Taq polymerase) remained at a constant volume.

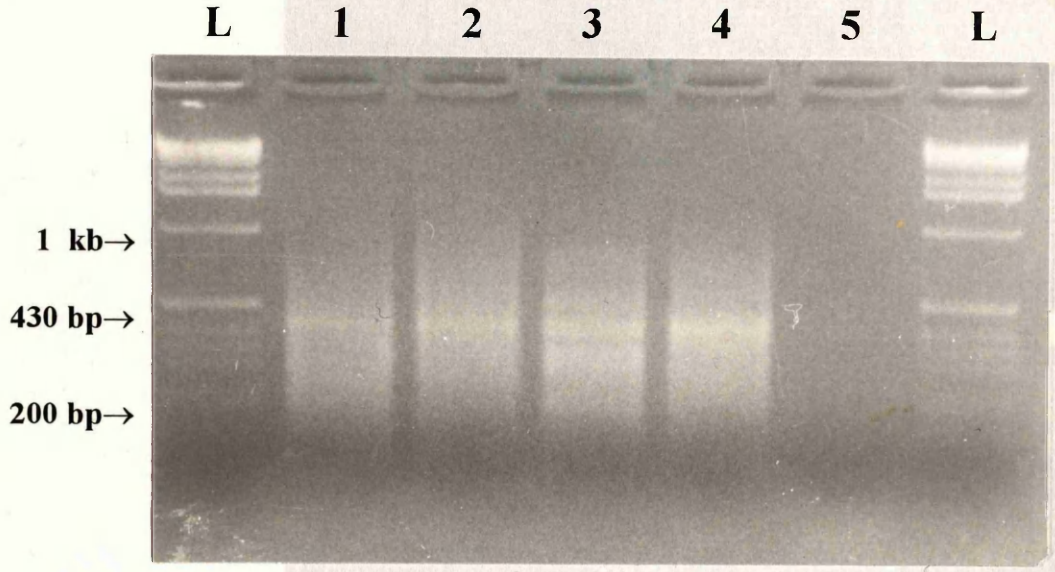


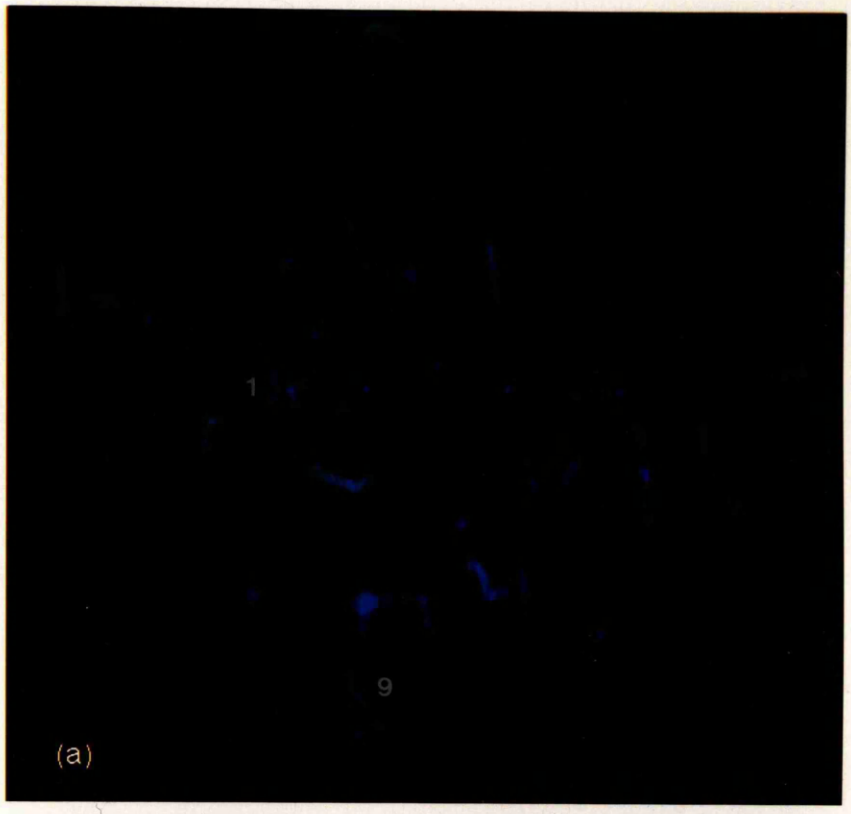
Figure 3.1.3 DOP- PCR products of human genomic DNA dilutions

L, 1kb ladder.

Lanes **1-4,** human genomic DNA dilutions (pg/μl): Lane **1,** 550 pg DNA. Lane **2,** 400 pg DNA. Lane **3,** 250 pg DNA. Lane **4,** 100 pg DNA.

Lane **5,** negative control (no template DNA).

L, 1 kb ladder.



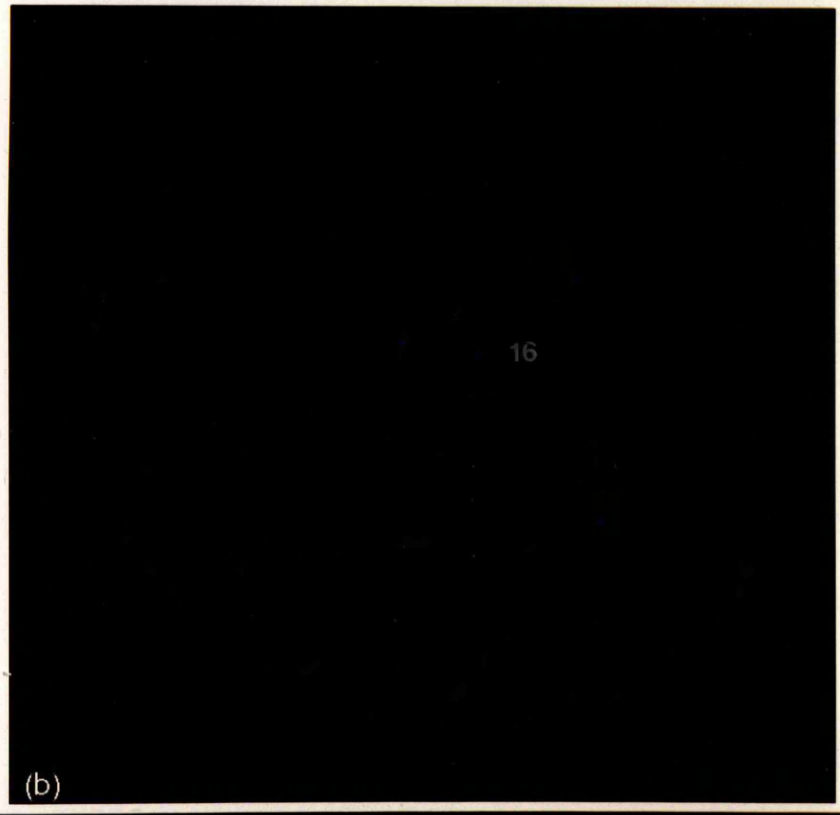


Figure 3.1.4 (a-b) : Hybridisation of human genomic DNA probes to normal lymphocyte cells. Biotin-labelled probes generated from the amplification of **(a)** 5 ng and **(b)** 0.8 ng human genomic DNA. Chromosomes are painted green (FITC) whereas unpainted parts are stained blue (DAPI).

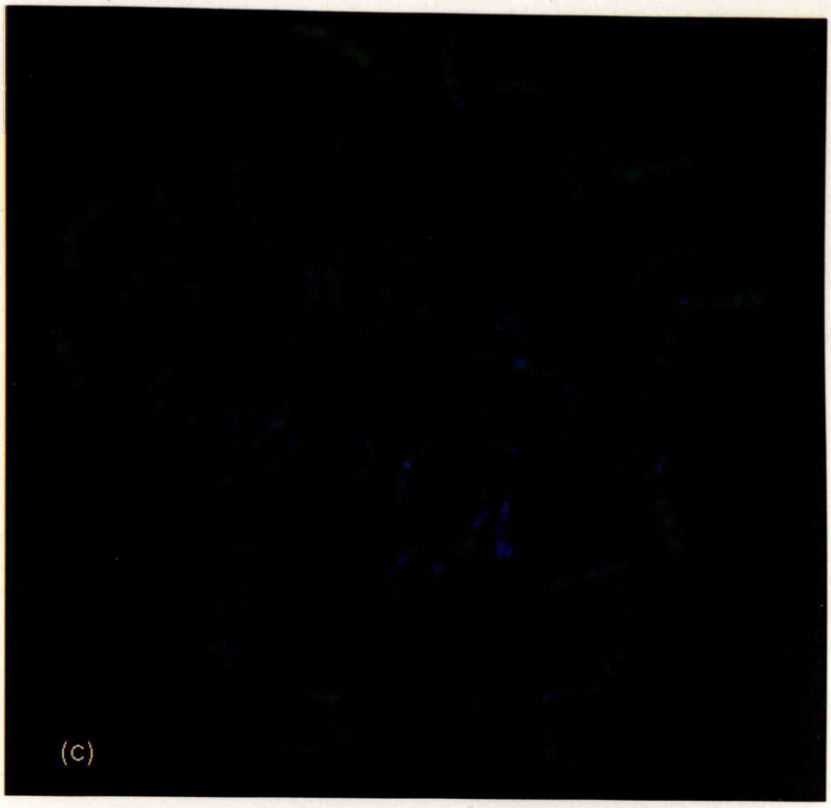


Figure 3.1.4 (c-d) : Hybridisation of human genomic DNA probes to normal lymphocyte cells. Biotin-labelled probes generated from the amplification of **(c)** 0.4 ng and **(d)** 0.1 ng human genomic DNA. Chromosomes are painted green (FITC) whereas unpainted parts are stained blue (DAPI).

Final DOP primer concentrations			
1μM	2μM	3μM	4μM
Human genomic DNA concentration			
1 ng/μl		0.5 ng/μl	

Table 3.1.2 DOP primer concentrations tested over dilutions of human genomic DNA.

Both DNA targets were successfully amplified under all four different DOP primer concentrations and with the absence of contamination (Figure 3.1.5). High molecular weight DNA fragments were produced in almost all amplifications (apart of lane 5; 0.5 ng target DNA), with a smear of 298 bp to above 12 kb. The intensity of the smear was slightly stronger for the reactions of 3μM and 4μM primer concentration. However, no difference was observed at the painting of the chromosomes produced by FISH under the four primer concentrations. It was therefore decided to use 2μM of the primer for all subsequent reactions as less would be insufficient for the PCR reaction whereas higher concentrations would increase the chances of non specific product formation such as primer artifacts or contaminants, especially over the presence of minute amounts of DNA template.

3.1.3.2 Magnesium concentrations

The concentration of magnesium (Mg^{++}) is considered to be an important factor that can influence primer annealing, formation of primer dimer artifacts and strand dissociation. For this purpose, a commercial PCR buffer that did not contain $MgCl_2$ was used so that the concentration of the $MgCl_2$ could be assessed separately. The amplification of diluted samples of human genomic DNA were investigated at four different $MgCl_2$ concentrations (Table 3.1.3).

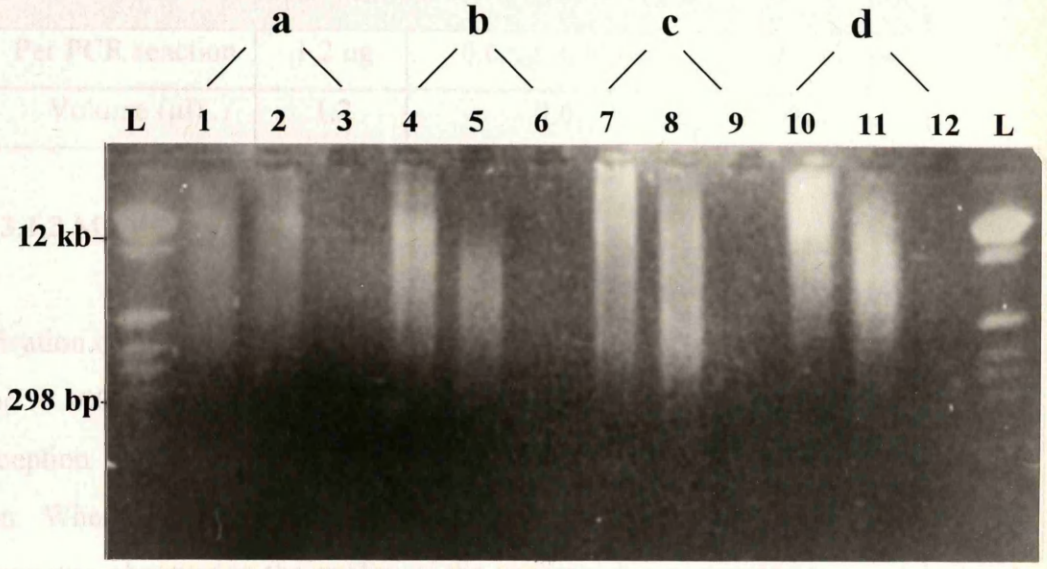


Figure 3.1.5 Gel electrophoresis of human genomic DNA amplified under four different DOP primer concentrations

L, 1 kb ladder.

(a) 1 mM DOP primer. **(b)** 2 mM DOP primer. **(c)** 3 mM DOP primer. **(d)** 4 mM DOP primer.

Lanes **1, 4, 7, 10,** 1 ng human genomic DNA template.

Lanes **2, 5, 8, 11,** 0.5 ng of DNA template.

Lanes **3, 6, 9, 12,** negative controls (no template DNA).

L, 1 kb ladder.

Final $MgCl_2$ concentrations				
Per PCR reaction	1.5 mM	2 mM	3 mM	4 mM
Volume (µl)	3	4	6	8
Human genomic DNA concentration				
Per PCR reaction	1.2 ng	0.6 ng (600pg)		0.3 ng (300 pg)
Volume (µl)	1.2	0.6		0.3

Table 3.1.3 $MgCl_2$ concentrations tested over dilutions of human genomic DNA.

Amplification of the target DNA was successful in all cases (Figure 3.1.6). A smear of ~400 bp to 1000 bp appeared in all lanes along with a distinct band of ~ 430 bp, with the exception of the negative control indicating absence of contamination from the reaction. When the amplified DNA samples were used as probes for FISH, no difference was observed in the quality of the painting. It was decided to use 1.5mM of $MgCl_2$ in the reaction, as higher concentrations can lead to generation of non-specific amplification products.

3.1.3.3 Primer annealing temperature

The low annealing temperature of the first PCR reaction remained constant in all amplification reactions performed. For the high annealing temperature cycles, three different temperatures (52°C, 56°C and 62°C) were tested in order to ensure sufficient amplification of low concentration DNA samples under such conditions. Both the first and the second round PCR reactions were subjected to three annealing temperatures while the number of cycles remained constant at thirty per reaction.

Human genomic DNA was diluted to 600 pg/µl and 300 pg/µl. Amplification was successful for all three annealing temperatures producing a smear of 140 bp up to several kbs in length (Figure 3.1.7). Multiple bands produced a very strong smear for all the samples amplified at the three annealing temperatures tested. Smear was absent from the negative controls of all three sets of annealing temperature indicating

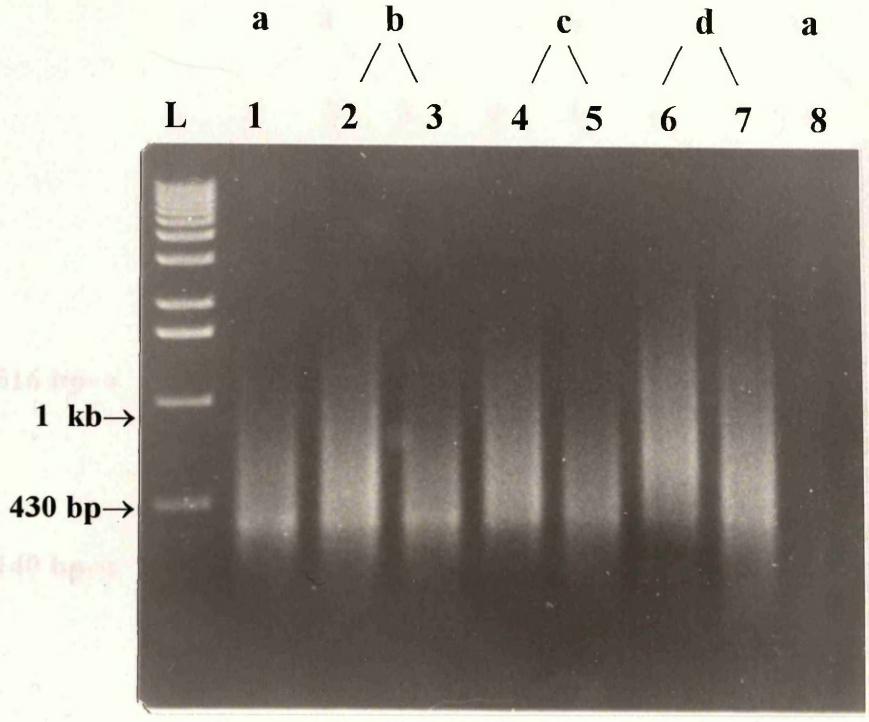


Figure 3.1.6 Gel electrophoresis of human DNA samples amplified under different $MgCl_2$ concentrations

L, 1 kb ladder.

(a) 1.5mM $MgCl_2$. **(b)** 2mM $MgCl_2$. **(c)** 3mM $MgCl_2$. **(d)** 4 mM $MgCl_2$.

Lane **1,** 1200 pg DNA.

Lanes **2, 4, 6,** 600 pg DNA.

Lanes **3, 5, 7,** 300 pg DNA.

Lane **8,** negative control (no template DNA).

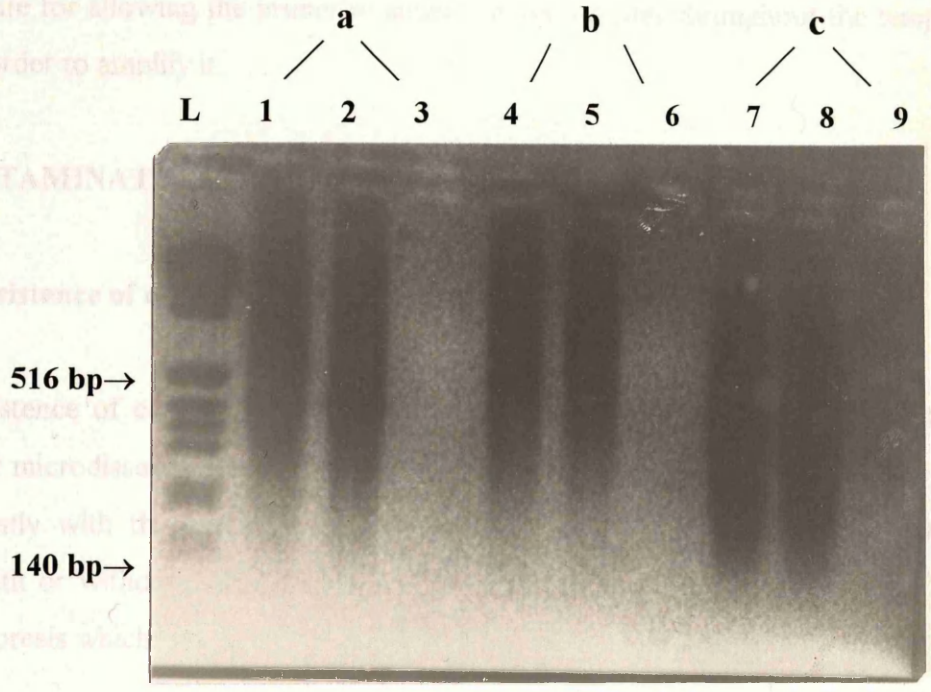


Figure 3.1.7 Gel electrophoresis of DNA samples amplified under three different annealing temperatures

L,1 kb ladder;

(a) 52°C, **(b)** 56°C and **(c)** 62°C annealing temperature.

Lanes **1, 4, 7,** 600 pg of total human DNA.

Lanes **2, 5, 8,** 300 pg of total human DNA .

Lanes **3, 6, 9,** Negative control samples (no target DNA) from each of the amplifications (i.e. 52°C, 56°C and 62°C respectively).

amplification in the absence of any contaminants. FISH results showed no difference at the chromosome painting when the probes that had been amplified under the three annealing temperatures were used and so, 56°C was considered as the appropriate temperature for allowing the primer to anneal on several sites throughout the template DNA in order to amplify it.

3.2 CONTAMINATION HAMPERING THE EFFICIENCY OF DOP-PCR

3.2.1 Persistence of contamination on the PCR reactions

The persistence of contamination in several experiments carried out on total human DNA and microdissected material interfered with the efficiency of the DOP-PCR and consequently with the micro-FISH results. Contamination could be visualised as a smear, with or without a distinct banding pattern on the negative control lane of gel electrophoresis which should otherwise be blank.

In a series of genomic DNA dilutions (1ng, 700 pg, 500 pg) contamination appeared as a smear of the same length, intensity and banding pattern as the positive controls (Figure 3.2.1).

Altering some of the critical PCR parameters did not alleviate the problem. The number of cycles at the first round of amplification was decreased from 35 to 25 cycles without any difference (Figure 3.2.2). The smear (200 to 800 bp) produced by the negative control was similar to the smear of the positive controls indicating strong levels of contamination.

In an attempt to prepare fresh aliquots from new stock solutions of all PCR reagents and test them on 1 ng of genomic DNA, contamination was clearly evident on the negative control lane. A very strong intensity smear (of higher density than of that of the positive control) was produced, however, being composed of fragments shorter than 298 bp that could be explained as products of primer artifacts such as concatamers (Figure 3.2.3).

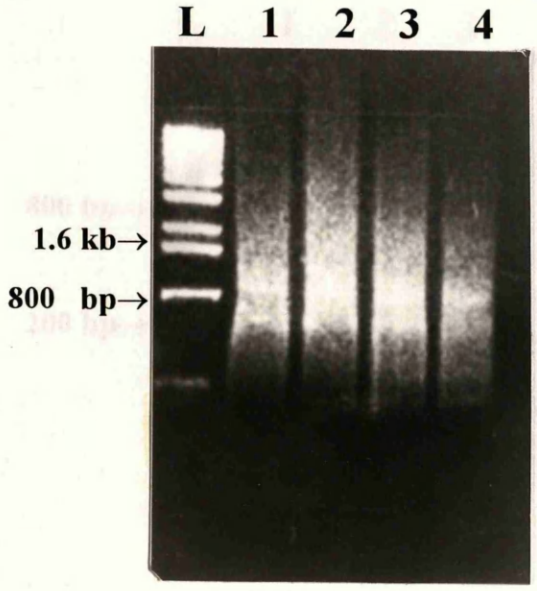


Figure 3.2.1 Gel electrophoresis showing contamination on the first round of DOP-PCR with human genomic DNA as the template in the reaction

L,1 kb ladder.

Lane **1,** 1ng of human genomic DNA.

Lane **2,** 700 pg of human genomic DNA.

Lane **3,** 500 pg of human genomic DNA.

Lane **4,** PCR negative control.

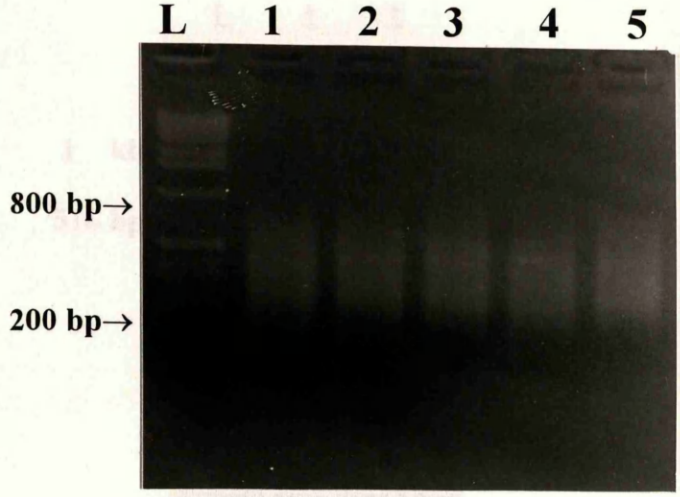


Figure 3.2.2 Gel electrophoresis showing contamination on a series of genomic DNA dilutions at the first round of DOP-PCR

L, 1 kb ladder.

Lanes **1-4,** dilutions (pg/μl) of target human DNA:

Lane **1,** 1000 pg. Lane **2,** 700 pg. Lane **3,** 500 pg. Lane **4,** 300 pg.

Lane **5,** PCR negative control.

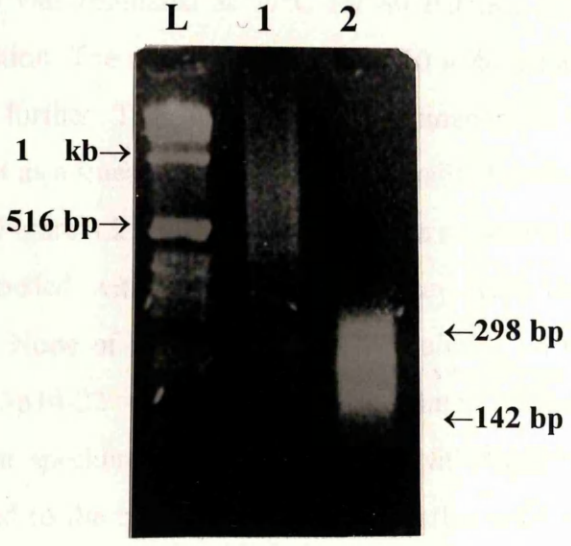


Figure 3.2.3 Gel electrophoresis of a human genomic PCR product in the presence of contamination

L, 1 kb ladder.

Lane **1,** 1 ng of human genomic DNA.

Lane **2,** PCR negative control sample with a smear indicative of contamination.

Several attempts to generate microdissected probes for FISH were hampered by the presence of contamination at the first round of amplification. Thirty to forty fragments of a certain region 3p14-22 (Appendix II) were dissected at each experiment and transferred into a tube containing 20 µl PCR reaction mix, by breaking the tip of the glass needle against the inside wall of the plastic tube. Proteinase K (50 µg/ml) was added to the mix (in order to dissolve any DNA proteins and inactivate endogenous nucleases) and the tube was incubated at 37°C for 40 minutes and at 90°C for 10 minutes to stop the reaction. The mix was made up to 50 µl by adding the rest of the PCR mix and amplified further. The presence of contamination on the first round of amplification was evident as a smear (154 bp-600 bp) visualised on the negative control lane of the agarose gel (Figure 3.2.4). Both products were subjected to a second round of amplification and labelled with biotin-11-dUTP. They were then hybridised on normal metaphase cells. None of the probes produced painting on the target specific regions. Chromosome 3p14-22 region was left unpainted, whereas both probes produced an insignificant speckling on the metaphases with some background. Such amplification attempts led to the conclusion that it was rather unlikely to get a micro-FISH result from PCR probes where contamination was clearly evident right from the first round of amplification. Having tested each of the reagents of the PCR reaction, it was necessary to thoroughly test both the purity and the efficiency of the DOP primer, the core component of the PCR mixture.

3.2.2 Testing the efficiency of a purified DOP primer

To ensure maximum efficiency of the DOP primer in the PCR reaction, the primer was synthesised with a dimethoxytrityl protecting group on its 5' hydroxyl end. This would increase the hydrophobicity of the primer thus retaining it longer when passed through a COP (Cruachem Oligonucleotide Purification) column, whereas any truncated sequences which do not possess the trityl-ON group of the 5' end would elute more readily.

Two PCR reactions were performed, one using a universal (DOP) primer synthesised according to the standard protocols and the other one using the purified DOP primer.

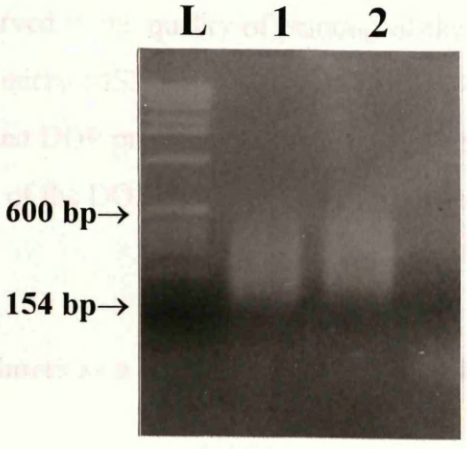


Figure 3.2.4 PCR amplification of the microdissected region 3p14-22

L, 1kb ladder.

Lane **1,** Amplified copies of the microdissected region 3p14-22.

Lane **2,** negative control with a smear (154bp-600bp) evident of contamination.

Total human DNA as the template was used in three different concentrations; 1 ng/μl, 0.8 ng/μl and 0.5 ng/μl. Amplification was successful for all target DNA samples, in both PCR reactions and in the absence of contamination (Figure 3.2.5).

The length of the smear produced by the standard DOP primer at 1 ng/μl target DNA (lane 1) was longer (>1kb) than of the smear produced by the purified primer (lane 2). Nevertheless, the intensity of the smear did not differ among the samples. The PCR products from both types of DOP primer were labelled and used as probes for FISH. No difference was observed in the quality of painting of the chromosomes. Moreover, inability to generate a micro-FISH probe when the COP-purified primer was used instead of the non-purified DOP primer for amplification of the microdissected material, indicated that the purity of the DOP primer was not the critical factor to affect the PCR efficiency.

3.2.3 Using specific primers as a control factor to the PCR components

The purity and efficiency of the PCR components was examined by using a specific set of control primers. Primers specific for repetitive alphoid sequences on chromosome 3 centromere were used to amplify this particular region from human genomic DNA. Thirty cycles were performed at 56°C annealing temperature. At the same time, a PCR reaction was performed on the same target DNA, using the same reagents but the DOP primer instead of the specific primer set.

