

**OPTIMISATION OF INTERPHASE FLUORESCENCE *IN SITU*
HYBRIDISATION FOR DETECTION OF COMMON ANEUPLOIDIES**

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Medicine in the University of Glasgow**

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....in loving memory of my mother

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.


S. Mojtaba Mohaddes

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List Of Abbreviations

µg	: Microgram
µl	: Microlitre
AAF	: 2-Acetylaminofluorene
AMCA	: Amino-methyl coumarin-acetic acid
APES	: 3-Aminopropyl-ttriethoxysilane
BCIP	: 5-Bromo-4 Chloro-3-indolylphosphate
BRL	: Bethesda Research Laboratories
CCD	: Charged Coupled Device
CEPH	: Centre d'Etude du polymorphisme Humain (Human polymorphism study centre)
CGH	: Coparative Genomic Hybridisation
CI	: Chlorophorm Isoamyl alcohol
CISS	: Chromosome In Situ Suppresion
Contig	: Contiguous sequence
DAB	: Diaminobenzidine
DAPI	: 4', 6-Diaminido-2-phenyl-indole
dATP	: Deoxyadenosine 5'-triphosphate
dCTP	: Deoxycytidine 5'-triphosphate
dGTP	: Deoxyguanosine 5'-triphosphate
Dig	: Digoxigenin
DNA	: Deoxyribonucleic acid
DNP	: Dinitrophenol
dNTP	: Deoxynucleotide 5'-triphosphate
dTTP	: Deoxythymidine 5'-triphosphate
e.g.	: exempla gratia (for example)
EDTA	: Ethylene Diamino Tetra-Acetic acid
et al.	: et alia
FISH	: Fluorescene In Situ Hybridisation
FITC	Fluoresceine isothiocyanate

G-banding	: Giensa banding
g	: gram
ISH	: In Situ Hybridisation
Kb	: Kilobase
LINEs	: Long Interspersed Nuclear Elements
Mb	: Megabase
mg	: milligram
ml	: millilitre
ng	: nanogram
NTB	: Nitrobluetetrazolium
OD	: Optical Density
p	: Short arm of a chromosome
PBS	: Phosphate-Buffered Saline
PCI	: Phenol Chlorophorm Isoamyl alcohol
PCR	: Polymerase Chain Reaction
PFGE	: Pulsed Field Gel Electrophoresis
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
SSC	: Saline Sodium Citrate
TE	: Tris-HCl/EDTA
Tm	: Melting temperature
UV	: Ultra Violet
v/v	: Volume per volume
w/v	: Weight per volume
YAC	: Yeast Artificial Chromosome

GLOSSARY

Antidigoxigenin	an anti body used for detection of digoxigenin labelled probes
Antidigoxin	a monoclonal antibody used for detection of digoxin and digoxigenin
Contigs	overlapping contiguous cloned DNA segments
Cosmid	a synthetic cloning vector with features of a phage and a plasmid which can accommodate large fragments of foreign DNA.
Competition principle	the binding of unlabelled human genomic DNA (e.g. sonicated human placental DNA) to complementary sequences on the probe and the target DNA (CISS)
DNA Complexity	the sum of the length of unique sequences plus the sum of the unit length of the repeatitive classes
Fab fragments	antigen binding Fragment of an antibody produced by chemical treatment (papain digestion)
Fluorochrome or Fluorophore	a fluorescence dye which is used for visualisation of a hybridisation area by a fluorescence microscope
Hapten or reporter	haptens are anti-body binding site of an antigen. In FISH the terms hapten and reporter are used for indirect labels which can be detected using a specific antibody
Probe Label	a hapten or a reporter molecule which is used for probe labelling
Pseudocolour	an artificial colour which can be produced by software to distinguish the closely related coloured signals
Pulsed-field gel electrophoresis	an apparatus which uses alternately pulsed, perpendicularly oriented electric fields and linear electrodes to obtain electrophoretic karyotypes of large DNA segments
Probe Sensitivity	the degree of signal to noise ratio for a specific probe
Probe Specificity	the degree of hybridisation of a specific probe to its specific target in comparison with its cross reactivity with non-specific sites
Ratio-labelling	simultaneous labelling of a probe with more than one label

Ratio-mixing	mixing of probes individually labelled with different labels
Target sequences	a specific sequence of DNA which is complementary to a labelled DNA probe

SUMMARY

The optimisation of a simple, reliable and practical method of interphase FISH which allows prenatal diagnosis of major chromosome aneuploidies using a minimum volume of amniotic fluid sample was the overall objective of this study. The alpha satellite DNA repeats DXZ1, pDP97 and L1.84 were used to detect the copy number of chromosomes X, Y and 18. These probes produced strong signals when hybridised to cultured and uncultured lymphocytes using uni-colour FISH.

For chromosomes 13 and 21 there was no access to reliable probes which could be used for interphase FISH analysis. A commercially available repeat probe which hybridises to both chromosomes 13 and 21 was available, however it could not be used in this project as it cannot distinguish between the two chromosomes. For this reason an Alu-PCR YAC probe (YAC 831B9) specific for chromosome 21, a cosmid contig composed of three overlapping cosmids and also an Alu-PCR YAC probe (YAC 744D11) specific for chromosome 13 were developed. Hybridisation of an unselected series of 20 uncultured lymphocytes and 23 uncultured amniocytes with Alu-PCR amplified YAC 831B9 showed that this probe can be reliably used for prenatal diagnosis of trisomy 21. Both the cosmid contig and the Alu-PCR amplified YAC 744D11, were shown to be useful probes for detection of chromosome 13 copy number in uncultured nuclei, however more intensive signals and less background fluorescence was generated when the YAC clone was used.

When all the probes required were available, the study continued by developing the technique of ratio-mixing FISH for simultaneous detection of the five major chromosome aneuploidies. The technique of five-colour ratio mixing FISH which has been presented here is simple and straightforward, since only two haptenisation and detection systems have been employed to visualise simultaneously five different targets in five distinguishable colours. The steps of denaturation, hybridisation and detection are the same as those used in a uni-colour FISH experiment. The results obtained from hybridisation of an unselected series of 20 uncultured lymphocytes and 27 uncultured amniocytes indicate that the technique is reliable and can be used for simultaneous detection of major chromosome aneuploidies. However a superior

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quality of amniocyte preparation was needed using this method, as 18.5 per cent of the samples failed to produce a result because of the poor quality of preparations. Another major disadvantage to a routine diagnostic laboratory is the prolonged time required for signal analysis, which is only possible using a digital imaging system.

In order to provide a practical strategy for clinical diagnostic purposes, the use of a three colour ratio-mixing FISH and a dual colour was investigated to visualise the five probe sets on two slides from the same sample. A total unselected series of 45 uncultured lymphocytes and 60 uncultured amniocytes were hybridised with different probe combinations using three colour ratio-mixing FISH. The results indicate that the major chromosome aneuploidies can be simply and reliably identified on two slides from the same sample, using a three colour ratio-mixing FISH to detect the chromosomes X, Y and 21 and a dual colour to detect chromosomes 13 and 18. The failure rate was reduced to 4 per cent using this approach. At present this strategy appears to be more accurate and practical than five colour detection approach. The time required for signal analysis is considerably less than that needed for five colour FISH and the method can be stabilised in most diagnostic laboratories which are equipped for FISH analysis, as it does not require the use of digital imaging system.

The reliability of interphase FISH for the prenatal detection of aneuploidy must now be investigated by clinical trials run in parallel with conventional cytogenetic analysis. At the same time, technical advances in clinical sample preparation, defined probe sets and automation of signal counting will extend the diagnostic limits of five colour ratio-mixing FISH, providing a powerful technique for rapid detection of multiple chromosomal abnormalities in a single hybridisation experiment.

1. INTRODUCTION

1.1 Historical Perspective Of *In Situ* Hybridisation

After the correct number of chromosomes for human somatic cells had been firmly established as 46 (Tjio and Levan, 1956; Ford *et al.*, 1956) and the correlation of trisomy 21 with Down's syndrome was found (Lejeune *et al.*, 1959), cytogenetic diagnostic techniques were introduced in routine clinical practice. A second generation of cytogenetics emerged when chromosome banding techniques were developed (Casperson *et al.*, 1968), allowing the recognition of each human chromosome individually and hence the identification of smaller chromosomal aberrations. The resolution of cytogenetic analysis was improved dramatically with the introduction of prometaphase preparation and banding (Yunis *et al.*, 1976). This technique, with an estimated resolution of 2-5 Megabases, has led to identification of an increasing number of small deletions causing specific syndromes such as del(13q13) for retinoblastoma and del(15q13) for Prader-Willi and Angelman syndromes. Despite the classical cytogenetic banding techniques having been extremely valuable in the assessment of chromosome structure, they have suffered from several limitations. First, detailed chromosome banding analysis can only be performed using high quality chromosome spreads which are not always available particularly in leukaemia and tumor cytogenetics. Secondly, the limit of resolution for the detection of deletions and the definition of breakpoints is several megabases at best. Thirdly, for prenatal diagnostic applications, diagnosis is labour-intensive and time-consuming as it depends on the culture of fetal cells and the analysis of metaphase chromosomes.

In 1969 Gall and Paradue and independently John *et al.* (1969) developed the technique of isotopic *in situ* hybridisation. This technique, which is the most direct way to study the chromosomal localisation of DNA sequences was established as a routine approach to gene mapping in many laboratories in the 1970s. In 1981 Gerhard *et al.*, Malcolm *et al.*, and Harper *et al.*, were the first to demonstrate the possibility of localising single-copy sequences cloned from individual genes by isotopic *in situ* hybridisation. In spite of high sensitivity and wide applicability of isotopic *in situ*

hybridisation techniques, their use has been limited to research laboratories. This is likely to be attributable to the problems associated with radioactive probes, such as the safety measures required, limited shelf life, and extensive time (3-4 hours or more) required for auto radiography. In addition, the track of the decaying particle emitted from radioactive probes imposes the disadvantage of limited spatial resolution.

Clearly the modification of nucleic acid probes with a stable nonradioactive label removes the major obstacles that hinder the general application of *in situ* hybridisation. Based on the developments in the early 1980s (For review, see Raap *et al.*, 1990) non-isotopic *in situ* hybridisation was introduced as a solution for these limitations. In this technique the isotopic probe labelling was replaced with non-radioactive haptens which could be detected immunologically by binding of the haptens with specific antibodies. The antibodies are cross-linked to either enzymes or fluorescent dyes.

Fluorescence *in situ* hybridisation (FISH) originally described by Landegent *et al.* (1984) has become increasingly popular during the last few years. A number of reasons account for this development, notably an increase in speed, improved signal resolution, the development of *in situ* suppression hybridisation (ISSH) protocols, and advanced optical equipment for 2-D and 3-D analysis of labelled specimens. FISH is presently revolutionising human genetics. In basic science it has strongly enhanced gene and genome analysis, enabling rapid mapping of genes (Landegent *et al.*, 1985; Wiegant *et al.*, 1991) and large genomic clones using the competition principle as well as chromosome painting (Landegent *et al.*, 1987; Lawrence *et al.*, 1988; Thompson *et al.*, 1990; Kievits *et al.*, 1990; Lichter *et al.*, 1990a; Lengauer *et al.*, 1990; Wada *et al.*, 1990; Trask *et al.*, 1989, 1991; Baldini *et al.*, 1991; Driesen *et al.*, 1991). Diagnostically, FISH allows identification of pathological chromosome abnormalities in oncology (Nederlof *et al.*, 1989a; Arnoldus *et al.*, 1990; Smith *et al.*, 1991; Dauwerse *et al.* 1990, 1992a), congenital malformations (Kuwano *et al.*, 1991) and single gene disorders (Ried *et al.*, 1990; Lupski *et al.*, 1991). Many of the detected abnormalities could otherwise not have been identified or only with great difficulty.

Fluorescence *in situ* hybridisation with chromosome specific probes can detect the number of copies of a particular chromosome present in interphase nuclei (Lichter *et al.*, 1988a; Pinkel *et al.*, 1988; Cremer *et al.*, 1990; Kuo *et al.*, 1991; Klinger *et al.*, 1992; Zheng *et al.*, 1992; Ward *et al.*, 1993), an approach which has been termed *interphase cytogenetics*. The major advantage of this technique is that there is no requirement for cell culture and hence the results can be available in two days. The technique has important applications for the aneuploidy analysis of fetal chromosome abnormalities if it can be shown to be reliable in uncultured amniotic fluid cells.

The potential of fluorescence *in situ* hybridisation has been greatly enhanced by simultaneous detection of multiple probes each labelled with a distinct hapten (Nederlof *et al.*, 1989b; Ried *et al.*, 1992a; Dawerse *et al.*, 1992b; Lengauer *et al.*, 1993). The number of sequences which can be simultaneously detected in a single hybridisation experiment can be further increased by ratio-labelling or ratio-mixing approaches (Nederlof *et al.*, 1990; Ried *et al.*, 1992b). Digital imaging systems, like the cooled CCD camera with powerful image processing capabilities, can considerably improve the overall detection sensitivity and exploit the full potential of multicolour fluorescence *in situ* hybridisation in clinical cytogenetics.

1.2 The Hereditary Material

In humans, as in other organisms, nucleic acid is the carrier of genetic information and has a structure which is ideally suited to this function. Nucleic acid is a complex substance composed of long chains of molecules called nucleotides. Each nucleotide is composed of a nitrogenous base, a sugar molecule, and a phosphate molecule. The nitrogenous bases are called purines and pyrimidines. The purines include adenine and guanine; the pyrimidines include cytosine, thymine and uracil. There are two different types of nucleic acids. One contains the sugar ribose, and is therefore called ribonucleic acid or RNA. The other contains a slightly different sugar called deoxyribose and is therefore called deoxyribonucleic acid or DNA. Both types of nucleic acids contain

cytosine and have the same purine bases, whereas thymine occurs only in DNA, uracil occurs only in RNA.

The DNA molecule is composed of two nucleotide chains which are coiled clockwise around one another to form a double stranded structure. The backbone of each chain is formed by sugar-phosphate molecules and the two chains are held together by hydrogen bonds between the nitrogenous bases which point in toward the centre of the helix. The arrangement of the bases in the DNA molecule is not random: a purine in one chain always pairs with a pyrimidine in the other chain. There is also specific base pairing: guanine in one chain always pairs with cytosine in the other chain and adenine always pairs with thymidine. This model of the DNA molecule is referred to as the Watson-Crick model. This model provides an answer to the question of how genetic information is transmitted from one cell generation to the next.

Genetic information is stored within the DNA molecule in the form of a triplet code, that is a sequence of three bases determining one amino acid. As each base in the triplet may be any of the four types of nucleotide (A, G, T, C), this results in 4^3 or 64 possible combinations or codons. All amino acids except methionine and tryptophan are coded by more than one codon. Three of the 64 codons designate the termination of a message (UAA, UGA, UAG). The AUG codon for methionine specifies the initiation of translation by N-formyl methionine at the beginning of a polypeptide chain. Proteins, whether structural components, enzymes, carrier molecules, hormones or receptors, are all composed of a series of amino acids. Twenty amino acids are known, and the sequence of these determines the form and function of the resulting protein. All proteins are encoded in DNA, and the unit of DNA which codes for a protein is by definition its gene. Genes vary greatly in size from small genes like the globins to medium sized genes of 15-45 kb to enormous genes such as dystrophin (more than 1 Mb).

1.3 Chromatin Structure

The total length of DNA in a haploid set of human chromosomes is 3000 Mb. There are an estimated 50 000-100 000 human structural genes encoded in the DNA.

Each structural gene usually has only one copy in the haploid genome, and if the average gene is 15 kb in size then this accounts for 1500 Mb or about one-half of the total DNA. Much of the remainder consists of repetitive DNA, which may be moderately repetitive with several hundred copies or highly repetitive with many thousands of copies.

The moderately repetitive DNA includes some functional genes which code for ribosomal and transfer RNAs and histones. These genes are necessary in all cells and in each phase of individual development. In contrast, the highly repetitive DNA is not transcribed and may be clustered or interspersed throughout the genome.

The DNA sequences adjacent to the centromeres of human chromosomes have been shown to consist of repeated DNA sequences of varying complexity, organisation, and abundance (Gosden *et al.*, 1975; Mitchell *et al.*, 1979). Some of these have been defined as satellite DNAs on the basis of different isopycnotic centrifugation techniques (Mitchell *et al.*, 1979). Part of the sequences have been shown to be simple sequences consisting of long, uninterrupted arrays of tandemly arranged units of 6-12 bp, whereas others are longer and organised in a more complex manner. One of these displays a basic repeating unit of 171 bp (Manuelidis, 1976) and is referred to as alphoid DNA because of its homology to the alpha component isolated from the African green monkey (Manuelidis and Wu, 1978).

The genomes of almost all higher eukaryotes also contain highly repetitive sequences that are not clustered together at centromeres as are satellite sequences. These sequences include short interspersed repeats (SINEs, unit repeat less than 500 bp), long interspersed repeats (LINEs, unit repeats of several kbp) and unclassified spacer sequences. In the human genome, the majority of SINEs belong to a single family called the *Alu* family. Its members are about 300 base pairs long and are recognisably related but not precisely conserved in sequence. Their name derives from the fact that most contain a single site of cleavage for the restriction enzyme *AluI*. Almost a million *Alu* sequences are present in the human genome, comprising up to 6 percent of the total DNA. Thus any particular DNA segment of 5000 base pairs or longer has a high

probability of containing an *Alu* sequence. Accordingly, many introns and sequences adjacent to genes (in both transcribed and non transcribed regions) harbour *Alu* sequences. In contrast to the SINEs the unit repeat of LINEs such as Kpn-LINE family found in primates is between 1.5 and 5 kbp long. LINE repeats have a different distribution from the SINEs and have considerable similarity with retroviral sequences.

The chromatin thread consists of repeat units called *nucleosomes*. Each nucleosome is made up of a set of histone molecules (positively charged alkaline proteins with a molecular weight of about 10 000-20 000) in association with about 200 DNA base pairs (Figure 1.1).

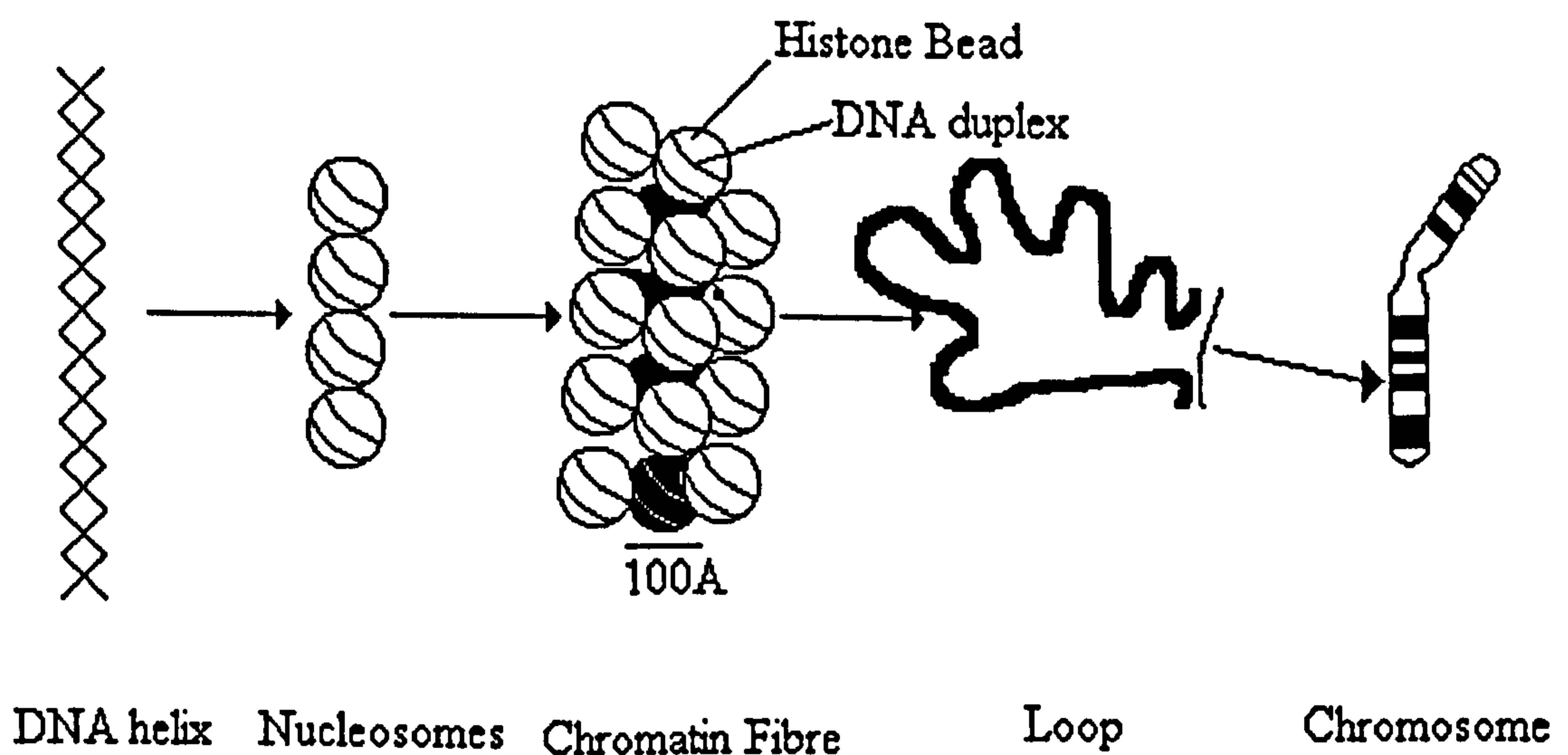


Figure 1.1. Simplified diagram of a proposed model of DNA coiling.