Visualisation of the PCR products from both amplifications revealed a strong smear in the negative control lane (lane 3) of the PCR with DOP primer (Figure 3.2.6). The PCR reaction using the chromosome 3 centromeric primers revealed a specific band at 1200 bp for both concentrations of the total human DNA (1 ng and 0.1 ng/μl), with no band present at the negative control lane (lane 6). This implies that since the PCR components were sufficient to amplify the target DNA in the PCR reaction driven by the specific set of primers, they should be equally efficient to yield a successful product in the DOP-PCR reaction. However, contamination present in the DOP-PCR reaction

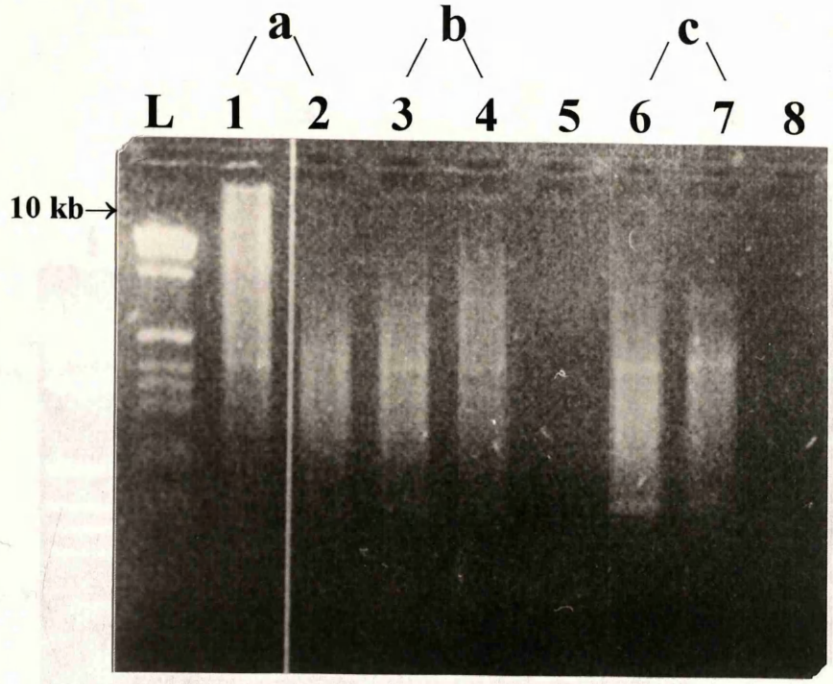


Figure 3.2.5 Total human DNA PCR products using a purified (COP) and a non purified DOP primer

L, 1 kb ladder.

Human DNA dilutions: **(a)** 1 ng/μl **(b)** 0.8 ng/μl and **(c)** 0.5 ng/μl.

Lanes **1, 3, 6,** non purified DOP primer (2μM).

Lanes **2, 4, 7,** purified (COP) -DOP primer (2μM) and

Lane **5,** DOP-PCR negative control sample (performed with a non purified DOP-primer).

Lane **8,** PCR negative control sample (performed with a COP-purified DOP primer).

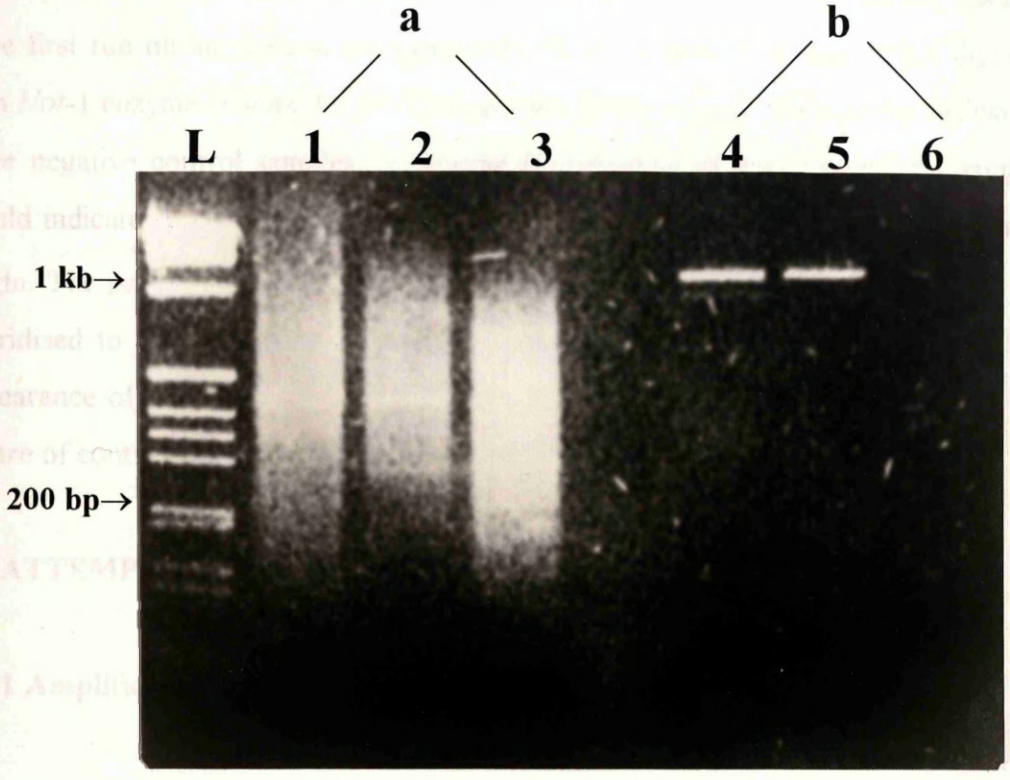


Figure 3.2.6 PCR products using **(a)** DOP primer and **(b)** chromosome 3 centromere specific primers

1 kb ladder.

(a) DOP primer (2 μM) and **(b)** centromere 3 specific primers (200 ng each)

Lanes **1, 4,** 1 ng/μl of total human DNA.

Lanes **2, 5,** 0.1 ng/μl of total human DNA

Lanes **3, 6,** PCR negative controls (no template DNA).

indicated that at least one of the reagents is of insufficient purity. Consequently, the source of contamination had to be investigated further.

3.2.4 Analysing contaminated samples by Southern blotting

Negative control samples from three PCR reactions which revealed a smear after gel electrophoresis were tested in order to analyse the source of contamination. Samples were first run on an agarose gel along with 10 μg of human genomic DNA digested with *Not*-1 enzyme (Figure 3.2.7a) The genomic DNA was used as a probe against the three negative control samples. Successful hybridisation of the probe to the samples would indicate the presence of contamination on the negative controls to be of human origin. The partially digested human genomic DNA was labelled with ^{32}P dCTP and hybridised to the filter on which the samples had been blotted (Figure 3.2.7b). The appearance of smear on all three samples and the human DNA could imply that the nature of contamination present in those samples was most likely human DNA.

3.3 ATTEMPTS TO PRODUCE MICRO-FISH PROBES

3.3.1 Amplification of microdissected material using region specific primers.

The centromeric region of chromosome 3 (Appendix II) was dissected. Twenty five copies instead of 40-50 copies dissected in previous attempts were added into a tube containing 20 μl of dH_2O in order to decrease the chances of introducing any contamination each time the tube lid was opened. The tip of each needle was broken inside the plastic tube. A negative control tube with no target DNA added to it, was used as a tester for contamination along with two positive controls containing 1 ng/μl and 0.1 ng/μl total human DNA respectively. A primer specific for the centromere of chromosome 3 was used for the amplification. The rest of the PCR components were added to the tube containing the microdissected material and covered with few drops of paraffin oil (Table 3.3.1).

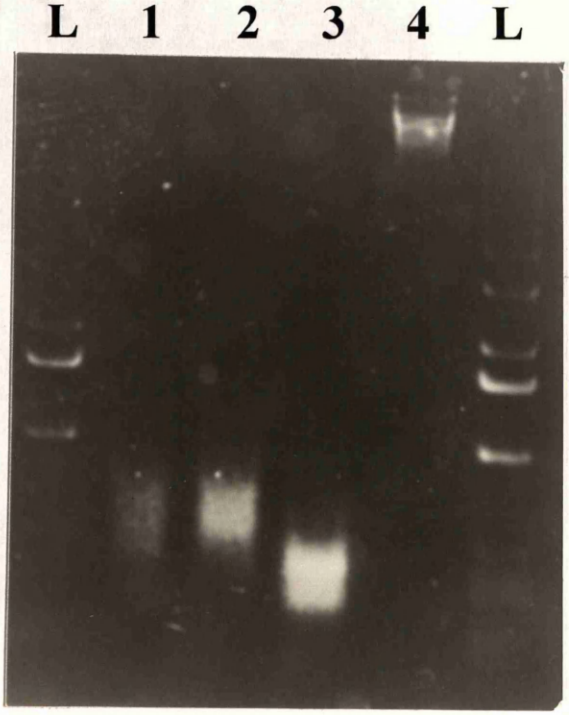


Figure 3.2.7a Gel electrophoresis of PCR negative control samples along with total human DNA

L, 1 kb ladder.

Lanes **1, 2, 3,** negative control samples from three different PCR amplifications, randomly selected.

Lane **4,** 10 μg of total human DNA (partially digested).

L, 1 kb ladder

Figure 3.2.7b Autoradiograph of PCR negative control samples hybridised to ^{32}P human genomic DNA

L, Ikb ladder.

Lanes **1, 2, 3,** negative control PCR products.

Lane **4,** digested ^{32}P human genomic DNA probe.

After the denaturation step of 95°C for 3 minutes, thirty cycles were performed at 95°C for 1 minute, 54°C for 1 minute and 70°C for 3 minutes, followed by an extension step at 72°C for 10 minutes. Gel electrophoresis did not reveal any smear from the microdissected material (lane 1), nor from the negative control (lane 2) after the first round of amplification, whereas both dilutions of human genomic DNA positive controls (1 ng/μl and 0.1 ng/μl respectively) showed the expected distinct band at 1200bp (Figure 3.3.1a).

	Target DNA (μl)	dNTPs 0.2μM (μl)	Buffer 1X (μl)	Primer 200ng each(μl)	H_2O (μl)	Taq polyme-rase	Total (μl)
Dissected material in H_2O	20	4	5	2	16.5	0.5	50
Negative control	-	4	5	2	35.5	0.5	50
Genomic DNA (1 ng/μl)	1	4	5	2	35.5	0.5	50
Genomic DNA (0.1 ng/μl)	1	4	5	2	35.5	0.5	50

Table 3.3.1 Volumes of reagents added to the PCR reaction mix

A second round of amplification was performed by repeating the same PCR conditions, using as a target DNA an aliquot of 5 μl from each of the previous amplification products but decreasing the number of cycles to 25. Both the microdissected PCR product and the negative control sample were visualised on a 2% agarose gel (Figure 3.3.1b). The presence of a distinct band at 1200 bp (lane 1) indicated successful amplification of the microdissected material, whereas the negative control remained clear of human DNA contamination.

The amplified dissected material was thereafter processed for FISH. Approximately 40 μl of the second PCR amplification were passed through a purification column

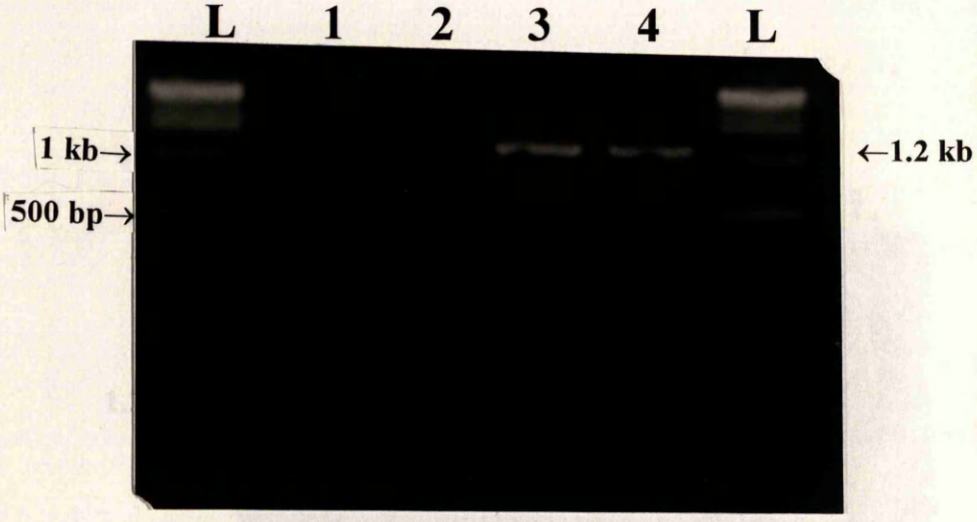


Figure 3.3.1a PCR products from the first amplification using centromere 3 specific primers

L, 1 kb ladder.

Lane **1,** centromere 3 microdissected material.

Lane **2,** negative control (no target DNA).

Lane **3,** 1 ng/μl total human DNA.

Lane **4,** 0.1 ng/μl total human DNA.

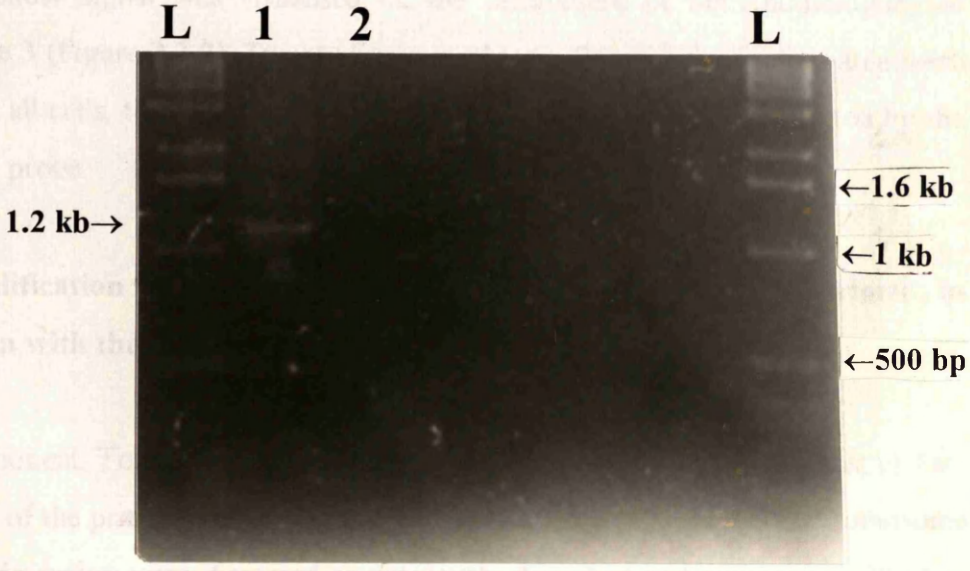


Figure 3.3.1b PCR products from the second amplification using centromere 3 specific primers

L, 1 kb ladder.

Lane **1,** amplified centromere 3 microdissected material.

Lane **2,** negative control sample containing only PCR reaction components.

(SephadexG-50). The eluent was ethanol precipitated and dissolved in 50 µl TE buffer. One microgram (1 µg) of the dissected probe was used for biotin labelling by nick translation. The labelled probe was once more ethanol precipitated in order to remove any unincorporated nucleotides. After dissolving in 25 µl TE buffer, 150 ng of the labelled probe were added into 10 µl of hybridisation buffer, mixed well and denatured at 90°C for 5 minutes. The probe mix was added to an already denatured slide and hybridised overnight at 39°C. For detection of the signal, two layers of anti FITC (5 µg/ml) were applied and chromosomes were stained with propidium iodide.

A bright yellow signal was visualised on the centromere of both homologues of chromosome 3 (Figure 3.3.2). Twenty five metaphase cells per hybridisation area were analysed. In all cells, the centromere of chromosome 3 was successfully painted by the micro-FISH probe.

3.3.2 Amplification of microdissected material using the DOP primer in combination with the TopoIsomerase enzyme.

A new component, TopoIsomerase I, was added into the PCR reaction in order to ease accessibility of the primer in the DNA template. Twenty five copies of the chromosome 3 centromeric region were dissected as previously described and collected in 20 µl of dH_2O. The volume was made up to 50 µl with the rest of the PCR component reagents and incubated for 30 minutes at 37°C with 2 units (0.2 µl) TopoIsomerase enzyme. After an inactivation step at 96°C for 10 minutes, eight cycles of low annealing temperature were carried out by adding T7 enzyme (0.3 units) just before the low annealing temperature step (30°C, 2 minutes). The PCR reaction was continued in the presence of Taq polymerase for 30 more cycles at 56°C. Two negative control tubes (one with broken needle tips and the other one with just the PCR components added to it) were used. After the first round of amplification a very faint smear (220 bp-500 bp) was observed on the lane loaded with the microdissected material indicating that the amount of template DNA was adequate enough to be observed right from the first amplification (Figure 3.3.3a). No smear was present on the negative control samples

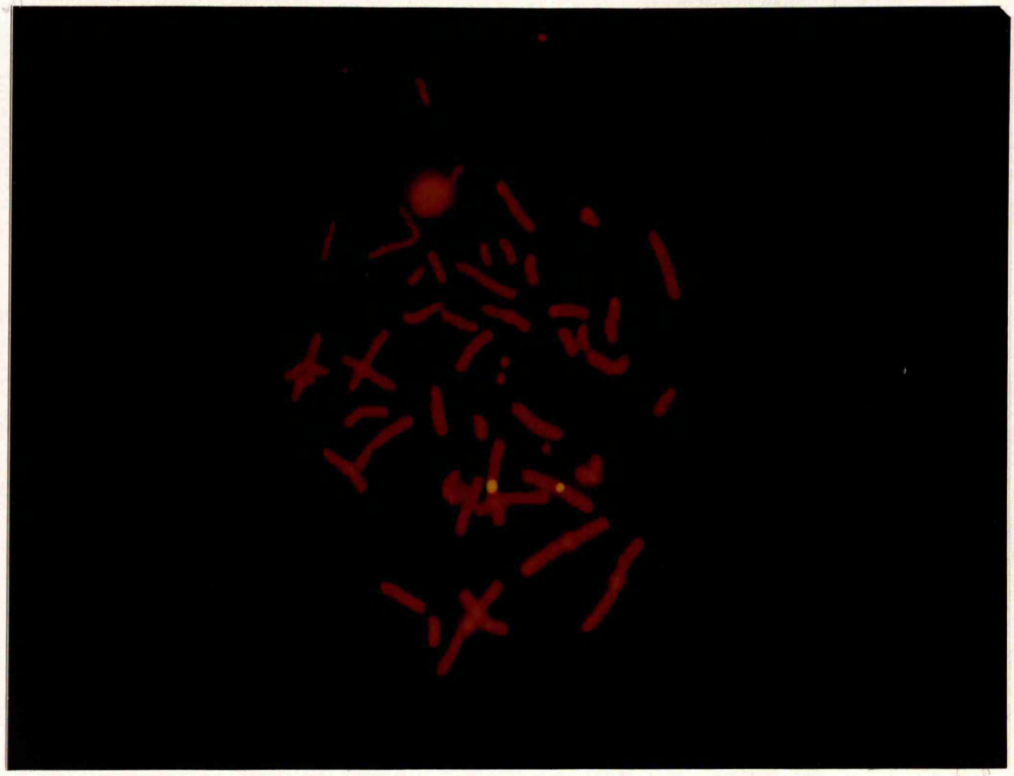


Figure 3.3.2 Centromere 3 micro-FISH probe hybridising to a metaphase cell

The microdissected material was amplified by chromosome 3 centromere specific primers. Bright yellow FITC signals appear on the centromeric region of both homologues of chromosome 3.

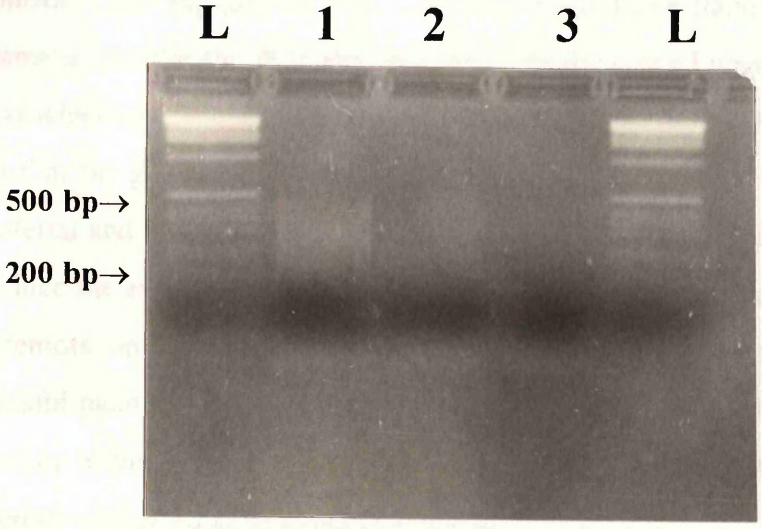


Figure 3.3.3a Gel electrophoresis of the amplified microdissected material (chromosome 3 centromere) and the negative control sample

L, 1 kb ladder.

Lane **1,** Amplified microdissected material (centromere 3) showing a smear of 220 bp-500 bp.

Lane **2, 3,** PCR negative controls (with and without the broken needle tips, respectively).

L, 1 kb ladder.

(with and without broken needles inside the tube, lanes 2 and 3 respectively), thus indicating successful amplification of microdissected DNA.

A second round of amplification was performed at 56°C for 30 cycles, by adding 5 µl aliquots of the first PCR amplification. Gel electrophoresis revealed a smear of 200 bp-800 bp as the product of amplification of the microdissected material (Figure 3.3.3b). The negative control containing just the PCR components was blank (lane 3), showing absence of contamination from the PCR mix, in contrast to the second negative control (with needle tips) which produced a smear of short length fragments (170 bp-360 bp) hence indicating that the glass needles had introduced the contamination. Labelling of the dissected material and hybridisation of the micro-FISH probe to normal metaphase cells did not produce the expected fluorescent signal on the centromere of chromosome three. Other attempts on different regions of several chromosomes also failed to produce a successful painting of the target region. As a result, the next steps involved testing the efficiency of the probe labelling and making further alterations to the actual technique of microdissection so as to avoid contamination being introduced by the glass needles inside the PCR tube.

3.3.3 Dot-blot assay

A Dot-blot assay was performed on human genomic DNA and microdissected material in order to check if biotin-11-dUTP had been successfully incorporated to each of the amplified probes.

As a control sample, a biotin-labelled probe (8 centromere) that had been successfully used before in FISH experiments was chosen. A series of dilutions (100 pg, 70 pg, 50 pg, 30 pg, 20 pg, 10 pg) were made on two microdissected probes and two PCR negative control samples that had produced a smear when visualised on an agarose gel. Each of the probe dilutions was added to a nitro-cellulose filter . The probes were incubated for 30 minutes at 37°C with streptavidin-alkaline phosphatase and after the necessary washing steps the filter was treated with developing solutions. A distinct dot would appear on each of the wells, where the probe was initially applied. This would

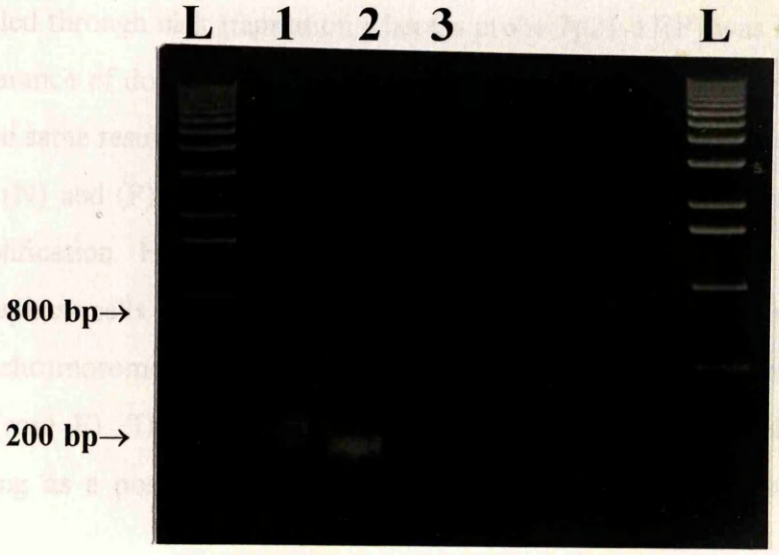


Figure 3.3.3b Visualisation of the second PCR products using the DOP primer

L, 1 kb ladder.

Lane **1,** microdissected centromere 3 target DNA.

Lane **2**, PCR negative control sample containing broken needle tips.

Lane **3,** PCR negative control sample containing only the reaction components.

L, 1 kb ladder.

indicate the presence of biotin-label in the probe , therefore confirming the success of the labelling procedure.

All diluted probes tested produced a white dot on the film (Figure 3.3.4). The human genomic DNA probe had previously painted all chromosomes of the cells examined by a FISH experiment. Two microdissected probes that had previously failed to produce a FISH result were tested. Both probes involved the same microdissected region 7p21-31 (Appendix II) and were treated in the same way with the exception that probe 7p21-31(N) was labelled through nick translation whereas probe 7p21-31(P) was labelled by PCR. The appearance of dots in all dilution samples revealed the presence of biotin in both probes. The same result was also obtained from labelled-negative control samples of the 7p21-31 (N) and (P) amplification reactions that had produced a strong smear after PCR amplification. However, one of them failed to produce any significant painting on metaphase cells when used as a probe for FISH (row F), whereas the other one painted all chromosomes present in the metaphase cell due to strong presence of contamination (row E). The presence of incorporated biotin in the probes tested excluded labelling as a possible factor responsible for the unsuccessful micro-FISH attempts.

3.3.4 Using isolated flow sorted chromosomes (FACS) as a control in all steps involved in the generation of micro-FISH probe

The need for a more thorough investigation of all steps involved in the micro-FISH procedure led to the use of a flow sorted chromosome samples as a reliable positive control that could resemble much more in terms of sensitivity the microdissected material. Flow sorted copies of chromosome 4 were used along with human genomic DNA as positive controls to all steps involved in producing micro-FISH probes. Approximately 350 copies of the isolated FACS chromosome 4 were obtained in a 25-30 μl solution. Dilutions were made on the basis of the number of chromosomes contained per volume, so that in 2.5 μl approximately 35 copies of the particular chromosome were contained. The aim was to obtain sufficient painting on both homologues of chromosome four, with the smallest possible number of chromosome

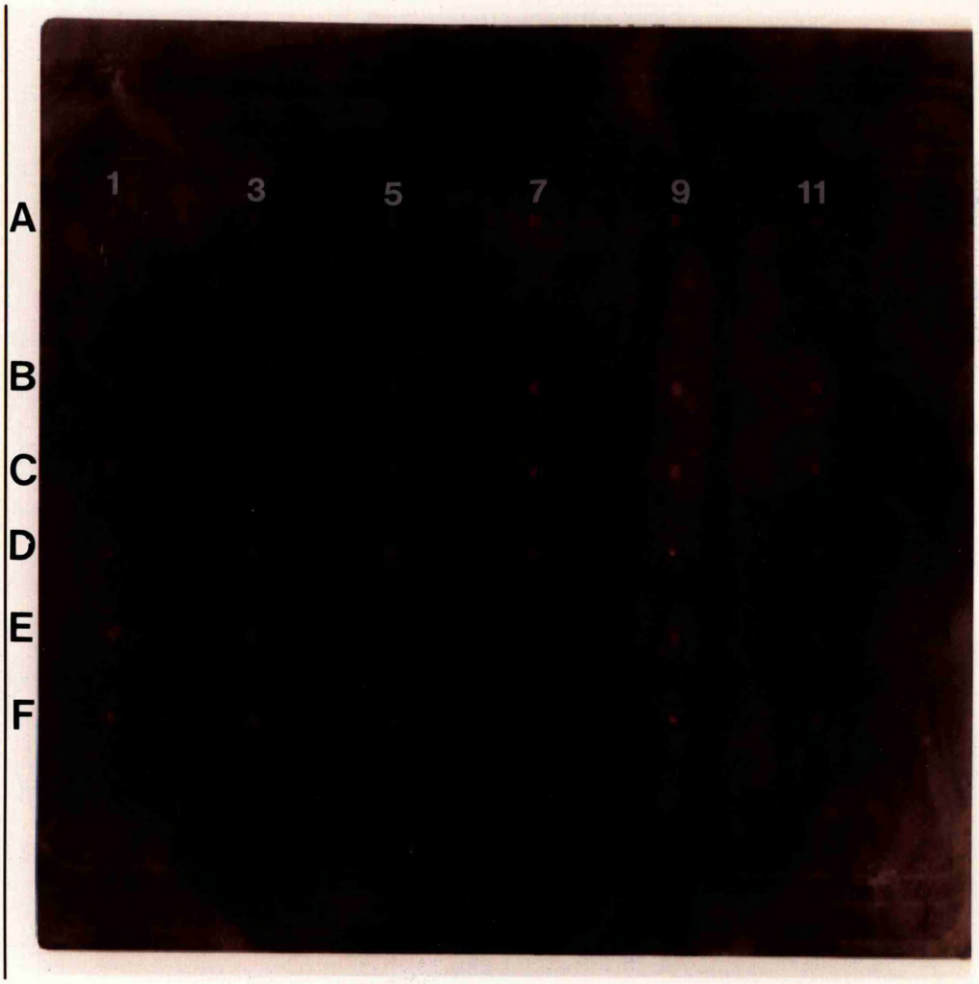


Figure 3.3.4 Detection of the biotin-11-dUTP label of diluted probes on a nitrocellulose filter after a dot-blot assay

Lanes **1-11**: Dilutions of the assessed probes, **1** (100pg), **3** (70pg), **5** (50pg), **7** (30pg), **9** (20 pg), **11** (10pg).

Row **A**, chromosome 8 centromere probe
Row **B**, human genomic DNA probe
Row **C**, microdissected 7p21-31 (N) DNA probe, labelled by nick translation
Row **D**, microdissected 7p21-31 (P) DNA probe labelled by PCR
Row **E**, PCR negative control (N)
Row **F**, PCR negative control (P)

copies contained in a certain volume of solution. That would prove that all parameters had been optimised at levels efficient for the amplification of very small amounts of template DNA (i.e. microdissected material).

A 5 μl collection drop containing the important PCR components (i.e. buffer, dNTPs, DOP primer and dH_2O) were added into each PCR tube. The appropriate volume of FACS solution, according to the number of copies, was added and the samples were treated with TopoIsomerase and T7 enzyme as previously described. Aliquots of 7 μl from the first amplification were added into a new tube containing 43 μl of the necessary PCR components and a second amplification of 35 cycles was performed.

The six volumetric dilutions prepared were estimated to contain the following copy number of chromosome 4: 140 copies (12 μl), 70 copies (6 μl), 50 copies (4.3 μl), 35 copies (3μl), 20 copies (1.7 μl) and finally 15 copies (1.5 μl). A very strong smear was obtained from the first four dilutions, whereas, the last two produced a very faint smear (Figure 3.3.5a). No contamination was observed from this first round of amplification. At the second round of amplification, a strong smear was observed in all dilutions, without any contamination being present in the negative control lane (Figure 3.3.5b).

Four of the PCR dilution products were labelled with biotin-11-dUTP and subsequently used as probes for FISH. 150 ng of each of the probes were added to the hybridisation buffer. Cross hybridisation was suppressed using two concentrations of Cot-1 DNA at 1 μg/μl and 0.5 μg/μl respectively, each tested on a single hybridisation area. A strong painting of both chromosome 4 homologues was observed in all metaphase cells examined, indicating clearly that the conditions of all steps involved in the experiment had been sufficiently optimised to allow amplification of small quantities of template DNA (Figures 3.3.6a-d).

3.3.5 DOP amplification of different microdissected regions

Several chromosome regions were dissected and fragments were added into a 5 μl collection drop containing a non commercial PCR buffer and 7 pmol of DOP primer.

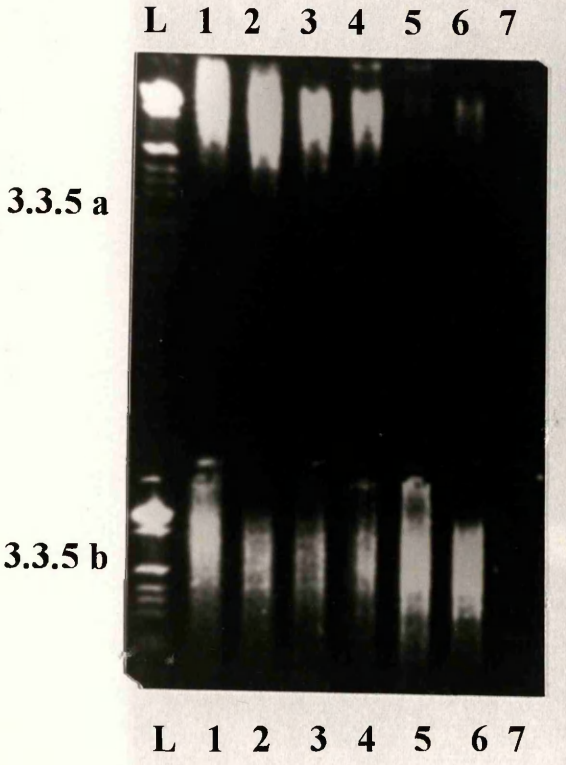


Figure 3.3.5a First PCR amplification products of chromosome 4 FACS human DNA dilutions

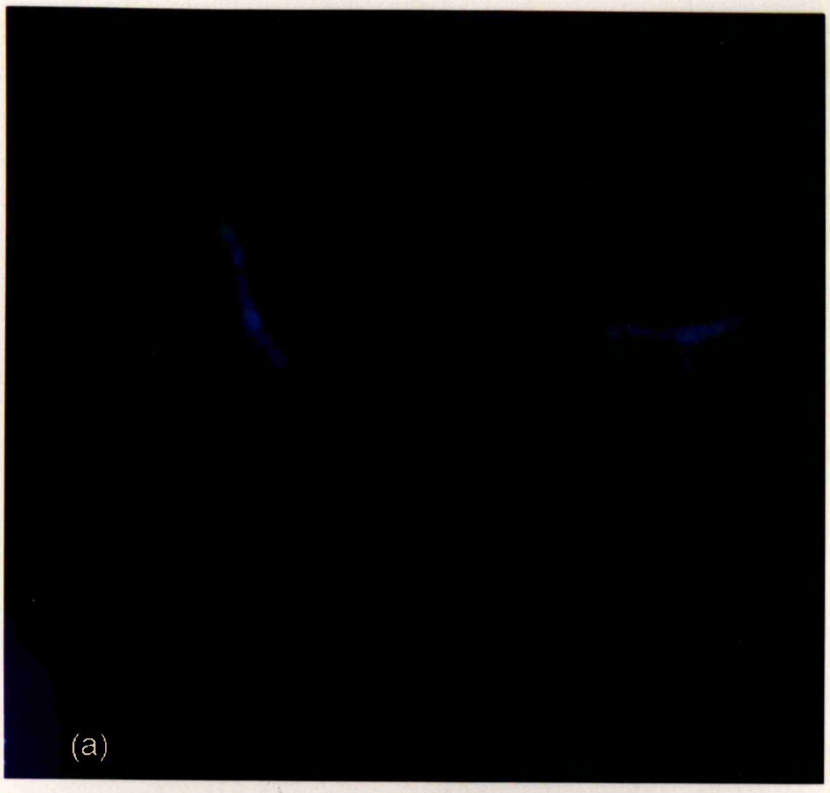
L, 1 kb ladder.

Lanes **1-6,** volumetric dilutions of 4 FACS human DNA. **1,** 140 copies (12 μl). **2,** 70 copies (6 μl). **3,** 50 copies (4.3 μl). **4,** 35 copies (3μl). **5,** 20 copies (1.7 μl). **6,** 15 copies (1.5 μl).

Figure 3.3.5b Second PCR amplification products of chromosome 4 FACS human DNA dilutions

L, 1 kb ladder.

Lanes **1-6,** volumetric dilutions of 4 FACS human DNA. **1,** 140 copies (12 μl). **2,** 70 copies (6 μl). **3,** 50 copies (4.3 μl). **4,** 35 copies (3μl). **5,** 20 copies (1.7 μl). **6,** 15 copies (1.5 μl). Lane 7, PCR negative control.



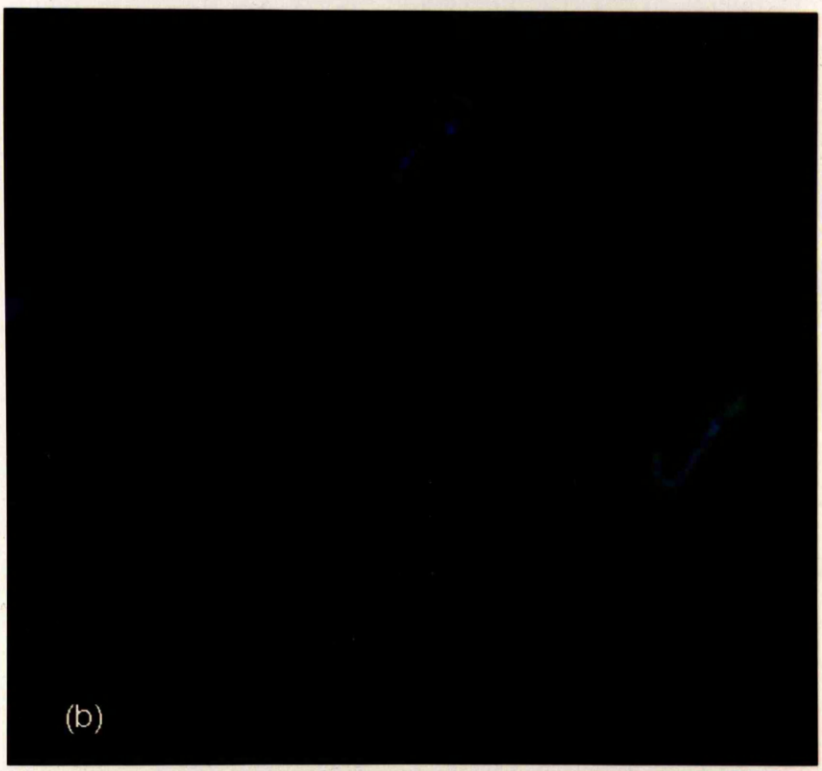
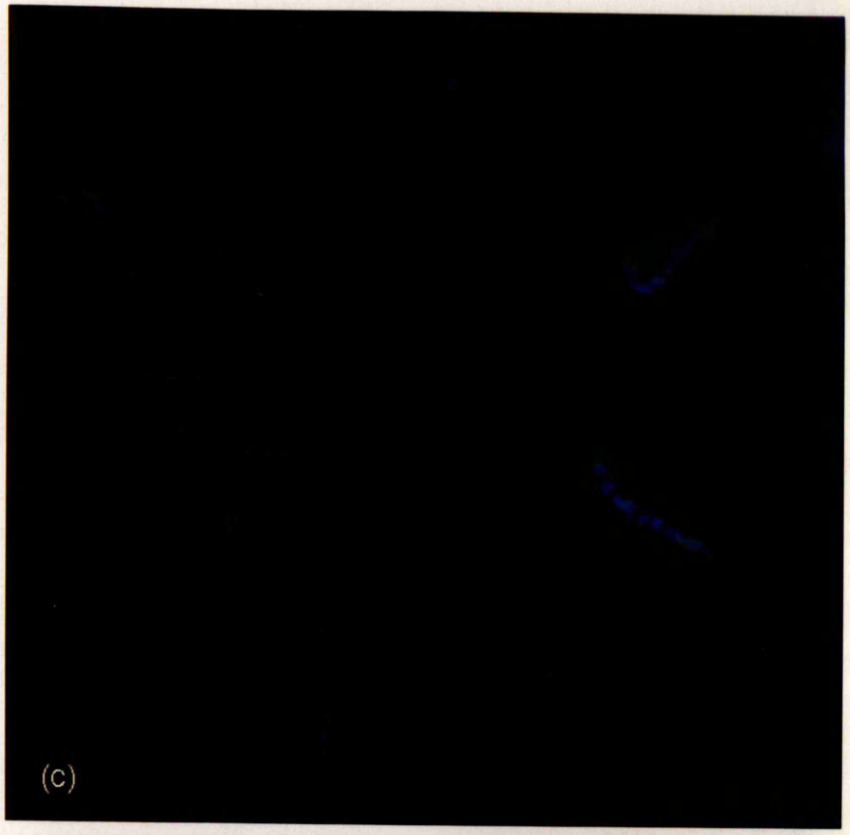


Figure 3.3.6 (a-b) : Hybridisation of chromosome 4 FACS- biotin labelled probe on normal metaphase cells. Chromosome 4 FACS probe derived from the amplification of **(a)** 50 copies and **(b)** 35 copies of chromosome 4. Both homologues are painted green, without any cross hybridisation being observed on the rest of the cell chromosomes. Cells are counterstained with DAPI (blue).



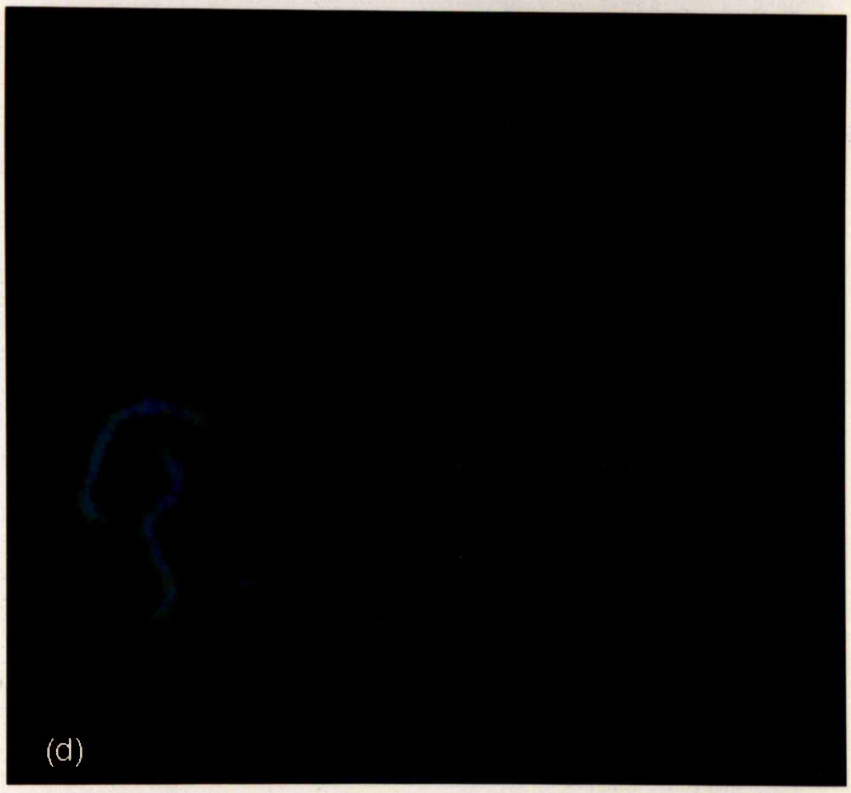


Figure 3.3.6 (c-d) : Hybridisation of chromosome 4 FACS- biotin labelled probe on normal metaphase cells. Chromosome 4 FACS probe derived from the amplification of **(c)** 20 copies and **(d)** 15 copies of chromosome 4. Both homologues are painted green, without any cross hybridisation being observed on the rest of the cell chromosomes. Cells are counterstained with DAPI (blue).

Ten to fifteen copies of a particular region were dissected at each experiment and transferred to a PCR tube by just inserting the tip of the needle to the collection drop to wash off the dissected fragment and then immediately removing it, instead of breaking it inside the tube as previously described.

On two different experiments, microdissected materials involved the following regions: 1q42 and Xp21 (Appendix II). Visualisation of the first amplification products did not show any smear and were free of contamination . The second amplification revealed in both experiments, successful amplification of the microdissected material along with chromosome 4 FACS (20 copies, 1.7 μl) used as a positive control, but with the presence of contamination (Figures 3.3.7 a and b, respectively). The intensity of the smears was stronger between 200 bp to 800 bp, with distinct multiple bands present in the negative control of Xp21.

All PCR products were labelled with biotin-11-dUTP and used as probes for FISH on normal metaphase cells. Chromosome 4 FACS probe from all three experiments painted fully both target homologues in all metaphase cells analysed. No painting was produced from the microdissected probe 1q42 and its negative controls (Figure 3.3.8a-b). On the contrary, a paint on almost all metaphase chromosomes was observed when the PCR products of the second micro-dissection experiment (Xp21) successfully hybridised to the metaphase cells due to the heavy contamination present in the PCR reaction mix.

The successful results obtained form the positive control samples in conjunction with the attempts and modifications made when microdissected material was used as a probe, suggested that the most critical step at that stage was to ensure successful transfer of the microdissected material into the collection tube. In consequence, more effort had to be made in obtaining needles of an appropriate shape and size that would allow the minute material first to adhere successfully to the needle and then easily come off once inside the PCR collection tube.

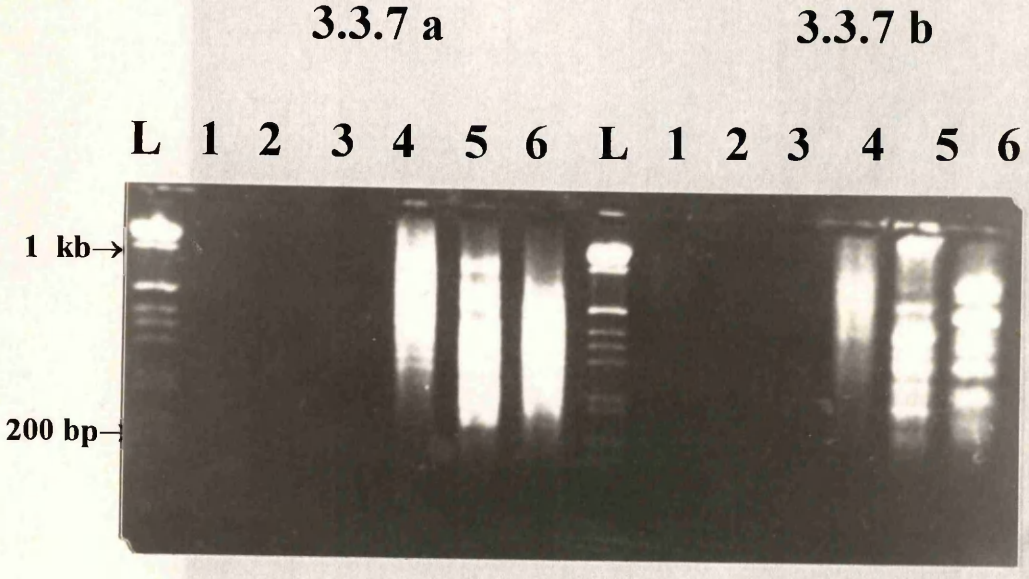


Figure 3.3.7a Gel electrophoresis of PCR products, including the microdissected region 1p41-42

L, 1 kb ladder. Lanes **1-3,** First round of amplification: Lane **1,** ~20 copies of FACS chromosome 4. Lane **2,** 10 copies of the microdissected region 1p41-42. Lane **3,** negative control. Lanes **4-6,** Second round of amplification with the same PCR products loaded in the same order.

Figure 3.3.7b Gel electrophoresis of PCR products, including the microdissected region Xp21.

L, 1 kb ladder. Lanes **1-3,** First round of amplification: Lane **1,** ~20 copies of FACS chromosome 4. Lane **2,** 10 copies of the microdissected region Xp21. Lane **3,** negative control. Lanes **4-6,** Second round of amplification with the same PCR products loaded in the same order.

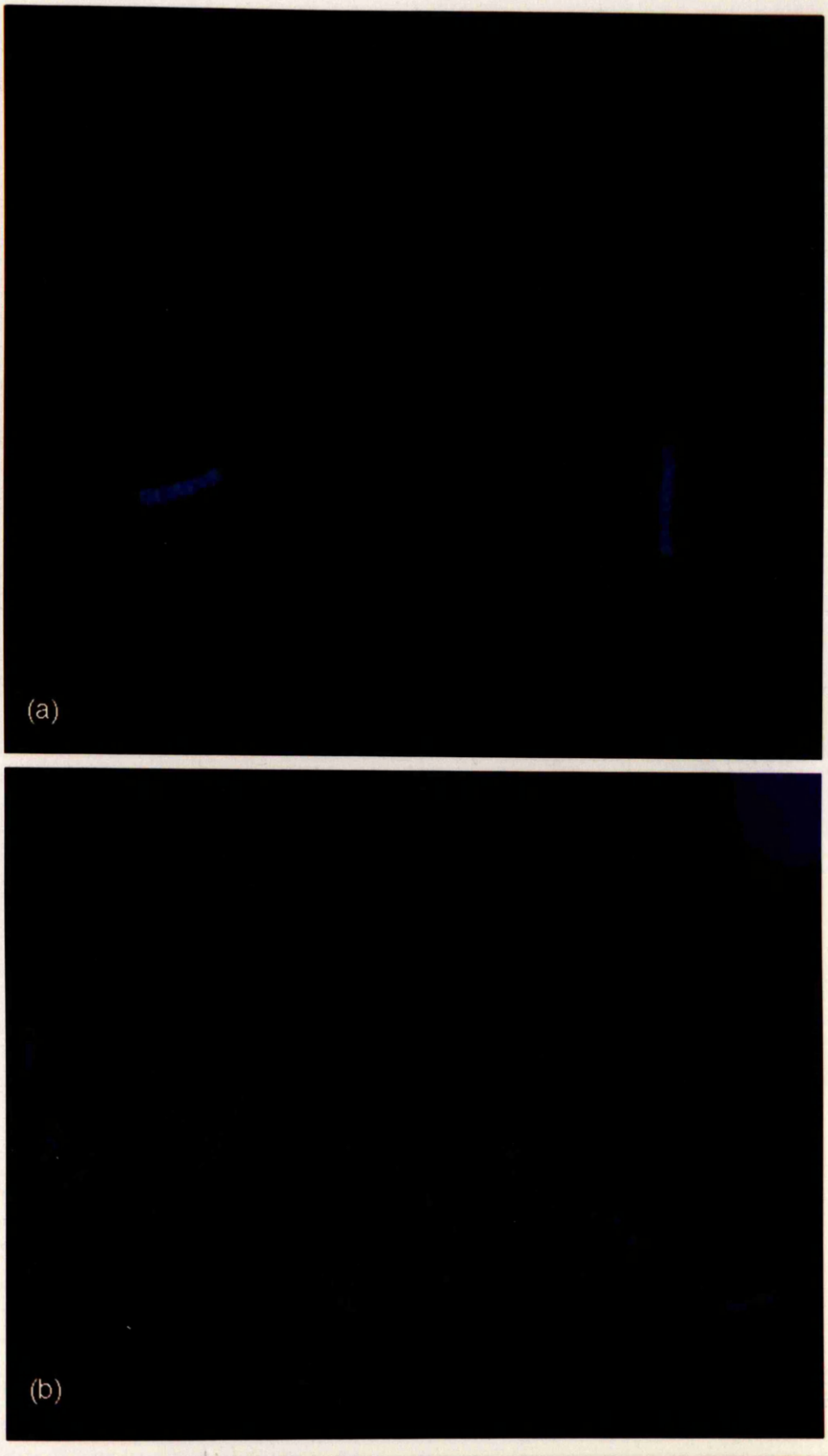


Figure 3.3.8 **(a-b)** : DOP-PCR probes hybridised to normal lymphocytes. Cells are counterstained with DAPI (blue). **(a)** Chromosome 4 FACS probe generated from the amplification of approx. 20 copies of chromosome 4. **(b)** 1q 42 micro-FISH probe : No specific painting is observed.

3.4 SUCCESSFUL GENERATION OF MICRO-FISH PROBES

3.4.1 Generation of a 7p21-22 micro-FISH probes

Six fragments of the region 7p21-22 (Appendix II) were dissected. The fragments were dissected and amplified as previously described. A 5 μl aliquot from the first amplification was used as the target DNA along with a negative control. The microdissected target DNA was labelled with spectrum orange dUTP which was incorporated during the second PCR amplification. A strong smear of 200 bp - 3 kb was produced from the second PCR amplification on both, the microdissected target DNA and the negative control lane, whereas no smear was evident on visualisation of the first PCR products (Figure 3.4.1).

The PCR products from the microdissected material and the negative control sample were purified, and co-precipitated with 1 μg of Cot-1 DNA. They were then dissolved in 1X TE buffer and 100 ng from each sample was used for FISH. Detection was performed by adding two layers of avidinFITC and the probes were visualised by staining the metaphase cells with DAPI (1 mg/ml) by adding 1 μl of it in 1 ml of antifade reagent. 10μl were added to each hybridisation area. The results revealed a strong painting of the 7p21-22 region on both chromosome 7 homologues in all cells examined indicating the successful generation of the micro-FISH probe from the microdissected material (Figures 3.4.2a and 3.4.2b). No painting was produced when the negative control PCR probe was hybridised to metaphase cells.

3.4.2 Reproducing the 7p21-22 micro-FISH probe

The above experiment was performed in a different laboratory and therefore it was decided to test all conditions and reagents involved in the generation of the micro-FISH probe by using an aliquot of the first PCR products in order to reamplify the target DNA and then use it as a probe for FISH.

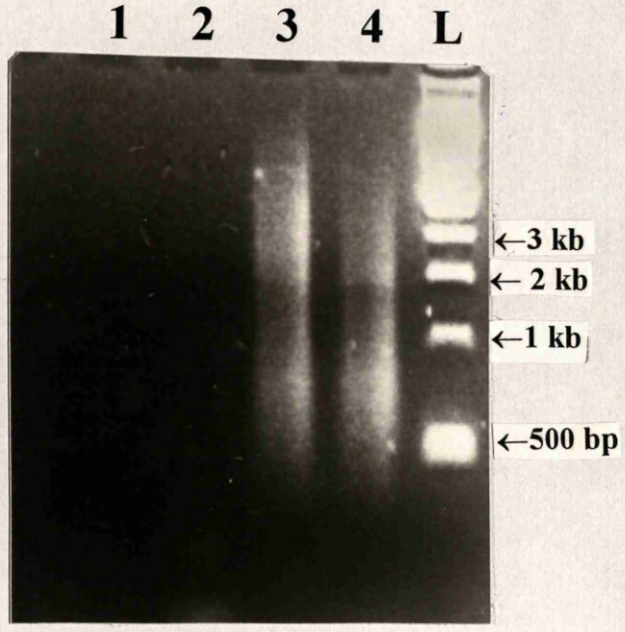


Figure 3.4.1 Gel electrophoresis of the DOP-PCR amplified 7p21-22 microdissected region

Lanes **1-2,** first PCR amplification products: **1,** 7p21-22. **2,** negative control. Lanes **3-4,** second PCR amplification products: **3,** 7p21-22. **4,** negative control. **L,** 1 kb ladder.

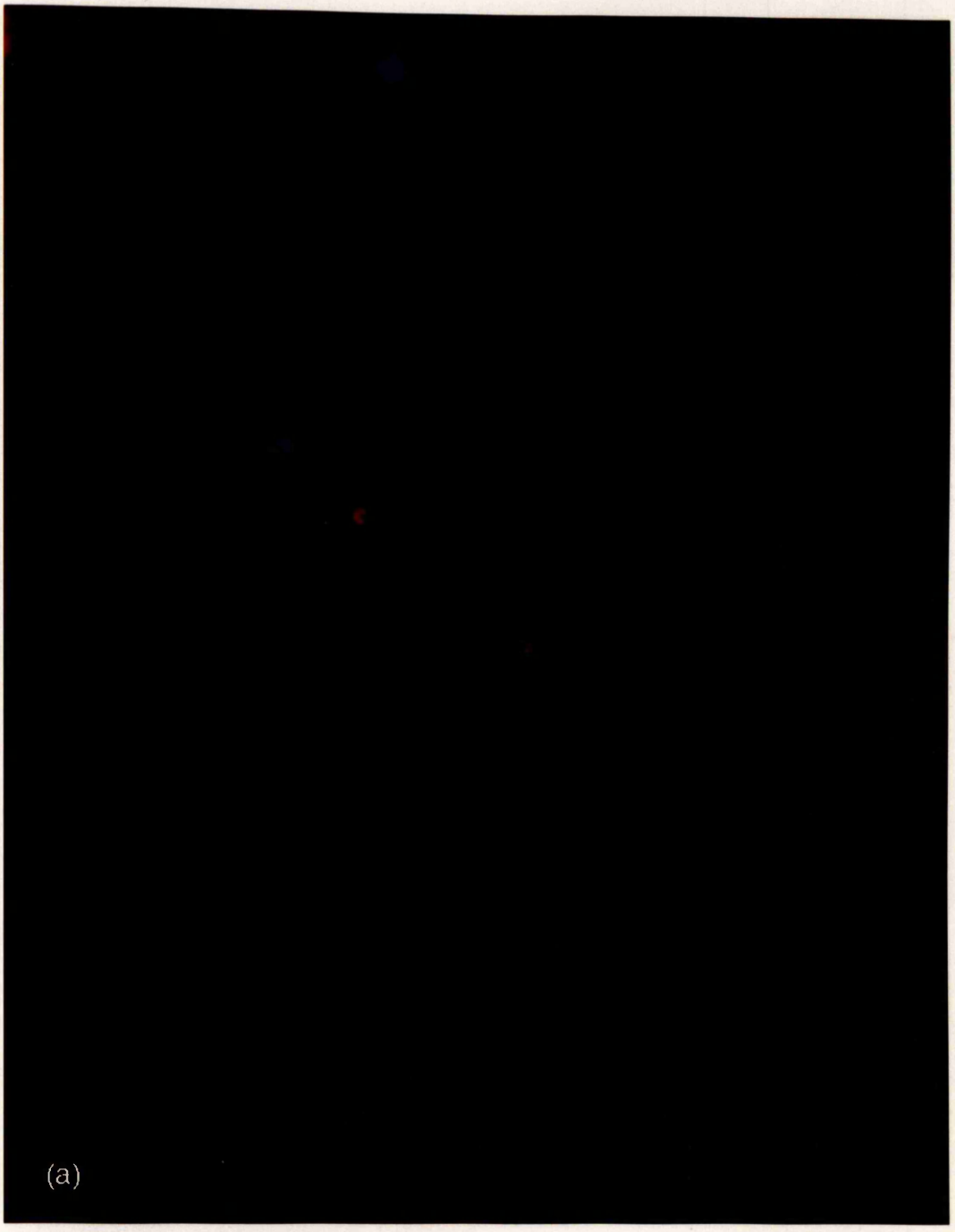


Figure 3.4.2 (a) Micro-FISH 7p21-22 probe hybridised to a normal metaphase cell. A distinct orange signal is observed on the target 7p21-22 region of both chromosome 7 homologues.

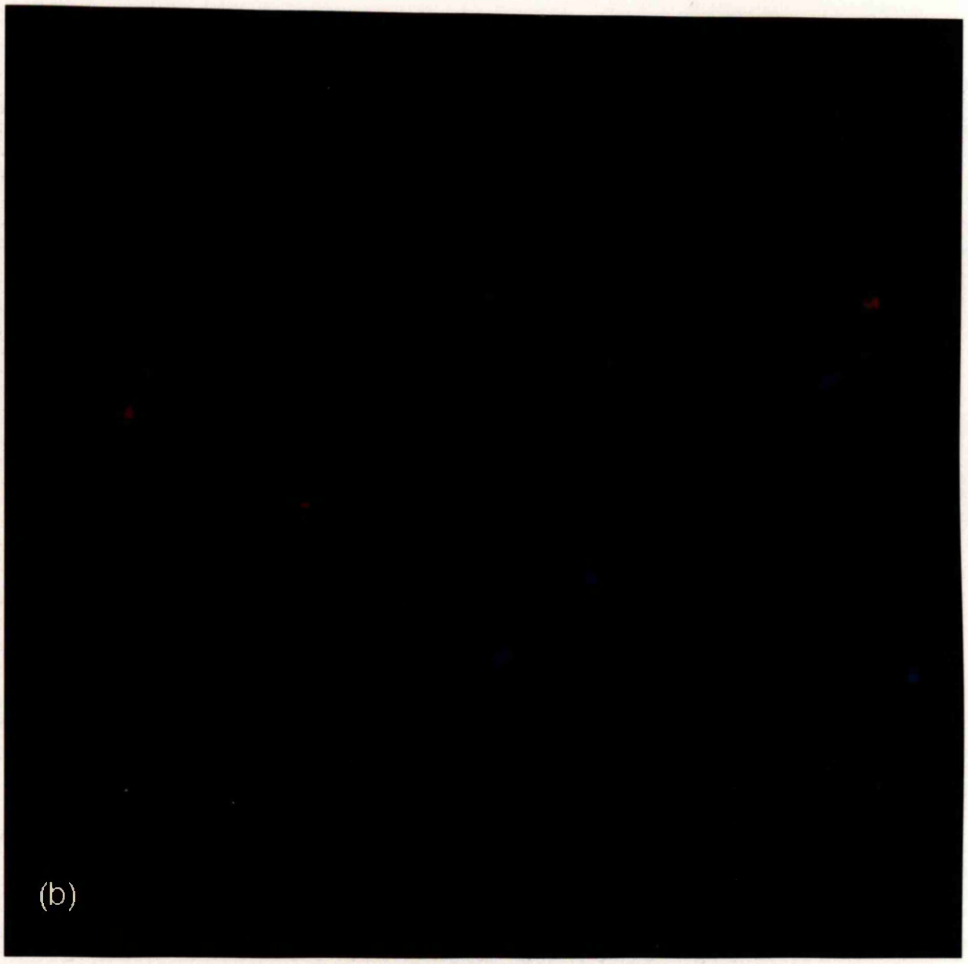


Figure 3.4.2 (b) Micro-FISH 7p21-22 probe hybridised to a normal metaphase cell. A distinct orange signal is observed on the target 7p21-22 region of both chromosome 7 homologues.

Five microlitre aliquots from the first PCR were added to the PCR mix. Two sets of amplifications were performed. One with the target DNA being labelled with biotin-11-dUTP during the amplification reaction and a second one with the target DNA being amplified and then labelled by nick translation.

Visualisation of the PCR products revealed a strong smear (150 bp-400 bp) on all samples, as well as the negative controls that had been reamplified (Figure 3.4.3). A negative control containing just the PCR components from the second amplification was also used to test if contamination was present in this PCR reaction. Absence of smear (lane1, of figure 3.4.3) indicated reactions to be free of contamination.