Other proteins which are involved in the structure of the chromatin, so-called non-histone proteins are present in varying but mostly smaller amounts. The non-histone fraction is heterogeneous. It includes, for example a number of enzymes. The DNA component of a nucleosome has two parts: a "core" of 140 base pairs and a "linker" which varies in length from about 15 to 100 base pairs, depending on the cell type. The chromosomes in interphase can be visualised as an elementary fibre that consists of a sequence of nucleosomes connected by linkers. Chromosomes in mitosis and meiosis show a much higher degree of coiling than in interphase.

1.4 Fluorescence *In Situ* Hybridisation-Basic Principles

In situ hybridisation (ISH) is the term used to describe the hybridisation of labelled nucleic acid probes to complementary sequences of chromosomes and chromatin throughout the cell cycle. The technique relies on the phenomenon that when double stranded DNA is heated, the strands dissociate or melt. If the temperature is reduced, the strands will reassociate or anneal. The sensitivity of ISH depends on the following variables: (1) the effect of tissue preparation on retention and accessibility of cellular (target) DNA or RNA; (2) type of probe construct, efficiency of probe labelling and sensitivity of the method used for signal detection; (3) the effect of hybridisation conditions on the efficiency of hybridisation and (4) spatial resolution and sensitivity of visualisation methods.

1.4.1 Specimen Preparation

Fluorescence *in situ* hybridisation can be successfully applied to a variety of cell types ranging from lymphocytes and cultured cells to uncultured amniocytes and cells from solid tumors. The most suitable specimens for FISH are isolated cells mounted on microscope slides. However, some studies have also reported successful hybridisation to cells in suspension (Trask *et al.* 1988) and to thin sections prepared from paraffin embedded material (Emmeirch *et al.* 1989).

The cultured cells or the samples which are used for interphase cell preparations are first treated with a hypotonic solution. This treatment swells the cells and spreads the chromosomes prior to fixation. A low concentration of hypotonic solution can be used to obtain well-swollen interphase cells. This is of major importance in multicolour interphase FISH as enough space is needed to simultaneously visualise multiple targets in uncultured cells. The cells are harvested by the appropriate fixative after treatment with hypotonic solution. The fixation preserves tissue morphology and minimises loss of nucleic acids. The morphology and permeability of the preparations are critical for *in situ* hybridisation experiments. There is an inverse relationship between the degree of tissue fixation and tissue permeability. Overfixed material may be well-preserved but may be less accessible

to the probe and detection reagents. Methanol and acetic acid (in a ratio of 3:1) are more frequently used as a fixative in FISH studies compared to other fixatives such as glutaraldehyde or formaldehyde, as they do not significantly reduce the tissue permeability (Leith *et al.*, 1994).

The samples are immobilised on glass microscope slides as fixed metaphase or interphase cells. The slides must be clean and free of oily residues as chromosomes and nuclei preparations do not adhere well to unclean slides and consequently can be lost during *in situ* hybridisation. The slides may also be coated with an adhesive to increase the adherence of fixed material. This step is particularly important if the sample size is limited as it prevents separation during post-hybridisation washings and detection steps. 3-Aminopropyl-triethoxysilane (APES) as an adhesive produces improved cell or tissue bonding over other commonly used adhesives such as poly-L-lysine, glycerin albumin, and gelatin. No background staining occurs using this adhesive and it does not interfere with immunostaining methods (Maddox *et al.* 1987). The fixed material is dropped on to the slides and may be slightly aged by leaving at room temperature overnight or by incubating in an 80°C oven for 2 hours, as the fresh slides do not maintain good morphology after hybridisation.

Prior to denaturation the slides are always fixed in methanol:acetic acid (3:1) to reduce diffusion and loss of cellular DNA during denaturation and post-hybridisation washings. Pretreatment of slides with RNase A results in reduced background, as it removes cytoplasmic and nuclear RNA and prevents their non-specific binding to the probe. The use of enzymes to digest proteins (e.g. proteinase K) can help with accessibility of probe and detection reagents. The enzymes are believed to act by unmasking nucleic acids from associated proteins (Leith *et al.*, 1994). If penetration of reagent is a problem, the concentration of enzyme can be increased, but if cell or chromosome morphology is poor the concentrations should be decreased.

1.4.2 Probes

DNA sequences are usually detected using labelled DNA probes, while RNA or DNA probes are used for RNA detection. The best probe length for *in situ* hybridisation

is about 100-300 bases. Shorter probes may result in lower nucleic acid hybrid stability and longer probes (especially more than 1 kb) may have tissue penetration problems.

In principle, recombinant DNA technology enables the cloning and purification of any DNA sequence. Alternatively, oligonucleotide sequences can be synthesised *de novo* or an organism's total genomic DNA used.

1.4.2.1 Cloned DNA Probes

To amplify a specific DNA sequence by cloning, the DNA is inserted into a vector and both vector and insert are amplified inside appropriate host cells. The amplified DNA is then extracted, labelled and used as probe in most *in situ* hybridisation experiments. In some cases the insert is excised from the vector prior to labelling, particularly when the inserted sequence is small in comparison with the vector. Commonly used vectors include bacterial plasmids containing up to 10 kb of foreign DNA insert, bacteriophages containing inserts of 5 to 25 kb, cosmids capable of retaining up to 45 kb inserts, and yeast artificial chromosomes (YACs) which may contain more than 1 Mbp of cloned DNA.

Many DNA sequences have been visualised by *in situ* hybridisation from single-copy to highly repeated sequences. Depending on the target, different probe selections can be applied. The most commonly used cloned DNA probes include:

- Chromosome specific library probes
- Alphoid DNA repeats
- Single copy probes (Cosmids, Cosmid contigs, Yeast Artificial Chromosomes)

I. Chromosome-Specific Library Probes

Collections of DNA sequences derived from a single human chromosome can highlight that chromosome in metaphase and interphase nuclei. This procedure has also been termed chromosome painting (Pinkel *et al.*, 1988). Collections can be derived from somatic cell hybrids carrying the desired chromosome as its only human material or from

suspensions of chromosomes purified by flow sorting (Lichter *et al.* 1988b and 1990b). In the case of hybrid cells the isolated DNA can be used directly for labelling. The sorted chromosomes are digested in solution, amplified by PCR between Alu or LINE sequence primers and labelled for use with FISH (Lichter *et al.* 1990b, Telenius *et al.*, 1992). The libraries used are either cloned in lambda phage vectors (Van Dilla *et al.*, 1986) or in pBS plasmid vectors (Fuscoe *et al.*, 1989). The latter are generally easier to use since the proportion of vector sequences is small compared to most of the presently available recombinant lambda libraries. Accordingly, background problems which may arise from high amounts of labelled vector can be eliminated. This problem could otherwise be overcome by isolating the library inserts (Lichter *et al.*, 1988b).

Whole chromosome probes comprise sequences distributed densely and more or less continuously over one or more chromosomes so that chromosomes targeted by the probe appear almost completely stained (painted) after hybridisation. Hence whole chromosome probes specific for human chromosomes can highlight the chromosomes in cultured and uncultured cells (Cremer *et al.* 1988; Pinkel *et al.* 1988). Specific chromosome paints have been made for almost all human chromosomes, and are proving extremely valuable in the cytogenetic analysis of chromosome rearrangements, whether constitutional or in neoplastic cells. However libraries usually contain repetitive elements such as the *Alu*, *LINE* and *KpnI* elements which can cross hybridise with other chromosomes. Chromosomal *in situ* suppression (CISS) hybridisation may provide a relatively simple method to suppress hybridisation of these repetitive elements to other chromosomes.

II. Alphoid DNA Repeat Probes

Alphoid DNA repeats are highly repetitive sequences clustered in the centromeric regions of human chromosomes. A variety of human alphoid sequences have been isolated, characterised and sequenced (McDermid *et al.*, 1986; Waye *et al.* 1985). Evidence has been presented that many, if not all, human chromosomes are characterised by specific subsets of alphoid satellite DNA, as revealed both by particular restriction

enzyme periodicities and by primary nucleotide sequence analysis (Mitchell *et al.* 1985; Jorgensen *et al.* 1986). Chromosome 21 and 13 share the same repeat sequences (Devilee *et al.*, 1986) and thus cannot be differentiated using the alphoid-satellite repeat probes. The molecular and evolutionary basis for the chromosome specificity of subsets of different satellite DNA, including alphoid satellite, is unclear.

Probes for these sequences have been used most commonly for analysis of chromosomal aneuploidy in cultured and uncultured cells. The sequences targeted by these probes are typically alphoid-satellite (Gray and Pinkel 1992). These probes produce hybridisation signals near to the centromeres or in heterochromatic regions of specific chromosomes and in compact domains in interphase chromatin when labelled and hybridised at sufficient stringency.

III. Single Copy Probes

Single copy targets in the genome can be detected by complementary unique-sequence probes in metaphase and interphase cells. Single copy probes have been important for a broad spectrum of cytogenetic and molecular genetic experiments. In clinical genetic applications unique-sequence probes are valuable tools to detect numerical and structural abnormalities such as deletions, inversions and translocations. There is a fairly good correlation between hybridisation efficiency and the size of the target DNA. When the size of the target DNA detected by a given probe decreases (e. g. using probe fragments cloned in phage or plasmid vectors), the percentage of successfully delineated target sites also decreases. With probes containing approximately 2 kb single-copy sequences, a maximum of 40% to 50% of all target sites can presently be detected. Although single-copy DNA fragments of about 1 kb have been successfully mapped by fluorescence *in situ* hybridisation, the discrimination of weak specific signals against background dots is likely to require statistical analysis. Since the size of DNA segments that can be carried in various types of vectors is different, the detection efficiency of probes hybridising to the single copy targets depends on the vector type used for cloning.

The two main types of vectors which have been widely used to produce single copy probes are cosmids and yeast artificial chromosomes (YACs).

Cosmids are hybrid vectors, derived from plasmids and λ phages, that facilitate genomic cloning by being able to carry approximately 45 kb of foreign DNA, three times more than that of phage vectors. The λ phage contains at each end single-stranded complementary stretches of DNA, the so-called *cos* sites. The *cos* sites are all that is necessary for packaging DNA into phage and cosmid vectors. To make a cosmid library, eukaryotic DNA is cleaved with a restriction enzyme under conditions that yield relatively large pieces of DNA. This DNA is then ligated to the cosmid, which has been cleaved with a restriction enzyme that leaves ends complementary to the cleaved genomic DNA. The ligated DNA is packaged *in vitro* into phages and introduced into *E. coli* by infection. Once inside the *E. coli* cell, the cosmid replicates and can be recovered from the cells in the same way as a plasmid.

Cosmid probes commonly contain 25-45 kb of cloned insert DNA and are efficient probes which yield highly specific signals on metaphase chromosomes. Usually more than 90% of all metaphases show signals on both chromatids of both chromosome homologs with virtually no background spots. Therefore, the need for statistical analysis is greatly reduced and the efficiency is high enough to use such probes for the diagnosis of chromosomal aberrations such as trisomies (Lichter *et al.* 1990c), translocations (Tkachuk *et al.* 1990; Rowley *et al.* 1990), inversions (Dauwerse *et al.* 1990) and deletions (Lux *et al.* 1990; Ried *et al.* 1990). Although single cosmids are efficient probes when used for peripheral lymphocytes, they result in weak signals when hybridised to uncultured amniocytes (Zheng *et al.*, 1992).

Cosmid contigs are overlapping cosmid clones specific for a single target DNA, which can overcome the limitations of single cosmids since they have high signal-to-noise ratios, acceptable spatial resolution of the fluorescence signals, and exhibit high hybridisation and detection efficiencies. Using these DNA probes it is possible to detect

the major chromosomal aneuploidies present in uncultured amniotic fluid samples in a routine prenatal diagnosis (Klinger *et al.* 1992; Zheng *et al.* 1992).

Yeast artificial chromosomes (YACs), with the ability to carry several kilobases of exogenous DNA have vastly improved the strategies for isolating and cloning genes important for human disease, as well as for constructing physical maps of regions of complex genomes. Burke *et al.* (1987) were the first to describe the use of YACs for isolation of fragments of human DNA in the size range of 100-1000 kb. Since this initial description substantial improvements have been made in methodology (Plinio *et al.* 1988; Pavan *et al.* 1990).

The vector system incorporates all necessary functions into a single plasmid that can replicate in *Escherichia coli*. This plasmid, called a “yeast artificial chromosome” (YAC) vector (fig. 1.2), supplies a cloning site within a gene (SUP4) whose interruption is phenotypically visible. It also contains an autonomous replication sequence (ARS1), a centromere (CEN4), selectable markers on both sides of the centromere (TRP1 and URA3), and two sequences that seed telomere formation *in vivo* (labelled TEL). Cleavage at the *Bam*HI sites adjacent to the TEL sequences produces termini that heal into functional telomeres *in vivo* (Burke *et al.* 1987). The overall cloning protocol is shown schematically in figure 1.2. Double digestion of the particular YAC vector shown, pYAC2, with *Bam*HI and *Sma*I yields three parts, which can be regarded as a left chromosome arm, including the centromere, a right chromosome arm and a throwaway region that separates the two TEL sequences in the circular plasmid. The two arms are treated by alkaline phosphatase to prevent religation, and then ligated on to large insert molecules derived from the source DNA by partial or complete digestion with an enzyme that leaves *Sma*I-compatible (that is blunt) ends. The ligation products are then transformed into yeast spheroplasts by standard methods, which involve embedding the transformed spheroplasts in agar on a selective medium. In the host yeast cell the artificial chromosome can replicate in the same way as a yeast chromosome and transfers from one cell generation to the next.

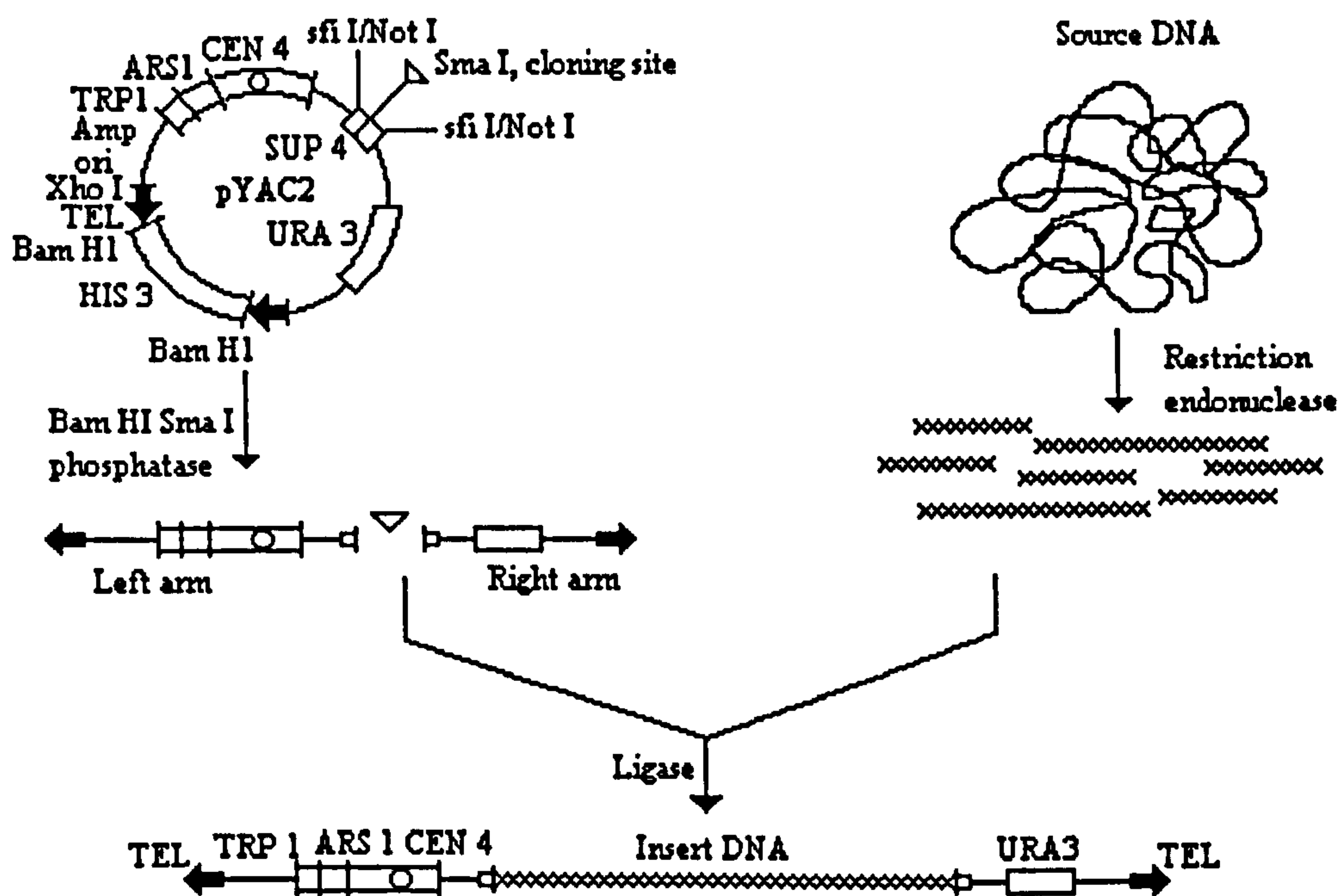


Figure 1.2. Diagrammatic representation of yeast artificial chromosome (YAC) cloning system.

Many problems of eukaryotic molecular biology require analysis of hundreds or thousands of kb either because of the size of individual genes or because available markers are located far from the genes or disease of interest (Koeing *et al.* 1987). Furthermore, global analysis of whole genomes such as that of humans will require analysis of millions of kb. DNA fragments several kb long have been successfully cloned in yeast artificial chromosomes (YACs) and the use of YAC clones for *in situ* hybridisation experiments offers significant advantages in gene mapping projects (Selleri *et al.* 1991) and also detection of aneuploidies on uncultured lymphocytes and amniocytes (Lengauer *et al.* 1992; Romana *et al.* 1993).

1.4.2.2 Synthetic Oligonucleotides

Synthetic oligonucleotides are short nucleotide sequences, usually between 10 and 50 bp long, prepared using a DNA synthesiser. The major advantage of synthetic

oligonucleotides is that they can be tailor made to hybridise to specific sequences. Oligonucleotides can be used as primers to specifically amplify DNA probes by polymerase chain reaction (PCR). Cloned DNA sequences can be amplified by this method using suitable primers that flank the insert. An important use of PCR amplification methods is to amplify specific sequences or classes of sequences from total genomic DNA or DNA isolated from flowsorted chromosomal material. Unlabelled oligonucleotides have been used as primers for direct DNA labelling on chromosomes, a process called primed *in situ* labelling (PRINS).

1.4.2.3 Total Genomic DNA Probes

Total genomic DNA (consisting of the entire DNA complement of an organism's genome) can be labelled and used as a probe to identify individual chromosomes in cell fusion hybrids. Human chromosomes in hybrid cell lines can be intensely and uniformly stained in metaphase and interphase nuclei when human genomic DNA is used as a probe. This method can be used to identify the interspecies chromosome rearrangements and such an ability suggests the utility of hybrid cell systems for studies of cell response to low-dose radiation or other reagents. More recently, comparative genomic hybridisation (CGH) has been used to identify chromosomal differences between normal cells and tumours. Labelled genomic DNA isolated from somatic cell hybrids containing a single human chromosome can be used to make chromosome specific paints.

1.4.3 DNA Probe Preparation

Recombinant DNA technology has greatly facilitated the preparation of DNA for molecular investigation. Segments of DNA are introduced into microbial systems for propagation and the synthesis of large amounts of material for analysis. Transformed bacterial or yeast cell stocks can be stored in 15-20% glycerol at -70°C for many years.

Bacterial cells carrying the probe DNA are streaked onto agar plates containing the appropriate selection antibiotic and several suitable single colonies are then grown up in liquid cultures. The overnight cultures are then used for the isolation of probe DNA. An

80 ml mini preparation culture will usually yield 100-200 µg of cloned DNA depending on the vector type and size of insert. Commercial DNA isolation kits are available for isolation of plasmid or cosmid DNA and are very quick and produce satisfactory DNA for FISH analysis.