Microdissected amplified products were processed further. The PCR labelled probe was purified, dissolved in 1X TE buffer and mixed appropriately in the hybridisation buffer with 1 μg Cot-1 DNA, whereas the unlabelled PCR product was labelled by nick translation and then processed accordingly for FISH. Both micro-FISH probes painted successfully the target 7p21-22 of both chromosome 7 homologues in all cell examined and in the absence of any cross hybridisation with other chromosomes (Figure 3.4.4a : probe labelled during PCR amplification and Figure 3.4.4b: probe labelled by nick translation).

3.4.3 Generation of a 21q22 micro-FISH probe

Nine fragments of the 21q22 (Appendix II) region were dissected. After the first PCR amplification, the PCR product was reamplified and labelled at the same time with biotin-11-dUTP (Figure 3.4.5) The first amplification did not show any smear on either the microdissected or the negative control lanes (lanes 1 and 2, respectively). A smear of 100 bp to 600 bp was visualised on the second PCR products with the negative control smear being more intense at the low region of 100 bp-142 bp. Both the 21q22 microdissected PCR probe and the negative control were labelled with biotin-11-dUTP during the second PCR and then used for FISH. 200 ng of each of the probes were mixed with 0.5 μg of Cot-1 DNA in the hybridisation mix and applied on normal metaphase cells which were counterstained with DAPI and PI mixed in the antifade

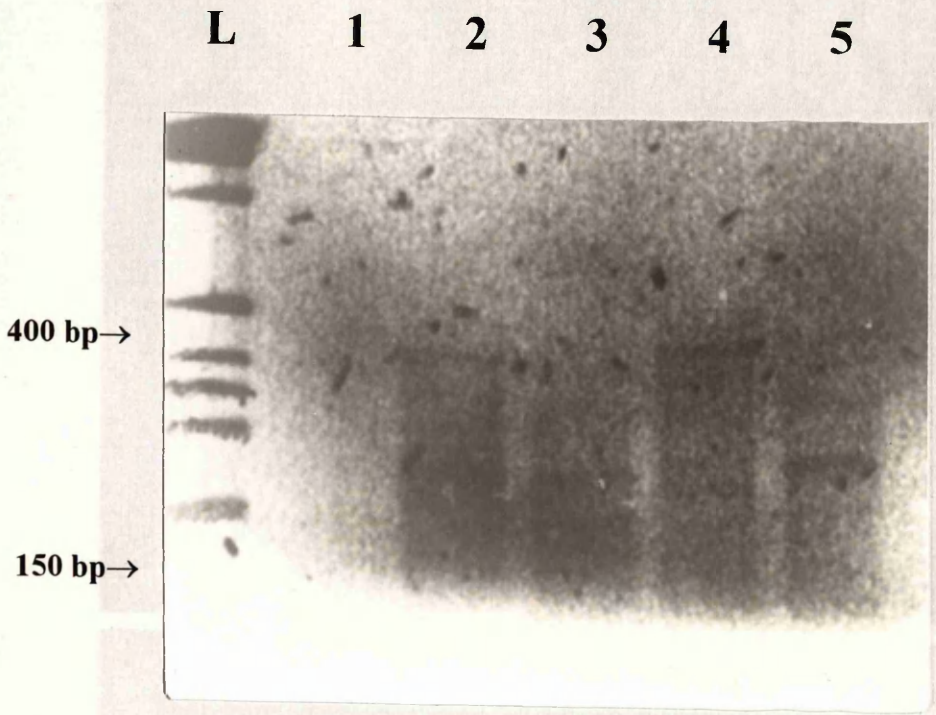


Figure 3.4.3 Second DOP-PCR amplification products of the 7p21-22 microdissected region

L, 1 kb ladder.

Lane **1,** negative control of the second PCR amplification

Lane **2,** 7p21-22 microdissected DNA, reamplified by DOP-PCR (unlabelled).

Lane **4,** 7p21-22 microdissected DNA, reamplified and labelled with biotin by DOP-PCR.

Lanes **3** and **5,** negative control products amplified from an aliquot of the first PCR negative control sample.

A strong smear of 150bp-400bp is present in all lanes but not the negative control for this amplification.

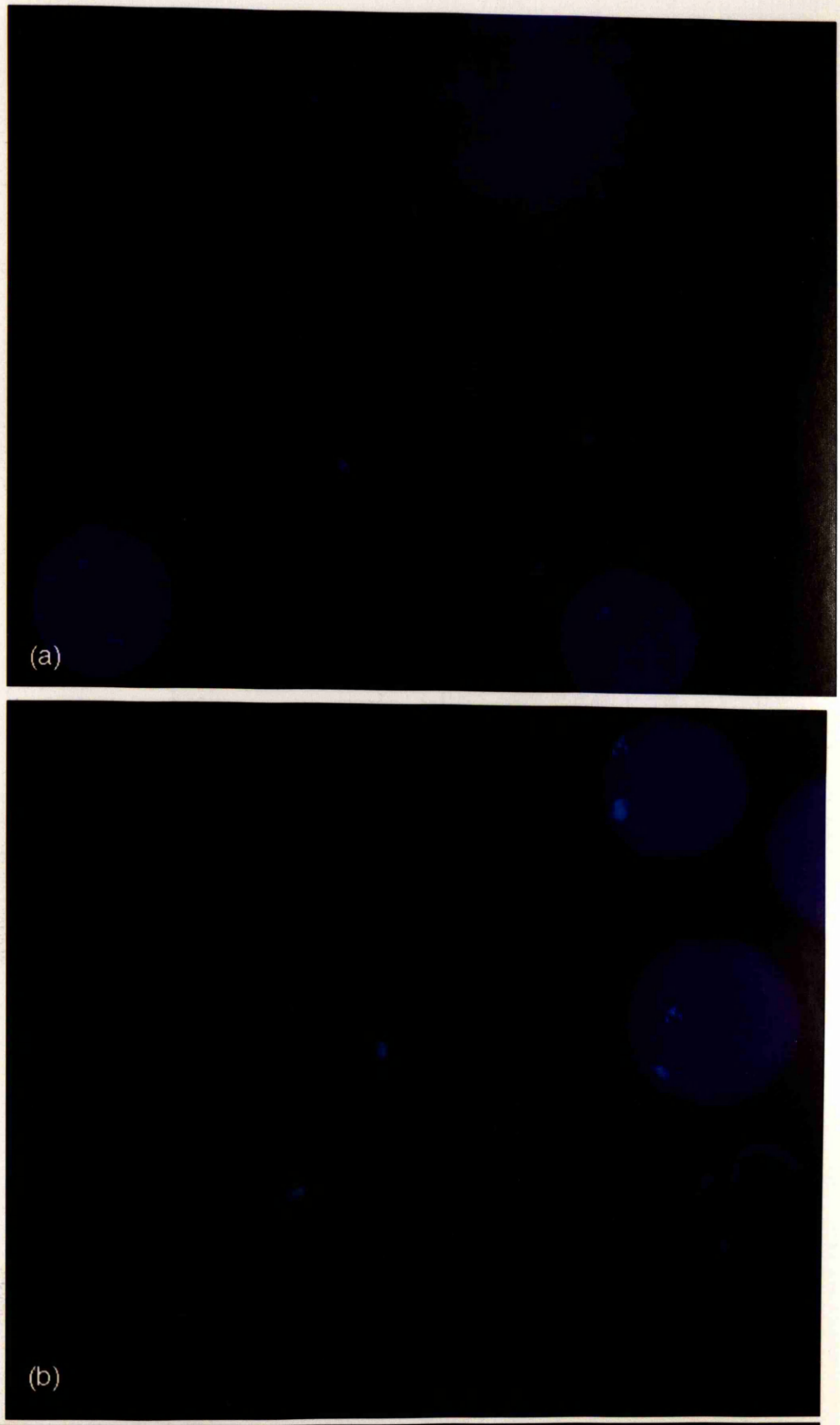


Figure 3.4.4 (a-b) : Hybridisation of the 7p21-22 micro-FISH probe labelled with biotin by **(a)** nick translation and **(b)** PCR, to normal metaphase cells. Two bright green signals are observed on the 7 p21-22 region of both homologues. Cells are counterstained with DAPI (blue).

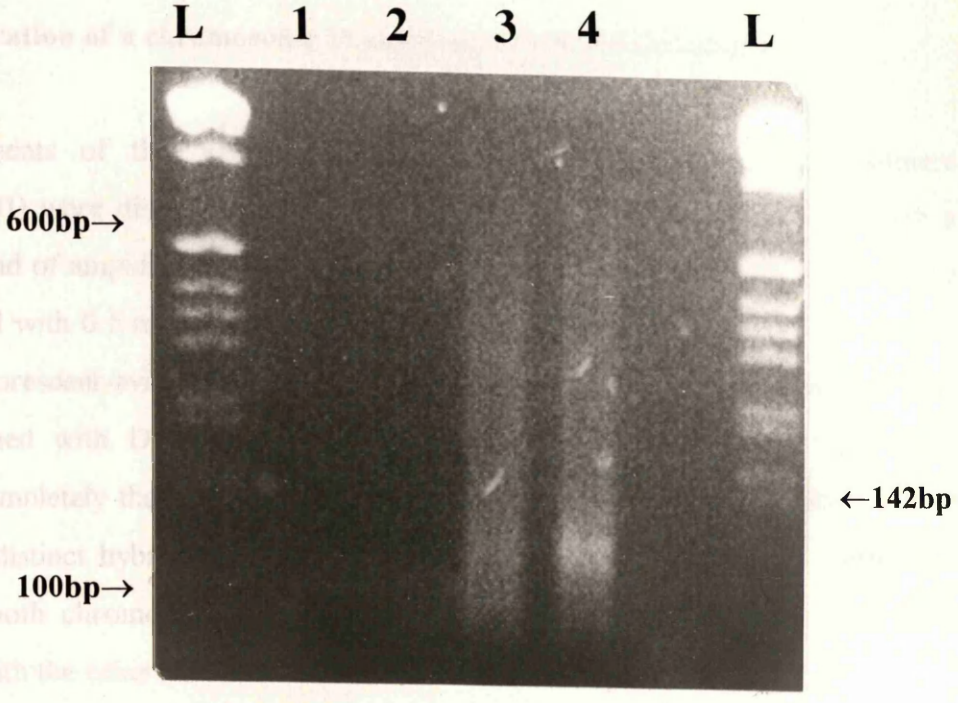


Figure 3.4.5 Gel electrophoresis of the microdissected 21q22 PCR product

L, 1 kb ladder.

Lanes **1** and **2,** first amplification of the microdissected 21q22 and the negative control samples, respectively.

Lanes **3** and **4,** second amplification products of the microdissected 21q22 and the negative control samples, respectively.

reagent. The micro-FISH probe successfully painted the 21q22 region of both chromosome 21 homologues in all cells examined, whereas no painting was observed in the cells hybridised to the biotin labelled negative control probe (Figure 3.4.6 a-d).

3.4.4 Generation of a chromosome 16 centromere micro-FISH probe

Nine fragments of the heterochromatic region of chromosome 16 centromere (Appendix II) were dissected, amplified and labelled with biotin-11-dUTP through a second round of amplification. FISH was performed with 200 ng of the labelled probe being mixed with 0.5 mg of Cot-1 DNA. Signals were amplified by applying a second layer of fluorescent-avidin on top of the biotinylated anti-avidin. Chromosomes were counterstained with DAPI. The micro-FISH probe produced a very bright signal covering completely the centromere of chromosome 16 (Fig. 3.4.7). All cells examined revealed a distinct hybridisation signal painting fully the heterochromatic centromeric region of both chromosome 16 homologues without any cross hybridisation being observed with the other chromosomes of the cells examined.

3.4.5 Generation of a micro-FISH painting probe, specific for the region 16cen→16q22

Nine fragments of the region 16cen→16q22 (Appendix II) were dissected, amplified by DOP-PCR and labelled by incorporating biotin-11-dUTP through a second PCR amplification. The first round of amplification revealed a very strong smear of the total human DNA (500pg) as a positive control, starting from few basepairs up to 350 bp (Fig 3.4.8). Both the microdissected material (lane 2) and the negative control sample (lane 3) revealed a very low intensity smear of very short fragments (few basepairs up to 130 bp), most probably primer artifacts or traces of contamination. Visualisation of the second PCR reaction products revealed successful amplification of all samples with a very strong intensity smear up to 400-500 bp in length (lanes 4, 5 and 6).

FISH was performed with 345 ng of the labelled microdissected material being mixed with 0.5 mg of Cot-1 DNA. Chromosomes were counterstained with DAPI. The micro-

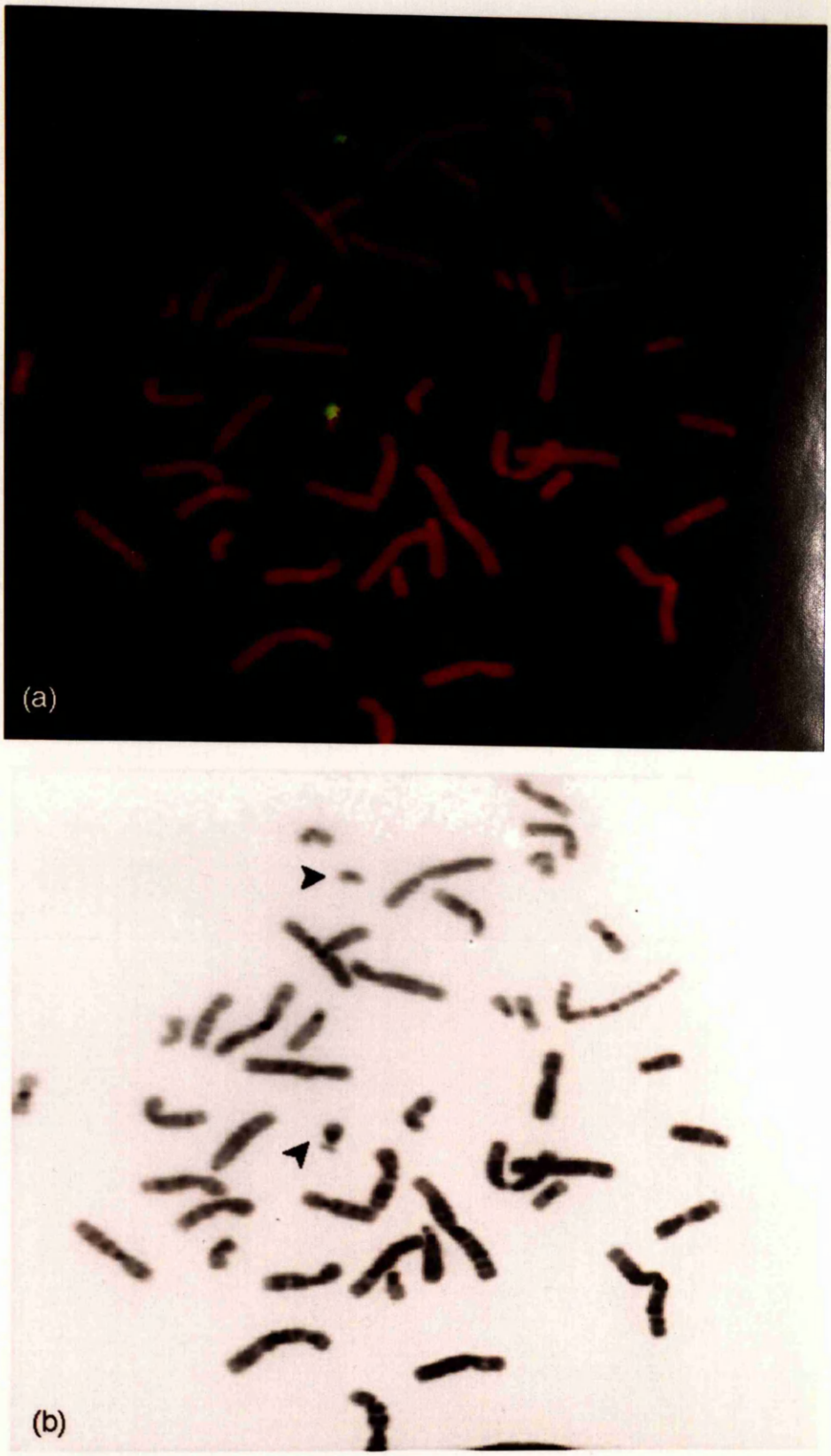


Figure 3.4.6 (a-b) : **(a)** Hybridisation of the PCR labelled 21q21-22 micro-FISH probe to a normal metaphase cell. Two bright yellow signals are observed on the 21q 21-22 region of both homologues. Cells are counterstained with PI (red). **(b)** Pseudo-coloured DAPI banding representation of the same metaphase . Two 21 chromosomes are indicated by arrows.

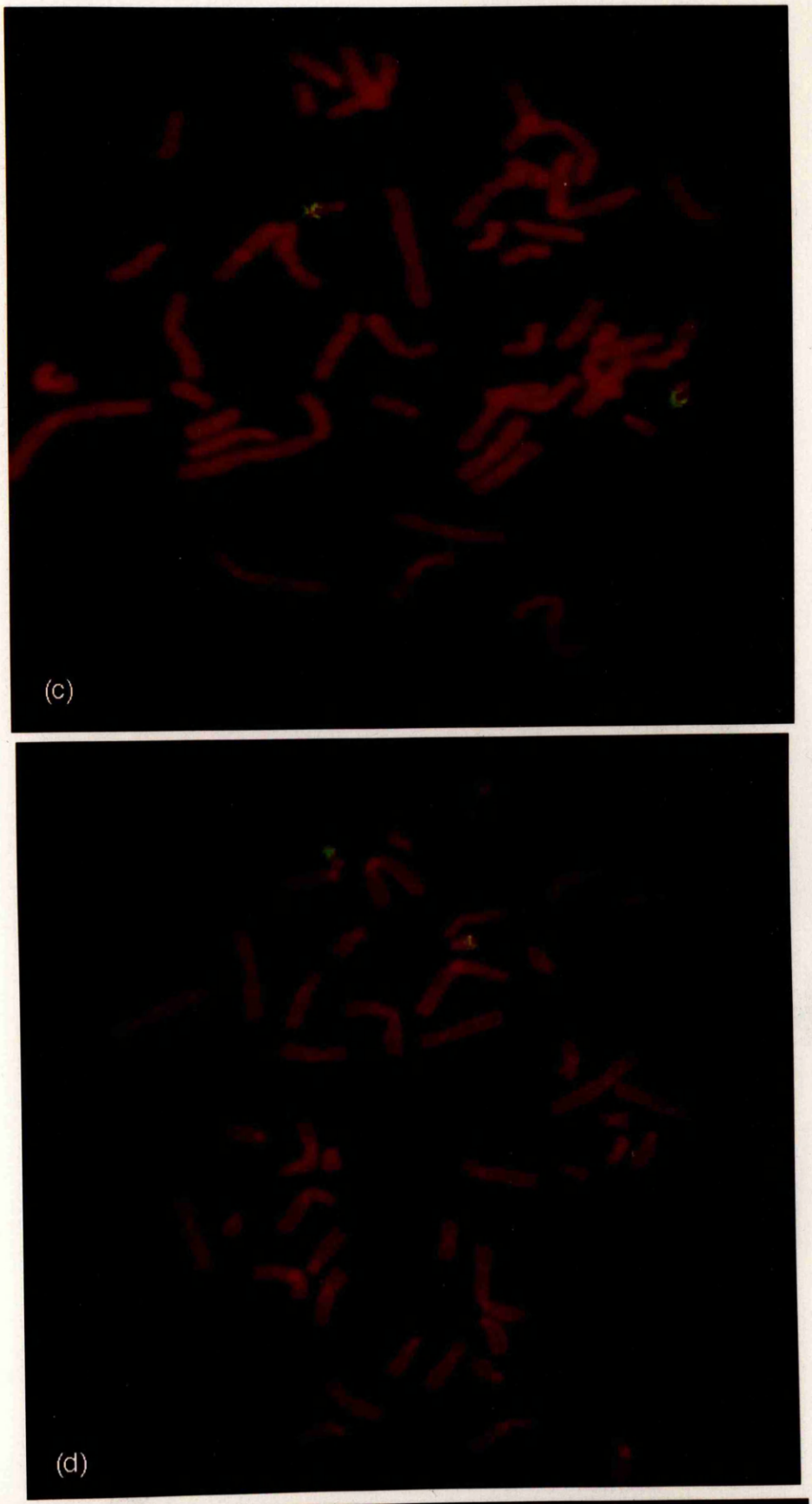


Figure 3.4.6 (c-d) : Hybridisation of the PCR labelled 21q 21-22 micro-FISH probe on normal metaphase cells.Two bright yellow signals are observed on the 21q 21-22 region of both homologues in each cell. Cells are counterstained with PI (red).

Figure 3.4.7: Hybridisation of the micro-FISH 16cen probe to a normal lymphocyte cell. A bright green signal is observed at the centromeres of both 16 homologues. One signal (shown by big arrow) has a stronger intensity than the other one (shown by small arrow), due to polymorphism of the heterochromatic region between the two homologues. Cells are counterstained with DAPI (blue).

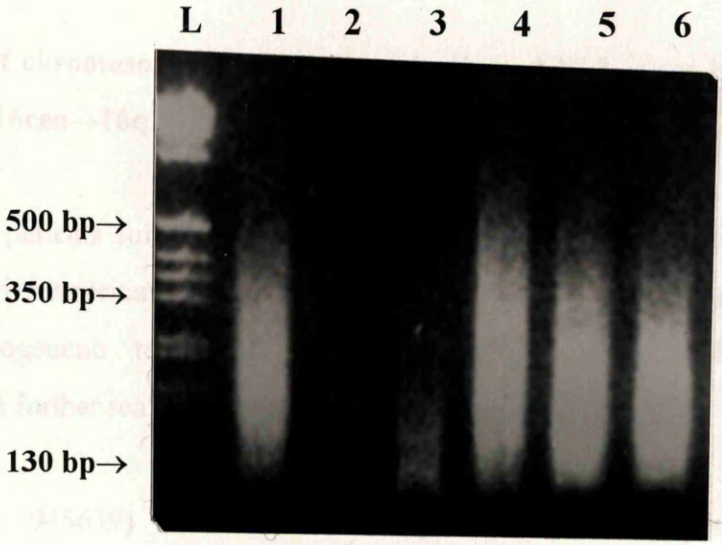


Figure 3.4.8 Gel electrophoresis of the DOP-PCR amplified 16cen→q22 microdissected region

L, 1 kb ladder

Lane **1,** 500 pg of amplified total human DNA

Lanes **2** and **3,** first amplification of the microdissected material 16cen→q22 and the negative control sample, respectively

Lane **4,** reamplified total human DNA

Lanes **5** and **6,** reamplification of the microdissected material 16cen→q22 and the negative control sample, respectively

FISH probe successfully painted the target region 16cen→16q22 of both chromosome 16 homologues in all cells of a normal peripheral blood sample examined (Fig. 3.4.9). Despite the fact that a high concentration of the probe was used for FISH, no cross hybridisation was observed with other chromosomes showing that 0.5 mg of Cot-1 DNA was adequate enough to suppress any cross reactivity.

3.4.6 Detection of chromosome 16 aberrations in three ANLL cases by applying the micro-FISH 16cen→16q22 probe

Three cases from patients suffering from ANLL were analysed in order to reconfirm the structural and numerical aberrations of chromosome 16 already reported by conventional cytogenetic techniques and to detect possible involvement of chromosome 16 in further rearrangements.

Case **1** (Lab.No. 945639) involved a patient with a pericentric inversion of chromosome 16, inv(16)(p13q22) being associated with ANLL-M4 with eosinophilic abnormalities (Fig. 3.5.1). G-banding analysis of bone marrow cell cultures revealed a karyotype with 46,XX, inv(16)(p13q22) present in eight cells, one cell with 47,XX,+8, inv(16)(p13q22) and eleven cells being normal 46,XX.

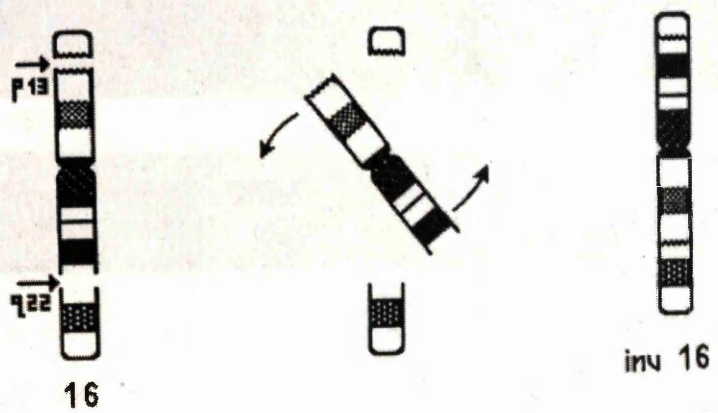


Figure 3.5.1 Diagrammatic representation of the pericentric inversion inv(16)(p13q22) in G-banding

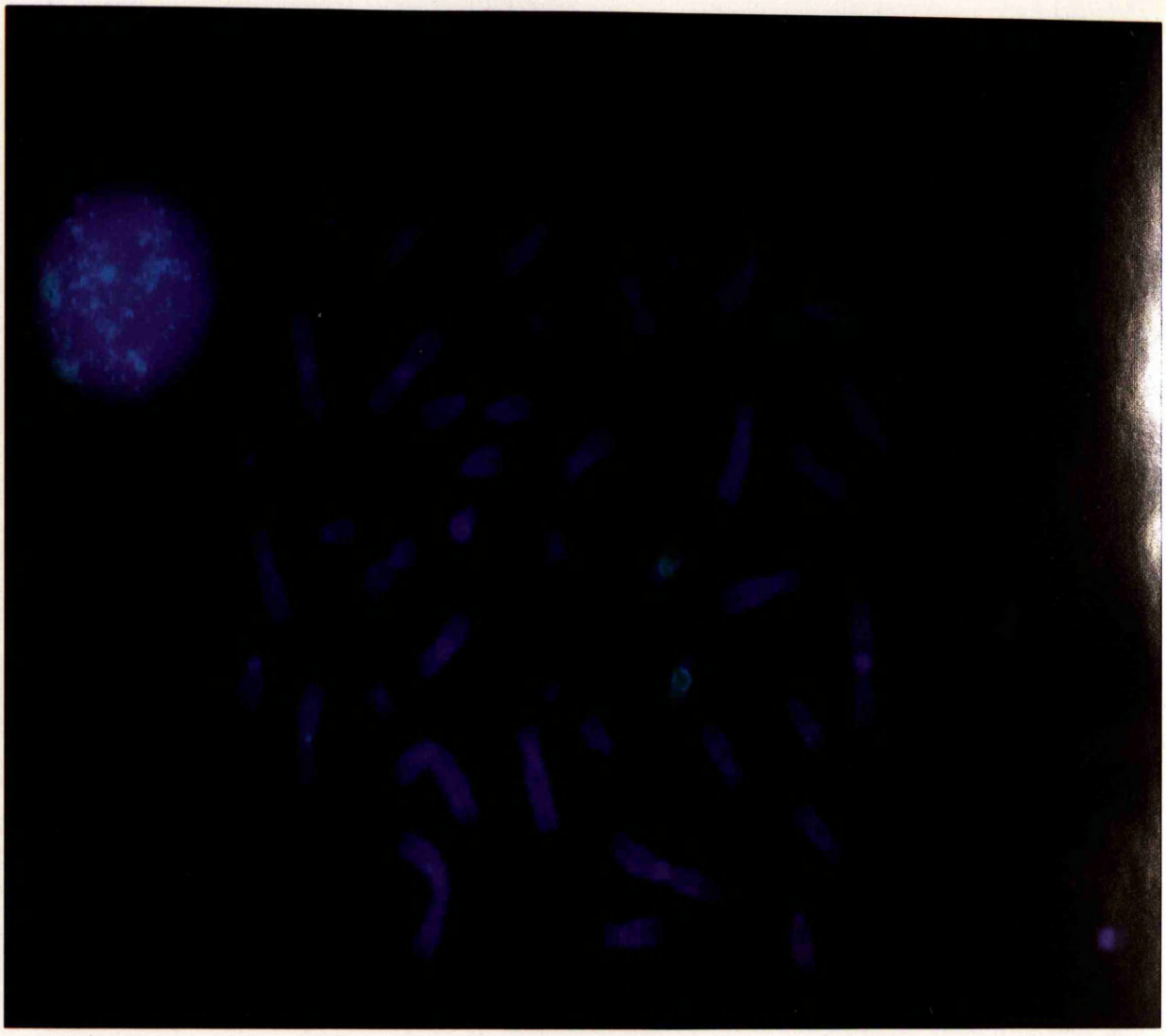


Figure 3.4.9: Hybridisation of the PCR biotin-labelled 16cen-q22 micro-FISH probe to a normal lymphocyte cell. Bright green painting of the 16cen-16q22 region is observed on both chromosome 16 homologues.

FISH analysis using the micro-FISH probe 16cen→16q22 revealed seven cells with both homologues of chromosome 16 appearing normal (Fig. 3.5.2), four cells with chromosomes of a very bad morphology where the signals obtained were not distinct and sharp enough to distinguish between the normal and the abnormal homologue, and four cells were found with the inv(16). In those cells, one homologue was normal with the 16cen→q22 region being fully painted and intact, whereas the abnormal homologue was inverted, with the breakpoint on the q arm occurring within the q22 band. As a result, following the diagram of Fig. 3.5.1, the painted q22 band would be expected to produce a split signal, part of it being transported as part of the inversion along with the rest of the painted q arm and the other part being observed as two dots (one for each chromatid) on the distal part of the unpainted-inverted 16p, along with the q23→tel, which was not involved in the inversion (Fig. 3.5.3a-c). Chromosomes had a very poor morphology, being contracted and fuzzy in appearance and hence differentiation between the p-arm and the q arm was done on basis of the DAPI banding pattern.

Case **2** (Lab.No. 965057) involved an AML patient with G-banding revealing a karyotype with multiple structural and numerical abnormalities: 41,X,-X, add(1)(q1?2), ?inv(5)(q11q33),-7,add(11)(q23),der(12)t(7;12)(p15;p12), -16, -17,-18. Fifteen mitotic divisions were counted and FISH analysis using the micro-FISH 16cen→16q22 probe revealed that all cells contained only one homologue 16 painted on the q arm, thus indicating absence of the second homologue from the metaphase cells (Fig. 3.5.4). Chromosomes counted on the cells were of a bad morphology, with a modal number of 38-41chromosomes.