Single colonies of yeast cells containing YAC which have been grown on agar plates, are transferred to a liquid selective medium to be amplified. The cultured cells are then used for isolation of yeast genomic DNA. The harvested cells can be embedded in agarose plugs prior to DNA isolation, to maintain the yeast chromosomes intact as described (Carle *et al.* 1987). The isolated DNA is used for labelling with desired reporter molecules or with an Alu-PCR reaction.

In fluorescence *in situ* hybridisation (FISH) experiments, the hybridisation efficiency is generally less than satisfactory when total yeast genomic DNA or YAC DNA isolated from pulsed-field gels is used as probes (Lengauer *et al.* 1992; Zheng *et al.* 1992). To overcome these problems Alu-PCR protocols first described by Nelson *et al.* (1989) have been optimised. Since Alu sequences are specific for the human genome and are not present in yeast genomic DNA, by using primers which are complementary to the Alu sequences only inter-Alu sequences of human insert will selectively be amplified. The generated probes are used for labelling and have been shown to have enough complexities to be used for fluorescence *in situ* hybridisation experiments. (Burke *et al.* 1987; Baldini *et al.* 1992; Lengauer *et al.* 1993; Romana *et al.* 1993).

1.4.3.1 DNA Labelling Techniques

Successful FISH is dependent on generating adequately labelled probes. Because of the intrinsic disadvantages of radioisotopes (such as personnel and disposal problems), isotopic labels have been replaced by fluorescent labels during recent years. In general, non-radioactive probes may be safer, although toxicity may not be fully known and contamination is less easy to detect than with radioactive probes. Non-radioactively labelled probes can be stored for long periods without loss of activity, the hybridisation

sites can be detected quickly (hours rather than days or weeks) and several sequences can be detected simultaneously by using different probe labels.

In fluorescence *in situ* hybridisation experiments the probes are either directly labelled with fluorescent molecules or with reporter molecules and broken into 200-400 base pair fragments, a size that maximises specific hybridisation and decreases background fluorescence (Lawrence *et al.* 1985). Fluorescent labels are directly visualised after post-hybridisation washings and mounting. In this procedure no immunocytochemical detection method is required. The main drawback of the direct labels is, however, that they can be less sensitive than indirect labels. This procedure is particularly useful when large regions are targeted, i.e. for staining of repetitive DNA or for chromosome painting. The most common fluorochrome which is used for direct *in situ* hybridisation experiments is fluorescein-dUTP (green fluorescent dye, Fig. 1.3).

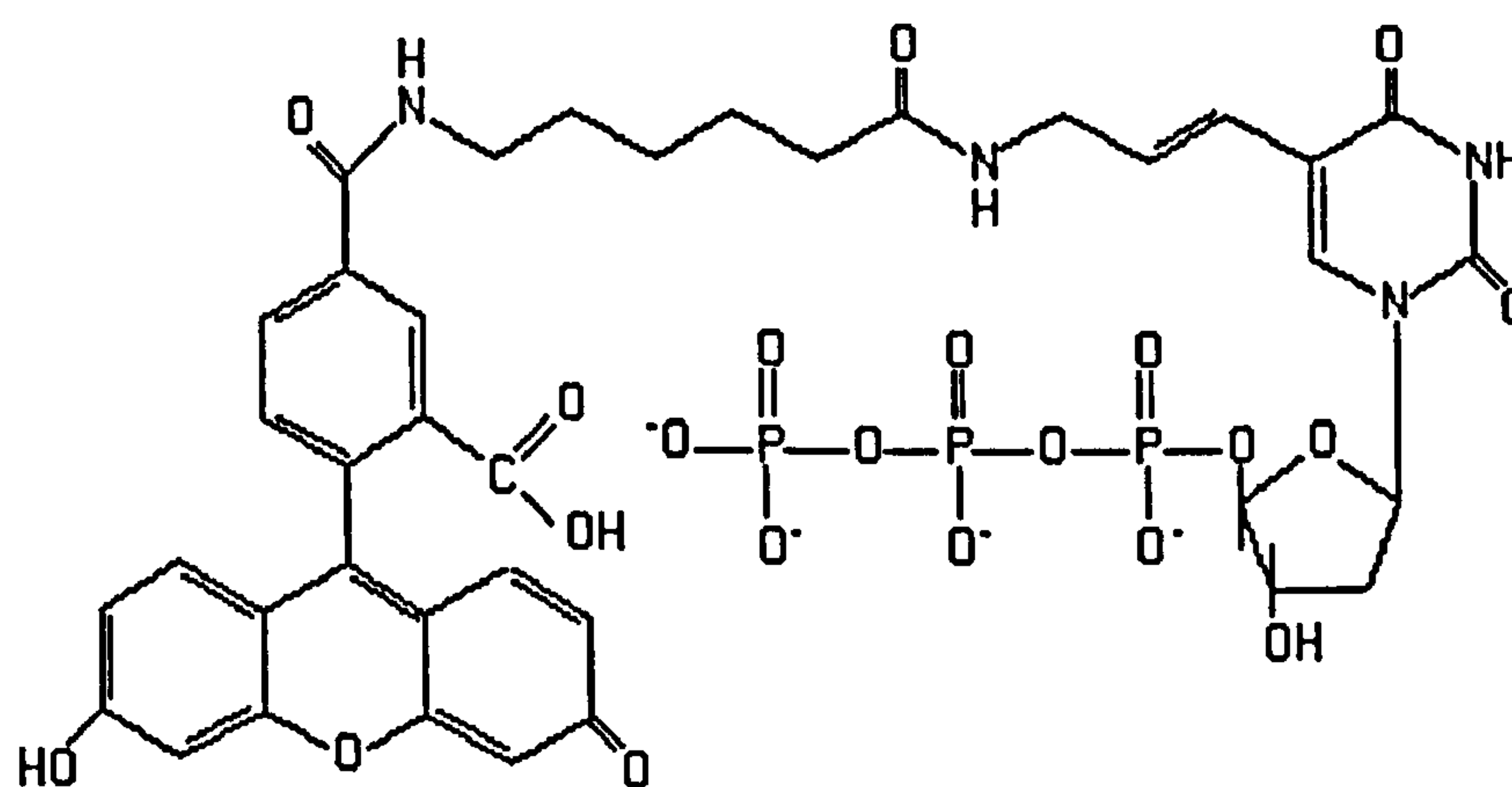


Figure 1.3. Fluorescein-dUTP

Resorufin-dUTP (red fluorescent dye) and Hydroxycoumarin-dUTP (blue fluorescent dye) are the some of other commercially available fluorochromes used for this purpose.

The most widespread approach however is to label probes with haptens that after hybridisation, bind fluorescent affinity reagents. Biotin and digoxigenin are the most commonly used reporter molecules in most laboratories. Biotin is a member of the vitamin B complex, also called vitamin H. The biotin molecule is covalently attached to

the pyrimidine ring to produce Biotin-dUTP which is an analogue of dUTP (Fig. 1.4). Biotin-11-dUTP is the most widely used biotin derivative, but other modified nucleotides are now available, e.g. biotin-16-dUTP, biotin-14-dATP and biotin-11-dCTP.

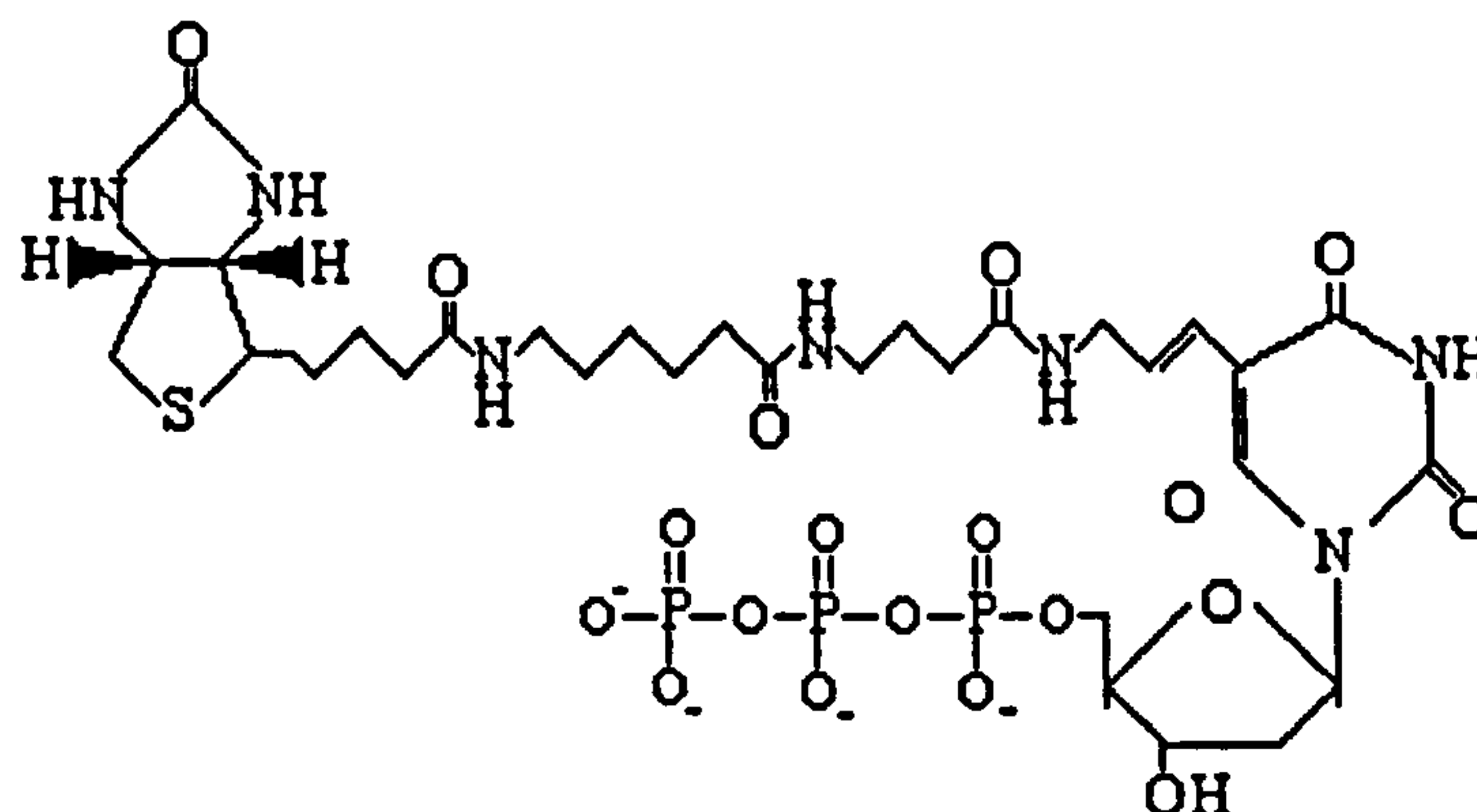


Figure 1.4. Biotin-dUTP

Each of the nucleotides is modified at a position that does not interfere with hydrogen bonding between the probe and the target nucleic acid. In addition, each contains a linker arm of at least 11 carbon atoms to ensure access of the detection reagents and to minimise steric hindrance during probe hybridisation. The enzymatic incorporation of biotin to nucleic acids was developed by David Ward and co-workers at Yale University (Langer *et al.*, 1981).

Digoxigenin is a steroid isolated from digitalis plants (*Digitalis purpurea* and *Digitalis lanata*). As the blossoms and the leaves of these plants are the only natural source of digoxigenin, no binding of the anti-DIG antibody in other biological material occurs. Digoxigenin is linked to uridine nucleotides at the number 5 position of the pyrimidine ring through an H-C atoms spacer arm (Fig. 1.5), and incorporated enzymatically, at a particular density in to nucleic acid probes. Dinitrophenyl (DNP), amino-acetyl fluorene (AAF), mercury and sulfonate are some of the other reporter molecules which have been used for nucleic acid labelling and are described in detail by Gray *et al.* (1989).

Radioactive and several non-radioactive labels (e.g. biotin, digoxigenin, fluorochromes) can be incorporated in to nucleic acids by enzymatic labelling systems

including nick translation, oligolabelling and polymerase chain reaction (PCR) using modified nucleotides. DNA can also be non-radioactively labelled by chemically modifying the DNA helix (e.g. with 2-acetylaminofluorene or mercury). The enzymatic labelling methods usually result in higher incorporation of modified nucleotides and can therefore generate the most sensitive probes.

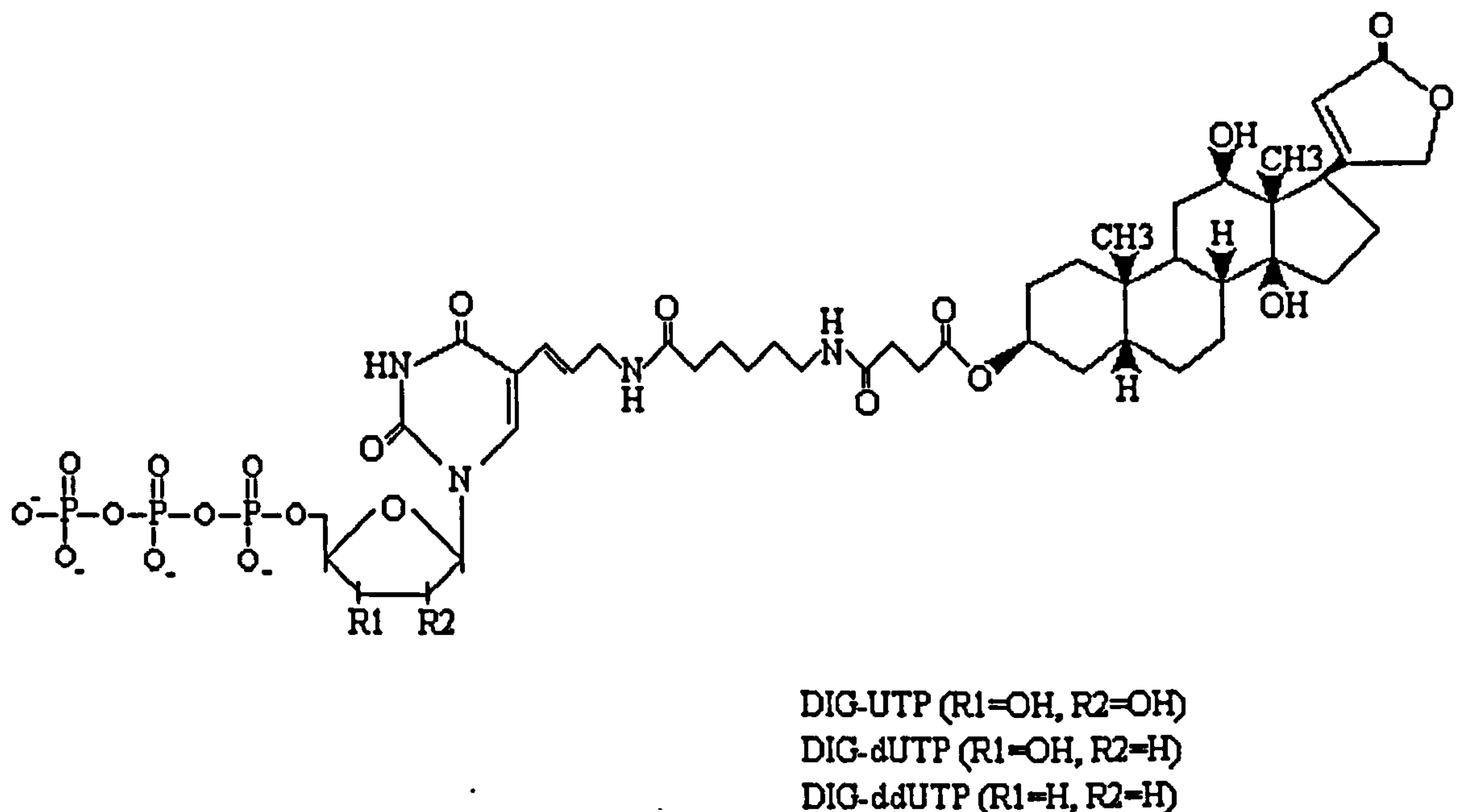


Figure 1.5. Digoxigenin-UTP/dUTP/ddUTP

I. Nick Translation

Nick translation first described by Rigby *et al.* (1977), is probably the most commonly used method for labelling hybridisation probes. The nick translation reaction employs two enzymes, bovine pancreatic deoxyribonuclease I (DNase I) and *E. coli* DNA polymerase I. (fig. 1.6). DNase I is an endonuclease that in the presence of Mg^{2+} attacks each strand of DNA independently and creates single stranded nicks. The sites of cleavage are distributed in a statistically random fashion. The number of nicks is critical and can be controlled by the concentration of DNase I in the reaction. Too little nicking can lead to inefficient incorporation of the label and probes that are too long. Too much nicking results in probes that are short.

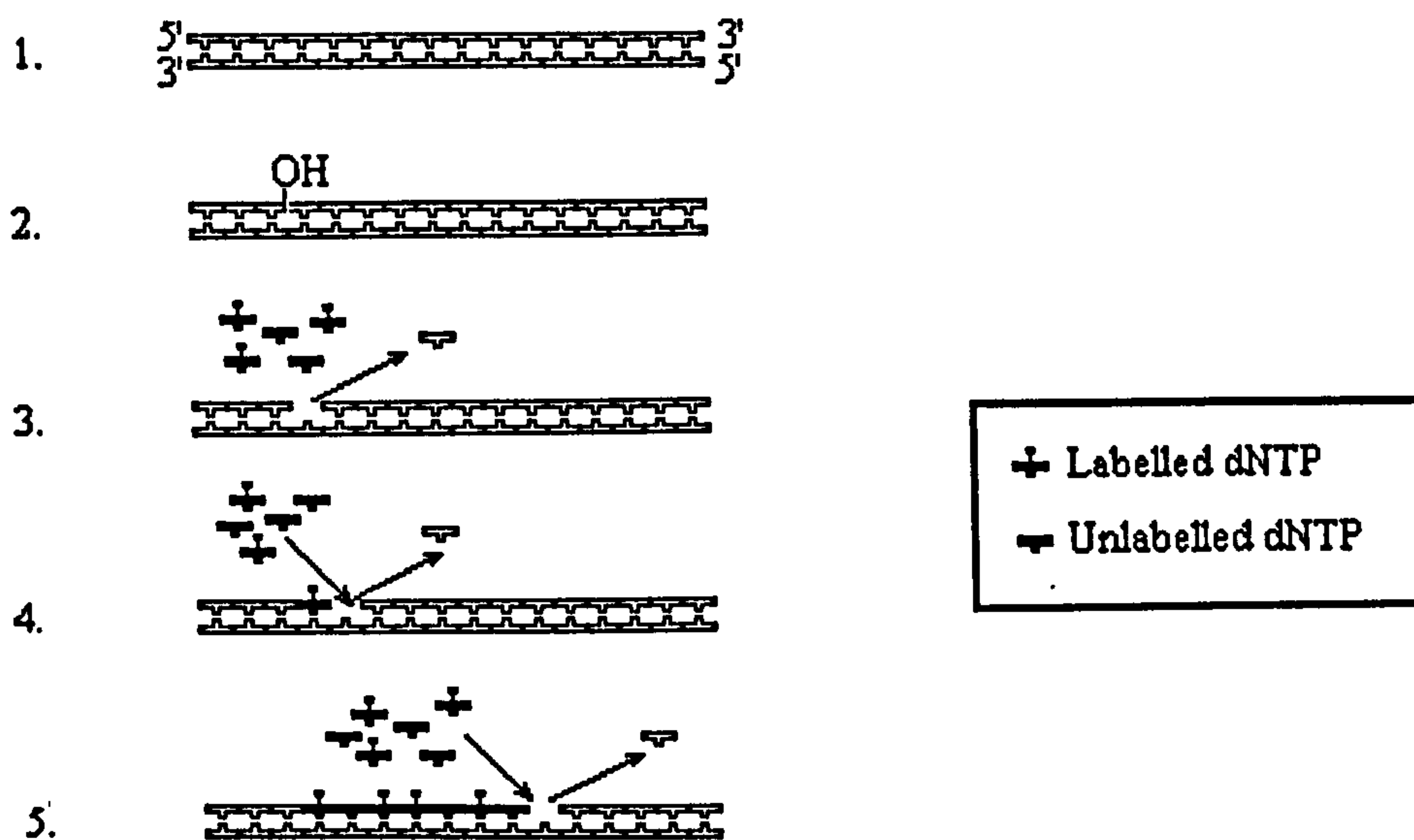


Figure 1.6. Diagrammatic representation of nick-translation reaction

The holoenzyme of DNA polymerase I has three different enzymatic activities: (a) 5'→3' polymerase, (b) 5'→3' exonuclease, (c) 3'→5' exonuclease. When there is single stranded nick in the DNA molecule, the exonuclease activity of DNA polymerase I hydrolyses the fragment 3' to the nick in 5'→3' direction. At the same time its polymerase activity sequentially incorporates nucleotides in the same direction and 5' to the nick, using the other strand as template. The result of these two reactions is the movement of the nick along the DNA strand (Sambrook *et al.*, 1989). The presence of labelled nucleotide in the reaction mixture results in production of labelled double stranded DNA. Because of the nicks produced by DNase I, different lengths of single stranded labelled DNA will be obtained after denaturation. The degree of label incorporation can be controlled by the concentration of labelled nucleotide, the concentration of DNA polymerase I and the duration of reaction time. The labelled DNA can be stored at -20°C for a minimum of one year.

II. Random-Primed Labelling

Random primed labelling which is also called oligolabelling was developed by Feinberg and Vogelstein (1983). The reaction uses the klenow fragment of *E. coli* DNA

polymerase I and random oligonucleotides (hexadeoxyribonucleotides) which serve as primers to initiate the polymerisation in the presence of all deoxyribonucleoside triphosphates (fig 1.7).

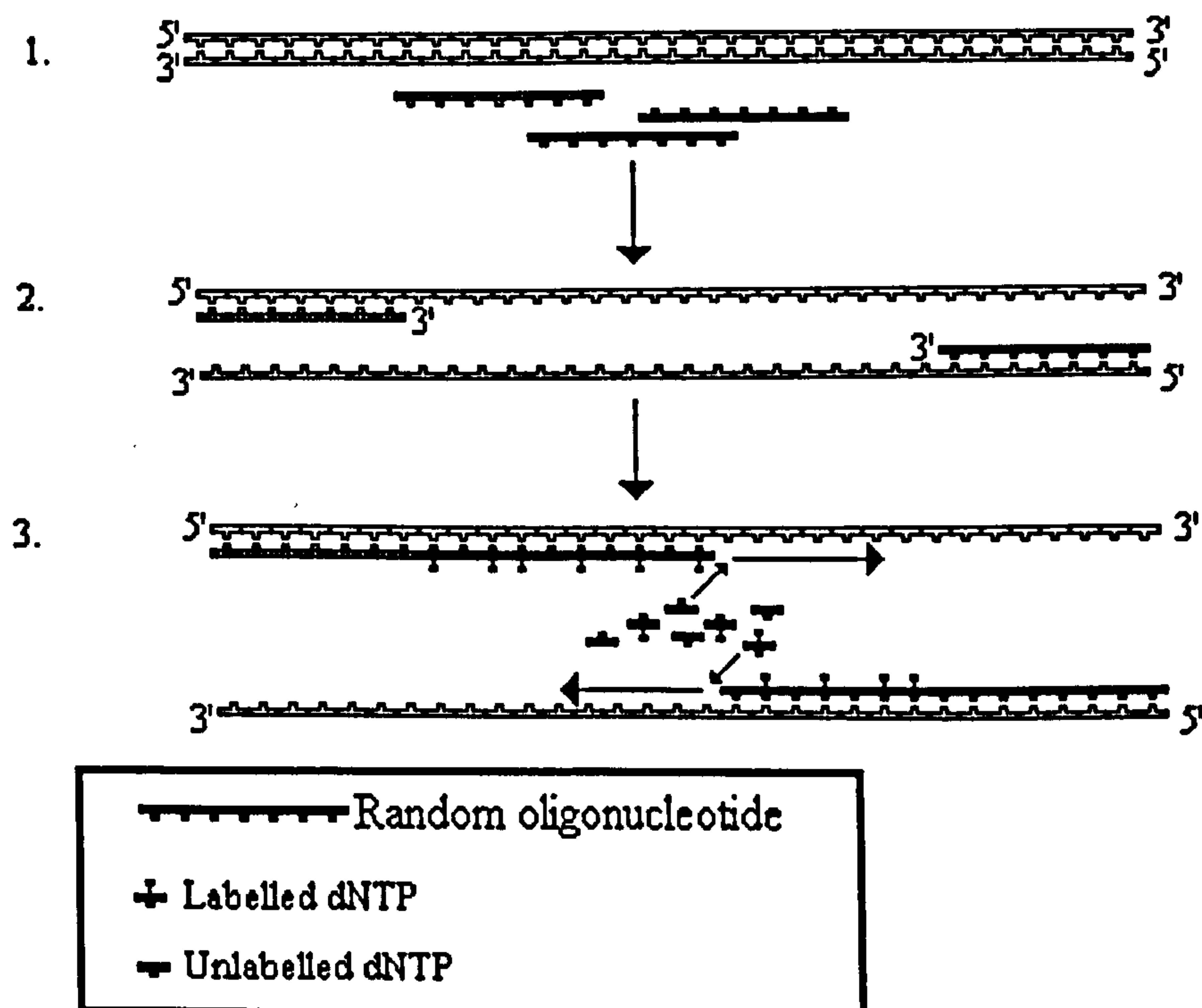


Figure 1.7. Schematic illustration of random primed DNA labelling reaction.

The klenow fragment differs from holoenzyme DNA polymerase I in that it lacks the 5'→3' exonuclease activity. In this method the DNA sample is first heat-denatured and the resulting single stranded DNA is used as template for the labelling reaction. In the appropriate conditions the short oligonucleotides randomly bind to the template and the klenow fragment incorporates nucleotide residues to the primer in 5'→3' direction. When a labelled nucleotide is present in the reaction mixture, the resulting DNA will be labelled. The length of labelled strand can be controlled by primer concentration. A high concentration of primer gives short labelled strands and vice versa. The method can be used to label any linear DNA molecule. Supercoiled DNA should be linearised before labelling since quick re-annealing of this type of DNA causes a rapid decrease in the concentration of template strands. The final product of the reaction contains both labelled

(synthesised) and unlabelled (template) strands. The labelled strands inevitably compete with each other during the hybridisation.

III. Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a modification of random primed labelling. The reaction employs a heat stable DNA polymerase isolated from the bacterium *Thermus aquaticus*, known as *Taq* polymerase. The enzyme exhibits highly processive 5' to 3' polymerase activity, which is at its maximum at around 72°C. The first step of the reaction is to denature the DNA template by heating to high temperatures. To eliminate the risk of damaging the enzyme, the DNA can be denatured before adding the enzyme. The denatured DNA is then annealed to a pair of specific oligomer primers, which are complementary to the flanking regions of template DNA. The annealed primers serve as a substrate for *Taq* polymerase and DNA synthesis. Following the first cycle of DNA synthesis the primers can anneal to both the original template DNA and the newly synthesised complementary strand of DNA. By repeating the cycles of denaturation, annealing and synthesis, the original target DNA can be amplified many times. Since *Taq* polymerase accepts nucleotides modified by radioactive or non-radioactive labels (Lo *et al.* 1988; Seibl *et al.* 1990), the PCR can be used not only to amplify DNA but also to produce large quantities of labelled probe DNA that is suitable for *in situ* hybridisation.

Primed *in situ* DNA synthesis (PRINS) is an alternative method which has been used to directly introduce the labelled nucleotides to the specific targets on the chromosomes (Koch *et al.* 1991). The technique is based on the annealing of sequence specific unlabelled oligonucleotide *in situ*, which serves as a primer for *in situ* chain elongation catalysed by *Taq* polymerase. In the presence of labelled nucleotides during the chain elongation the newly synthesised DNA will be labelled. The use of cyclic amplification of the synthesised products *in situ* (cycling PRINS) has also been reported (Gosdon *et al.* 1994). Where the labelled product is too small or the copy number too

low to be readily seen after the single extension reaction, this technique enables clear visualisation of the signal.

Double-stranded or single-stranded DNA can also be labelled with 2-acetylaminofluorene (AAF), mercury or similar reagents by chemical labelling procedures (Verdlov *et al.* 1974; Landegent *et al.* 1984; Hopman *et al.* 1987). The reaction is simple and produces stable probes, however its use has been limited due to the toxicity of the procedure.

1.4.3.2 Ratio-Labeling

The nucleotides modified by different haptens can also be incorporated simultaneously to a nucleic acid probe using nick translation or PCR. This approach termed combinatorial probe labelling can markedly increase the number of distinguishable targets relative to the number of available fluorophore detectors (Ried *et al.* 1992b). Using variable ratios of each hapten can further increase the number of probes which can be detected (Dauwerse *et al.* 1992b). This technique facilitates simultaneous visualisation of multiple targets in metaphase spreads and interphase nuclei from peripheral blood or amniotic fluid samples and can be used for detection of numerical as well as structural chromosome abnormalities.

1.4.3.3 Purification Of Labelled Probes

The probe mixture resulting from a labelling reaction usually contains unincorporated labelled nucleotides that may increase the background. For this reason different methods have been employed to purify the labelled probes before use in hybridisations. A simple method commonly used by different research groups is Sephadex G-50 column filtration. The unincorporated labelled nucleotides in this procedure are trapped in the matrix (sephadex G-50) and the labelled probes, which are excluded from the pores of the matrix, are spun through the column and collected in the eluate. The incorporation of the reporter (biotin or digoxigenin) can be assessed by dot blot analysis (Saiki *et al.* 1987).

1.4.4 Denaturation, Hybridisation And Washing

Nucleic acid hybridisation is the reaction in which single-stranded target sequences re-anneal to complementary probes to form double-stranded hybrid molecules. In general, the target sequences are part of a complex population of heterogeneous species of nucleic acids, and labelled probes must therefore search for and anneal to their complementary targets. DNA probes are usually double-stranded, so they require denaturation to single stranded molecules before hybridisation. This is most simply achieved by heating the hybridisation mixture containing probe to 65-70°C. However denaturation conditions for target DNA are more critical than those for probes, as poor denaturation conditions may lead to insufficient hybridisation or loss of target DNA and morphology. Several approaches have been used to denature target DNA including acids, alkali or formamide treatments and thermal denaturation. Thermal denaturation is usually done in formamide containing ionic buffers. Formamide alters the dielectric constant of a solution and thereby lowers the melting temperature of DNA approximately 0.75°C for each 1% of added formamide (Leitch *et al.*, 1994). This decrease in the temperature required for chromosomal DNA denaturation helps to maintain good morphology of target DNA.

The probe and the target can also be denatured simultaneously under a sealed coverslip by heat. This method is simple and moderately safe as it does not require handling of large amounts of hot formamide. However the procedure may result in poor morphology of the target chromosomes and nuclei due to the high temperature which is used for denaturation.

Once the probe and the target DNA are denatured, the hybridisation mixture containing probe is applied to the slides and incubated under conditions that promote the formation of duplex molecules. The most frequently manipulated variable which can either promote or prevent hybridisation is the temperature. A measure of the stability of hybrids is the melting temperature (T_m), defined as the temperature at which 50% of all hybrids are denatured. The T_m can be calculated by measuring the absorption of ultraviolet light at a wave length of 260 nm. The maximum rate of hybridisation is

typically observed at about 20°C below the calculated T_m in a system. The inclusion of formamide and moderate salt concentrations modify this parameter and permit hybridisation at moderate temperatures. Dextran sulphate is another component of the hybridisation buffer which is used to increase the rate of hybridisation. It is a polyanion of high molecular weight which functions by forming a matrix in the hybridisation mixture and concentrates probe without affecting the stringency. The *in situ* hybridisation shows 10 to 20 percent increased rate when the buffer contains 10 percent dextran sulfate. Other factors which may influence the rate and the stability of *in situ* hybridisation are guanine and cytosine content of the hybrids and probe length, concentration, complexity and complementarity (for review see Farrell *et al.* 1993).

The presence of interspersed repetitive sequences such as LINE and Alu elements within the cloned genomic fragments results in cross-hybridisation of the probes with non-specific sites on the genome. These repetitive sequences can be suppressed by the use of unlabelled competitor DNA, a procedure which has been called chromosomal *in situ* suppression (CISS) hybridisation (Landegent *et al.* 1987; Lichter *et al.* 1988b). The labelled probe in this protocol is denatured together with an excess of unlabelled competitor DNA and allowed to pre-anneal for a given period of time. During preannealing, the repetitive elements within the probe hybridise with the excess repetitive sequences of the competitor DNA, while most of the unique or low copy sequences remain single stranded. As a result double stranded repetitive elements in the preannealed probe can no longer hybridise to their chromosomal targets.

For dual-colour fluorescence *in situ* hybridisation, two differently labelled probes (e.g. with biotin or digoxigenin), are mixed at the correct concentrations into the same probe hybridisation mix. Simultaneous visualisation of more than two targets can be achieved by incorporating more than one label in some probes in different ratios, and hybridisation of differently labelled probes to their specific targets (ratio-labelling FISH). Further some probes can be separately labelled with more than one label and mixed in variable ratios prior to hybridisation (ratio-mixing FISH). The final concentration of each

probe in this procedure should be kept within the optimal range which is normally used for uni-colour experiments. The principles of denaturation and hybridisation of multi-colour FISH is as described for uni-colour experiments.

After hybridisation, excess unhybridised probe must be removed by extensive washing of the slides. The fidelity of the hybrids is assured by washing the slides under conditions that permit retention of actual hybrids only. In general, greater specificity is obtained when hybridisation is performed at a high stringency and washing at similar or lower stringency. The stringency required for optimal signal must be determined empirically. The hybridisation and stringent washing steps are always followed by a blocking step to minimise the background signals. The blocking reagent (human serum, non fat milk or any other blocking reagent) contains antibodies which can bind to cellular and nuclear proteins and suppress them from binding to the antibodies conjugated to the fluorescent dyes during detection steps.

1.4.5 Detection

Probes which are directly labelled with fluorochromes such as fluorescein (FITC) can be visualised immediately after hybridisation without requiring blocking and detection steps. However the probes conjugated with a hapten require to be detected with an appropriate detection system. The method depends on the type of label which has been incorporated into the probe.

Biotin-labelled probes can be detected by anti-biotin antibodies or (strept) avidin conjugated to an enzyme or a fluorochrome. However avidin or streptavidin is more frequently used because of the high binding capacity of the interaction. Avidin is a positively charged glycoprotein found in the egg whites of birds, reptiles and amphibians. This molecule has a high affinity for biotin and the two molecules bind through a strong noncovalent chemical bond. Streptavidin is an alternative for avidin which is an uncharged protein derived from the bacterium *Streptococcus avidini*. The use of streptavidin for detection of biotin labelled probes may result in less background as the

non specific binding will be reduced. However its affinity for biotin is less than that of avidin and it is less stable.

In enzyme mediated systems the avidin molecule is conjugated to an enzyme. The most commonly used enzymes are horseradish peroxidase and alkaline phosphatase. The enzyme-coupled avidin requires the use of substrates which generate a precipitating, coloured product. The most common substrate for horseradish peroxidase is diaminobenzidine (DAB, brown) and for alkaline phosphatase is 5-Bromo-4chloro-3-indolyl phosphate (BCIP, enzyme substrate) and nitro blue tetrazolium (NTB, blue chromogen). The signals produced by this system are stable and can be visualised by a light microscope.

Detection of biotin-labelled probes with fluorochrome-conjugated avidin is the most widely used procedure. Various types of fluorochromes are commercially available that are attached to avidin or antibodies. The common fluorochromes are fluorescein isothiocyanate (FITC, green), Texas red, rhodamine (red) and amino-methyl coumarin acetic acid (AMCA, blue). Biotin-labelled probes can be detected in a single layer incubation with fluorochrome-avidin complex. However the signals can be amplified using biotinylated anti-avidin as a second layer and incubation with a further layer of fluorochrome-avidin conjugate (Fig. 1.8). A modified two layer protocol can also be used by employing avidin-fluorochrome and fluorochrome attached biotinylated anti-avidin. A further modification of this method adds the two components to the slides simultaneously in a single hybridisation experiment.

Digoxigenin labelled probes can be detected in a similar way as biotin using anti-digoxigenin antibodies conjugated to enzymes or fluorochromes. The signals can be amplified using a second antibody raised against the species which produced the anti-digoxigenin and has bound to the same fluorochrome.

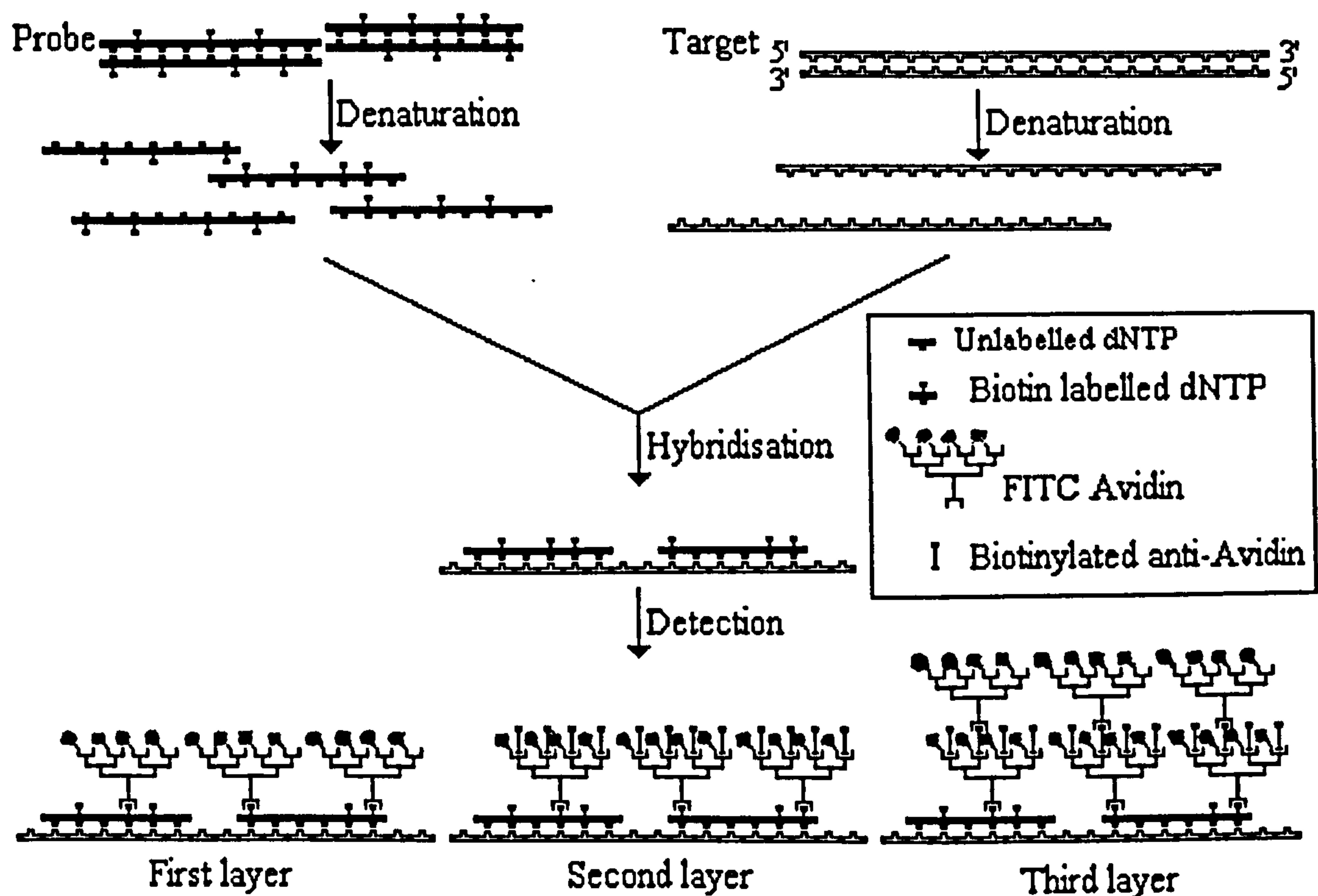


Figure 1.8. Diagrammatic representation of detection of biotin-labelled probes with FITC-avidin/biotinylated anti-avidin system using a three layer approach.

Several other methods for detecting DNA probes by nonradioactive *in situ* hybridisation have also been developed. However the immunocytochemical methods are more commonly used procedures for fluorescence *in situ* hybridisation experiments and the availability of different labels and related detection systems permits simultaneous detection of multiple targets in a single hybridisation area.

1.4.6 Simultaneous Detection Of Multiple Sequences

Multi-colour detection of differently labelled probes requires a combination of two or more detection systems to be used on a single hybridisation area. The procedure relies on the phenomenon that each hapten can be detected with a specific antibody conjugated with one of the available fluorescent dyes. In a dual colour FISH experiment, the detection mixtures contain the specific antibodies for each label differentially conjugated with fluorescent dyes (fig. 1.9). As three sets of distinguishable fluorophores are

available, three separate chromosomal DNA sequences can be delineated simultaneously by combining appropriate fluorophores with three differentially labelled probes (Nederlof *et al.* 1989b).