Case **3** (Lab.No. 972017) involved a patient with multiple chromosomal aberrations including three marker chromosomes. G-banding analysis revealed a composite karyotype of 44-46,XY,-9,add(10)(p1?2),del(17)(11.2),-16,-18,add(21)(q22),+idic(?), +mar 1,+mar 2,+mar 3. In one cell (Fig. 3.5.5) two normal homologues 16 were scored along with a group-size E marker (m1), in addition to other numerical and structural abnormalities present in the cell. In a second cell (Fig. 3.5.6) only one normal homologue 16 was identified along with a group-size C marker (m2) and other aberrant

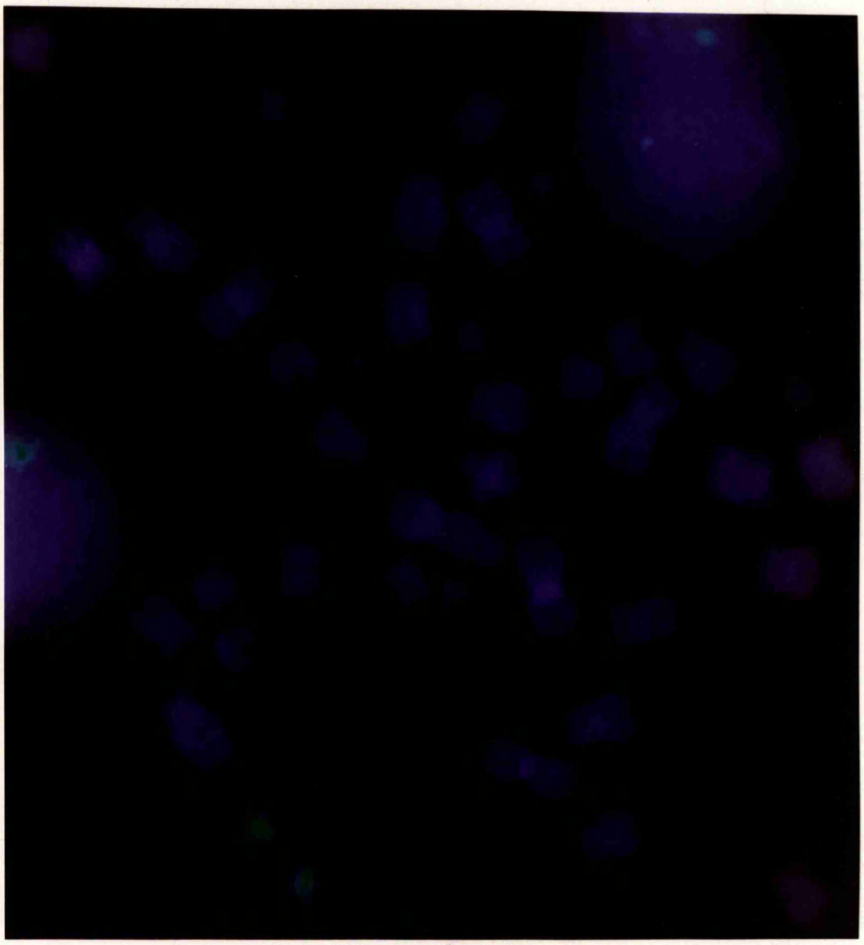


Figure 3.5.2: Hybridisation of the micro-FISH 16cen-16q22 probe to a bone marrow metaphase cell from an ANLL patient (case 1). Both homologues 16 appear normal with the centromere and the q arm being painted green (FITC) by the micro-FISH probe.

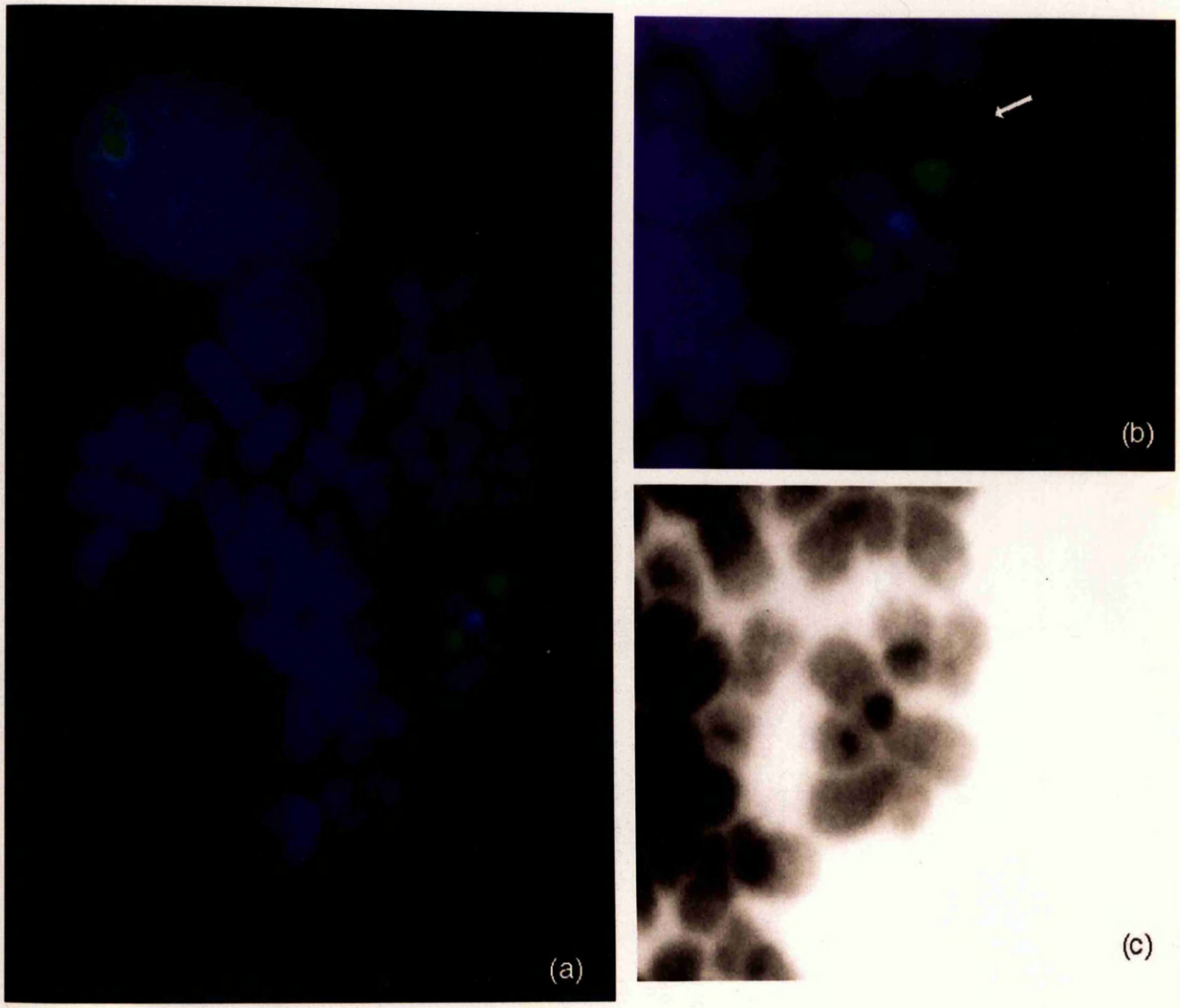


Figure 3.5.3(a-c): Hybridisation of the micro-FISH 16cen-16q22 probe to a bone marrow metaphase cell from an ANLL patient (case 1) with an inv(16)(p13q22). **(a-b)** The split signal (arrow) corresponds to the distal half of the q22 band which remaind uninverted. **(c)** Grey scale image of (b).

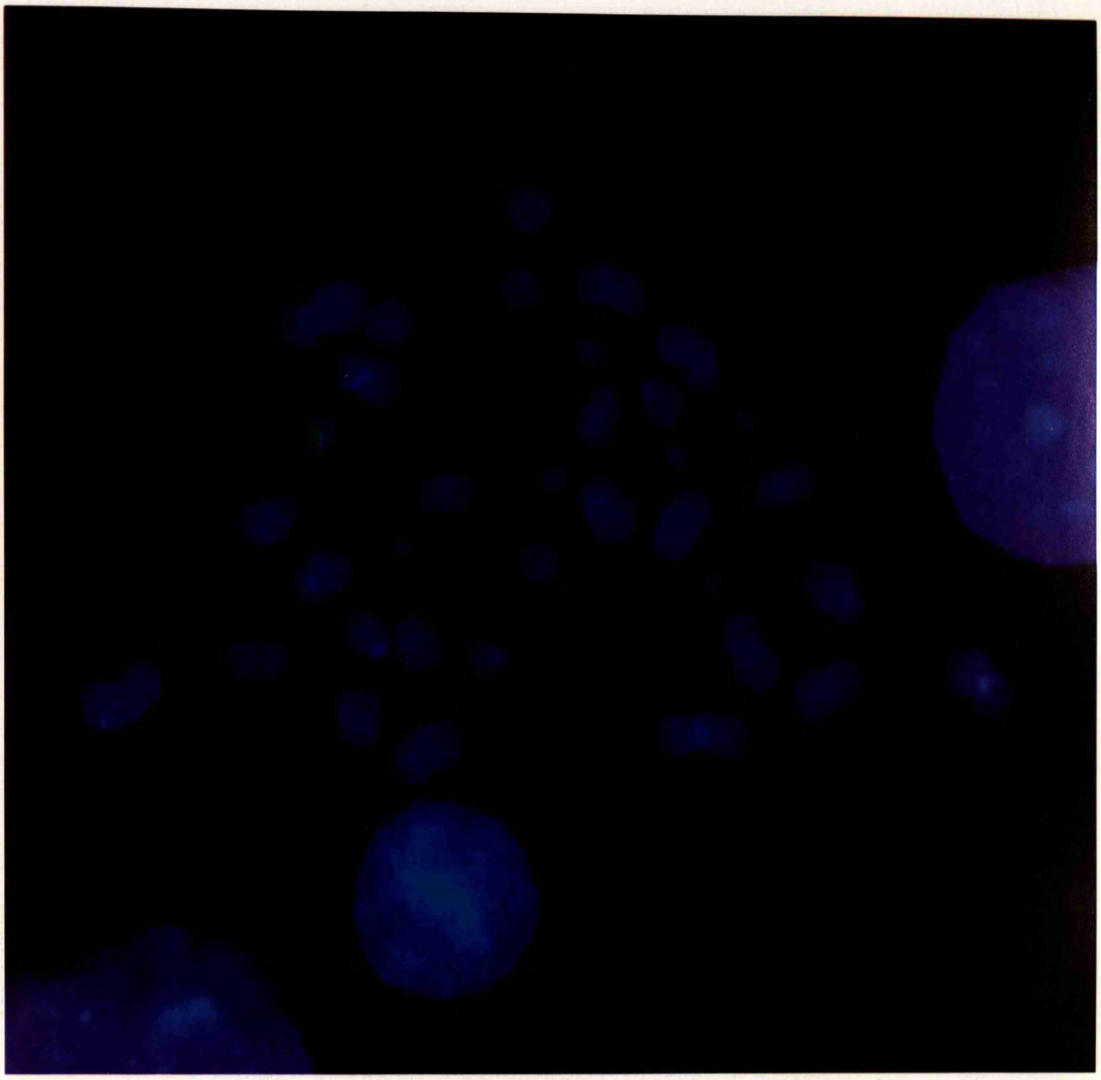


Figure 3.5.4 : Hybridisation of the micro-FISH 16cen-q22 probe to a bone marrow metaphase cell from an AML patient (case 2). Only one chromosome 16 is painted, indicating monosomy 16 as one of the numerical abnormalities present in the cell population.

Figure 3.5.5: Photomicrograph of a G-banded metaphase from an ANLL patient (case 3). Numerical and structural aberrations are present in the cell, including a group-size E marker (m1) and an abnormal chromosome with additional material 21q+.

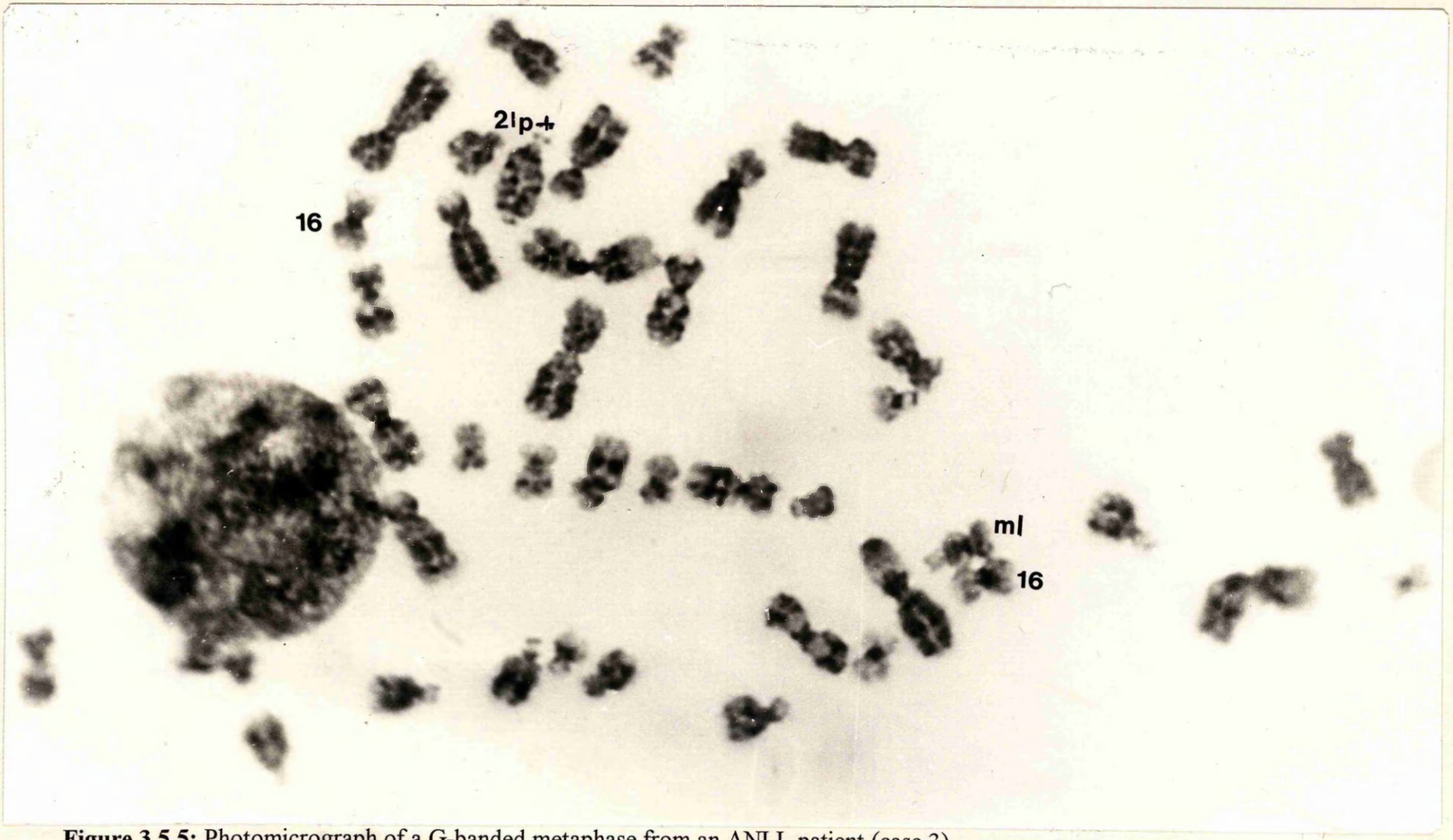


Figure 3.5.5: Photomicrograph of a G-banded metaphase from an ANLL patient (case 3).

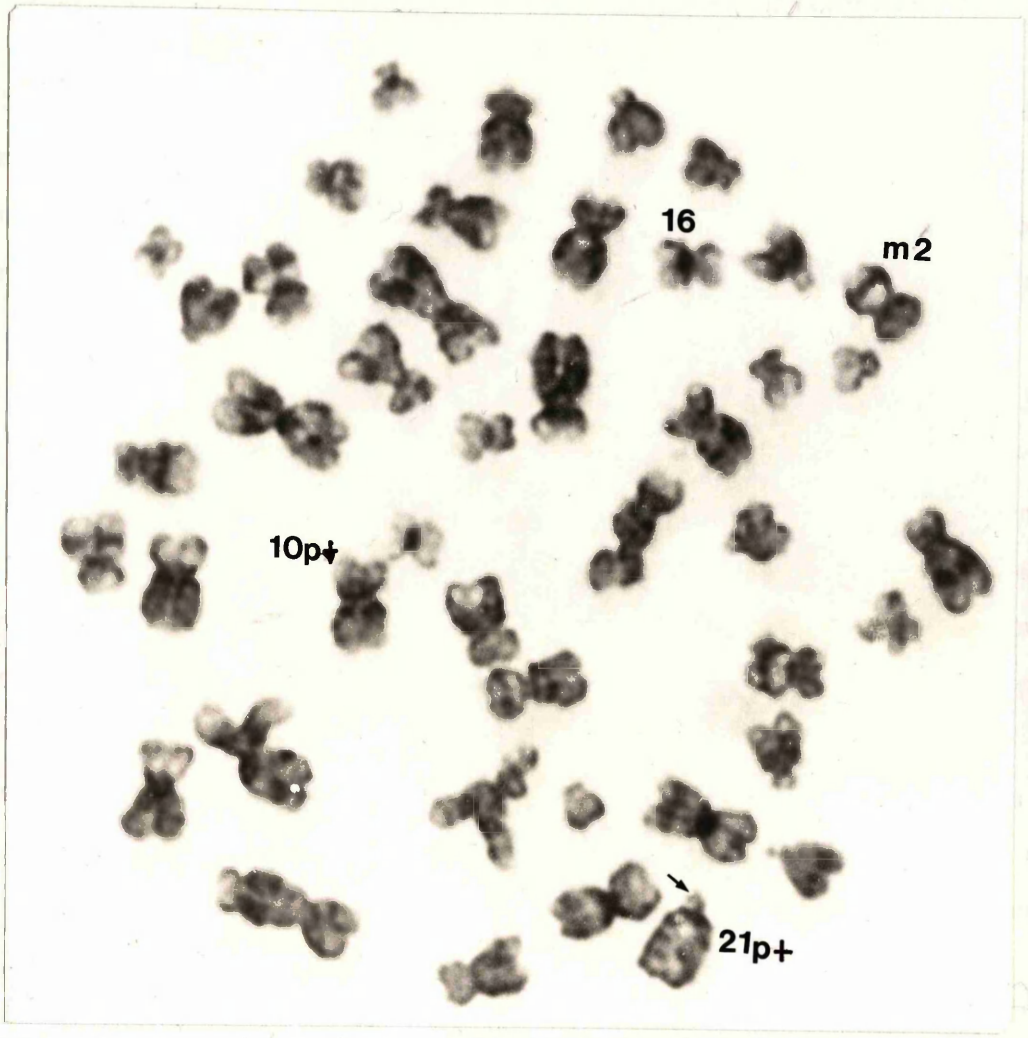


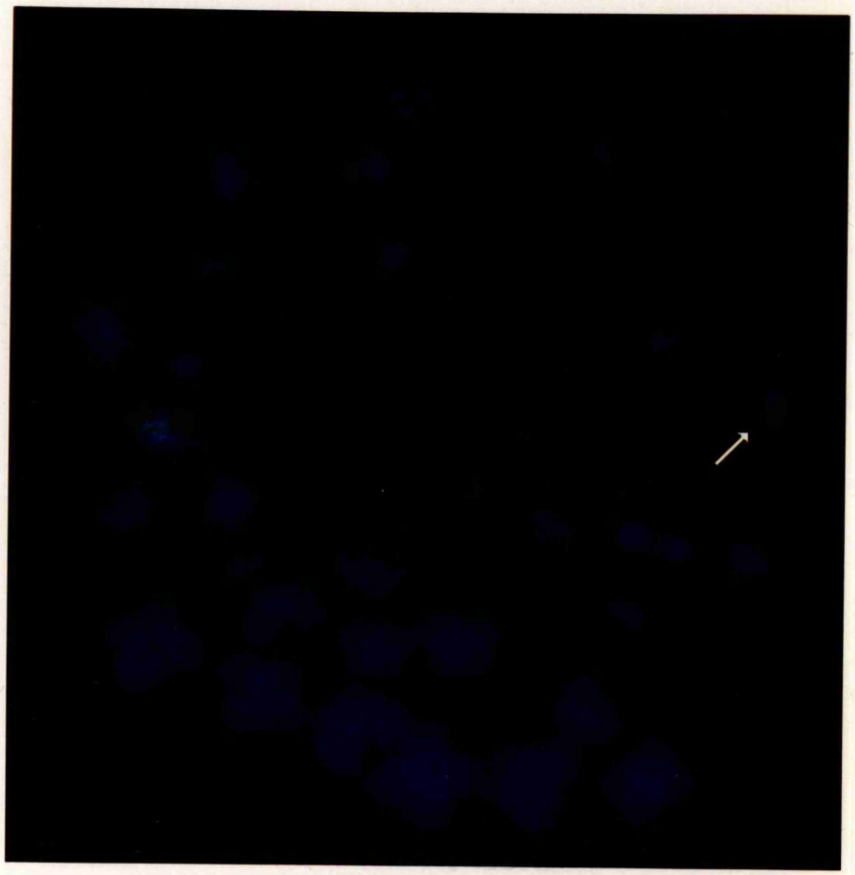
Figure 3.5.6: Photomicrograph of a G-banded metaphase from an ANLL patient (case 3). Numerical and structural aberrations including a marker (m2), an abnormal chromosome with 21q+ and a aberrant homologue 10 (10p+) are present in the cell.

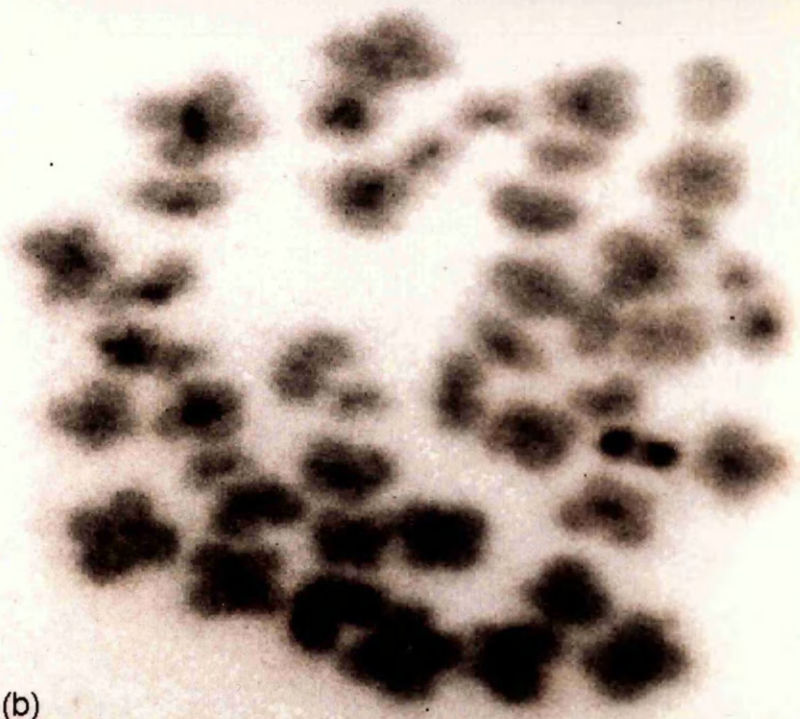
chromosomes including an abnormal homologue 10 with extra material on its p arm (10p+).

FISH analysis was performed using the micro-FISH probe 16cen→16q22 and 21 metaphases were countable. In 18 cells, chromosome 16 appeared to be normal with both homologues having their q arm painted. The remaining three cells were abnormal for chromosome 16. In one of them three chromosomes, apparently being of a similar size, were observed painted with the micro-FISH probe 16cen→16q22, thus demonstrating that these chromosomes carried material from the 16q arm (Fig. 3.5.7a-b). Due to the poor morphology of the cell, it was difficult to distinguish between the normal homologue(s) 16 and the abnormal chromosome(s) carrying material from the 16q arm. However, one of the chromosomes (shown by arrow) revealed a hybridisation signal that was slightly smaller than the signal observed on the other two chromosomes. From this finding it could be assumed that the two similar size signals might correspond to the two normal homologues 16 present in the cell, whereas the third one corresponded to a size E marker carrying material from part of the 16q region on one of its arms. This would be consistent with the G-banding analysis of one of the cells (Fig. 3.5.5) where two normal homologues 16 and an E size marker were all present in the same cell.

The two other cells revealed two chromosomes painted with micro-FISH probe. Chromosomes were of a very poor morphology and thus DAPI-pseudocolour stain was used in order to define the possible size group in which these chromosomes belonged. DAPI stain was not informative at all as to the banding pattern. However, it was clear that on both cells, one painted chromosome was of a small size group and this was defined as the single homologue 16 present in the cell.

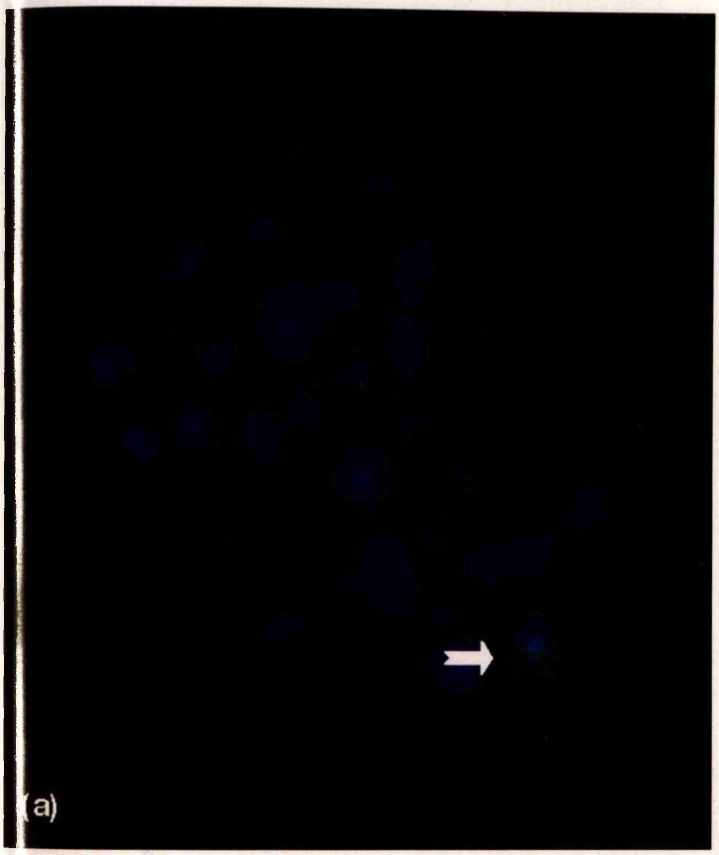
In one of these cells (Fig. 3.5.8 a-c) the large size chromosome appeared to belong to group C with its centromere and one arm being fully painted with the 16cen→16q22 probe. The other cell (Fig. 3.5.9 a-c) had a large size chromosome with painting on one of its arms, however, the hybridisation signal did not cover the heterochromatic region of the centromere as in the previous cell. Two possibilities exist here. Firstly, it could

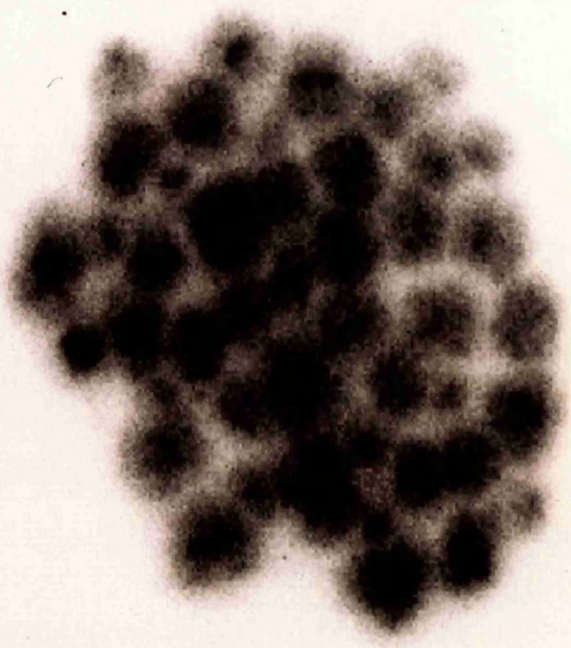




(b)

Figure 3.5.7 (a-b): (a) Hybridisation of the micro-FISH 16cen-16q22 probe to a bone marrow metaphase cell from an ANLL patient (case 3). Three chromosomes of a similar size are painted with the probe.One of them (arrow) thought to represent an abnormal chromosome carrying 16q material **(b)** Gray scale image of (a)





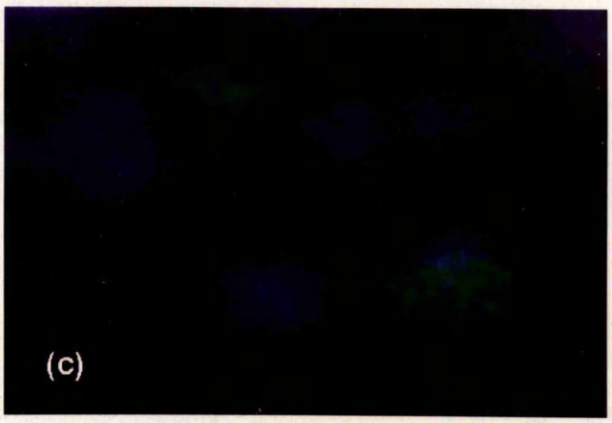
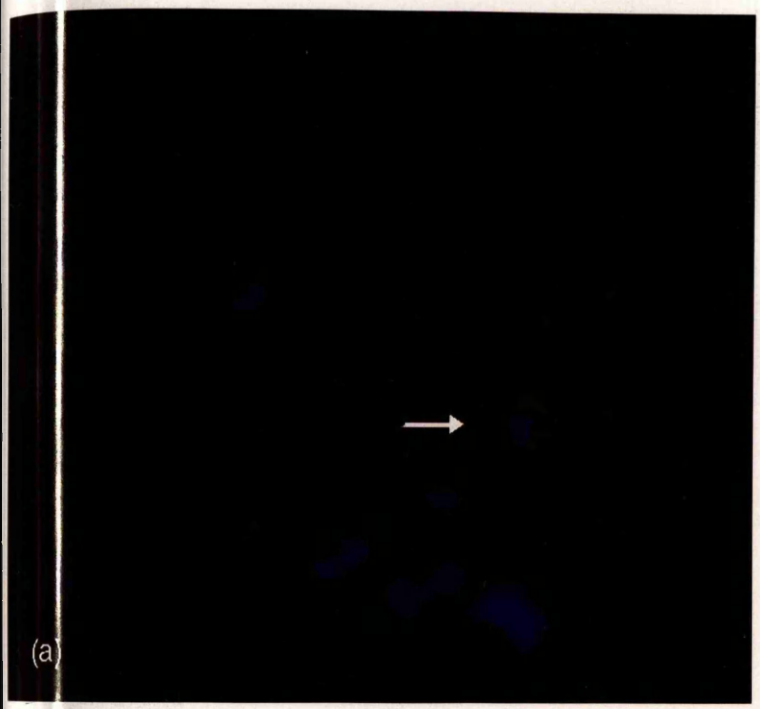
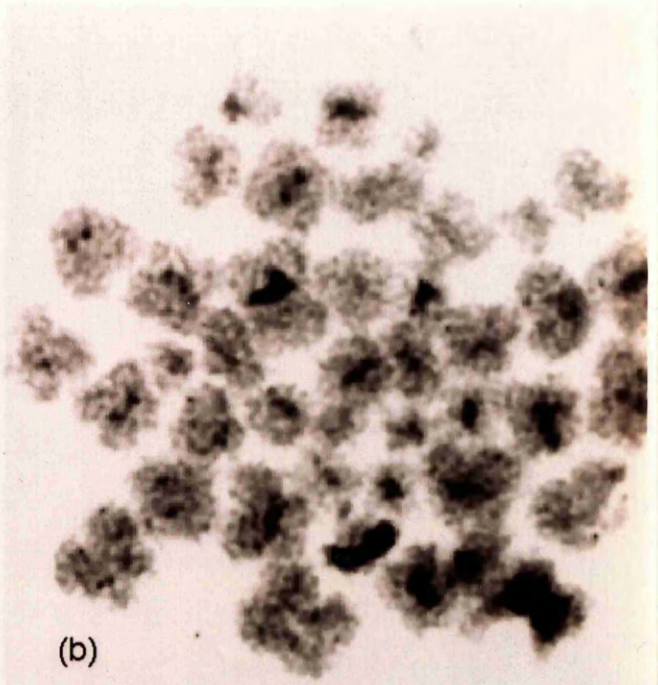


Figure 3.5.8 (a-b): Hybridisation of the micro-FISH 16cen-16q22 probe to a bone marrow metaphase cell from an ANLL patient (case 3). One large chromosome carrying material from the 16q arm (arrow) is observed, whereas the smaller size painted chromosome is thought to represent the normal homologue 16 present in the cell. **(b)** Grey scale image of (a). **(c)** Partial magnification of (a).