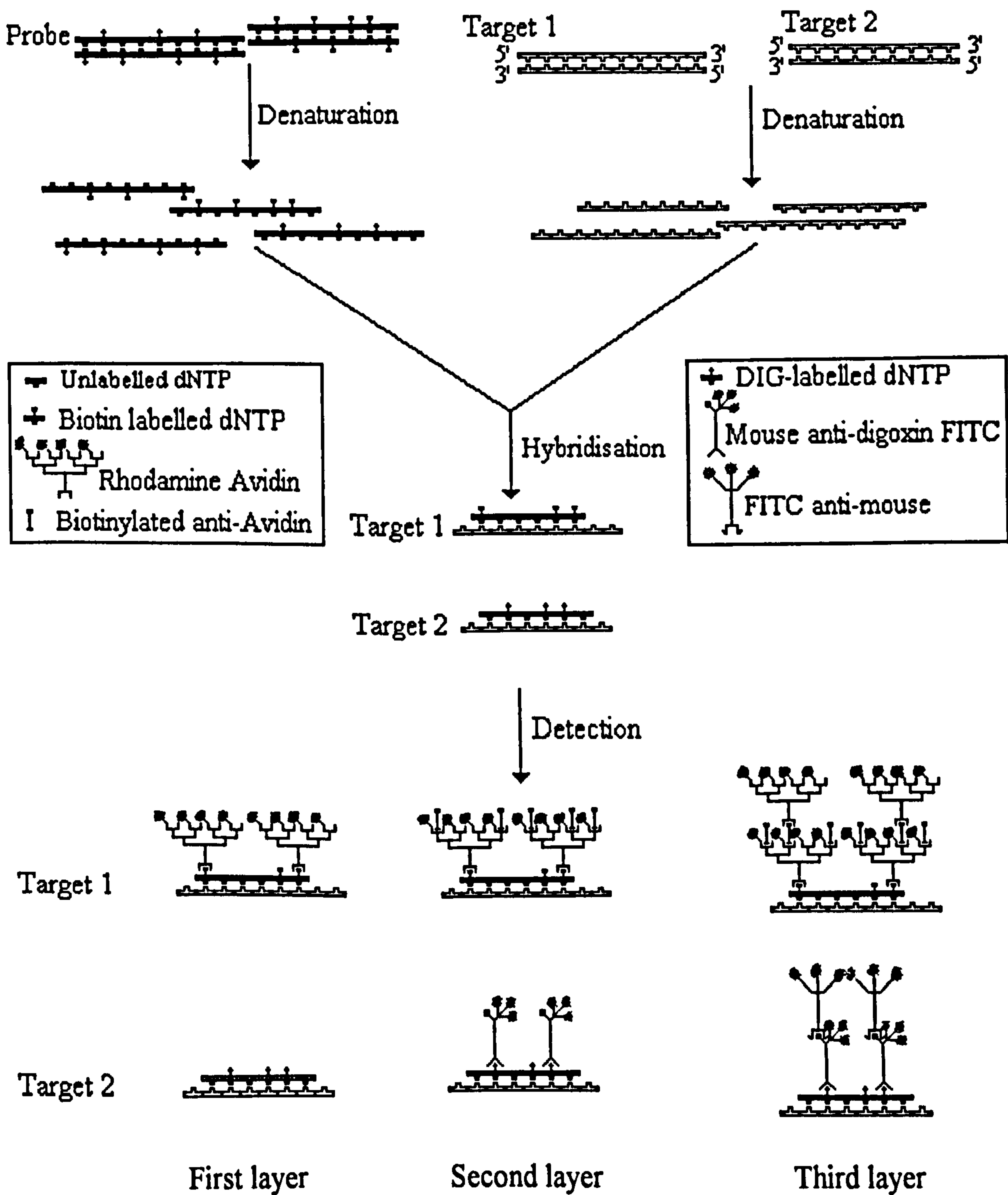


Figure 1.9. Diagramatic illustration of dual-colour detection system using rhodamine and FITC conjugated anti-bodies to detect simultaneously biotin labelled and digoxigenin labelled probes.

The number of sequences which can be simultaneously detected in a single experiment is further increased by detection of ratio-labelled or ratio-mixed probes with a combination of two or more detection systems (fig 1.10). In this method, the sequences hybridised with ratio-labelled or ratio-mixed probes display intermediate coloured signals between the main colours. Five different target sequences can be detected in a single hybridisation area using only two labelling and detection systems. By the use of three labelling and detection systems the number of simultaneously distinguishable signals can be further improved.

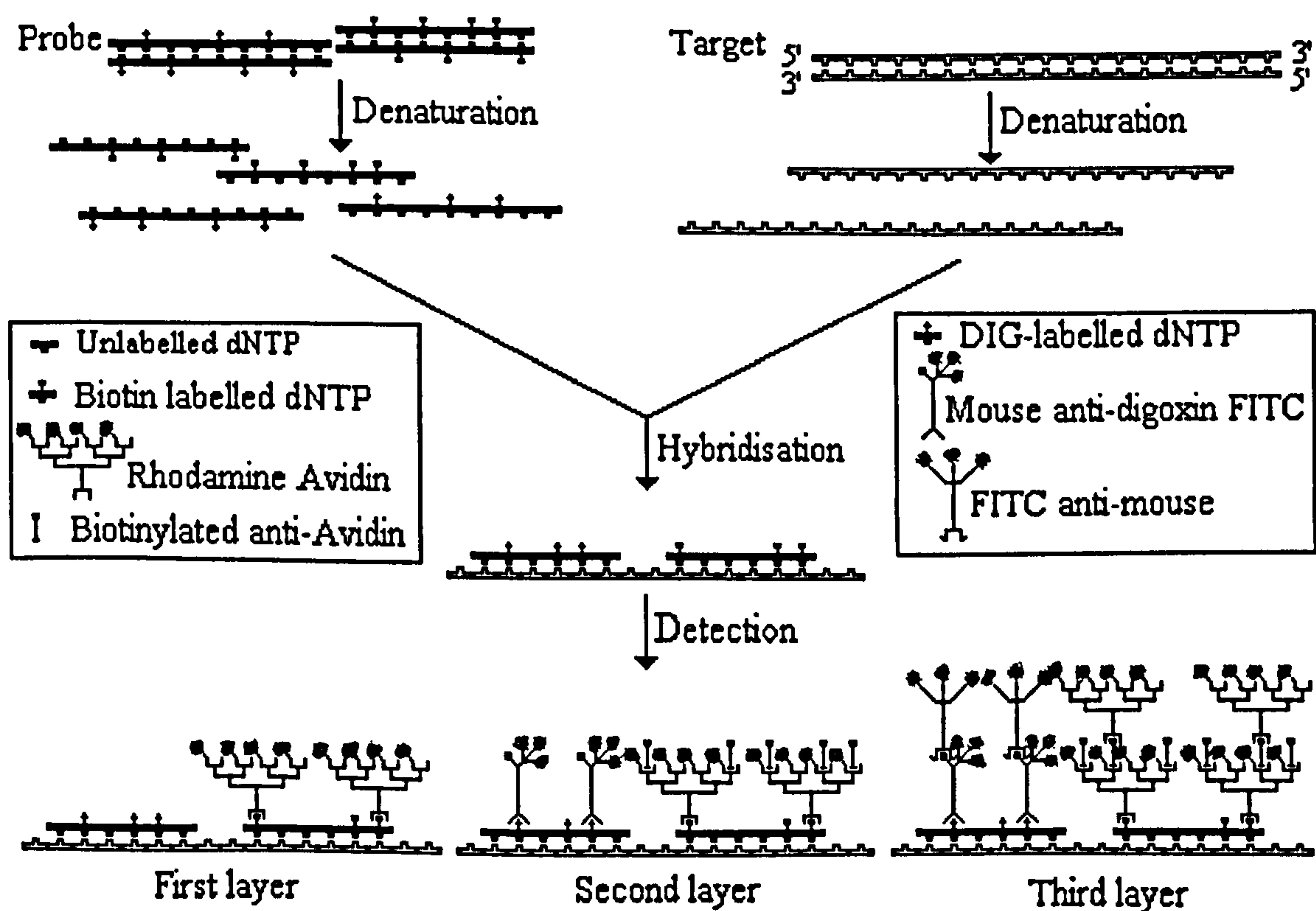


Figure 1.10. Schematic representation of detection of ratio-mixed probes with rodamine conjugated avidin and FITC conjugated mouse anti-digoxin using a three layer approach.

The principle of multicolour detection is almost the same as unicolour detection procedures, however in simultaneous detection systems care must be taken to choose a detection strategy avoiding cross-reacting antibodies.

1.4.7 Counterstaining And Visualisation

Following detection steps the slides must be prepared for visualisation with appropriate systems. In enzyme-mediated detection methods the slides are covered with a conventional mounting reagent and are usually visualised by transmitted light microscopy. However hybridised cells which have been detected with fluorescence attached antibodies are mounted in an antifade medium containing appropriate counterstain. Citifluor is a commercially available antifade medium which preserves signals against rapid fading by fluorescent light.

The use of counterstain facilitates easy visualisation of metaphase spreads and interphase nuclei. DAPI (4',6-diaminido-2-phenylindole) is a blue counterstain which can be used with both green (FITC) and red (rhodamine or texas red) fluorescent dyes. Propidium iodide (red) is the other counterstain which can be used separately or in combination with DAPI to counterstain the cells displaying green signals. Propidium iodide can mask part of the green background on chromosomes or nuclei and increases the quality of preparations. However in Texas red or rhodamine involved detection systems, the cells cannot be counterstained by propidium iodide, as red signals will be obscured by red background colour produced by propidium iodide.

The hybridisation sites detected with fluorochromes are analysed with an epifluorescence microscope equipped with filter sets specific for visualisation of blue (for DAPI), green (for FITC) and red (for propidium iodide, rhodamine and texas red) fluorochromes. Double band-pass filter sets which allow the simultaneous visualisation of fluorochromes, such as FITC and rhodamine are suitable for analysis of dual-colour detected signals.

Digital imaging microscopy can provide a further improvement in signal detection compared to the conventional microscopes. The material under investigation is located and focused by eye and then the image is recorded by a camera and displayed on a monitor. The system has the advantage of image processing which facilitates preparation of quality pictures. Currently, the most sensitive system is the cooled CCD (charged

coupled device) camera. This system allows detection of fluorescence signals which are not visible to the observer's eyes. However the system does not appear to be essential in a diagnostic laboratory. A major use of digital imaging systems is to produce pseudocoloured signals after a ratio-labelling or ratio-mixing fluorescence *in situ* hybridisation approach. This allows the enhancement of differences between intermediate colours which can hardly be distinguished by eye.

1.5 Aims Of The Project

Work began at a time when other workers had shown the potential of FISH in rapid diagnosis of major chromosome aneuploidies on uncultured lymphocytes but there was no consensus on the best probes to use for each chromosome. Alpha satellite DNA probes had been described as suitable probes for interphase analysis. However such probes were not available for chromosomes 13 and 21 as these chromosomes share the same repeat sequences and thus, could not be differentiated using alpha-satellite repeat probes. Furthermore the necessity for FISH to be used as an adjunct to classical cytogenetic analysis was making the analysis of all five chromosomes difficult, as the excess amniotic fluid samples remaining after the sample requirements for cytogenetic analysis were insufficient.

The overall aim of the present study is to develop the technique of ratio-mixing fluorescence *in situ* hybridisation (FISH) for simultaneous visualisation of the chromosomes X, Y, 13, 18 and 21 in uncultured lymphocytes and amniocytes. This would allow rapid diagnosis of clinically important aneuploidies without the need for cell culture. The specific goals are:

- (i) Optimisation of FISH with alphoid DNA repeat probes specific for chromosomes X, Y and 18.
- (ii) Development of reliable probes for chromosomes 13 and 21.

Having achieved the above goals the method of simultaneous detection and visualisation of multiple chromosomes on a single hybridisation experiment will be investigated. This

method has the advantage of a smaller sample requirement than uni-colour FISH. Various strategies of probe labelling, probe mixing and detection are needed to be compared to achieve a reliable and practical way to visualise simultaneously the five clinically important chromosomes in a single hybridisation area. Finally the optimised method will be applied on uncultured amniocytes to demonstrate that it is suitable for prenatal diagnosis. The present study suggests that five colour ratio-mixing FISH has the potential for the rapid diagnosis of major chromosome aneuploidies and provides a more practical approach to minimize the number of probe labelling and detection systems and sample requirement.

2. MATERIALS AND METHODS

1. MEDIA AND SOLUTIONS

1. Luria-Bertani Medium

The following were dissolved in 450 ml of deionised water:

bacto-tryptone	5 g
bacto-yeast extract	2.5 g
NaCl	5 g

The pH was adjusted to 7.0 with concentrated NaOH. The volume was adjusted to 500 ml with deionised water and then sterilised by autoclaving at 121 °C for 20 minutes. The medium was cooled and then an appropriate antibiotic with final concentration of 25 µg/ml was added under sterile conditions and kept at 4°C until used.

2. Terrific-Broth

The following were added to 450 ml of deionised water:

bacto tryptone	6 g
bacto yeast extract	12g
glycerol	2ml

The solution was shaken to dissolve and then was autoclaved at 121°C for 20 minutes. 50 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 and 1 ml of 60 mg/ml kanamycin was added, when the solution was cooled to 60°C or less. The medium was stored at 4°C until used.

3. Isocoves medium

Using an aseptic technique the following were added to 100 ml of Isocoves medium.

Heparin (5 000 units/ml)	1ml
L-glutamine (100X)	1ml
Phytohaemagglutinin	2ml

Penicillin/Streptomycin 1ml

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

4. AHC Medium

The followings were dissolved in 450 ml of deionised water:

yeast nitrogen base w/o 3.35 g

acid hydrolysed casein 5 g

ammonium sulfate 2.5 g

adenine hemisulfate 10 mg

The pH was adjusted to 5.8 and then the volume was adjusted to 475 ml with deionised water. The solution was autoclaved at 121°C for 20 minutes and mixed with 25 ml of sterile 40% w/v D-glucose. The medium were stored at 4°C until used.

5. Sorbitol solution (1M)

91 g of sorbitol were dissolved in distilled water adjusting the volume to 500 ml.

6. Sodium citrate (1M)

29.4 g of sodium citrate was dissolved in 90 ml of distilled water. The pH was adjusted to 5.8 and then the volume was adjusted to 100 ml with distilled water.

7. EDTA (0.1 M)

3.2 g of EDTA was dissolved in 90 ml of distilled water. The pH was adjusted to 8 and then the volume was adjusted to 100 ml with distilled water.

8. SCE solution

The following were mixed under sterile condition:

Sorbitol (1M) 400 ml

Na citrate (1M, pH = 5.8) 50 ml

EDTA (0.1M, pH = 8) 50ml

The solution was autoclaved at 121°C for 20 minutes and stored at 4°C until used. A final concentration of 30 mM β -mercaptoethanol was added to the solution immediately before use.

9. DLS lysis buffer

The following were dissolved in 90 ml of deionised water:

dodecyl lithium sulphate	1 g
sodium chloride	0.29 g

The solution was mixed with 10 ml of 0.1 M Tris-HCl, pH = 7.8 and stored at room temperature until used.

10. 5M Potassium acetate

4.9 g of Potassium acetate was dissolved in 10 ml of deionised water. The solution was filtered and then stored at room temperature.

11. T.E. buffer

10 mM Tris-HCl was mixed with 1mM EDTA, pH = 8.6. The obtained solution was filter sterilised and stored at room temperature.

12. T. B. E. Buffer (10X)

To make 1 liter of (10X) T. B. E. buffer:

Tris base	108 g
boric acid	55 g
EDTA	9.3 g

was dissolved in 800 ml of dH₂O. The pH was adjusted to 8.1-8.2 using solid boric acid or NaOH. The volume was adjusted to 1 liter by dH₂O and stored at room temperature.

13. Agrose gel (1.3%)

0.65 g of agarose was dissolved in 50ml of T.B.E buffer and heated in a microwave oven for 3 minutes. The solution was cooled to 60°C and then 2 μ l of

10mg/ml ethidium bromide was added. The gel was then immediately poured in to the electrophoresis gel casting unit.

14. Loading mix

To prepare 100 ml of gel loading buffer:

bromophenol blue	0.25 mg
Ficoll (type 400)	25mg

were dissolved in 100 ml of distilled water and stored at room temperature.

15. DNAase free RNAase A (100µg/ml)

80µl of concentrated stock (25 mg/ml) were diluted in 20 ml of 2×SSC. The diluted stock was boiled on a hot plate for 10 minutes to destroy any contaminating DNAase and dispensed into volumes of 1 ml and stored at -20°C until used.

16. Salmon Sperm DNA (20 mg/ml)

200 mg salmon sperm DNA was dissolved in 10 ml of distilled water and incubated in a 70°C waterbath. The solution was then sheered by repeatedly passing through a needle (steril 21G11/2 0,8×40 Nr.2T.W.P.M.) attached to a 10 ml syringe. The tube was placed on ice and sonicated for 3×4 minutes at 4 volts. The fragment size of sonicated DNA was checked by gel electrophoresis in 0.8% agarose gel and stored at 4°C.

17. Hybridisation buffer

2 g of Dextran sulfate was dissolved in 4ml of deionised water and then the following were added:

Fluka formamide	10 ml
20X SSC	2 ml
Salmon sperm DNA (10mg/ml)	200 µl

The contents were mixed very well, aliquoted in 1ml volumes in ependorf tubes and stored at -20°C until used.

18. 20X SSC

Tri-sodium citrate	44.1 g
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Sodium chloride	87.6 g
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The volume was adjusted to 500ml with distilled water and stored at room temperature.

19. 4X SSC, Tween-20

The volume of 100 ml of 20X SSC was adjusted to 500 ml by dH₂O, then 250µl of tween-20 was added. The solution was mixed and stored at room temperature.

20. Wash A solution

20X SSC	100 ml
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Tween-20	250 µl
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dried skimmed milk	25g
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The volume was adjusted to 500 ml with dH₂O, mixed on low heat until dissolved and spun in 50 ml volumes at 2000 rpm. The solution was freshly prepared for every experiment.

21. DAPI (4,6-Diamidino-2-Phenyl-Indole) working solution (40 µg/ml)

1µl of DAPI (1 mg / ml) was diluted in 24µl of distilled water, mixed and stored in dark at 4 °C until used.

22. Propidium iodide working solution (20 µg /ml)

2 µl of propidium iodide (10 mg/ml) was diluted in 988 µl of distilled water, mixed and stored in dark at 4 °C.

23. CIRCLEPREP® Kit (BIO 101; Cat No. CP-100),

Description:

Pre-lysis buffer (Tris/EDTA/glucose solution)

Neutralizing solution (3M Potassium acetate)
 Lithium Chloride solution (near saturated solution)
 CIRCLEPREP GLASSMILK
 Binding buffer (KBr/NaI/Tris mixture)
 Wash solution
 Sieve material

24. Insta-Mini-Prep™ Kit (5prime→3prime Inc., Cat. No. p1-678901)

Component

Insta-Mini-Prep™ Tubes	50 Each
PCI (50 Phenol : 49 chloroform : 1 Isoamyl Alcohol)	25 ml
CI (49 chlorophorm : 1 Isoamyl Alcohol)	15 ml
TE, pH 8 (10 mM Tris-Cl, 1 mM EDTA, pH 8)	5 ml
10X RNase Plus™ Gel loading Buffe	110µl

25. BRL Nick Translation Kit (GIBCO BRL, Cat. No. 8160SB)

Description:

Solution A4 (0.2mM nucleotides C, G and A in 500 mM Tris-HCl (pH 7.8) 50mM Magnesium Chloride).

Solution C (0.4 units/µl DNA polymerase I, 40 pg/µl DNA Pol I/DNase I, 50mM Tris-HCl, pH 7.5), 5 mM Magnesium acetate, mM 2-mercaptoethanol, 0.1 mM phenylmethysulphonyl fluoride, 50% glycerol and 100 µg/µl bovine serum albumin).

Solution D (300mM disodium EDTA, pH 8).

Solution E dH2O

26. Digoxigenin DNA labelling Kit (Boehringer Mannheim Biochemica; Cat. No. 1175033)

Description:

Unlabelled control-DNA 1 (One vial with 20 µl pBR328 DNA, 100 µg/ml)
Unlabelled control-DNA 2 (One vial with 20 µl pBR328, 200 µg/ml, linearised with Bam HI)
DNA dilution buffer (Two vials with 1ml of 50 µg/ml herring sperm DNA in 10 mmol/l Tris-HCl and 1mmol/l EDTA, pH 8.0 each)
Labelled control DNA (One vial with 50 µl linearised pBR328 DNA, labelled with digoxigenin)
Hexanucleotide Mixture (One vial with 80 µl 10X concentrated hexanucleotide reaction mixture)
dNTP Labelling Mixture (One vial with 80 µl of 10X concentrated dNTP labelling mixture containing dATP, 1mmol/l; dCTP, 1mMol/l; dGTP, 1mMol/l; dTTP, 0.65 mmol/l; Dig-dUTP, 0.35 mMol/l; pH 7.5)
Klenow Enzyme, labelling grade (One vial with 40 µl klenow enzyme labelling grade, 2units/µl)

2. METHODS

2.1 Environmental Conditions And Safety

The methods used in the present study included handling of transformed microbial host cells and also human cells from peripheral blood or amniotic fluid. As many microorganisms grow more rapidly than human cells in tissue culture, and may produce toxins that are harmful to the cultured cells, care had to be taken to avoid any contamination. Culture of bacterial and yeast cells was carried out in a containment lab equipped for this purpose. To isolate the DNA, the host cells were lysed in the same lab and then were transferred to the *in situ* lab for further treatment and purification. Disposable culture flasks, universals, petri dishes and pipettes used for culturing and DNA isolation, were placed in an autoclavable disposal bag (sterilin) to be sent for sterilisation.