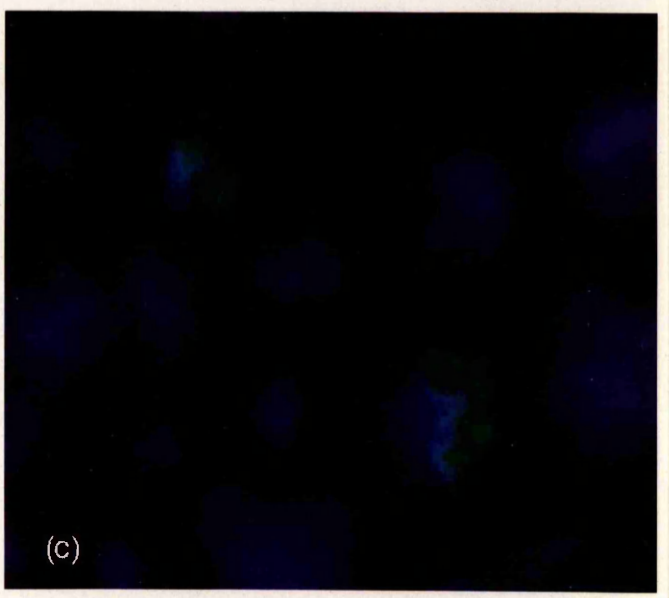


Figure 3.5.9 (a-b): Hybridisation of the micro-FISH 16cen-16q22 probe to a bone marrow metaphase cell from an ANLL patient (case 3). One large chromosome carrying material from the 16q arm (arrow) is observed, whereas the smaller size painted chromosome is thought to represent the normal homologue 16 present in the cell. (b) Grey scale image of (a). (c) Partial magnification of (a).

be that the two cells carried the same marker (m2) indicating that the painting at the centromere of the marker of one cell (Fig. 3.5.8) was only a fluorescent diffusion of the signal which had expanded from the arm to the centromere rather than a true hybridisation signal. In this case the size C chromosome identified by micro-FISH on both cells could represent the marker m2 of the cell analysed by G-banding (Fig. 3.5.6) containing material from the 16q region on one of its arms. Secondly, it could be that the two cells carried a different marker each. The size C chromosome carrying material from the 16cen→16q22 (Fig. 3.5.8) could correspond to the abnormal homologue 10 (Fig. 3.5.6) which had lost its p arm, containing instead material from the centromere and q arm of chromosome 16. That would rule out the possibility of both the abnormal homologue 10 and the m2 carrying 16q material since two C size chromosomes being painted by the micro-FISH probe would be expected instead of only one. On the other hand, if the derivative 10 chromosome did not contain 16q material, then this leaves as an alternative that the C size chromosome with its unpainted centromere corresponded to the m2 chromosome identified by G-banding (Fig. 3.5.6). Another possibility could be that in both cells analysed by micro-FISH, the size C chromosome could represent another chromosome in the cell population (different from the derivatives and markers observed by G-banding) that had remained unidentified by conventional cytogenetics. In both cells, the C size chromosome produced a slightly larger signal than the expected 16cen→16q22 signal observed on the normal homologue 16. This could lead to the assumption that the arm of the C size chromosome consisted of material from the 16q region that had probably duplicated thus composing the arm of this large chromosome.

3.4.7 Identification of the origin of Double-Minutes (dms) present in an AML case by chromosome microdissection

Chromosome analysis of unstimulated peripheral blood cell cultures of a patient previously diagnosed with MDS (Myelodysplastic syndrome) revealed an abnormal karyotype with 46-47,XY,-5?der(9)del(9)(p13)inv(9)(p13q21.1), der(17)t(5;17)(p13;p11.2) +r, 8-13 dms (Fig. 3.6.1). Few mitotic cells were present in the culture, with very poor morphology chromosomes.

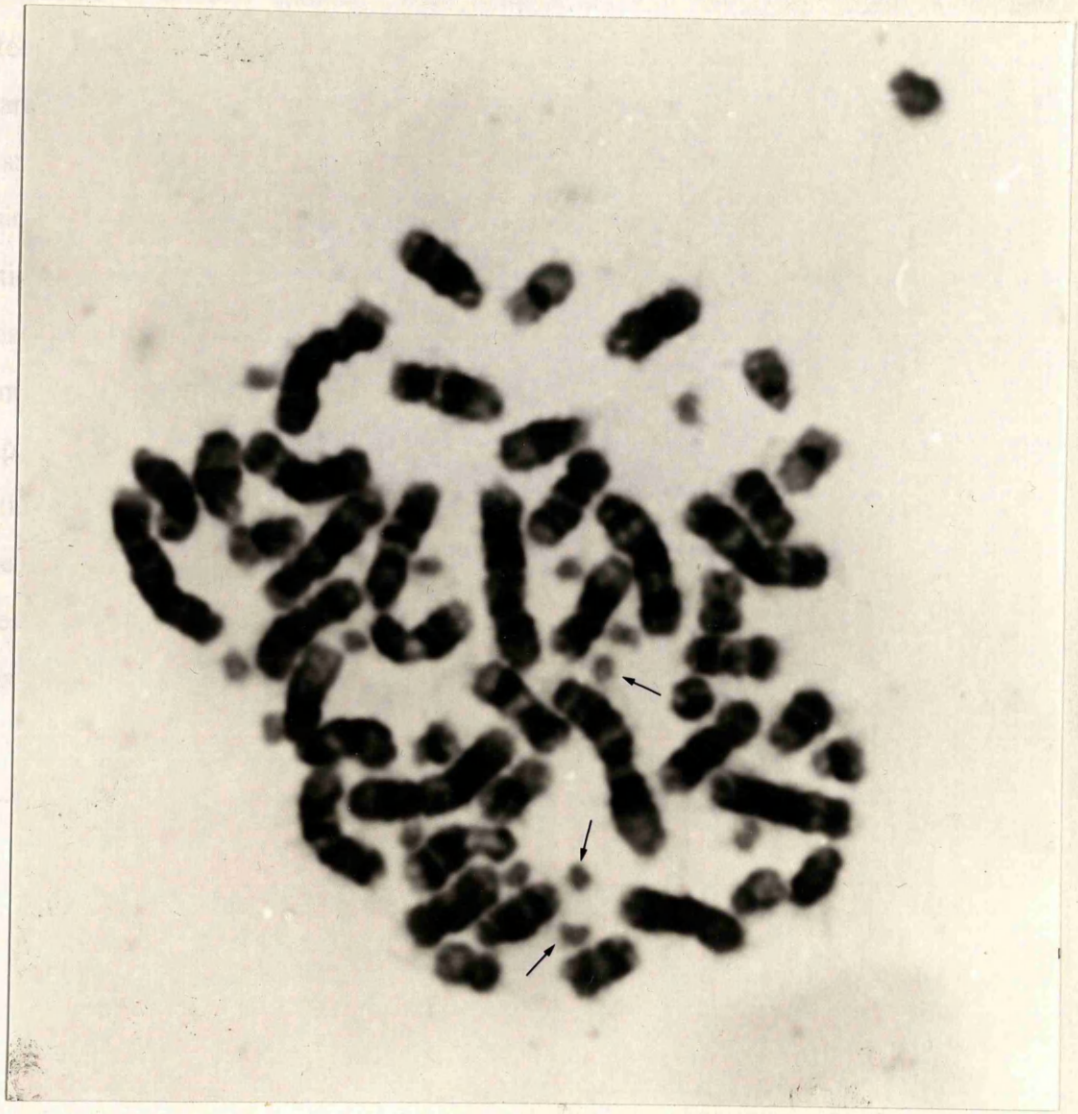


Figure 3.6.1: Photomicrograph of a G-banded metaphase from an AML patient containing Double Minutes (dms) shown by arrows.

CGH analysis revealed amplification of the 8q24 region, the location of the c-myc gene. FISH studies using whole chromosome 8 library painted both homologues of chromosome 8. Double minutes were present in very few cells. These were also painted by the chromosome 8 library, however, due to the bad quality of the cell preparation, the quality of the hybridisation signals on the double minutes was poor. At that stage, microdissection was performed on the double minutes in order to confirm the site of chromosomal origin. A total of seven dms was microdissected from three mitotic cells, transferred into a 0.2 ml collection tube and successfully amplified by means of DOP-PCR. The PCR product was labelled with biotin-11-dUTP through a second amplification and used as a micro-FISH probe for hybridisation to normal metaphase chromosomes (reverse chromosome painting). The results clearly showed that the dms contained sequences derived from the 8q24 region where the c-myc gene resides (Fig. 3.6.2), thus proving the potential of the microdissection technique in successfully identifying the origin of aberrant chromosomal structures present in genetic diseases such as cancer.

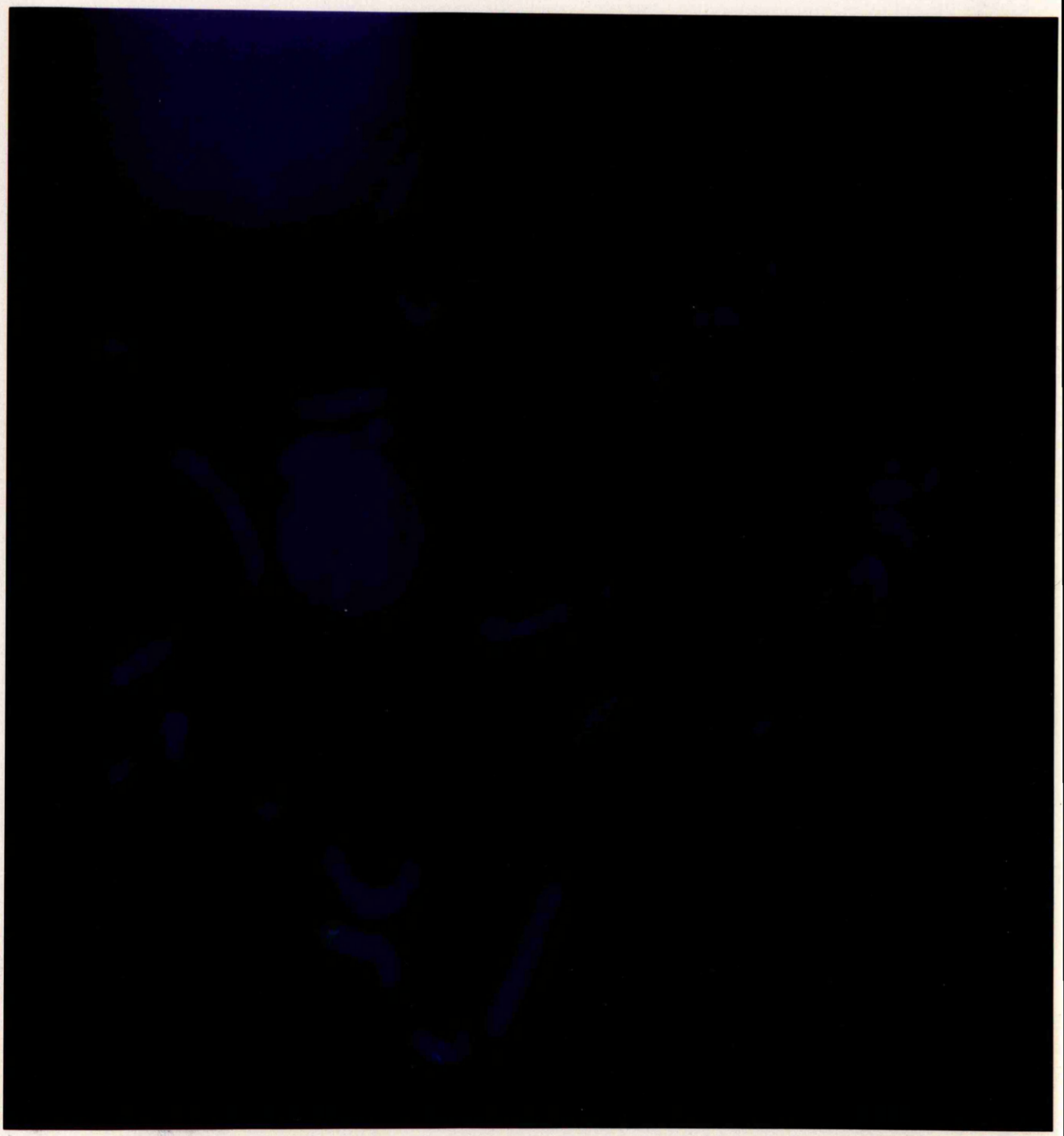


Figure 3.6.2: Hybridisation of the micro-FISH dms probe on a normal lymphocyte cell. The probe painted the 8q24 region, thus revealing the chromosome origin of the amplification unit.

CHAPTER 4

DISCUSSION

CHAPTER 4: DISCUSSION

4.1 DOP-PCR ON HUMAN GENOMIC DNA

The development of a six degenerated bases oligonucleotide-primed PCR (DOP-PCR) for genome mapping studies was first introduced in 1992 by Telenius et al. (1992b). Previous studies on the amplification of target DNA with an unknown sequence had been carried out using either Alu primers or primers with less than six degenerated bases. The universal primer (UN-1) with six degenerated bases on its 3'end has the great advantage of providing a pool of 4^6 primers of different sequences as opposed to a single sequence-unique primer. Its random priming throughout the genome makes it favourable to the Alu primers, the repeats of which are not found uniformly distributed within the human genome, but preferentially in G-light bands.

Several factors that can affect the efficiency of the PCR amplification were first tested on human genomic DNA which was used as a positive control in all experiments carried out. The aim was to amplify total human DNA of low concentrations and use these as probes for FISH. Ensuring successful efficiency of the DOP-PCR on low concentrations of total human DNA samples would provide the appropriate conditions for the amplification of microdissected DNA where the starting material (30 fg = $30x10^{-9}$ μg per dissected fragment) does not exceed 600 fg in total of DNA template.

Total human DNA was added into a 50 μl PCR mixture. One round of amplification consisting of both low and high annealing temperature cycles (30°C and 56°C respectively) was sufficient to amplify DNA of a few nanograms concentration (1 ng, 0.9 ng and 0.8 ng). Below that concentration (e.g. below 600 pg), the PCR products visualised by gel electrophoresis had a very faint intensity smear or no smear at all. Consequently, aliquots of the first amplification were reamplified by omitting the low temperature cycles. The smear obtained from all samples had a strong intensity starting from 200 bp to 516 bp with some distinct bands especially at the 430 bp region. Such bands could indicate the preferential amplification of the DOP primer

towards certain regions of the genome, the repetitive sequences of which share the same sequence with the degenerated sequence of the primer.

Total human DNA PCR products were labelled with biotin-11-dUTP and used as probes for FISH. An even painting on all chromosomes was produced. All low concentration probes tested, painted the target chromosomes successfully (100 pg starting DNA material). However, certain chromosome regions were left unpainted such as the centromeres of chromosomes 1, 9 and 16 as well as the short (p) arms of the acrocentric chromosomes. These heterochromatic regions consist of highly repetitive sequences (satellite DNA I and II) that should produce signals of a stronger intensity than the intensity of painting on the rest of the chromosomes. Absence of painting was due to the presence of Cot-1 DNA in the hybridisation mixture. Repetitive sequences of the template DNA were suppressed by CISS when an adequate concentration of Cot-1 DNA was added. Failure of certain chromosomal regions to become painted could occur. This would be because the DOP primer consists of a 3'end with a six base (ATGTGG) repetitive sequence estimated to occur every 4 kb throughout the human genome. Subsequently, low or no content of such a repetitive DNA would prevent the DOP primer from annealing successfully, therefore, preventing the amplification of the target region which will consequently remain unpainted after FISH.

The concentration of the primer is a critical factor in the PCR reaction. Primer concentrations higher than the optimal point may increase the probability of generating template-independent artifacts, known as primer-dimers. Such artifacts may themselves become substrates for PCR and hence compete with the desired product for dNTPs, primers and enzymes. Consequently, if the template of the target DNA is low in concentration and the primer concentration is high, self priming of the DOP primer could take place thus resulting in a very low yield of the desired product. Four concentrations of the DOP primer were evaluated (1mM, 2mM, 3mM and 4 mM). Satisfactory PCR product smears were obtained from all products, however, a stronger intensity smear was present at 3mM and 4mM of the DOP primer. This result showed no significance since no difference was observed on the painting of the chromosomes when FISH was performed with the PCR probes obtained under those concentrations. In result, 2mM (final concentration) of DOP primer was used as the

optimal concentration since higher concentrations could lead to formation of primer artifacts, especially over the presence of minute amounts of DNA template, whereas lower concentrations may have proved insufficient for amplifying the target DNA.

$MgCl_2$ can dramatically affect the yield of the PCR reaction since the level of divalent cations is critical to the reactivity of the Taq polymerase in the reaction. In addition, dNTPs tend to bind to Mg^{++} cations and consequently decrease the quantity of $MgCl_2$ available for the Taq polymerase. It is therefore important that the appropriate amount of Mg^{++} cations is added into the reaction mixture to ensure sufficient quantity for the polymerase activity. In the present study, different concentrations of $MgCl_2$ that were tested resulted in PCR products of a similar intensity and length of smear. Although the smear obtained from 1.5mM $MgCl_2$ was shorter and of less intensity than for 4 mM, no difference was observed in the quality of chromosome painting following a FISH experiment. According to the general PCR recommendations, 0.5mM to 2.5mM of magnesium should be added in the PCR reaction. Using 1.5mM $MgCl_2$ and 3.5 units of Taq polymerase satisfactory results were obtained. The recommended concentration of $MgCl_2$ (20mM) in the 5µl collection drop buffer used for the first eight critical cycles of amplification remained constant for all subsequent experiments. The concentration over the following 30 cycles of high annealing temperature was tested and 2mM was decided to be used since lower concentrations of $MgCl_2$ might have been insufficient for the Taq polymerase activity whereas a higher concentration may have increased the chances of amplifying minute amounts of contamination present in the PCR reaction.

Annealing of the DOP primer at the high temperature cycles could take place between 52°C and 62°C. Amplification was successful at all three temperatures tested although it is generally accepted that increasing the annealing temperature could enhance discrimination against incorrectly annealed primers and increase the specificity of the primer whereas low annealing temperatures could enhance amplification of any contaminants present in the reaction mix. High temperature cycles were kept constant at 56°C, to allow amplification of the entire target DNA present in the reaction mixture.

4.2 CONTAMINATION PRESENT IN THE PCR NEGATIVE CONTROL SAMPLES

Due to its six degenerate bases, the DOP primer can amplify any template DNA present in the reaction mixture. This makes the PCR itself very sensitive but also very prone to the consequences of DNA contamination. Moreover, the total DNA template from the microdissection of 30 fragments of an average size chromosome band does not exceed 1000 fg (1 pg) in terms of concentration (one fragment of 10 - 30 Mb equals 20 fg - 50 fg). Consequently, the effects of contamination over such a minute amount of template DNA, in the PCR mix, are increased.

Contamination was a negative factor, preventing successful amplification of the target DNA. The smear on the negative control samples varied from one experiment to another. Its intensity, length and band pattern would either match the positive DNA control or the dissected material smear or would otherwise consist of short fragments, not exceeding 200 bp in length. The presence of contamination on the first PCR round did not result in a successfully amplified probe following the second PCR round with the high annealing temperature cycles. Such experiments where a product smear was present on the negative control of the first PCR round were not processed further. However, if the smear of the positive control on the first round of amplification was strong, whereas the smear of the negative control was short and of a very faint intensity which was very difficult to observe on an agarose gel, the experiment was processed till the end, since this would indicate low levels of contamination that would not be sufficient to interfere with the PCR efficiency.

The persistence of contamination over numerous PCR reactions made it necessary for several precautions to be taken. To minimise contamination it proved necessary for all steps involved in the preparation of the PCR mix to be performed in a sterile safety cabinet so to avoid any contamination from the air (e.g. dust aerosols). PCR reagents were aliquoted into small quantities, allowing only a single use of each aliquot per experiment. Barrier-pipette tips were used all the time to prevent any aerosols from the barrel of the pipette being introduced into the sample, and all solutions were autoclaved. However, persistence of the contamination made it necessary to test each of the PCR reagents in order to identify the contaminated component responsible.

4.3 TESTING THE EFFICIENCY AND PURITY OF THE DOP PRIMER

The efficiency and purity of the PCR components were tested by using a set of specific primers for the centromere of chromosome 3 in parallel to the DOP-PCR reaction. Using a low concentration of human genomic DNA (1 ng and 0.1 ng/μl) DOP primers would be expected to give a smear of 200 bp to 800 bp, whereas primers specific for the centromere of chromosome 3 would form a distinct band at 1200 bp. Regarding the efficiency of the PCR components, the PCR reaction with the specific set of primers yielded a successfully amplified product. Consequently, the same PCR components should be equally sufficient to amplify the target DNA in the reaction driven by the DOP primer. Regarding the purity of the PCR reagents, contamination was observed but only in the DOP-PCR reaction. If any contaminants were present in the mix of both PCR reactions (e.g. plasmid DNA), they would be expected to be amplified by the DOP-PCR reaction and not by the reaction with the specific set of primers, since the specific primers would not have amplified any extraneous DNA due to their sequence specificity. Therefore, although the efficiency of the PCR reagents (including the DOP primer) was high, their purity had to be investigated further.

Fresh stock solutions for each of the PCR components were prepared once more and at the same time, the purity of the DOP primer was tested. During synthesis of the primer, many incomplete oligos are formed. Such sequences of a short length, could interact with the nucleotides of the target DNA in the presence of Taq polymerase thus preventing the DOP primer from binding to target DNA sequences. To avoid interference of the truncated short sequences with the PCR components, a COP column purification system was used. Both the purified COP primer and the non purified DOP primer were synthesised at the same time and used for the amplification of total human DNA. The PCR reaction carried out produced smears of a similar length and intensity and in the absence of contamination. However, when both primers were used for the amplification of the same microdissected region, neither of them produced any specific painting after labelling for FISH. Moreover, the reappearance of contamination on the PCR reaction of both DOP primers led to the observation that utilising a purified form of the DOP primer would not increase the

efficiency of the amplification sufficiently to allow amplification of minute amounts of DNA and would not alleviate the problem of contamination.

4.4 EXPLORING THE SOURCE OF CONTAMINATION

The persistence of contamination made it necessary to try to identify the source of it, so that by extra precautions its presence in the PCR mix could be eliminated. For this, negative control PCR samples from reactions that had been contaminated were randomly selected. The aim was to either exclude or attribute the source of contamination to human genomic DNA, since most of the steps involved were performed in an environment free of particular types of exogenous DNA. This was accomplished by hybridising the negative control samples to labelled ^{32}P human genomic DNA which was used as a probe for Southern blotting.

The blotting revealed smears on all three negative control samples tested, thus indicating hybridisation of the labelled human genomic DNA probe. The outcome of the experiment may lead to the conclusion that the DNA material present in the negative control was of human origin, thus confirming the source of contamination. However, the nature of the DOP primer is such that it allows amplification of DNA from any other species. Assuming that the DNA present in the negative control sample was of a different species (e.g. mouse), this would not prevent it from hybridising to the human DNA probe at low stringency as both species share sequences of homology. Moreover, the short fragment smears could correspond to primer artifact products such as concatamers which could be formed during the PCR reaction and in the absence of target DNA. Concatamers can be formed as a result of sequence homology between two DOP primer molecules. This could happen if the pool of DOP primers for a single PCR reaction contains strands that have complementary bases within the degenerated sequence. As a result, products of a few hundred base pairs in length could be formed, thus producing a smear or a distinct band noticeable in the negative control smears. In conclusion the best possible way to identify the origin of persistent contamination would ideally be to digest and clone randomly selected PCR negative control samples with the view to sequencing some of the clones and thus obtain information as to the

origin (i.e. species) of contamination by comparison with Genome databases. However, despite the outcome of such studies, the only effective way to overcome contamination would again be to take specific precautions for the several steps involved in the preparation of the PCR samples, like the ones already mentioned.

4.5 CHANGES IN THE STRATEGY OF MICRODISSECTION AND DOP-PCR

The initial approach on microdissection was to dissect at least thirty-five fragments of a particular chromosomal region and transfer them into a tube containing 20 µl of dH_2O or PCR mix. The first change was to omit the 60 minutes incubation in Proteinase K at 37°C. Proteinase K is an enzyme that rapidly inactivates endogenous nucleases such as DNases and RNases, thus preventing them from digesting the DNA between the nucleosome 'beads' in the chromatin structure. Treatment with proteinase K would protect the DNA from undergoing such a digestion and hence preserve its quality. However, no improvement in the efficiency of the PCR and consequently in the quality of the PCR product was observed. Following that, an experiment where 25 fragments of the chromosome 3 centromere region were dissected and amplified by using region specific primers was performed. After a second round of amplification the PCR product was labelled and used for FISH. Successful hybridisation of the microdissected probe painted the target region in all cells. Most importantly, the experiment was free of any source of contamination. Although the result indicated successful amplification and painting of the micro-FISH probe, the absence from the experiment of a negative control containing broken tips of unused needles prevented verification of the success of the experiment since in the presence of contamination (human DNA) introduced by the tips of the needles, the specific centromere 3 primers would have been able to amplify the relevant region of the extraneous (human) DNA and hence produce a successful specific signal by FISH. All needles used for microdissection had been treated with UV light prior to the experiment to ensure 'nicking' of any DNA contaminant present on them. Any other more drastic treatment such as rinsing with hydrochloric acid (HCl) that would effectively destroy any DNA contamination was avoided since the

effect would be outweighed by the fact that any traces of HCl remaining inside the very fine tips of the needles (after drying) could immediately damage the minute microdissected fragment. Since the heat needed to pull the needles was high enough to sterilise them, immediate transfer and storage of the needles inside a sealed chamber would prevent them from becoming contaminated. However, dissecting in an open-air environment would allow any contamination particles (dust, extraneous DNA) to contaminate either the needles or the tube used for collection of the microdissected fragments.

A new step was introduced, the addition of Topoisomerase I in the reaction mixture as described by Guan et al. (1993). Its ability to relax the highly condensed and supercoiled strands of the DNA molecule increased the efficiency of the PCR reaction by making the target-template DNA more accessible to the DOP primer and the other PCR reagents. The development of this strategy did not any longer require the dissection of a high number of fragments which could be time consuming. Ten to twenty fragments from a particular target region (or even less) were enough to provide a sufficient template for the PCR reaction. In addition, decreasing the number of dissections implied less exposure of the PCR mix to any exogenous DNA introduced while opening the PCR tube to insert the dissected DNA fragment.

Topoisomerase I was introduced in the microdissection experiments. It was then decided to dissect the same region (centromere 3) that had previously produced a promising result, but using this time the DOP primer instead of the region specific primers. A second negative control containing broken tips of unused needles was included. Although after gel electrophoresis a faint smear, but of an expected length, was visualised showing successful amplification of the microdissected material in the absence of contamination right from the first round of amplification, the product of the second PCR did not paint the target chromosomal region.

Not being able to produce a micro-FISH probe led to another change which allowed collection of the DNA fragments into just 5 µl of the PCR mix instead of the 20 µl used before. This was necessary since adding the minute

amount of microdissected material DNA into such a large volume (relative to the DNA amount) might over-dilute the sample and inhibit the efficiency of the reaction.

Further to that, transferring of the microdissected fragment into the PCR solution was achieved by touching the needle to the solution so that the fragment would detach from the needle and readily dissolve into the collection drop instead of breaking the tip of the needle inside the tube. This was done so as to avoid possible contamination introduced by the glass of the needle inside the PCR solution.

4.6 EVALUATION OF THE PROBE LABELLING

Having tested the different parameters involved in the PCR reaction, it became necessary to evaluate the labelling of the probe by a Dot-blot assay, although results by FISH were successful when control samples were used.

Both the centromere 8 specific probe and the human genomic DNA probe that had been labelled by nick translation successfully painted the target regions by FISH and therefore detection of the incorporated biotin by the Dot-blot assay was expected.

The assay on the two micro-FISH probes which originated from the same microdissected material 7p21-31 but which were labelled differently -the 7p21-31(N) by nick translation and the 7p21-31(P) by PCR - revealed the presence of biotin, despite the fact that the particular probes failed to produce any specific signal after FISH. This could be attributed to the fact that the DNA template of the starting material was not enough to produce a sufficiently amplified probe. In effect, the incorporation of biotin labelled nucleotides was insufficient to produce any painting after hybridisation. Considering the fact that contamination was always present in the second amplification reaction, biotin would incorporate into the nucleotides of the contaminant DNA as well. Although the levels of such an incorporation would be sufficient to be detected by the Dot-blot assay, the DNA template would consist of genomic sequences

rather than region specific sequences, thus failing to produce any specific FISH signals.

This could also explain the fact that although biotin was detectable in the negative control sample of the 7p21-31(P) amplification reaction, hence indicating incorporation of it into the DNA that had acted as a template in the labelling procedure, the levels of contamination and consequently of any exogenous DNA were not sufficient enough to hybridise evenly and produce painting on the metaphase chromosomes. In contrast, the negative control PCR product that had been labelled by nick translation, did create a paint in all chromosomes present in the metaphase cells, as a result of a high level contamination present at the second PCR amplification.

Different concentrations of biotin-11-dUTP were assessed (20μM, 40μM and 100μM) for both labelling procedures (nick translation and PCR). 20μM of biotin was decided to be used as a standard concentration since satisfactory results had been produced when tested on several positive control samples.

4.7 ATTEMPTS TO PRODUCE MICRO-FISH PROBES

4.7.1 Using isolated FACS chromosomes as a positive control sample

Human genomic DNA samples of low concentrations had been previously used as a positive control at all steps involved in chromosome painting using microdissected material. It has been estimated that in 1 μg DNA there are 152,000 copies of human genomic DNA and therefore 15 copies of total human DNA (46 chromosomes per copy) are contained in 100 pg of DNA. If we consider that about 10 copies of any chromosomal region could produce a successful FISH probe after amplification, then, for total genomic DNA , 75 pg (corresponding to 10 copies of the whole genome) should be the minimum concentration at which an even painting of all chromosomes could be obtained. As far as the microdissected material is concerned, since an average band size of 10 - 30 Mb is 15 - 50 fg, ten copies of such a band should be approximately 300 fg in terms of DNA concentration. Apparently, there is a

difference of 250X in the concentration corresponding to ten copies of target DNA between total human DNA and the microdissected material.

Therefore, using isolated FACS chromosomes as a control sample would resemble much more the microdissected material in terms of sensitivity and quantity of the DNA starting material (i.e. copies of a single isolated chromosome as opposed to copies of the whole genome). Chromosome 4 isolated by FACS was used in all experiments. Although the starting material consisted of approximately 350 copies of chromosome 4 in 25 - 30 µl volume, dilutions made it possible to reach the number of 10 copies of the isolated chromosome, corresponding to a concentration of approximately nine picograms, which was only 30X higher the concentration of ten copies of a microdissected material as opposed to the total human DNA control (i.e. 250 times higher).

Successful painting of both chromosome 4 homologues using as a starting material 10 pg in terms of concentration corresponding to 15 copies of the target DNA, indicated that all parameters involved in the PCR and FISH steps had been optimised successfully to produce probes generated from only few copies of the chromosomal region of interest.

4.7.2 Producing micro-FISH probes

Having evaluated the factors that could alter the efficiency of the amplification and hybridisation steps involved in the generation of a micro-FISH probe, the question remained as to whether the quantity of the microdissected material was adequate to achieve efficient amplification and hybridisation.

At first, the DNA amount of the first PCR amplification aliquoted into the second PCR mix was increased. Instead of adding 5 µl of the first PCR product, 12 µl were added so as to increase the DNA template present in the second PCR amplification. Alternatively, multiple reamplifications were initiated by using almost all of the first amplification product. All products from the different PCR reactions were eventually co-precipitated in a single reaction tube

to ensure higher concentrations of the DNA. No difference was observed at the end of each of the experiments performed.

Increasing the number of cycles during PCR would favour the amplification of DNA contaminants and would increase the number of artificial DNA sequences. Consequently, such an approach was ruled out. The findings led to the questioning of the successful transfer of the dissected material inside to the collection tube. Successful transfer of the dissected fragment is dependent on the needles used for performing the dissection. Needles appropriate for microdissection should be fairly closed at the end of their tip so to avoid drowing up any solution once the needle holding the fragment had been inserted into the PCR buffer. Therefore, providing that the tip of the needle had a very small opening, the fragment was picked up on the side of the tip by electrostatic attraction. Once the tip of the needle was inside the collection drop, the minute quantities of DNA should readily dissolve inside the vast amount (in relation to the microdissected fragment) of the collection drop.

Difficulties in making the DNA stick on the needle meant that the electrical forces built up between the DNA and the needle were not appropriate. Such an event relies on the principle of equal signs of charge being repelled whereas opposite signs of charge are attracted. DNA is negatively charged because of its phosphate groups (PO_4^{-2}). Therefore, the DNA fragment will be attracted to a positive charged surface. Ensuring that the glass needle has a constant positive charge would facilitate attraction between the needle and the DNA fragment . The fragment would eventually fall off the needle, inside the collection drop which neutralises the charge and hence disrupts the electrostatic attraction.

Treating needles with silicone, a solution that prevents DNA from sticking firmly to and remaining on the needles so that the fragment can easily come off the needle, did not make any difference. An antistatic instrument was used in a way that emits a stream of positive ionised air over the surface of the needles to maintain their positive charge.

Transfer of the microdissected fragment to the PCR tube was successfully achieved only when the needle had approached the DNA fragment at a specific angle. This allowed a very small surface area of the fragment to adhere to the tip of the needle, thus making detachment feasible. In contrast, touching the DNA fragment with the side of the needle did not allow successful transfer to occur, as observation under the microscope showed that the DNA fragment remained attached on the needle. In some cases the tip of the needle accidentally broke inside the tube, thus drawing in some of the collection drop but this did not affect the quality of the experiment providing that it occurred only occasionally and without decreasing the amount of the collection drop dramatically.

The successful micro-FISH results were obtained from chromosome preparations that had been stored at -20°C for several months. Fixation of the chromosomes in methanol/acetic acid (3:1) did not affect the quality of the experiments. Dissected chromosomes had been banded at least ten days prior to the microdissection and kept at room temperature, thus indicating that subsequent ageing of the DNA did not affect the yield of the experiments.

Evaluation of the amplification efficiency by visualising the PCR products on an agarose gel, proved important only at the first round of amplification to assess the presence or absence of contamination. The micro-FISH probes produced from the microdissection of the chromosome regions 7p21, 21q22 and 16cen were obtained under contamination free conditions. However, the micro-FISH probes produced from the microdissection of the16cen→16q22 region and the double minutes were obtained by amplification of the microdissected material under the presence of low levels of contamination as observed by gel electrophoresis. Although the intensity of the negative control sample smear was slightly stronger than that of the microdissected material, this did not seem to affect the yield of the PCR reaction. In fact the quality and quantity of the microdissected material was high enough to be observed right from the first amplification indicating high yield of the PCR reaction. It is therefore concluded that the faint smears were more likely to be primer artifacts, due to possible excess of the DOP primer on the particular reactions, rather than contamination. If contamination was present on the first round of amplification a successful micro-FISH result would be unlikely unless the levels of contamination

were very low lower than the PCR yield for the microdissected material. Visualising the products of the second amplification was not informative as to the success of amplification since a strong intensity smear was always observed in all PCR products as well as the negative control samples. It was therefore decided that regardless of the outcome of the second amplification, the experiments should carry on till the end, proving that the most reliable way to confirm the success of the microdissection attempts was by FISH.

Adding Cot-1 DNA (0.5-1.0 μg) to the hybridisation mixture was necessary to avoid any cross reactivity. In fact, no cross hybridisation was observed with any of the other chromosomes in the metaphase spread due to the complete suppression of the repetitive sequences present in the microdissected regions. Diffusion of the signal could be minimised by adjusting the probe concentration. The signals obtained were very distinct and bright and painted fully the target region on all cells analysed. Due to the probe specificity and high signal intensity, it was not necessary to assess but only few cells thus making the analysis time effective.

4.8 CONCLUSIONS

4.8.1 Overall evaluation of the contamination effects

In this project the technical difficulties often encountered in the steps associated with the generation of micro-FISH probes were thoroughly investigated. DNA contamination hampered the interpretation of the PCR results and consequently the outcome of the FISH hence, it was necessary to limit it to the lowest possible levels. Contamination was always evident as a strong intensity smear at the second DOP-PCR amplification. This led to the conclusion that testing the products of the second PCR by gel electrophoresis was not at all informative as to the successful amplification of micro-FISH probes and therefore, the results of this analysis should be completely ignored.

On the other hand, it is very crucial to test for presence of contamination on the first DOP-PCR since the efficiency of the amplification and the presence of contamination at this stage could drammatically affect the experiments to follow. In the absence of

smear from both the microdissected product and the negative control sample lanes, the experiments should be continued. However, if both lanes show a smear with the negative control sample having a smear of a stronger intensity that that of the microdissected product, then it is unlikely that the generation of micro-FISH probe will be successful. On the other hand, if a very faint smear is present at the negative control lane and with a low molecular wight two possibilities exist. First, this smear can be due to contamination present at very low levels, most probably not sufficient to interfer with the re-amplification and generation of the micro-FISH probe. In this case, the experiments should continue until the end (i.e. FISH). Second, the smear of the negative control sample could be nothing else but primer artifacts formed in the absence of a DNA template. This again would mean that the experiments should be proccessed further.

All different parameters that could affect the techniques of microdissection and efficiency of the PCR were assessed. Controlling the contamination (i.e. preparation of the PCR being performed in a sterile safety cabinet and using only barrier-pipette tips etc.), keeping the rest of the PCR and FISH parameters to the optimal levels and most importantly, assuring successful transfer of the microdissected fragment inside the collection drop led to the successful production of micro-FISH probes by dissecting specific regions of normal lymphocyte cells and aberrant chromosome structures (7p21-31, 21q21-22, 16cen, 16cen→16q22 and double-minutes).

4.8.2 Analysis of chromosome aberrations using microdissection techniques

Recent advances in molecular cytogenetics, especially in FISH and PCR have made it possible to ascertain a large number of aberrant chromosomes present in both clinical and cancer cytogenetics. Reverse chromosome painting using chromosome microdissection has been a powerful tool in investigating the origin of abnormal chromosomes.

The potential value of reverse chromosome painting using microdissection techniques has been demonstrated by the application of the 16cen→16q22 microdissected probe to a number of leukaemic bone marrow samples and the deliniation of the origin of a dm.

The pericentric inversion of chromosome 16, inv(16)(p13q22) is characteristic of acute nonlymphocytic leukaemia, subtype M4 and is associated with abnormal marrow eosinophils (eosinophilic granulation). It is believed to occur because of sequence cross-homology between the 16p and 16q regions, thus facilitating such a rearrangement. It has been reported that this subgroup of ANLL has a good response to intensive therapy with most of the patients achieving complete remission. Hence it is important to be able to identify cells of ANLL patients carrying such an aberration.

The inv(16) is a subtle aberration that is difficult to detect with certainty on contracted chromosomes with inferior banding quality which is a common feature of metaphase chromosomes in leukaemic cells. The use of cosmids and YAC clones as probes for FISH has facilitated the detection of inv(16) (Dauwerse et al. 1993) and this study shows that the micro-FISH probe 16cen→16q22 is also an efficient aid to detection of the inversion. Chromosomes were of very poor morphology. Both the normal and the abnormal (with a pericentric inversion) homologues of chromosome 16 were correctly identified using the micro-FISH probe which contained sequences from the centromeric region. A split hybridisation signal representing the distal half of the 16q22 which was not involved in the inversion clearly indicated the presence of the inversion chromosome inspite of the quality of metaphases. In one other case chromosomes from three metaphase cells revealed 16q material being present on a C group size chromosome and on an E group size chromosome in addition to the normal homologue 16 present in the cells.

The quality of the chromosomes was very poor, thus allowing only speculation as to the genetic composition of the aberrant chromosomes identified by micro-FISH. However, when comparing with the chromosome preparations used for G-banded analysis, the latter presented with a better chromosome morphology despite the fact that in both cases, cells derived from the same sample. It is clear that the routine FISH protocol results in the morphology of the chromosomes being distorted, perhaps due to the hybridisation conditions such as the denaturation temperature. More emphasis should be given to the optimisation of the FISH technique in order to overcome the problem. Obtaining cells with a reasonably good quality could greatly assist interpretation of the FISH results. Furthermore, for the markers and derivative

chromosomes the genetic composition of which could not be fully delineated in the case analysed, reverse chromosome painting of the rest of the abnormal chromosomes would prove the most certain approach for fully characterising and karyotyping each of the abnormal cells.

Marker chromosomes in tumour cytogenetics, are often observed as supernumerary chromosomes which remain unidentifiable, thus preventing complete karyotypic analysis. Ascertaining the origin of these chromosomes by microdissection is much to be preferred to forward chromosome painting ,which is a time consuming and an expensive approach, since a battery of probes must be tested empirically in each de novo chromosome abnormality. Using FACS as a approach to isolate the chromosome of interest, presents difficulties in clearly distinguishing constitutional markers of a very small size (smaller than one third of chromosome 22), thus limiting its application. Moreover, the presence of inadequate number of cells in division can hamper analysis of chromosomes since numerous chromosomes are needed for sorting. This can be overcome by microdissection. Recent advances have made it possible to create a micro-FISH probe from just a single microdissected DNA fragment. Further optimisation of this sensitive technique would be of tremendous value in analysing cases where the material available is very limited and the chromosome preparations of a poor quality.

The presence of unknown supernumerary markers (usually small and with an obscure banding) and ring chromosomes is a problem encountered during prenatal cytogenetic analysis with an incidence of 0.4/1000 to 1.5/1000 for marker chromosomes. Identification of such chromosomes, especially when they occur de novo is a crucial step in the successful evaluation of possible phenotypic effects to the carrier. Depending on the size of the marker and the amount of euchromatin present, the clinical findings may range from normal to severe mental retardation in association with congenital abnormalities. Therefore, it is of great importance to be able to identify their origin, so that a karyotype-phenotype correlation is established, thus allowing reliable risk estimates to be obtained which can be used in genetic prenatal counselling. In both tumour cytogenetics and prenatal diagnosis microdissection techniques have proved to be efficient in revealing the composition of these aberrant chromosomes (Viersbach et al. 1994, Thangavelu et al. 1994, Muller -Navia et al.

1995). The more marker chromosomes identified and associated with recognisable syndromes, the better it will be to assess the prognostic value and significance of these small extra chromosomes.

Regions of amplification such as dms and HSRs have been shown to contribute to the multiple process of carcinogenesis and overexpression of amplified genes harboured by these amplification units can contribute to the malignant phenotype of several solid tumours such as breast, lung and ovarian cancers. Working on both fresh and established cell line samples could help identify oncogenic enhancement in specific tumours. Further studies by utilising standard molecular techniques such as Southern blotting for confirming the involvement of particular genes and Northern blotting for detecting possible changes at the RNA level can provide valuable information.

DNA sequence amplification is described as an abnormal process in human cells and is considered to play a role in the multiple process of carcinogenesis in a variety of human cancers such as malignant melanomas, ovarian cancer, osteosarcomas and several other solid tumours. Two main types of cytological markers for DNA amplification are known to be present in malignant cells. The HSRs (homogeneously stained regions), which are integrated within the linear DNA of chromosomes and the dms (double minutes), which are small-spherical, extrachromosomal DNA structures. Dms lack a functional centromere. Their number can very greatly from cell to cell with cell lines containing one or two dms or sometimes up to 1000 per cell. Their average size is 0.3-0.5μm and they are estimated to contain about 1-5Mb of DNA in length. They are frequently observed in solid tumours such as neuroblastoma and rarely found in leukaemia.

Several reports have shown the amplification unit of these structures to harbour genes, the overexpression of which could possibly be linked with the progression of the disease. It is thus important to be able to identify the exact origin of the amplified DNA present in these cytological structures and this can be achieved by microdissecting the amplicons and reverse painting onto normal metaphase cells. In this way, Zhang et al. (1993) reported for the first time a link between an overexpressed gene (IGF1R) within the HSR of 15q26-origin and the malignant

melanoma case of study. Similar studies on breast cancer cell lines, not only provided further insights into the mechanism of formation of HSRs, but also identified regions likely to include genes amplified in breast cancer (Guan et al. 1994b). Identification of cryptic sites of DNA amplification by the same approach could provide important information on the development and progression of ovarian cancer (Guan et al. 1995) as well as small cell lung cancer (Xu et al. 1996).

In this study, the chromosomal origin of dms present in the cell population of an AML patient was identified by successfully isolating the extrachromosomal structures using microdissection techniques. The ampification unit revealed to contain sequences that localised at region 8q24 where the c-myc gene resides. C-myc is a proto-oncogene coding for a product that acts in the nucleus and appears to be involved in regulating the transition of cells from a resting to a proliferating state. Dysregulation of c-myc expression as a result of a translocation may contribute to malignant transformation or to the high proliferative rate that is characteristic of neoplasia such as Burkitt's lymphoma. Although gene amplification is unlikely to contribute to leukemogenesis in most cases, its detection in the clinical setting can provide an important means for improving the staging of patients with certain types of solid tumours. Moreover, once the amplified gene has been identified, molecular techniques such as Southern blotting can be used to confirm its involvement in amplification. Levels of its expression can be studied by detecting changes in the RNA using Northern blotting. It has been previously proposed that the length of dms can far exceed the size of the known genes amplified on them (Sen et al. 1994). Therefore, it is of great interest to investigate if the sequences harboured on dms are derived from one or multiple chromosome segments. Moreover, this can be done by hybridising the micro-FISH probe generated from the dms to the cell population containing the dms (forward chromosome painting). This could also reveal possible reintegration of the dm sequences at specific chromosomal sites following prolonged maintenance in the cancer cell lines.

The identification and genetic characterisation of new areas of amplification in human malignacies remains an important goal in understanding the underlying genetic lesion within these tissue. This can be greatly facilitated by utilising

microdissection techniques, thus assisting in the identification of novel amplified genes important in neoplastic development and progression.

4.8.3 Applications of microdissection and micro-FISH

Lack of sufficient numbers of polymorphic markers has hindered in the past the systematic analysis of loci involved in genetic disorders. This has now been circumvented by constructing region-specific libraries, following microdissection of the region of interest. This approach has provided numerous probes for the characterisation of loci associated with several types of cancer such as the 3p14-p23 linked to renal cell carcinomas (Bardenheuer et al. 1994), the 6q16-q21 linked to malignant melanoma (Guan et al. 1992), the 9p21-p22 associated with acute lymphoblastic leukaemia, lymphomas, bladder cancer and lung cancer (Bohlander et al. 1994), the 11p15.5 linked with Wilm's tumour and Beckwith-Wiedemann syndrome (Newsham et al. 1991) and several others. Large numbers of unique sequence clones from microdissection derived libraries have been isolated, thus, enabling the construction of resolution physical maps for the identification and elucidation of disease related genes such as the Langer-Giedion syndrome at 8q23-q24.1 (Ludecke et al 1989) and the Prader-Willi syndrome (Buiting et al. 1990). Moreover, these microclones have been used as probes for screening genomic libraries that contain larger DNA inserts, such as cosmid or YAC libraries (Yu et al. 1992). Screening of cDNA libraries and consequently isolating cDNA clones as candidate genes can facilitate cloning of disease-related genes mapped to the region of interest.

It is important to be able to investigate the molecular structure and function of important chromosomal entities such as telomeres and centromeres by using microclone sequences from these regions. Fragile sites have been associated with genetic disorders and cancer. The molecular characterisation of such structures can be facilitated by microdissection and subsequent microcloning of the sequences present in those regions. Microdissection and microcloning can also provide information on the distribution of coding and noncoding DNA sequences within the G-light and G-dark bands of the genome, thus helping to elucidate the molecular

mechanisms underlying the formation of these banding patterns. The presence or absence of genes or coding sequences other than ribosomal genes in the short arms of the acrocentric chromosomes (13, 14, 15, 21 and 22) can be assessed by the same approach.

Microdissection and enzymatic amplification has enabled the mapping of various translocation breakpoints involved in cancer chromosome rearrangements. This approach has offered a rapid alternative to mapping by hybridising probes either in situ to chromosomes or to somatic cell hybrids containing the derivative chromosome of interest. Cotter et al (1991) successfully demonstrated the precise location of the leukaemia-associated translocation breakpoints involving the band 11q23 (reported to be associated with cytogenetic alterations in many malignacies) in relation to the genes known to be present. This was accomplished by microdissecting the relevant translocation regions and then amplifying them by using PCR gene specific primers. In addition, Zhang et al (1993) defined the breakpoints involved in the constitution of the Philadelphia (Ph) chromosome present in complex variant chromosome rearrangements of CML cases. Their important findings, by applying unique, band-specific, microdissection probes for dual colour FISH (which spanned over both sites of both translocation breakpoints), offered direct information on the Ph-producing rearrangements as the probes used previously (Tkachuk et al. 1990) could recognise only one side of the translocation breakpoint. Several other groups have reported identification of chromosome rearrangements, previously unidentified by conventional cytogenetics, by combining microdissection with forward chromosome painting, thus facilitating the analysis of obscure aberrations present in clinical and tumour cytogenetics (Lengauer et al. 1991, Xu et al. 1995).

Microdissection of chromosome specific sub-regions has generated unique "bar codes" which can paint by micro-FISH multiple regions of the particular chromosome (Guan et al. 1995), making plausible the analysis of rearrangements within such a chromosome. The development of whole chromosome painting probes (WCPs) can greatly facilitate the cytogenetic analysis of complex aberrations found in several cancers (Guan et al. 1994a). Moreover, the generation of chromosome

arm painting probes (CAPs) could detect not only the chromosome involved in a particular rearrangement, but also the specific arm, hence, enabling the limitations of conventional banding analysis to be overcome (Guan et al. 1996). The availability of such probes in this department will greatly facilitate to identify aberrations such as pericentric inversions, cryptic translocations and the delineation of big markers with a complex genetic composition.

Finally, studies using microdissection followed by micro-injection of the dissected DNA fragment into mouse eggs have also been performed. By this approach, Richa et al. (1989) introduced human fragments containing more than 10 Mb of DNA as opposed to previous experiments where cloned sequences that were injected into mouse eggs, did not exceed 100 kb in size. Such experiments, where microdissected human fragments of certain interest are introduced into mouse eggs, could provide us with animal models that could help define at the molecular and biochemical level diseases that have just been cytogenetically localised.

Previous investigations have demonstrated the tremendous importance of chromosome microdissection in clinical and cancer cytogenetics. In this study, micro-FISH probes were generated from less than 10 fragments. Optimising further the PCR technique (ensuring that contamination is strictly kept to the least possible levels), would allow the generation of probes from less than 5 fragments with the ultimate goal of achieving amplification from a single dissected fragment. This could greatly facilitate the analysis of cases where only a few mitotic cells are present or in the case of solid tumours where the primary abnormality is present in only one or two cells. In terms of diagnosis, the whole procedure of generating micro-FISH probes does not exceed a period of 3 days (first day: microdissection and amplification; second day: re-amplification and hybridisation; third day: detection-FISH). The already mentioned technical improvements will make the microdissection procedure itself less time consuming, thus allowing for more cases to be analysed at a single microdissection experiment and consequently making it a powerful molecular cytogenetic tool in the diagnostic laboratory. The availability of a wide size range of needles could permit microdissection of any required chromosomal region , thus leading to the generation of FISH probes which are more specific to the region of interest. This would allow

the building up of a bank of inexpensive probes as opposed to commercial probes which are expensive and limited to a small number of tests. Micro-FISH probes which are band specific may in some cases be more useful for diagnosis than the chromosome arm libraries available at present.

The results of this project have confirmed the ability of the micro-FISH probes to analyse chromosome abnormalities that could not be detected by conventional cytogenetics. The availability of this technique in our department can undoubtedly improve the accuracy in cytogenetic analysis, thus providing further insights into the pathogenesis of several disorders.

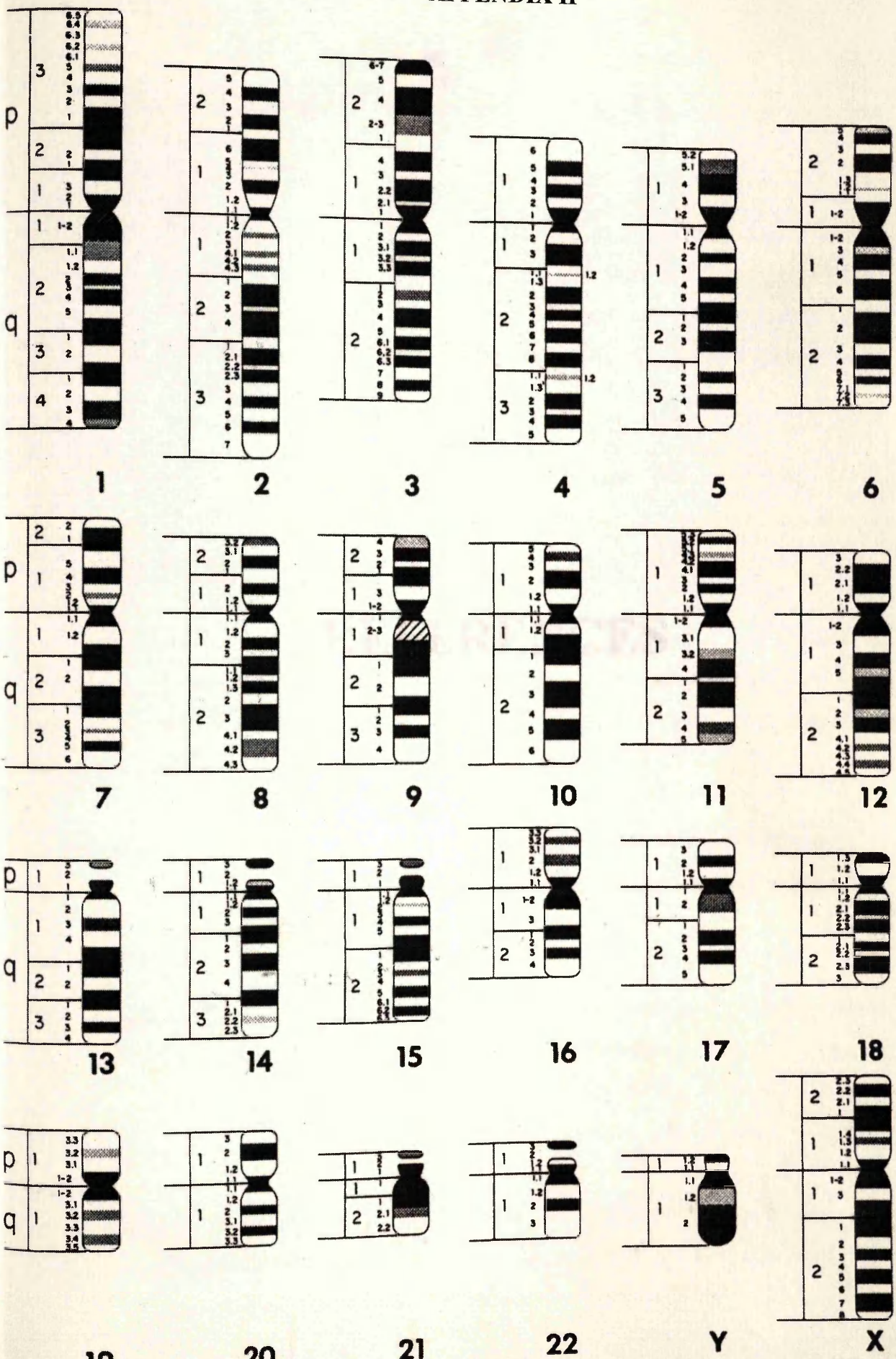
APPENDICES

APPENDIX 1: Source of reagents

1. LymphoprepTM (Nycomed Pharma AS., Cat No. 1001967)
2. Iscove's Modified Dulbeco's Medium (Gibco BRL; Cat No. 21980-32)
3. RPMI 1640 medium (Gibco BRL; Cat No. 21875-042)
4. Phytohaematogglutinin, lyophilised (Gibco BRL; Cat No. 10576-015)
5. Heparin sodium, 5000 units/ml (LEO Laboratories)
6. KCl (BDH analar)
7. Thymidime (Sigma; T-5018)
8. 2-deoxycytidine (Sigma; D-3897)
9. KaryoMAX Colcemide® solution (10 μg/ml), liquid (Gibco BRL; Cat No. 15212-012)
10. Penicillin/streptomycin (Gibco BRL; Cat No. 15140-114)
11. Ethanol 99.7-100% ('Absolute') (Analar; Cat No. 10107 5G)
12. Nuclease free water (Promega; Cat No. P1191)
13. 10X PCR Buffer (Perkin Elmer; Cat No. N808 0006)
14. 10X PCR Buffer II (Perkin Elmer; Cat No.N808 0010)
15. $MgCl_2$ (Perkin Elmer; Cat No. N808 0010)
16. Ultrapure dNTP set, 2'-deoxynucleoside 5'-triphosphate (Pharmacia Biotech; Cat No. 27-2035-0)
17. Topoisomerase I (Promega; Cat No. M 285/1,2)
18. T7 DNA polymerase- Version 2.0 (Amersham-Life Science; Cat No. E70775Y/Z)
19. AmpliTaq®DNA polymerase,250 units(Perkin Elmer, Cat No. N801-0060)
20. AmpliTaq®DNA polymerase, LD, 250 units (Perkin Elmer, Cat No. N808-0107)
21. Biotin-11-dUTP (Sigma; Cat No. B6780)
22. 1Kb DNA Ladder (Gibco BRL, Cat No. 15615-016)
23. Ethidium bromide, 10mg/ml (Sigma; E-1510)
24. Bio Spin Chromatography Columns, BP6 (Bio-Rad Laboratories; Cat No. 732-6002)

25. Glycogen (Boehringer Mannheim, Cat No. 901 393)
26. Human Cot-1™ DNA (Gibco BRL; Cat No. 15279-011)
25. Phosphate buffered saline, Dulbecco 'A' tablets (Oxoid; Cat No. BR14a)
28. RNase, DNAse free, 100mg (Sigma; Cat No. R-4875)
29. Tween 20 (BDH; BDH 66368)
30. Human AB serum (Sigma; S-7148)
31. Fluorescein Avidin DCS, 0.2 mg/ml (Vector Laboratories; Cat No. A2011)
32. Biotinylaetd Antiavidin D (Affinity putified) 2 mg/ml (Vector Laboratories; Cat No. BA0300)
33. DAPI, 1mg/ml (Sigma Chemical Co; Cat No. D-9542)
34. Propidium Iodide 95-98% (TLC) 10 mg/ml (Sigma Chemical Co; Cat No. P4170)
35. QuikHyb (Stratagene, Cat. No. 201220)
36. SDS (BDH, Cat. No. 30175 4L)

APPENDIX II



REFERENCES

REFERENCES

Allanson, J., Bixrnman, H. (1985) A new generation of chromosomal syndromes. *Karyogram* **11,** 71-76