The human cells were cultured and harvested in a tissue culture lab and a biological safety cabinet was used when harvesting the human peripheral blood cells. To minimize the risk of contamination, a laboratory coat, gloves and other protectives were worn at all times during the experiments and all the tips and eppendorf tubes were sterilised before use.

Many chemicals used for *in situ* hybridisation are dangerous and include toxic, allergenic, carcinogenic and teratogenic substances. Procedures involving these materials, especially those that were heated (for example formamide) were carried out in a fume hood.

2.2 Preparation Of Samples

The cultured and uncultured lymphocytes were mounted on clean microscope slides. To clean the slides they were soaked in Decon overnight and then rinsed in running tap water for 2-5 hours. The cleaned slides were stored in 70 % ethanol until used. To remove the alcohol, slides were rinsed in running tap water prior to cell preparation and mounting.

The uncultured amniocytes were mounted on glass microscope slides coated by 3-aminopropyltriethoxysilane (APES).

2.2.1 Preparation Of “APES” Coated Slides

APES coated slides was prepared according to the method described by Klinger et al. (1992).

1. Precleaned and dry slides were immersed in 100% ethanol for 10 minutes.
2. Slides were then immersed in distilled water for 10 minutes and dried.
3. A fresh solution of 2% (V/V) APES in acetone was prepared and the slides were then submerged for 2 minutes in this solution.
4. The covered slides were immersed in distilled water for 5 minutes, air dried and stored in a dust free place at room temperature.

2.2.2 Peripheral Blood Cultures

Chromosome elongation method was used to prepare metaphase spreads from human peripheral blood:

1. Using an aseptic technique 1ml of heparinised whole blood was added to 9ml of Isocoves medium in a culture tube and incubated at 37°C for 48 hours.
2. 0.1ml of thymidine solution (30 mg/ml) was added to each 10ml culture and the incubation was continued for a further 18 hours at 37°C.
3. 0.1 ml of 2-deoxycytidine (1M) was added and incubated for 3 hours and 55 minutes at 37°C.

2.2.3 Harvesting Of Blood Cultures And Slide Preparation

1. 0.1ml of colcemide (10µg/ml) was added, mixed by gently shaking the tube and incubated for an additional 20 minutes.
2. The tubes were centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded leaving approximately 1ml and the pellet was resuspended by gently tapping the tube.
3. 9ml of fresh fixative (3:1 methanol acetic acid) was added. The first 0.5ml was added drop by drop and mixed by gently tapping the tube.
4. The cell suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded leaving about 1ml and the pellet was resuspended as described above.
5. The steps 3 and 4 were repeated. The pellet was resuspended in 5ml of fresh fixative and stored at 4°C for a minimum of 30 minutes.
6. The suspension was centrifuged at 1000 rpm for 10 minutes, the supernatant was pipetted off and then the pellet was resuspended in .05-1ml of fresh fixative.
7. 3-4 drops of the cell suspension were dropped onto a precleaned microscope slide and dried on a hot plate. The slides were stored in a dust free place at room temperature.

2.2.4 Preparation Of Uncultured Lymphocytes

1. 10ml of hypotonic solution (0.0375 M KCl) prewarmed to 37°C, was added to 1ml heparinised whole blood and incubated at 37°C for 5 minutes.
2. The cells were precipitated by spinning at 1200 rpm for 8 minutes.
3. The supernatant was discarded, leaving a small volume (about 1ml) and the pellet was thoroughly resuspended by gently tapping the tube.
4. The cells were fixed using 9ml of cold and fresh fixative (3:1 methanol:acetic acid). The first 0.5ml was added drop by drop.
5. The tubes were spun at 1200 rpm for 8 minutes.
6. The steps 3, 4 and 5 were repeated until the solution was clear.
7. The fixed cells were dropped onto a precleaned microscope slide and allowed to dry.
8. The slides were then dehydrated through an ethanol series (50%, 70%, 90% and 100%), air dried and aged by storing at room temperature overnight.
9. For long term storage the slides were transferred to -20°C.

2.2.5 Preparation Of Uncultured Amniocytes

The uncultured amniocytes were prepared according to the method described by Klinger et al. (1992) with small modifications.

1. Slides were preheated at 37°C on a hot plate.
2. 1-2ml of fresh amniotic fluid was spun at 1200 rpm for 10 minutes.
3. The fluid was removed and replaced with PBS prewarmed at 37°C.
4. The resuspended cells (35µl vol/slide) were transferred on to the prewarmed slides coated by APES.
5. To make the PBS solution hypotonic (150mM → 50mM) two volumes of distilled water (70 µl) prewarmed at 37°C was added.
6. The slides were then incubated at 37°C for 15 minutes (The evaporation of water was compensated by adding 10 µl/slide at 5 minutes intervals).

7. The hypotonic solution was carefully decanted and replaced by 100 µl of 30% fixative (3:1 methanol : acetic acid) in 75 mM KCL, for 5 minutes at room temperature.
8. The solution was carefully decanted and fresh 3:1 fixative was dropped on to slides from a height of 50-60 cm.
9. Excess fix was decanted and slides were dried for 5 minutes at 60°C.
10. The slides were dehydrated through alcohol series (50%, 70%, 90% and 100%), air dried and Stored at -20°C until used.

2.3 Probes

The probes used in this study consisted of alpha satellite DNA repeats specific for the centromeric regions of chromosomes X, Y and 18 respectively, two YAC clones specific for chromosome 21, a combination of three cosmids and also a YAC clone specific for chromosome 13.

- ◆ The commercial alpha satellite DNA probe DXZ1 (Oncor), is a 2.0 kb *Bam* H1 fragment which hybridises to highly repeated alphoid DNA located at the centromere of chromosome X (Waye and Willard, 1985).
- ◆ The alpha satellite DNA probe pDP97 is a subclone derived from cosmid Y97 (Wolfe et al. 1985). At high stringency it detects a repeated Y-specific EcoR1 fragment of 5.5 kb (DYZ3), a marker for the centromere of the Y chromosome.
- ◆ Probe L1.84 is a human DNA fragment cloned in the plasmid pAT153 which hybridises to the centromeric region of chromosome 18. It is 684bp long and represents a variant of the human alphoid DNA repeat with an estimated copy number of about 2 000 per haploid genome (Devilee et al. 1986).
- ◆ Three cosmids 29G3, 121F8 and 167H1 which were kindly provided by Dr. I. S. Edelman, (Colombia University/Bio.Chemistry, 630 West 168th Street, New York, NY USA 10040), were used individually or in combination to detect the copy

number of chromosome 13. The cosmids extend across most of the length of CEPH YAC 744D11 and maps to 13q12.3 (Fischer *et al.* 1994). The YAC 744D11 was subsequently received from Dr. Edelman, I. S. and was compared to cosmid probes for intensity and specificity. The chromosome 13 specific sequences carried by 744D11 are 0.9 Mb long and are located in 13q12.3 (Fischer *et al.* 1994; Warburton *et al.* 1993).

- ◆ YACs 745H11 and 831B9 were kindly provided by Dr. Denis Le Paslier (27, rue juleitte Dodu, F-75010 Paris-France) and were used as chromosome 21 specific probes in interphase FISH experiments. YAC 745H11 carries DNA sequences from 21q22.2, while sequences carried by YAC 831B9 are located in 21q22.1-q22.2 (Chumakov *et al.* 1992; Soeda *et al.* 1995).

Biotin-labelled and Digoxigenin-labelled DXZ1 were commercially available (Oncor). Probes pDP97, L1.84 and cosmids were amplified by growing in appropriate culture media and used for DNA isolation. The yeast cells containing YAC were cultured in a selective medium (AHC) and the DNA was isolated and used for Alu-PCR amplification to selectively amplify the human insert within the YACs.

2.4 Preparation Of Probes

All host cells containing the recombinant vector were cultured in an appropriate culture medium and then the amplified cells were used for DNA isolation.

2.4.1 Growing Of Transformed Bacterial And Yeast Host Cells

The bacterial cells containing the probes pDP97 and L1.84 were initially cultured on agar plates containing L-broth and 25-50 µg/ml ampicillin. A single colony was then selected to be cultured in 100 ml of L-broth at 37°C overnight in a shaker incubator. The overnight culture was then used for DNA isolation.

The bacterial host cells containing cosmids were received as stab cultures which were transferred into 100 ml of T-broth containing 30 µg/ml kanamycin and cultured in a

shaking incubator at 37°C overnight. The amplified cells were used either for DNA isolation or for colony selection as described for probes pDP97 or L1.84.

The yeast cells containing YAC were cultured in 20 ml of AHC medium at 30°C until saturation. The amplified cells were then used for DNA isolation. Culture of all host cells were carried out in a shaker incubator.

2.4.2 Isolation Of Plasmid And Cosmid DNA Using CIRCLEPREP Kit

Isolation of plasmid and cosmid DNA were carried out using CIRCLEPREP kit. The method of DNA isolation was according to supplier's protocol with small modifications.

1. The overnight culture was transferred to a 50ml plastic conical tube and centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded and the tube was air dried.
2. The pellet was resuspended with 4ml of pre-lysis buffer and mixed until a homogeneous suspension was obtained.
3. 4ml of alkaline lysis reagent was then added to the suspension and mixed until all the cells were uniformly lysed.
4. 4ml of neutralising solution was added and mixed until the contents became essentially two phases, consisting of a white precipitate suspended in a clear liquid. The mixture was centrifuged at 12 000 rpm for 5 minutes and the supernatant was transferred through the sieve material provided with the kit to a new conical tube.
5. The filtered supernatant was mixed with an equal volume of isopropanol (approximately 12ml) until a fine precipitate was formed. The suspension was centrifuged at 12 000 rpm for 5 minutes, the supernatant was discarded and the residual liquid was removed by inverting the tube and wiping inside the walls with a tissue.
6. The pellet was dissolved in 0.5ml of sterile distilled water and transferred to a microcentrifuge tube. As the resulting solution consisted of RNA, plasmid DNA and some cellular chromosomal DNA, further purification was necessary.

7. The linear cellular DNA was denatured by placing the tube on a hot plate which allowed the solution to boil for 3-5 minutes. To avoid evaporation, the lid of tube was vented before placing on the hot plate. The tube was immediately cooled on ice for at least 1 minute and then 300 μ l of LiCl was added to precipitate the single stranded DNA and ribosomal RNA. The suspension was mixed and allowed to sit at room temperature for 5 minutes. The mixture was centrifuged for 2 minutes and the supernatant was transferred to a new tube.
8. 600-700 μ l of isopropanol was added and the suspension was centrifuged for 2 minutes. The supernatant was discarded and the drained pellet was dissolved in 0.5ml of filtered distilled water. 300 μ l of LiCl was again added, mixed and allowed to stand at room temperature for 5 minutes.
9. 75 μ l of Circleprep Glassmilk (vortexed before use) was added to the LiCl solution and incubated at room temperature for 5 minutes with occasional mixing. The mixture was centrifuged for 2 minutes to precipitate the Glassmilk/DNA complex and then the supernatant was transferred to a new microcentrifuge tube which could be used for more DNA isolation if required.
10. The pellet was washed 2-3 times with 1ml of Binding buffer which selectively maintains binding of DNA (but not RNA and proteins) to Circleprep Glassmilk. The last Binding buffer was removed with a small micropipette tip.
11. The pellet was washed twice with wash solution and vacuum dried. The plasmid or cosmid DNA was then eluted from the Glassmilk by resuspending the pellet in 100-300 μ l of filtered distilled water. The suspension was mixed and incubated in a 65°C waterbath for 5 minutes. The tube was centrifuged for 2 minutes and the supernatant was transferred into a new microcentrifuge tube.
12. The resulting solution which contained plasmid or cosmid DNA was incubated in a 37°C waterbath for 30 minutes and then stored at -20°C until used.

2.4.3 Rapid Isolation Of Plasmid DNA Using Insta-Mini-Prep Kit

Rapid isolation of plasmid DNA was achieved using Insta-Mini-Prep Kit according to the protocol provided by supplier:

1. The Insta-Mini-Prep gel was precipitated by spinning the unopened Insta-Mini-Prep tube at 12 000 rpm for 10 seconds.
2. 1.5 ml of the bacterial culture was transferred to a 1.5 ml eppendorf tube and centrifuged at 12 000 rpm for 30 seconds.
3. The supernatant was carefully decanted and the pellet was resuspended in 50 µl of TE buffer.
4. 300 µl of shaken PCI (Phenol, chloroform and Isoamyl Alcohol solution) was added to the bacterial cell suspension under a fume hood and mixed by repeated inversion.
5. The contents of the eppendorf tube were transferred to the pre-spun Insta-Mini-Prep tube using a micropipette.
6. The tube containing the bacterial lysate was centrifuged at 12 000 rpm for 30 seconds.
7. 300 µl of CI (chloroform and Isoamyl Alcohol solution) was added to the tube and the two liquid upper phases in the tube were mixed by repeated inversion.
8. The tube was centrifuged at 12 000 rpm for 30 seconds and then the isolated plasmid DNA was transferred to a fresh microcentrifuge tube.

2.4.4. Isolation of yeast genomic DNA containing YAC

The isolation of yeast genomic DNA was basically according to Sherman et al. (1986), with following modifications:

1. The amplified yeast cells containing YAC were transferred into a plastic conical tube and centrifuged at 10 000 rpm for 5 minutes.
2. The supernatant was discarded and the pellet was drained and resuspended in 1 ml of SCE solution. The cell suspension was then transferred into a 1.5 ml microcentrifuge tube.

3. The cells were then lysed by adding 10 μ l of zymolyase (100 mg/ml) and incubated in a 37°C waterbath for 1 hour.
4. The mixture was centrifuged at 12 000 rpm for 1 minute. The supernatant was discarded and the pellet resuspended in 0.5 ml of DLS (dodecyl lithium sulfate) lysis buffer. The suspension was mixed well and incubated at 65°C for 30-60 minutes.
5. 200 μ l of 5M potassium acetate was added and then the tube was placed on ice for 60 minutes.
6. The mixture was centrifuged at 12 000 rpm for 5 minutes. The supernatant was transferred to a fresh microcentrifuge tube and then an equal volume of isopropanol was added. The mixture was allowed to sit at room temperature for 5 minutes.
7. The tube was centrifuged at 12 000 for 10 seconds, the supernatant was discarded and the pellet was air dried (chromosomal DNA precipitation).
8. The pellet was resuspended in 300 μ l of T.E buffer, and then 15 μ l of RNase-A (1 mg/ml) was added and incubated in a 37°C waterbath for 1 hour.
9. 30 μ l of 3M sodium acetate and 200 μ l of isopropanol were added to the suspension, mixed and the tube briefly spun to precipitate the yeast genomic DNA containing YAC.
10. The supernatant was discarded and the pellet was vacuum dried and resuspend in 100- 300 μ l of filtered distilled water. The DNA solution was incubated in a 37°C waterbath for 30 minutes and then stored at -20°C until used.

2.4.5 Estimation Of DNA Concentration

10 μ l of purified DNA was added to 990 μ l of deionised water in 1.5ml Eppendorf tube. The suspension was transferred into a quartz tube and the optical density was measured at 260nm in a UV/VIS spectrophotometer (PU 8820 - Philips UK). As one OD₂₆₀ unit corresponds to 50 μ g of DNA and the DNA was diluted by a factor of 100, the OD₂₆₀ reading of the DNA was multiplied by 5000 to give the concentration in μ g/ml.

2.4.6 Alu-PCR Amplification Of The Human Inserts Within YAC Clones

The inter-Alu sequences in human insert were amplified using yeast genomic DNA containing YAC as a template and primers complementary to the 3' and 5' ends of Alu sequences. The procedure allowed selective amplification of the human insert within the YACs. The amplified DNA was precipitated and used as a probe after labelling by nick translation.

I. Oligonucleotide Synthesis

Two Alu primers: BK-33 (5'-CTGGGATTACAGGCGTGAGC-3') priming to the 5' end of the Alu-sequences (nt positions 15-34), and SR1 (5'-CCACTGCACTCCAGCCTGGG-3') close to the 3' end (nt positions 241-261) (Romana et al. 1993) were used to selectively amplify the human inserts within the YAC clones. The primers were prepared by a "391 DNA synthesizer PCR-MATE" (Applied Biosystems) and extracted from the column as follows:

1. 1ml of ammonium hydroxide was drawn into a polypropylene (blue) syringe and connected to one end of the column.
2. An empty syringe was attached to the other end of column.
3. The ammonium hydroxide was gently pushed through the column, displacing the barrel of the second syringe. This was repeated 2-3 times periodically for 2-3 hours until the colour of the column was changed from yellow to white.
4. The ammonium hydroxide was withdrawn and expelled into a small Nunc (round-bottomed, screw cap) tube and the volume was adjusted to 2.5 ml.
5. The tube was incubated at 55°C overnight and then stored at -20°C.

II. Ethanol Precipitation Of Oligonucleotide

1. 0.45ml of oligonucleotide in ammonium hydroxide was aliquoted in to 1.5ml eppendorf tubes.
2. 50µl of 3M sodium acetate and two volumes of filtered ethanol were added.

3. The mixture was maintained at -70°C for 1.5-2 hours
4. The tube was centrifuged at 12 000 rpm for 10 minutes
5. The supernatant was discarded, the pellet was washed with 85% ethanol and air dried.
6. The drained pellet was resuspended in 200µl filtered distilled water and then the O.D. was measured at 260nm.
7. Finally the oligonucleotide solution was diluted to 5µM concentration and stored at 4°C.

III. Alu-PCR Method

The PCR assay was performed as described by Lengauer et al. (1992) with small modifications. 100 ng of purified total genomic yeast DNA (including YAC) was used as template. The two primers were each used at a concentration of 0.25 µM in a total volume of 50µl PCR buffer containing 250 µM of each of the four dNTPs, and 2.5 units of Taq polymerase. Distilled water, dNTPs, primers and the PCR buffer were added sequentially to a small eppendorf tube and the tube was placed under the UV light to be sterilised for 10 minutes. The yeast genomic DNA was then added and the mixture was covered by mineral oil and placed in the PCR machine (Hybaid-OmniGene). After an initial denaturation at 96°C for 5 min the Taq polymerase was added and then the reaction was continued with 30 cycles of PCR with denaturation at 96°C for 1 min, annealing at 37°C for 30s and extension at 72°C for 6 min. A 10 min extension was performed at the end of the last cycle. When the PCR reaction was completed, the PCR product was separated from the mineral oil, transferred to a new 1.5 ml eppendorf tube and then examined by gel electrophoresis.

IV. Gel Electrophoresis Of PCR Products

Ten-microliter aliquots of amplified DNA sequences and 5-6µl of 1kb ladder were individually mixed with 2µl of loading mixture and loaded on a 1.3% agarose gel at 100

mA for 1 hour. When the DNA fragments were fractionated, the bands were visualised using a UV light (UVP Image store 5000 ultraviolet products) coupled with a black and white camera and monitor. The images were printed by a sony video graphic printer (UP-860CE).

V. Ethanol Precipitation Of PCR Products

The PCR product was mixed with 1/10 volume of 3M sodium acetate, two volumes of cooled ethanol and 1µl of glycogen and then maintained at -20°C for 30 minutes. The suspension was then centrifuged at 12 000 rpm for 30 minutes. The supernatant was discarded, the pellet was vacuum desiccated and dissolved in 12µl of filtered distilled water. The concentration of DNA yield was calculated by measuring the optical density at 260nm and stored at -20°C until used.

2.4.7 Biotin-labelling by Nick Translation

Biotin-labelling of the probes was carried out by nick-translation Kit according to the procedure provided by the supplier with small modifications.

1. The followings were sequentially added to a 1.5ml eppendorf tube:

Solution A4	5µl
DNA to be labelled	1µg
Biotin-11-dUT	2.5µl

The volume was adjusted to 45µl by filtered distilled water, then 5µl of solution C was added and the contents were mixed well and incubated at 15°C for 90 minutes

2. The reaction was stopped by adding 5µl of solution D and then the following were added in order to precipitate the labelled DNA:

3M sodium acetate (pH, 5.2)	4.6µl
Glycogen (20 mg/ml)	1µl

Iced ethanol 122µl

The suspension was mixed and briefly spun.