Bardenheuer, W., Szymanski, S., Lux, A., Ludecke, H.-J., Hrsthemke, B., Claussen, U., Senger, G., Smith, D.I., Wang, N.-D., LePaslier, D., Cohen, D., Heppell-Parton, A., Rabbitts, P., Schutte, J. and Opalka, B. (1994) Characterization of a microdissection library from human chromosome region 3p14. *Genomics* **19,** 291-294

Bates, G.P., Wainwright, B.J., Williamson, R. and Brown, S.D.M. (1986) Microdissection of and microcloning from the short arm of human chromosome 2. *Molecular and Cellular Biology* **6,** 3826-3830

Bauman, J., Wiegant, J., Borst, P., van Duijn, P. (1980) A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochrome labelled RNA. *Experimentall Cell Research* **128,** 485-490

Bhatt, B., Burns, J., Flannery, D. and O'D. McDee, J. (1988) Direct visualization of single copy genes of banded metaphase chromosomes by non-isotopic in situ hybridization. *Nucleic Acid Research* **16**(9), 3951-3961

Blennow, E., Telenius, H., Larsson, C., de Vos, D., Bajalica, S., Ponder, B.A.J., Nordenskjold, M. (1992) Complete characterization of a large marker chromosome by reverse and forward chromosome painting. *Human Genetics* **90,** 371-374

Bohlander, S.K., Espinosa III, R., Le Beau, M.M., Rowley, J.D. and Diaz, M.O. (1992) A method for the rapid sequence-independent amplification of microdissected chromosomal material. *Genomics* **13,** 1332-1324

Bohlander, S.K., Dreyling, M.H., Hsgos, F., Sveen, L., Olopade, O.I. and Diaz, M.O. (1994) Mapping a putative tumor suppressor gene on chromosome 9 bands p21-p22 with microdissection probes. *Genomics* **24,** 211-217

Buehler, E. (1984) The tricho-rhino-phalangeal syndrome(s): Chromosome 8 long arm deletion:is there a shortest region of overlap between reported cases? *American Journal of Medical Genetics* **19,** 113-119

Buiting, K., Neumann, M., Ludecke, H.-J., Senger, G., Claussen, U., Antich, J., Passarge, E. and Horsthemke, B. (1990) Microdissection of the Prader-Willi syndrome chromosome region and identification of potential gene sequences. *Genomics* **6,** 521-527

Carter, N.P., Ferguson-Smith, M.A., Perryman, M.T., Telenius, H., Pelmear, A.H., Leversha, M.A., Glancy, M.T., Wood, S.L., Cook, K., Dyson, H.M., Ferguson-Smith, M.E., Willatt, L.R. (1992) Reverse chromosome painting: a method for the rapid analysis of aberrant chtomosomes in clinical cytogenetics. *Journal of Medical Genetics* **29**, 299-307

Caspersson, T., Farber, S., Foley, G.E., Kudynowski,J., Modest, E.J., Simonsson, E., Wagh, U. and Zech, L. (1968) Chemical differentiation along metaphase chromosomes. *Journal of Experimental Cell Research* **49,** 219-222

Cinti, C., Santi, S. and Maraldi, N.M. (1993) Localization of single copy genes by PRINS technique. *Nucleic Acid Research* **21**(24), 5799-5800

Choo, K.H., Vissel, B., Nagy, A., Earle, E. and Kalitsis P. (1991) A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and derivation of a new consensus sequence. *Nucleic Acid Research* **19**(6), 1179-1182

de Albuquerque Coelho, K-E.F., Egashira, M., Kato, R., Fujimoto, M., Matsumoto, N., Rerkamnuaychoke, B., Abe, K., Harada, N., Ohashi, H., Fukushima, Y. and Niiikawa, N. (1996) Diagnosis of four chromosome abnormalities of unkown origin by using chromosome microdissection and subsequent reverse and forward painting. *American Journal of Medical Genetics* **63,** 468-471

Collins, C., Kuo, W.L., Segraves, R., Fuscoe, J., Pinkel, D. and Gray, J.W. (1991) Construction and characterization of plasmid libraries enriched in sequences from single human chromosomes. *Genomics* **11,** 997-1006

Cotter, F.E., Lillington, D., Riddle, P., Nasipuri, S., Gibbons, B. and Young, B.D. (1991) Gene mapping by microdissection and enzymatic amplification: Heterogeneity in leukaemia associated breakpoints on chromosome 11. *Genes, Chromosomes and Cancer* **3,** 8-15

Cremer, T., Lichter, P., Borden, J., Ward, D.C., and Manuelidis, L. (1988) Fluorescence in situ hybridisation with human chromosome-specific libraries: Detection of trisomy 21 and translocation of chromosome 4. *Human Genetics* **80,** 235-246

Curry, C., Lanman, J., Magenis, R., Brown, M., Bergner, E., Shapiro, L. (1982) X linked chrondrodystrophia punctata with ichthyosis and chromosomal localization to Xp. *American Journal of Human Genetics* **34,** 122A

Dauwerse, J.G., Wiegant, J., Raap, A.K., Breuning, M.H. and van Ommen, G.J.B., (1992) Multiple colors by FISH ratio labelled DNA probes created a molecular karyotype. *Human Molecular Genetics* **1**(8), 593-598

Dauwerse, J.G., Wessels, J.W., Giles, R.H., van der Reijden, B.A., Fugazza, G., Jumelet, E.A., Smit, E., Baas, F., Hagemeijer, A., Beverstock, G.C., van Ommen, G.J.B. and Breuning, M.H. (1993) Cloning the breakpoint cluster region of the

inv(16) in acute nonlymphocytic leukamia M4 Eo. *Human Molecular Genetics* **2**(10), 1328-1333

Deininger, P.L and Daniels, G.R. (1986) The recent evolution of mammalian repetitive DNA elements. *Trends in Genetics* **2,** 76-86

Dirks, R.W., Van Gijlswijk, R.P.M., Vooijs, M.A., Smit, A.B., Bogerd, J., van Minnen, J., Raap, A.K. and Van der Ploeg, M. (1991) 3'-end fluorochromized and haptenized oligonucleotides as in situ hybridization probes for multiple, simultaneous RNA detection *Experimental Cell Research* **194,** 310-315

Djabali, M., Nguyen, C., Biunno, I., Oostra, B.A., Mattei, M-G., Ikeda, J.-E. and Jordan, B. (1991) Laser microdissection of the fragile X region: Identification of cosmid clones and of conserved sequences in this region. *Genomics* **10,** 1053-1060

Dyer, K. and Meyne, J. Molecular Cytogenetics: Use of DNA probes as an adjunct to classical clinical cytogenetics. In: Barrch, J.M. ed. (The Association of Cytogenetic Technologists) *The ACT Cytogenetics Laboratory Manual. 2nd ed.* Raven Press, LTD., New York, (1991), **12,** 525-532

Fan, Y-S., Daris, L.M. and Shows, T.B. (1990) Mapping small DNA sequences by FISH directly on banded metaphase chromosomes. *Proceedings of the National Academy of Science* **87,** 6223-6227

Fuscoe, J.C., Clark, L.M. and van Dilla, M.A. (1986) Construction of fifteen human chromosome specific DNA libraries from flow-purified chromosomes. *Cytogenetics Cell Genetics* **43,** 79-86

Fuscoe, J.C., Collins, C.C., Pinkel, D. and Gray, J.W. (1989) An efficient method for selecting unique-sequence clones from DNA libraries and its application to fluorescent staining of human chromosome 21 using in situ. *Genomics* **5,** 100-109

Gall, J.G and Pardue, M.C. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *PNAS* **63,** 378-383

Gray, J.W. Flow Cytogenetics. Academic Press, California, USA, 1989, 276-301

Gray, J.W. and Pinkel, D. (1992) Molecular cytogenetics in human cancer diagnosis. *Cancer Suppl* **69**(6), 1536-1542

Guan, X.-Y., Meltzer, P.S., Cao, J. and Trent, J.M. (1992) Rapid generation of region-specific genomic clones by chromosome microdissection: isolation of DNA from a region frequently deleted in malignant melanoma. *Genomics* **14,** 680-684

Guan, X.-Y., Trent, J.M. and Meltzer, P.S. (1993) Generation of band-specific painting probes from a single microdissected chromosome. *Human Molecular Genetics* **2**(8), 1117-1121

Guan, X.-Y., Meltzer, P.S. and Trent, J.M. (1994a) Rapid generation of whole chromosome painting probes (WCPs) by chromosome microdissection. *Genomics* **22,** 101-107

Guan, X.-Y., Meltzer, P.S., Dalton, W.S. and Trent, J.M. (1994b) Identification of cryptic sites of DNA sequence amplification in human breast cancer by chromosome microdissection. Nature *Genetics* **8,** 155-160

Guan, X.-Y., Meltzer, P.S., Burgess, A.C., Trent, J.M. (1995) Coverage of chromosome 6 by chromosome microdissection: generation of 14 subregion-specific probes. *Human Genetics* **95,** 637-640

Guan, X.-Y., Cargile, C., Anzick, S.L., Thompson, F.H., Meltzer, P.S., Bittner, M.L., Taetle, R., McGill, J.R. and Trent, J.M. (1995) Chromosome microdissection identifies cryptic sites of DNA sequence amplification in human ovarian carcinoma. *Cancer Research* **55,** 3380-3385

Guan, X.-Y., Zhang, H., Bittner, M., Jiang, Y., Meltzer, P. and Trent, J. (1996) Chromosome arm painting probes. *Nature Genetics* **12,** 10-11

Hadano, S., Watanabe, M., Yokoi, H., Kogi, M., Kondo, I., Tsuchiya, H., Kananawa, I., Wakasa, K. and Ikeda, J.-E. (1991) Laser microdissection and single unique primer PCR allow generation of regional chromosome DNA clones from a single human chromosome. *Genomics* **11,** 364-373

Hagemeijer, A., Smit, E.M.E. and Bootsma, D. (1979) Improved identification of chromosomes of leukemic cells in methotrexate-treated cultures. *Cytogenetics, Cell Genetics* **23,** 208-212

Harper, M.E. and Saunders, G.F. (1981) Localization of single copy DNA sequences on G-banded human chromosomes by in situ hybridization. *Chromosoma(Berl)* **83,** 135-141

Herrington, C.S., Burns, J., Graham, A.K., Evans, M., McGee, J.O'D. (1989) Interphase cytogenetics using biotin and digoxigenin labelled probes I: relative sensitivity of both reporter molecules for detection of HPV16 in CaSki cells. *Journal of Clinical Pathology* **42,** 592-600

Holtke, H.-J., Burg, J., Muhlegger, K., Kessler, C. (1990) Non-radioactive labelling and detection of nucleic acids-optimization of the digoxigenin system. *Biology Chemistry Hoppe-Seyler* **371,** 929-938

Hsu, T.C. (1952) Mammalian chromosomes in vitro. 1. The karyotype of man. *Journal of Heredity* **43,** 187-172

Hulten, M.A., Gould, C.P., Goldman, A.S.H., Waters, J.J. (1991) Chromosome in situ suppression hybridization in clinical cytogenetics. *Journal of Medical Genetics* **28,** 557-582

ISCN.(1981) An international system for human cytogenetic nomeclature - high-resolution banding. *Cytogenetics Cell Genetics* **31,** 1-23

John, H.A., Birnstiel, M.L and Jones, K.W. (1969) RNA-DNA hybrids at the cytological level. *Nature* (London) **233,** 582-587

Johnson, D.H. (1990) Molecular cloning of DNA from specific chromosomal regions by microdissection and sequence-independent amplification of DNA. *Genomics* **6,** 243-251

Kallioniemi, A., Kallioniemi, O.-P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F. and Pinkel, D. (1992) Comparative Genomic Hybridisation for molecular cytogenetic analysis of solid tumours. *Science* **258,** 818-821

Kao, F.-T. (1987) Chromosome microdissection an microcloning in human molecular genetics. *Somatic Cell and Molecular Genetics* **13**(4), 357-380

Kao, F.-T. and Yu, J.-W. (1991) Chromosome microdissection and cloning in human genome and genetic disease analysis. *Proceedings of the National Academy of Science USA* **88,** 1844- 1848

Koch, E., Kolvraa, J.S., Petersen, K.B., Gregersen, N. and Bolund, L. (1989) Oligonucleotide-priming methods for chromosome-specific labeling of alpha satellite DNA in situ. *Chromosoma (Berl)* **98,** 259-265

Kozma, R. and Adinolfi, M. (1987) In situ hybridization and the detection of biotinylated DNA probes. *Molecular Biology and Medicine* **4,** 357-364

Kuwano, A., Ledbetter, S.A., Dobyns, W.B., Emanuel, B.S. and Ledbetter, D.H. (1991) Detection of deletions and cryptic translocations in Miller-Dieker

syndromes by in situ hybridisation. *American Journal of Human Genetics* **49,** 707-714

Landegent, J.E., Jansen in de Wal, N., van Ommen, G.-J.B., Baas, F., de Vijlder, J.J.M., van Duijn, P. and van der Ploeg, M. (1985) Chromosomal localization of a unique gene by non-autoradiographic in situ hybridization. *Nature* **317,** 175-177

Landegent, J.E., Jansen in de Wal, N., G.-J.B., Baas, Dirks, R.W., Baas, F.M. and van der Ploeg, M. (1987) Use of whole cosmid cloned genomic sequences for chromosomal localisation by non radioactive in situ hybridisation. *Human Genetics* **77,** 366-370

Landegren, U., Kaiser, R., Caskey, C.T., Hood, L. (1988) DNA Diagnostics-Molecular Techniques and automation. *Science* **242,** 229-237

Langer, P.R., Waldrop, A., Ward, D. (1981) Enzymatic synthesis of biotin-labelled polynucleotides: Novel nucleic acid affinity probes. *Proceedings of the National Academy of Science* **78,** 6633-6637

Safer-Langer, P.R., Levine, M. and Ward, D.C. (1982) Immunological method for mapping genes on Drosophilla polytene chromosomes. *Proceedings of the National Academy of Science* **79,** 4381-4385

Lawrence, J.B. and Singer, R.H. (1985) Quantitative analysis of in situ hybridisation methods for the detection of actin gene expression. *Nucleic Acids Research* **13**(5), 1777-1798

Lawrence, J.B., Villnare, C.A. and Singer, R.H. (1988) Sensitive, high-resolution chromatin and chromosome mapping in situ: Presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell* **52,** 51-61

Lawrence, J.B., Singer, R.H., McNeil, J.A. (1990) Interphase and metaphase resolution of different distances within the human Dystorphin gene. *Science* **249,** 928-932

Lejeune, J., Gatier, M. and Turpin, R. (1959) Les chromosomes somatique des enfants mongolines. *Compts Rend. Acad des Sc. Paris* **248,** 1721

Lengauer, C., Riethman. H. and Cremer, T. (1990) Painting of human chromosomes with probes generated from hybrid cell lines by PCR with Alu and L1 primers. *Human Genetics* **86,** 1-6

Lengauer, C., Eckelt, A., Weith, A., Endlich, N., Ponelies, N., Lichter, P., Greulich, K.O. and Cremer, T. (1991) Painting of defined chromosomal regions by in situ suppression hybridization of libraries from laser-microdissected chromosomes. *Cytogenetics Cell Genetics* **56,** 27-30

Ledbetter, D., Ricardi, V., Airhart, S., Stobel, R., Keenan, B., Crawford, J. (1981) Deletions of chromosome 15 as a course of the Prader-Willi syndrome. *New England Journal of Medicine* **37,** 1-23

Ledbetter, S.A., Nelson, D.L., Warren, S.T. and Ledbetter, D.H. (1990) Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase chain reaction. *Genomics* **6,** 475-481

Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D.C. (1988) Delineation of individual human chromosomes in metaphase and intrephase cells by in situ suppression hybridization using recombinant DNA libreries. *Human Genetics* **80,** 224-234

Lichter, P., Ledbetter, S.A., Ledbetter, S.A. and Ward, D.C. (1990) Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines. *Proceedings of the National Academy of Science* **87,** 6634-6638

Lichter, P. and Cremer, T. Chromosome analysis by non isotopic in situ hybridisation. In: Rooney, D.E., Czepulkowski, B.H. Human Cytogenetics: A Practical Approach, Volume I,Chapter 12, 2nd ed., Oxford University Press.1992

Lo, M.D., Mehal, W.Z. and Fleming, K.A. (1988) Rapid production of vector-free biotinylated probes using the polymerase chain reaction. *Nucleic Acid Research* **16**(17), 8719

Ludecke, H.-J., Senger, G., Claussen, U. and Horsthemke, B. (1989) Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification. *Nature* **388,** 348-350

Ludecke, H.-J., Senger, G., Claussen, U. and Horsthemke, B. (1990) Construction and characterization of band-specific DNA libraries. *Human Genetics* **84,** 512-516

Mahler, V., Vogt, P., Blonden, L., van Ommen, G.-J.B., Cremer, T. and Cremer, M. (1990) Direct carrier detection by in situ suppression hybridization with cosmid clones of the Duchenne/Becker muscular dystrophy locus. *Human Genetics* **85,** 581-586

Malcolm, S., Barton, P., Murphy, C. and Ferguson-Smith, M.A. (1981) Chromosomal localization of a single copy gene by *in situ* hybridization- human , globin genes on the short arm of chromosome 11. *Annals of Human Genetics* **45,** 135-141

du Manoir, S., Soeicher, M.R., Joos, S., Schrock, E., Popp, S., Dohner, H., Kovacs, G., Robert-Nicoud, M., Lichter, P. and Cremer, T. (1993) Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridisation. *Human Genetics* **90,** 590-610

Marmur, J. and Lane, D. (1960) Strand separation and specific recombination in deoxyribonucleic acids: biological studies. *Proceedings of the National Academy of Science* **46,** 453-463

Meltzer, P.S., Guan, X.-Y., Burgess, A. and Trent, J.M. (1992) Rapid generation of region specific probes by chromosome microdissection and their application. *Nature Genetics* **1,** 24-28

Mohaddes, S.M., Boyd. E., Morris, A., Morrison, N. and Connor, J.M. (1996) A practical strategy for detection of major chromosome aneuploidies using ratiomixing fluorescence in situ hybridization. *Molecular and Cellular Probes* **10,** 147-154

Monajembashi, S., Cremer, C., Cremer, T., Wolfrum, J. and Greulich, K.O. (1986) Microdissection of human chromosomes by a laser microbeam. *Experimental Cell Research* **167,** 262-265

Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M., Hungerford, D.A. (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. *Experimental Cell Research* **20,** 613-616

Navia-Muller, J., Nebel, A., Schleiermacher, E. (1995) Complete and precise characterization of marker chromosomes by application of microdissection in prenatal diagnosis. Human *Genetics* **96,** 661-667

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology* **L1,** 263-273

Nederlof, P.M., van der Flier, S., Wiegant, J., Raap, A.K., Tanke, H.J., Ploem, J.S., van der Ploeg, M. (1990) Multiple fluorescence in situ hybridization. *Cytometry* **27,** 126-131

Nelson, D.L., Ledbetter, S.A., Corbo, L., Victoria, M.F., Ramirez-Solis, R., Webster, T.D., Ledbetter, D.H. and Caskey, C.T. (1989) Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proceedings of the National Academy of Science* **86,** 6686-6690

Newsham, I., Claussen, U., Ludecke, H.-J., Mason, M., Senger, G., Horsthemke, B. and Cavenee, W. (1991) Microdissection of chromosome band 11p15.5: characterization of probes mapping distal to the HBBC locus. *Genes, Chromosomes and Cancer* **3,** 108-116

Pinkel, D., Landegent, C., Collins, C., Fuscoe, J., Segraves, R., Lucas, J. and Gray, J. (1988) Fluorescence *in situ* hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocation of chromosome 4. *Proceedings of the National Academy of Science* **85,** 9138-9142

Ponelies, N., Bautz, E.K.F., Monajembashi, S., Wolfrum, J. and Greulich, K.O. (1989) Telomeric sequences derived from laser-microdissected polytene chromosomes. *Chromosoma* **98,** 351-357

Raap, A.K., Dirks, R.W., Jiwa, N.M., Nederlof, P.M., van der Ploeg, M. (1990) In situ hybridizatiom with hapten-modified DNA probes. Pacz, P., Haase, A.T., Gluckman, J.C. (eds) *Modern Pathology of AIDS and Other Retroviral Infections. Basel, Karger.* pp17-28

Raap, A.K., Florijn, R.J., Blonden, L.A., Wiegant, J., Vaandrager, J.-W., Vrolijk,H., den Dunnen, J., Tanke, H.J. and van Ommen, G.-J. (1996) Fiber FISH as a mapping tool. *METHODS: A companion to Methods in Enzymology* **9,** 67-73

Rack, K.A., Harris, P.C., MacCarthy, A.B., Boone, R., Raynham, H., McKinley, M., Fitchett, M., Towe, R., Reidd, P., Armour, J.A.L., Lindenbanm, R.H. and Buckle, V.J. (1993) Characterization of three de novo derivative chromosomes

16 by "reverse chromosome painting" and molecular analysis. *American Journal of Human Genetics* **52,** 987-997

Richa, J. and Lo, C.W. (1989) Introduction of human DNA into mouse eggs by injection of dissected chromosome fragments. *Science* **245,** 175-177

Ried, T., Mahler, V., Vogt, P., van Ommen, G.-J.B. (1990) Direct carrier detection by in situ hybridization with cosmid clones of the Duchenne/Becker muscular dystrophy locus. *Human Genetics* **85,** 581-586

Ried, T., Landes, G., Dackowski, W., Klinger, K. and Ward, D. (1992a) Multicolor FISH for the simultaneous detection of probe sets for chromosomes 13, 18, 21, X and Y in uncultered amniotic fluid cells. *Human Molecular Genetics* **1**(5), 307-313

Ried, T., Baldini, A., Rand, T.C. and Ward, D.C. (1992b) Simultaneous visualisation of seven different DNA probes by FISH using combinatorial fluorescent and digoxigenin imaging microscopes. *Proceedings of the National Academy of Science* **89,** 1388-1397

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Lsbeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113,** 237-251

Rudkin, G.T. and Stollar, B.D. (1977) High resolution detection of DNA-RNA hybrids in situ by indirect immunofluorescence. *Nature* **265,** 472-473

Saunders, R.D., Glover, D.M., Ashburner, M., Siden-Kiamos, I., Louis, C., Monastirioti, M., Savakis, C. and Kafatos, F. (1989) PCR amplification of DNA microdissected from a single polytene chromosome band: a comparison with conventional microcloning. *Nucleic Acids Research* **17**(22), 9027-9037

Scalenghe, F., Turco, E., Edstrom, J.E., Pirrotta, V. and Melli, M. (1989) Microdissection and cloning of DNA from a specific region of Drosophila melanogaster polytene chromosomes. *Chromosoma (Berl.)* **82,** 205-216

Schrock, E., DuManoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferrguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Baram, I., Soenksen, D., Garini, Y. ans Ried, T. (1996) Multicolour spectral karyotyping of human chromosomes. *Science* **273,** 494-497

Seibl, R., Holtke, H-J., Ruger, R., Meindl, A., Zachau, H.G., Rabhoffer, R., Roggendorf. M., Wolf, H., Arnold, N., Wienberg, J. and Kessler, C. (1990) Non-radiographic labelling and detection of nucleic acids III. Application of the digoxigenin system. *Biology Chemistry Hoppe-Seyler* **371,** 939-951

Selleri, L., Hermanson, G.G., Eubanks, J.H., Lewis, K.A. and Evans, G.A. (1991) Molecular localization of the t(11;22)(q24;q12) translocation of Ewing sarcoma by chromosomal *in situ* suppression hybridization. *Proceedings of the National Academy of the Science* **88,** 887-891

Sen, S., Sen, S., Mulac-Jericevic, B., Zhou, H., Pirrotta, V. and Stass, S.A. (1994) Microdissected double-minute DNA detects variable patterns of chromosomal localisations and multiple abundantly expressed transcripts in normal and leukaemic cells. *Genomics* **19,** 542-551

Senger, G., Ludecke, H-J., Horsthemke, B. and Claussen, U. (1990) Micredissection of banded human chromosomes. *Human Genetics* **84,** 507-511

Smit, V.T.H.B.M., Wessels, J.W., Mollevanger, P., Dauwerse, J.G., van Vliet, M., Beverstock, G.C., Breuning, M.H., Devilee, P., Raap, A.K. and Cornelisse, C.J. (1991) Improved interpretation of complex chromosomal rearrangements by combined GTG banding and in situ suppression hybridization using chromosome-

specific libraries and cosmid probes. *Genes, Chromosomes and Cancer* **3,** 239-248

Speicher, M.R., Stephen G.B. and Ward, D.C. (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genetics* **12,** 368-375

Suijkerbuijk, R.F., Matthopoulos, D., Kearney, L., Monard, S., Susheela, D., Cotter, F.E., Herbergs, J., van Kessel, A.G. and Young, B.D. (1992) Fluorescence in situ hybridization of human marker chromosomes using flow sorting and Alu-element-mediated PCR. *Genomics* **13,** 355-362

Tagaro, I., Fernandez-Peralta, A.M., Gonzalez-Aquilera, J.J. (1994) Chromosomal localisation of human satellites 2 and 3 by a FISH method using oligonucleotides as probes. *Human Genetics* **93,** 383-388

Telenius, H., Pelmear, A.H., Tunnacliffe, A., Carter, N.P., Bhmel, A., Ferguson-Smith, M.A., Nordenskjold, M., Pfragner, R. and Ponder, B.A.J. (1992a) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes, Chromosomes and Cancer* **4,** 257-263

Telenius, H., Carter, N.P., Bebb, C.E., Nordenskjold, M., Ponder, B.A. and Tunnacliffe, A. (1992b) Degenarate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* **13,** 718-725

Thangavelu, M., Pergament, E., Espinosa, R. and Bohlander, S.K. (1994) Characterization of marker chromosomes by microdissection and fluorescence *in situ* hybridization. *Prenatal Diagnosis* **14,** 583-588

Tjio, J. and Levan, A. (1953) The chromosome number of man. *Hereditas* **42,** 1-5

Tkachuk, D.C., Westbrook, C.A., Andreeff, M., Donlon, T.A., Cleary, M.L., Suryanarayan, K., Homge,M., Redner, A., Gray, J., Pinkel, D. (1990) Detection of bcr-abl fusion in chronic myelogeneous leukemia by in sity hybridization. *Science* **250,** 559-562

Van Dilla, M.A., Deaven, L.L., Albright, K.L., Allen, N.A., Aubuchon, M.R., Batholdi, M.F., Brown, N.C., Campbell, E.W., Carrano, A.V., Clark, L.M., Cram, L.S., Crawford, B.D., Fuscoe, J.C., Gray, J.W., Hildebrand, C.E., Jackson, P.J., Jett, J.H., Longmire, J.L., Lozes, C.R., Luedemann, M.L., Martin, J.C., McNinch, J.S., Meincke, L.J., Mendelsohn, M.L., Meyne, J., Moyzis, R.K., Munk, A.C. Perlman, J., Peters, D.C., Silva, A.J. and Trask, B.J. (1986) Human chromosome-specific libraries: construction and availability. *Biotechnology* **4,** 537-553

Verlinsky, Y., Cieslak, J., Lifchez, A., Strom, C., Kuliev, A. (1996) Birth of healthy children after preimplantation diagnosis of common aneuploidies by polar body fluorescent in situ hybridization analysis. Fertility and Sterility **66** (1), 126-129

Viersbach, R., Schwanitz, G., Nåothen, M.M. (1994) Delineation of marker chromosomes by reverse chromosome using only asmall number of DOP-PCR amplified microdissected chromosomes. *Human Genetics* **93,** 663-667

Vogt, P. (1990) Potential genetic functions of tandem repeated DNA sequence blocks in the human genome are based on a highly conserved "chromatin folding code". *Human Genetics* **84,** 301-336

Vooijs, M., Yu, L.-C., Tkachuk, D., Pinkel, D., Johnson, D. and Gray, J.W. (1993) Libraries for each human chromosome, constructed from sorted-enriched chromosomes by using linker-adaptor PCR. *American Journal of Human Genetics* **52,** 586-597

Waye J.S. and Willard H.F. (1989) "Human β satellite DNA: Genomic organisation and sequence definition of a class of highly repetitive tandem DNA". *Proceedings of the National Academy of the Science* **86,** 6250-6254

Weber, J., Weith, A., Kaiser, R., Grzeschik, K.-H. and Olek, K. (1990) Microdissection and microcloning of human chromosome 7q22-23 region. *Somatic Cell and Molecular Genetics* **16**(2), 123-128

Wesley, C.S., Ben, M., Kreitman, M., Hagag, N. and Eanes, W.F (1990) Cloning regions of the Drosophila genome by microdissection of polytene chromosome DNA and PCR with nonspecific primer. *Nucleic Acid Research* **18**(3), 599-603

Xu, J., Cedrone, E., Roberts, M., Wu, G., Gershagen, S. and Wang, N. (1995) The characterization of chromosomal rearrangements by a combined micro-FISH approach in a patient with myelodysplastic syndrome. *Cancer Genetics Cytogenetics* **83,** 105-110

Xu, J., Tyan, T., Cedrone, E., Savaraj, N. and Wang, N. (1996) Detection of 11q13 amplification as the origin of a homogeneously staining region in small cell lung cancer by chromosome microdissection. *Genes, Chromosomes and Cancer* **17,** 172-178

Yu, J., Hartz, J., Xu, Y., Gemmill, R.M., Korenberg, J.R., Patterson, D. and Kao, F.-T. (1992) Isolation, characterization, and regional mapping of microclones from a human chromosome 21 microdissection library. *American Journal of Human Genetics* **51,** 263-271

Yunis, J.J. (1976) High resolution of human chromosomes. *Science* **191,** 1268-1270

Yunis, J.J., Pamsay, N. (1978) Retinoblastoma and sub-band deletion of chromosome 13. *American Journal of Diseases of Children* **132,** 161-163

Yunis, J.J. (1981) New chromosome techniques in the study of human neoplasia. *Human Pathology* **12,** 540-549

Zhang, J., Meltzer, P., Jenkins, R., Guan, X.-Y. and Trent, J. (1993a) Application of chromosome microdissection probes for elucidation of BCR-ABL fusion and variant Philadelphia chromosome translocations in chronic myelogenous leukaemia. *Blood* **81**(12), 3365-3371

Zhang, J., Trent, J.M. and Meltzer, P.S. (1993b) Rapid isolation and characterization of amplified DNA by chromosome microdissection: identification of IGF1R amplification in malignant melanoma. *Oncogene* **8,** 2827-2831

Zheng, Y.L., Ferguson-Smith, M.A., Warner, J.P., Ferguson-Smith, M.E., Sargent, C.A. and Carter, N.P. (1992) Analysis of chromosome 21 copy number in uncultured amniocytes by FISH using a cosmid contig. *Prenatal Diagnosis* **12,** 931-943

GLASGOW UNIVERSITY LIBRARY