3. The supernatant was discarded and the pellet was vacuum desiccated and dissolved in 10 µl of T.E buffer to give a concentration of 100 ng/µl.
4. The DNA solution was incubated in a 37°C waterbath for 30 min and then stored at -20°C.

2.4.8 Digoxigenin-Labelling By Nick Translation

Digoxigenin labelling of probes was carried out using digoxigenin DNA labelling mixture and nick translation kit. To obtain 1µg of labelled DNA the following were added sequentially:

Probe DNA	1µg
Labelled dNTPs	2µl

the volume was adjusted to 25µl with filtered distilled water and then 5µl of enzyme mixture (solution C, Nick Translation kit) was added. The contents were mixed and briefly spun and then incubated at 15°C for 90 minutes. The subsequent stages for digoxigenin labelling were followed as described for biotin-labelling, using nick translation kit.

2.4.9. Random Primed DNA Labelling With Digoxigenin-dUTP

Random primed DNA labelling was carried out using digoxigenin DNA labelling kit (Boehringer Mannheim Biochemica) according to supplier's protocol with the following modifications.

1. 1µg of probe DNA was added to a 1.5ml eppendorf tube and denatured at 100°C for 10 minutes on a hot plate. The tube was transferred immediately on dried ice to prevent reannealing of denatured DNA.

The following was then added:

Hexanucleotide mixture (vial 5)	2µl
dNTP mixture (vial 6)	2µl

2. The volume was adjusted to 19µl with filtered distilled water and 1µl of klenow enzyme was then added. The contents were mixed and briefly spun and incubated in a 37°C waterbath for at least 1 hour. Longer incubation (up to 20h) can increase the yield of labelled DNA.
3. The reaction was stopped by 2µl of EDTA solution (0.2 mol/l; pH 8.0).
4. The labelled DNA was then precipitated by adding 4.6µl of 3M sodium acetate, 1µl glycogen and 75µl of precooled filtered ethanol.
5. The suspension was mixed and briefly spun and then incubated at -20°C for at least 30 minutes.
6. The tube was centrifuged at 12 000 rpm for 30 minutes. The supernatant was discarded and the pellet was vacuum desiccated and dissolved in 10 µl of T.E buffer to obtain the concentration of 100ng/µl..
7. The labelled DNA was stored at -20°C until used.

2.5 Uni-Colour Fluorescence *In Situ* Hybridisation

Uni-colour fluorescence *in situ* hybridisation was used to detect single targets in uncultured lymphocytes and amniocytes. Both the biotin labelled and digoxigenin labelled probes were used in this series of experiments. The experimental procedure was the same for both biotin labelled and digoxigenin labelled probes except for detection where two different detection systems were employed. The biotin labelled probes were detected with either fluorescein conjugated or rhodamine conjugated avidin and the digoxigenin labelled probes were detected with fluorescein conjugated anti-digoxigenin or monoclonal anti-digoxin labelled with fluorescein.

2.5.1 Pretreatment Of Slides

Before denaturation the fixed material on microscope slides was examined under a phase contrast microscope and the desired area on each slide was marked. The

preparations were then fixed in a fresh fixative (3:1 methanol:acetic acid) for 1 hour. The slides were dried and then immersed in acetone for 10 minutes. After air drying the slides, peripheral blood preparations were dehydrated through an ethanol series and then directly used for denaturation and hybridisation. However for uncultured amniocytes the pretreatments were continued by adding 100 µl of RNase-A (100 µl/ml) on each hybridisation area. The area was covered by a coverslip and incubated in a 37°C waterbath for 30-40 minutes. The coverslips were removed in 2XSSC and then the slides were dehydrated through an ethanol series and used for denaturation.

2.5.2 Preparation Of Hybridisation Mixture For Uni-Colour FISH

Hybridisation mixtures were prepared using desired concentrations of labelled probes and hybridisation buffer immediately before use. The concentration of a given probe could be varied depending on the labelling efficiency. In chromosome *in situ* suppression hybridisation experiments the probe DNA was preannealed with relevant concentration of human placental DNA to suppress the repetitive sequences on both probes and targets. The concentrations that were commonly used to prepare hybridisation mixtures of biotin-labelled alpha satellite DNA probes, are given in table 2.1.

<i>Biotin-labelled probe</i>	<i>Probe DNA (µl)</i>	<i>Human placental DNA (µg)</i>	<i>Hybridisation buffer (µl)</i>	<i>Total volume (µl)</i>
DXZ1 (1ng/µl)	0.5	—	11.5	12
pDP97(100ng/µl)	0.5	—	11.5	12
L1.84 (10ng/µl)	0.5	—	11.5	12

Table 2.1. Preparation of hybridisation mixture using alpha satellite DNA probes in uni-colour fluorescence *in situ* hybridisation experiments.

The concentration of digoxigenin labelled DXZ1 used for detection of the copy number of chromosome X was the same as used for biotin labelled DXZ1 (0.5ng for each hybridisation area). However a higher concentration of digoxigenin labelled pDP97 and

L1.84 were used (80-100 ng and 7-10 ng respectively) to detect the copy number of chromosomes Y and 18.

To allow the hybridisation of individual biotin labelled cosmids to chromosome 13, the hybridisation mixtures were prepared according to table 2.2.

<i>Biotin-labelled cosmid probe</i>	<i>Probe DNA (μl)</i>	<i>Human placental DNA (μl)</i>	<i>Hybridisation buffer (μl)</i>	<i>Total volume (μl)</i>
29 G3 (100ng/μl)	1	0.6	10.4	12
121 F8 (100ng/μl)	1	0.5	10.5	12
167 H1 (100ng/μl)	1	0.5	10.5	12

Table 2.2. Preparation of hybridisation mixture using cosmid probes specific for chromosome 13 in uni-colour fluorescence *in situ* hybridisation.

However a combination of cosmids 121 F8 and 167 H1 and a contig of all three cosmids were used to detect the copy number of chromosome 13. For combination of two cosmids 80ng of each 121 F8 and 167 H1 were mixed with 0.6μl of human placental DNA (10mg/ml) and the volume were adjusted to 12μl by hybridisation buffer. To use a combination of three cosmids, 0.8μl of each cosmid were mixed with 1μl of human placental DNA (10 mg/ml) and the volume was adjusted to 12μl by hybridisation buffer. The cosmids were mainly used as biotin-labelled probes. However in a limited number of experiments which digoxigenin labelling was used a higher concentration of digoxigenin labelled cosmids was needed.

The Alu-PCR products of YAC 744D11 specific for chromosome 13 and YACs 745 H11 and 831B9 specific for chromosome 21 were labelled with biotin or digoxigenin and used for detection of chromosomes 13 and 21 copy number. The volumes that were

used to prepare the hybridisation mixtures using biotin labelled Alu-PCR products are described in table 2.3.

<i>Biotin-labelled probe (µl)</i>	<i>Probe DNA (µl)</i>	<i>Human placental DNA (µl)</i>	<i>Hybridisation buffer (µl)</i>	<i>Total volume (µl)</i>
745 H11 (100ng/µl)	1	0.5	10.5	12
831 B9 (100ng/µl)	1	0.5	10.5	12
744 D11 (100ng/µl)	1	0.5	10.5	12

Table 2.3. Volumes used to prepare hybridisation mixture of biotin labelled PCR-products of chromosomes 13 and 21 YACs.

The digoxigenin labelled 831 B9 and 744 D11 were both used at concentration of 150 ng for each hybridisation area in combination with 0.5µl of human placental DNA (10 mg/ml) which was adjusted to 12 µl with hybridisation buffer.

2.5.3 Denaturation And Hybridisation

Two methods of denaturation were used in this study:

- I. Simultaneous denaturation of probe and target DNA;** Using this method the hybridisation mixture was added on to the marked area on the microscope slide and covered with a coverslip. The coverslip was sealed with cowgum and then the probe and target DNA were denatured simultaneously in an 80°C oven for 8-10 minutes.
- II. Separate denaturation of probe and target DNA;** Probes were denatured by incubating the hybridisation mixture in a 65-70°C waterbath for 10 minutes and pre annealed for 30-60 minutes at 37°C. The target DNA was denatured by immersing slides in 70% formamide/2×SSC for 2-3 minutes in a 65-70°C waterbath. Slides were quenched immediately in cold 70% ethanol and dehydrated in an ethanol series (70%,

90% and 100 percent). The pre annealed probe mixture was applied to slides and covered by a coverslip.

The denatured probe and target DNA were allowed to hybridise at 42°C overnight (12-16 hours) under a sealed coverslip.

2.5.4 Post-Hybridisation Washes

The non-specifically bound and weakly hybridised probes were removed in this stage as described bellow:

1. The cowgum was carefully removed and the coverslips were floated off by rinsing the slides in 2×SSC at 42°C.
2. The slides were washed twice in 50% formamide/2×SSC and twice in 2×SSC at 42°C for 5 minutes each.
3. The slides were then blocked in wash-A solution for 30 minutes at 37°C.

2.5.5. Detection Of Biotin-Labelled Probes

Biotin-labelled probes were detected using fluorescein conjugated avidin/anti-avidin system as described bellow:

1. 100µl of 5µg/ml FITC conjugated avidin (diluted in wash-A solution) was added for each slide, the area was covered with parafilm and incubated in a 37°C waterbath for 15-20 minutes.
2. Slides were washed 3×5 minutes in wash-A at 42°C.
3. 100µl of 5µg/ml biotinylated anti-avidin D (diluted in wash A solution) was added, the area was again covered with parafilm and incubated for 15-20 min at room temperature.
4. Slides were washed 2×5 minutes in wash-A at 42°C and then incubated with 100µl of 5µg/ml FITC conjugated avidin (diluted in wash A) for 15-20 min at room temperature.

5. Slides were washed 2×5 min at 42°C in wash-A and 2×5 min in 4×SSC, 0.5% Tween-20 at room temperature, dehydrated through an ethanol series (50, 70, 90 and 100 %) and air dried.

To detect the biotin labelled probes with rhodamine, the slides were incubated with 100µl of 5µg/ml rhodamine-avidin in steps 1 and 4 instead of FITC-avidin.

2.5.6 An Alternative Method For Detection Of Biotin Labelled Probes

Biotin labelled probes were alternatively detected using a one layer detection procedure. After removing the coverslips the slides were washed 2×5 minutes in 50 percent formamide (diluted in 2×SSC) at 42°C followed by 2×5 minutes washes in 2×SSC at 42°C. Using this method the slides were blocked in 15 percent human AB serum (diluted in 4×SSC, 0.05 percent Tween 20) for 30 minutes at 37°C. The detection reagents used in this method consisted of fluorescence conjugated avidin and fluorescence conjugated anti-avidin. A 1:250 dilution of FITC conjugated avidin and 1:125 dilution of FITC labelled antiavidin were prepared individually and then mixed in equal volumes prior to detection. 100 µl of the mixture was applied for each slide, covered with parafilm and incubated for 20-30 minutes in a 37°C waterbath. The coverslips were removed and the slides were washed 2×5 minutes in 4×SSC, 0.05 percent Tween 20 in a 42°C waterbath. The slides were then dehydrated through an ethanol series (50%, 70%, 90% and 100%) and allowed to dry at room temperature.

2.5.7 Detection Of Digoxigenin Labelled Probes

Immunocytochemical detection of digoxigenin-labelled probes was carried out as follows:

1. 100µl of 0.6µg/ml anti-digoxigenin fluorescein Fab fragments prepared in sheep (diluted in wash-A solution) was added to each slide, the hybridisation area was covered with parafilm and incubated at 37°C for 15-20 minutes. Slides were washed

- 3×5 minutes at 42°C in wash-A solution and then were incubated with 100µl of 2.25µg/ml Fluorescein anti-sheep IgG for 15-20 minutes at room temperature.
2. Slides were washed 2×5 minutes at 42°C in wash-A solution, 2×5 minutes in 4×SSC, 0.5% Tween-20 at room temperature, dehydrated through an ethanol series (50, 70, 90 and 100 %) and air dried.

2.5.8 Counterstaining In Uni-Colour FISH Experiments

- I. The cells displaying FITC (green) signals were counterstained using both DAPI and Propidium iodide as follows: 1µl of DAPI (40µg/ml) and 2µl of Propidium iodide (20µg/ml) were resuspended in 97µl of mounting medium AF1 (Citifluor Ltd). 10-12µl of the suspension were applied on each slide, the area were covered with a 22×22mm coverslip and sealed with nail polish. Slides were stored at 4°C in dark until used.
- II. The cells illustrating rhodamine (red) signals were conterstained with only DAPI. 1µl of DAPI (40µg/ml) was resuspended in 99µl of mounting medium AF1. 10-12µl of the suspension was applied to each slide and mounted as described above.

2.6 Multi-Colour Fluorescence *In Situ* Hybridisation

A dual colour fluorescence *in situ* hybridisation approach was used to detect simultaneously the probe sets specific for chromosomes X and Y as well as 13 and 18. In a further study probe sets specific for chromosomes X, Y, 18; X,Y, 21 and 13, 18, 21 were simultaneously detected using a three colour ratio-mixing FISH. Finally a five colour ratio-mixing FISH was used to simultaneously visualise the probe sets specific for chromosomes 13, 18, 21 and both sex chromosomes in a single hybridisation experiment. The pretreatment of uncultured lymphocyte and amniocyte preparations and denaturation of target DNA in multi-colour FISH experiments were performed as described for uni-colour FISH. However the procedures that were used for preparation of hybridisation mixtures and detection were different.

2.6.1 Preparation Of Hybridisation Mixture For Multi-Colour FISH

The hybridisation mixtures used in dual colour FISH experiments contained two specific probes of which one was labelled with biotin-11-dUTP and the other with digoxigenin-11-dUTP. In ratio-mixing FISH experiments some of the probes were labelled with either biotin-11-dUTP or digoxigenin-11-dUTP and mixed with desired ratios to produce intermediate colours.

I. Preparation Of Hybridisation Mixture In Dual Colour FISH

In dual colour FISH experiments using chromosomes X and Y specific probes , for each slide 0.5ng of biotin labelled DXZ1 and 50ng of digoxigenin labelled pDP97 were resuspended in 12µl of hybridisation buffer and used as a hybridisation mixture. To detect simultaneously probe sets specific for chromosomes 13 and 18, for each slide 150 ng of digoxigenin labelled 744D11 YAC and 5µg of human placental DNA were resuspended in 5 µl of hybridisation buffer (50% formamide, 2xSSC, 10% dextran sulphate and 5 µg of salmon sperm DNA) and mixed with 5 µl of hybridisation mixture containing 0.5 ng of biotin labelled L1.84 immediately before hybridisation.

II. Preparation Of Hybridisation Mixture In Three Colour Ratio-Mixing FISH

The concept of ratio-mixing FISH was achieved from Dauwerse *et al* (1992b). To detect simultaneously probe sets specific for chromosomes X, Y and 18, 0.5ng of biotin labelled DXZ1, 5ng of digoxigenin labelled L1.84 and 50 ng each of biotin labelled and digoxigenin labelled pDP97 (1 : 1 ratio) were resuspended in 12µl of hybridisation buffer and hybridised to the specific targets after denaturation. In some experiments a 1 : 1 ratio of biotin labelled and digoxigenin labelled L1.84 was used instead of Y.

The simultaneous detection of chromosomes X, Y and 21 specific probes was carried out as follows: For each slide 100 ng of digoxigenin labelled 831B9 YAC and 5 µg of human placental DNA were resuspended in 6µl of hybridisation buffer and mixed with 6µl of

hybridisation mixture containing 0.5 ng of biotin labelled DXZ1 and 50 ng each of biotin labelled and digoxigenin labelled pDP97 (1:1 ratio mixing) prior to hybridisation.

Three colour ratio-mixing FISH was also used to simultaneously detect probe sets specific for the three autosomes 13, 18 and 21. For this, 100ng of biotin labelled 831B9 YAC, 150ng of 744D11 YAC and 10µg of human placental DNA were resuspended in 6 µl of hybridisation buffer and mixed with 6µl hybridisation mixture containing 5ng each of biotin labelled and digoxigenin labelled L1.84 (1 :1 ratio mixing).

III. Preparation Of Hybridisation Mixture In Five Colour Ratio-Mixing FISH

In five colour ratio-mixing FISH experiments for each slide 2ng of DXZ1, 10 ng of L1.84 and 120 ng of pDP97 were resuspended in 7µl of hybridisation buffer and mixed with 7µl of hybridisation mixture cotaining 100 ng of 831B9 YAC and 150 ng of 744D11 YAC immediately before hybridisation. The mixing ratio of the probes was as shown in table 2.4.

<i>probe</i>	<i>Biotin</i>	<i>Digoxigenin</i>	<i>Detection</i>	<i>Pseudocolour</i>
DXZ1	1	3	Rhodamine + FITC	Pink
pDP97	3	1	Rhodamine + FITC	Yellow
744D11	-	1	FITC	Green
L1.84	1	1	Rhodamine + FITC	White
831B9	1	-	Rhodamine	Red

Table 2.4. Mixing ratio of the probes, detection system and the pseudocolours used for simultaneous five colour detection

The target DNA was denatured by immersing slides in 70% formamide/2xSSC for 2 min at 65°C. Slides were quenched immediately in cold 70% ethanol and dehydrated in an ethanol series (50%, 70%, 90% and 100 percent). All the probes were denatured at 65

°C for 10 min and pre annealed for 30-60 min at 37°C. Individually pre annealed probe mixtures were mixed and spun briefly immediately before hybridisation. The preannealed probe mixtures were applied to slides, covered with a 22×22 mm coverslip and sealed with cowgum and allowed to hybridise at 42°C overnight.

2.6.2 Post Hybridisation Washes And Detection In Multi-Colour FISH

The post hybridisation washes in multi-colour FISH experiments were performed as described for uni-colour FISH. The same immunocytochemical detection method was used in all dual colour and ratio-mixing FISH experiments. In all multi-colour FISH experiments the biotin labelled probes were detected with avidin conjugated with rhodamine and the signals were enhanced by using biotinylated anti-avidin and adding an extra layer of avidin conjugated with rhodamine. However to detect the digoxigenin labelled probes in a multi-colour FISH experiment the use of two detection systems was investigated. Initially the system of anti-digoxigenin fluorescein fab fragments (prepared in sheep) and fluorescein anti-sheep IgG were used and then the experiments were carried out using FITC conjugated monoclonal anti digoxin prepared in mouse and FITC conjugated anti mouse IgG. The procedure of detection was as described bellow:

1. 100µl of 5µg/ml FITC conjugated avidin (diluted in wash-A solution) was added for each slide, the area was covered with parafilm and incubated in a 37°C waterbath for 15-20 minutes.
2. Slides were washed 3×5 minutes in wash-A at 42°C.
3. 100µl of a mixture of 5µg/ml biotinylated anti-avidin D and 0.6µg/ml anti-digoxigenin fluorescein Fab fragments prepared in sheep (diluted in wash A solution) was added, the area was again covered with parafilm and incubated for 15-20 minutes at room temperature.
4. Slides were washed 2×5 minutes in wash-A at 42°C and then incubated with 100µl of a mixture of 5µg/ml FITC conjugated avidin and 2.25µg/ml fluorescein anti-sheep IgG (diluted in wash A) for 15-20 minutes at room temperature.

5. Slides were washed 2×5 minutes at 42°C in wash-A and 2×5 minutes in 4×SSC, 0.5% Tween-20 at room temperature, dehydrated through an ethanol series (50, 70, 90 and 100 %) and air dried.

In an alternative method the simultaneously hybridised probe sets were detected using 100 µl of wash A containing 5µg/ml of goat anti-avidin D and 5µg/ml of FITC conjugated monoclonal anti-digoxin prepared in mouse at step 3. The detection was then followed by incubating the slides with 100 µl of a mixture of rhodamine avidin (5µg/ml) and FITC conjugated anti-mouse IgG (5µg/ml) in wash-A at room temperature as described at step 5 above.

2.6.3 Counterstaining Of Simultaneously Detected Probes

In rhodamine (red fluorochrome) involved detection procedures (dual colour and ratio-mixing FISH experiments) the slides were mounted using only DAPI. Two alternative methods were used:

- I. 1µl of DAPI (40µg/ml) was resuspended in 99µl of mounting medium AF1 (Citifluor Ltd). 10-12 µl of the suspension was then applied for each slide. The area was covered with a 22×22mm coverslip, sealed with nail polish and stored at 4°C until used.
- II. 4µl of DAPI (1mg/ml) was resuspended in 50ml of 2×SSC. The slides were immersed in the suspension for 5 minutes at room temperature, followed by 5 minutes wash in 2×SSC at room temperature. After air drying, slides were mounted with 10-12µl of mounting medium AF1, covered with coverslip and sealed with nail polish. The slides were stored at 4°C until used.

2.7. Visualisation

In uni-colour FISH experiments the results were analysed using an epifluorescence microscope (Zeiss Axioplan) equipped with suitable filter sets (table 2. 5). Metaphase spreads and interphase nuclei were visualised by a DAPI filter. The use of Zeiss filter set

9 allowed simultaneous visualisation of FITC signals (green fluorochrome) and rhodamine (red fluorochrome) stained chromosome spreads or interphase nuclei.

<i>Fluorochrome</i>	<i>Excitation wavelength (nm)</i>	<i>Emission wavelength (nm)</i>	<i>Zeiss filter set</i>	<i>colour of fluorescence</i>
DAPI	345	425	1 , 2	Blue
Propidium iodide	520	610	9	Red
FITC	490	525	9 , 10	Green
Rhodamine	540-560	580	15	red

Table 2.5. Filters used to visualise fluorochromes by epifluorescence microscopy

The results obtained from multi-colour FISH experiments were analysed by an epifluorescence microscope (Zeiss Axioskop) equipped with a cooled CCD camera (Photometrics, KAF 1400) which was controlled by an Apple Macintosh computer (Quadra 950) and Smartcapture software (Digital Scientific). In this system the excitation filter sets specific for DAPI, FITC and rhodamine were mounted in a motorised filter wheel which was controlled by computer software. The images were visualised under the microscope using the motorised excitation filter set and an appropriate emission filter block. The Smartcapture software was used to capture and process the images. This software has been designed for automated colour image capturing. Using this system the image under the microscope is automatically exposed by green, red and blue fluorescence lights to produce a coloured image. The images can be processed by normalising the colour and removing the background fluorescence. Analysis of the results in dual colour and three colour FISH experiments was mainly carried out by visualisation of the images under the fluorescence microscope of the CCD system using the filter wheel and a triple dichromic emission filter block (Pinkel No. 1, Chroma technology). The images were

captured where required for accurate signal interpretation or for recording. However to analyse the signal constitution of the hybridised cells in a five colour ratio-mixing FISH experiment, the images were captured and the signals were pseudocoloured using the Smartcapture programme (table 2. 4). In all experiments the captured images were printed on sublimation printing papers (A4-SPW), using a Mitsubishi colour printer.

2.8 Analytical Protocol

Peripheral blood and amniotic fluid samples were obtained from Duncan Guthrie Institute of Medical Genetics (blood lab and prenatal lab respectively). 1 ml of each peripheral blood sample and 1-3 ml of each amniotic fluid sample were used for *in situ* hybridisation experiments. A brief information about the status in which each amniotic fluid sample arrived was recorded to be used for data interpretation. The techniques were originally optimised using normal blood samples and then applied to unselected series of uncultured lymphocytes and amniocytes to assess the reliability and sensitivity of each technique. For each sample 25-100 hybridised nuclei were analysed. In multi-colour FISH experiments each nucleus was searched for all probe sets that were simultaneously hybridised. Nuclei without signal, clumped nuclei and nuclei covered with cytoplasm or cellular membrane were not scored. The results were finally compared to the results obtained from cytogenetic analysis.

3. RESULTS

3.1 Uni-Colour FISH Using Alpha Satellite DNA Probes

3.1.1 Detection Of Chromosome X Copy Number Using Probe DXZ1

As a start for the study of chromosome aneuploidies in uncultured cells using fluorescence *in situ* hybridisation, probe DXZ1 specific for the centromeric region of chromosome X was used to optimise the technique of uni-colour FISH. The probe was biotin-labelled and was obtained commercially. A series of cultured peripheral blood samples was initially hybridised with this probe. The probe and target DNA were simultaneously denatured under a sealed coverslip in an 80°C oven and hybridised at 42°C overnight. As the probe is highly specific, no competitor DNA was needed. The hybridised probe was detected with avidin conjugated with FITC and the intensity of the signals was enhanced using biotin labelled anti-avidin as a second layer and again FITC-avidin as a third layer. Under stringency conditions used in this study, specific green signals were produced on centromeric region of chromosome X using 0.5-2 ng of labelled probe DNA. However, the procedure resulted in damaged morphology of chromosomes and nuclei due to the high temperature of denaturation used in this series of experiments.

To maintain the morphology of targets the slides were treated with fresh fixative (3:1 methanol : acetic acid) and acetone. The target DNA was denatured at 65-70°C in 70 per cent formamide for 2-3 minutes. Probe DNA was denatured in a 65-70°C waterbath for 10 minutes and then the denatured probe and target DNA were allowed to hybridise under a sealed coverslip at 42°C overnight. Using this method intense and specific signals with minimum background and a superior quality of target morphology were obtained. Ten cultured lymphocyte samples were then hybridised with the probe DXZ1 and analysed in a blind fashion using a Zeiss epifluorescence microscope equipped with appropriate filter sets. A minimum of 25 metaphase spreads were analysed for each sample. Five samples showed two signals on all metaphase cells and were correctly

identified as normal female samples (Fig. 3.1a). Four samples displayed one signal specific for chromosome X on all of the metaphase cells and were correctly detected as normal male samples (Fig. 3.1b). One of the samples displayed three signals in 34 metaphase spreads out of 35 cells which were analysed and was correctly identified as 47, XXX (Fig. 3.1c).

The experiments were then carried out using uncultured lymphocyte preparations. The technique was first applied on a number of normal uncultured lymphocytes and then a series of 15 uncultured peripheral blood samples was hybridised with the probe set and analysed in a blind fashion. A minimum of 50 nuclei were scored for each sample and the results were compared to those of cytogenetic analysis. Nine samples were correctly detected as normal male displaying one signal in an average of 94.9 per cent of the hybridised cells (Fig. 3.1d). Six samples displayed two signals in an average of 94.4 per cent of the hybridised cells and correctly identified as normal female samples. In a number of experiments rhodamine labelled avidin was used instead of FITC labelled avidin and the slides were counterstained by DAPI diluted in anti-fade medium. The hybridised cells displayed red signals when analysed using Zeiss filter set number 15 specific for rhodamine and propidium iodide (Figs. 3.1e).

Digoxigenin labelled DXZ1 was also used to detect the copy number of chromosome X in a number of uncultured lymphocytes. The digoxigenin labelled probe was detected by anti-digoxigenin fluorescein fab fragments prepared in sheep and the signal was amplified using FITC anti-sheep as the second layer. The signals produced by digoxigenin labelled DXZ1 were strong and specific and could be used as an alternative for biotin-labelled DXZ1 (Fig. 3.1f).

3.1.2 Detection of chromosomes Y and 18 copy number using probes pDP97 and L1.84

Probes pDP97 and L1.84 specific for the centromeric regions of chromosomes Y and 18 respectively were available as glycerol stocks in this department. The bacterial host

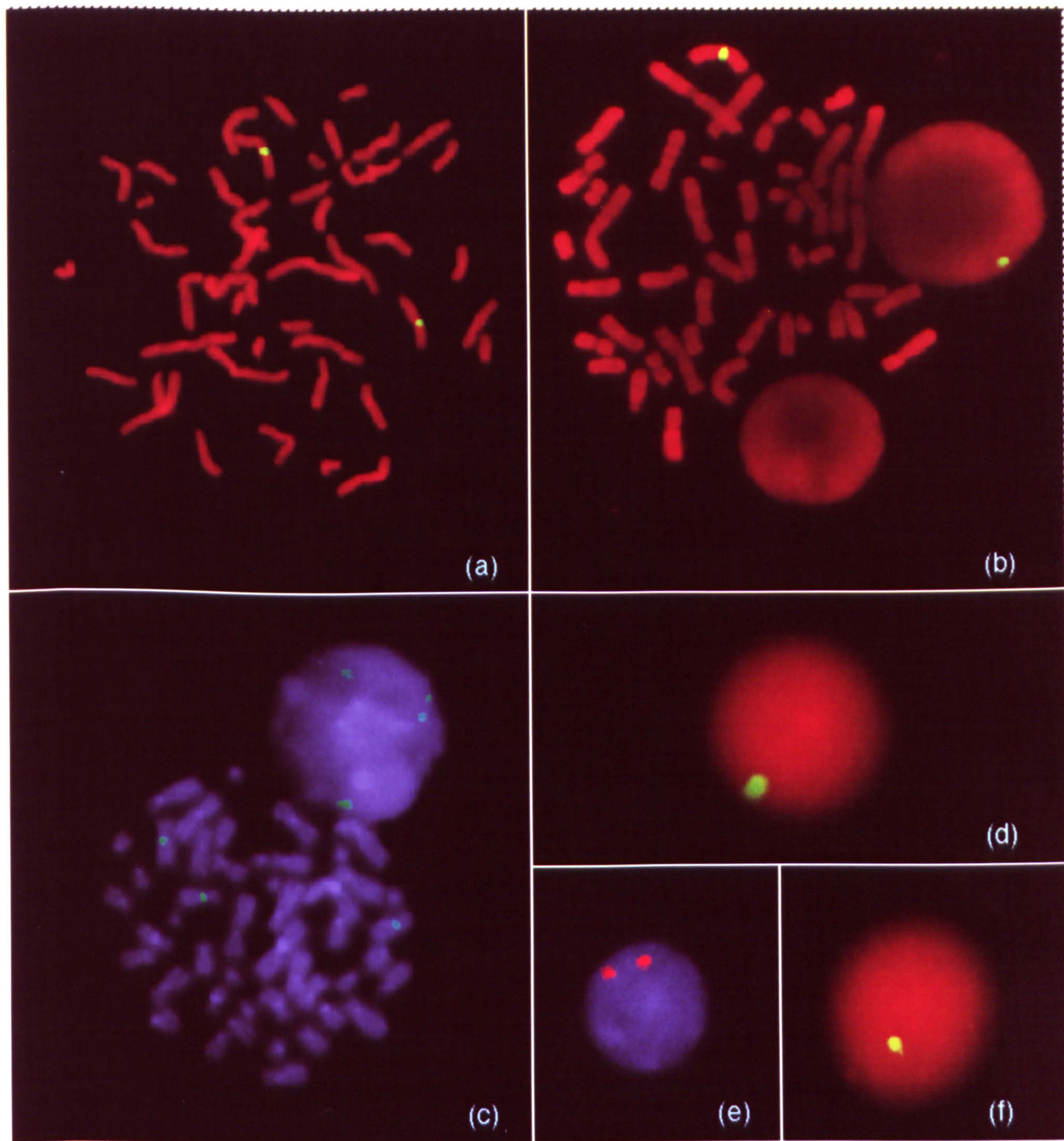


Figure 3.1. Cultured and uncultured lymphocytes hybridised with biotin-labelled and digoxigenin-labelled DXZ1. (a-c) Normal female, normal male and 47, XXX metaphase spreads respectively, hybridised with biotin-labelled DXZ1 and detected with FITC; (d) a normal male interphase nucleus hybridised with biotin-labelled DXZ1 and detected with FITC; (e) a normal female interphase nucleus hybridised with biotin-labelled DXZ1 and detected with rhodamine; (f) a normal male interphase nucleus hybridised with digoxigenin-labelled DXZ1 and detected with FITC.

cells carrying the probes were cultured in Luria Bertani medium containing ampicillin and the plasmid DNA including the human insert was isolated from amplified bacterial cells using Circle prep kit. A high concentration of plasmid DNA (0.5-1 mg/ml) was obtained using this kit. In some experiments the Insta prep kit was used which allowed rapid isolation of plasmid DNA within 15 minutes. Each Insta prep tube produced pure DNA sufficient for 20-25 labelling reactions. The plasmid DNA containing human insert was directly used for labelling and no isolation of human insert from plasmid DNA was carried out. The probes were labelled with biotin-11-dUTP using standard nick-translation method. The labelled DNA was precipitated by 3M sodium acetate and cooled ethanol. Incubation of precipitated DNA at -20°C for a minimum of 1 hour appeared to increase the efficiency of labelled probes. The suspension was centrifuged and the pellet was dissolved in 10µl of T.E. buffer or hybridisation buffer to give a concentration of 100 ng/µl. The labelled probes were then hybridised to cultured cells from peripheral blood samples. Prior to denaturation the slides were treated with fresh fixative and acetone, the probe and target DNA were denatured individually and hybridised at 42°C overnight. Hybridisation of both pDP97 and L1.84 was carried out without suppression with competitor DNA. The concentrations required to produce intense and specific signals using each of the probes were mainly dependent on labelling efficiency. Approximately 30-50 ng of probe pDP97 and 5-10 ng of probe L1.84 produced strong and specific signals on the centromeric regions of chromosomes Y and 18 respectively. The male samples were readily distinguished from females when hybridised with probe pDP97. Most of the metaphase cells displayed one signal on each chromatid of chromosome Y (Fig. 3.2a). The signals produced by probe pDP97 were characteristically smaller than the signals produced by probes DXZ1 and L1.84. The cells hybridised with probe L1.84 displayed two large signals on almost all of the metaphase spreads from each sample indicating the normal number of chromosome 18 (Fig. 3.2b).

The optimised protocol was used to study the chromosomes Y and 18 in interphase nuclei preparations from uncultured lymphocytes. Fifteen uncultured

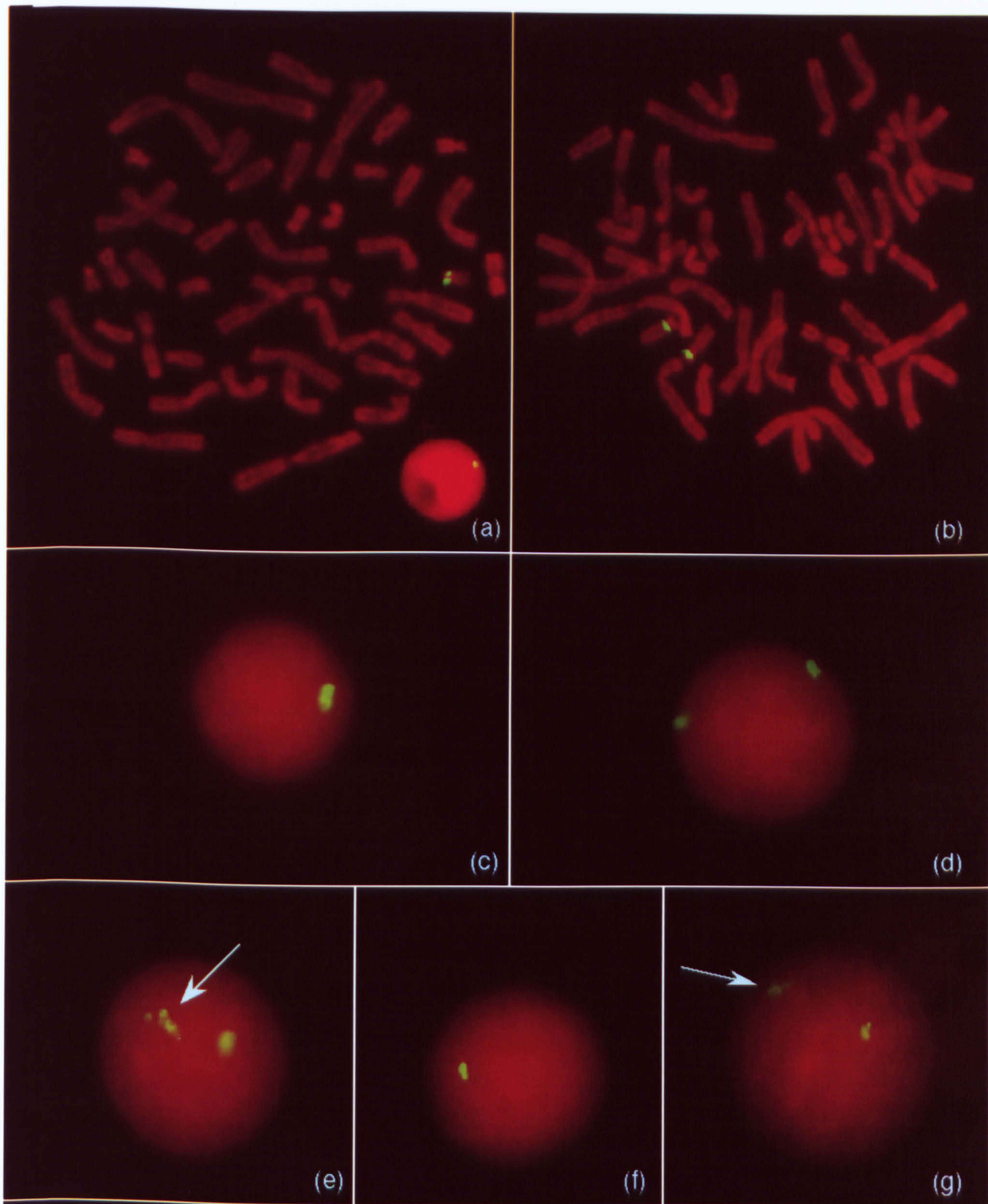


Figure 3.2. Cultured and uncultured lymphocytes hybridised with probes pDP97 and L1.84 specific for chromosomes Y and 18 respectively. (a) Biotin-labelled pDP97; (b) biotin-labelled L1.84; (c) biotin-labelled pDP97; (d) biotin-labelled L1.84; (e) an uncultured lymphocyte displaying a diffuse signal for chromosome 18 (arrowed); (f) digoxigenin-labelled pDP97; (g) digoxigenin-labelled L1.84 with a diffuse signal specific for chromosome 18 (arrowed).

lymphocyte preparations were hybridised to the probe pDP97 and analysed in a blind fashion. A minimum of 50 nuclei were analysed for each sample. Unhybridised and clumped cells were excluded from counting. Nine samples displayed one distinct signal specific for chromosome Y on an average of 93.9 per cent of the cells and were correctly identified as male samples (Fig. 3.2c). Six samples did not show any signal and correctly detected as female samples. Twelve uncultured lymphocytes were also hybridised to probe L1.84 and analysed by scoring a minimum of 50 nuclei on each hybridisation area. An average of 92.6% of the hybridised cells showed two signals specific for chromosome 18 (Fig. 3.2d). A percentage of the hybridised cells displayed diffuse signals for chromosome 18 (Fig. 3.2e). These cells were not included in signal analysis.

In a number of experiments the probes were labelled with digoxigenin and used to detect the copy number of chromosomes Y and 18 on uncultured lymphocytes. Random primed DNA labelling or nick translation procedures were used to label the probes with digoxigenin according to the procedures described earlier in materials and methods. The results revealed that nick translation method is more suitable for digoxigenin labelling than random primed labelling. The digoxigenin labelled pDP97 and L1.84 were then hybridised to uncultured lymphocytes. Detection of digoxigenin labelled probes hybridised to the specific targets was carried out according to the procedures described for digoxigenin labelled DXZ1. Both digoxigenin labelled probes produced intense and specific signals with high hybridisation and detection efficiency and less background compared to the biotin -labelled probes (3.2f and 2g).

3.2 Dual Colour Fluorescence *In Situ* Hybridisation

Following optimisation of uni-colour FISH to detect the copy number of chromosomes X, Y and 18 using biotin labelled and digoxigenin labelled probes, a dual-colour FISH approach was employed to simultaneously detect two chromosomes in a single hybridisation experiment. The major purpose of dual colour FISH experiments was to investigate the reliability and sensitivity of the probes when detected simultaneously in interphase nuclei, and optimise a method for simultaneous detection of differently labelled

probes which could be used in ratio-mixing FISH experiments. For this reason a limited number of samples were analysed for each probe combination when the technique was optimised.

To simultaneously detect the copy number of chromosomes X and Y in metaphase and interphase cells, a mixture of biotin labelled DXZ1 and digoxigenin labelled pDP97 was hybridised to the specific targets. Biotin labelled probe was detected with rhodamine (red) and digoxigenin labelled probe was detected with FITC (green). Each of the red and green signals was amplified using an extra layer of rhodamine and FITC. The detection solutions which were initially used consisted of a mixture of rhodamine avidin and anti-digoxigenin fluorescein fab fragments as first layer, biotin labelled anti-avidin and fluorescein conjugated anti-sheep for second layer and rhodamine avidin as third layer. This method of detection resulted in weak FITC signals probably as a consequence of extra washes after third layer of detection with rhodamine. As a result the sequence of application of the detection solutions was changed to rhodamine avidin for first layer of detection, a mixture of biotin labelled anti-avidin and anti-digoxigenin fluorescein fab fragments as second layer and a mixture of rhodamine avidin and fluorescein conjugated anti-sheep as third layer. The cells were counterstained with DAPI which allowed visualisation of both red and green signals on a blue background. The technique was used to enumerate chromosomes X and Y simultaneously on a number of cultured and uncultured lymphocytes. Signal analysis was initially carried out using an epifluorescence microscope with filter sets specific for each of the DAPI, rhodamine and FITC fluorochromes. The signals produced by each of the probes were strong and distinct and could be easily distinguished. However, frequent manual changing of the filter sets was required to score a dual colour experiment. Analysis of the results were also carried out using a CCD system. The excitation filter sets in this system are mounted in a motorised filter wheel. Selection of an appropriate excitation filter in the filter wheel used in combination with the triple dichromic emission filter allowed simultaneous visualisation of all three fluorochromes under the microscope or on the screen. The

signals were clearly distinguished and rapidly analysed using this system. However, a slight cross reactivity was observed between green and red fluorochromes when the images were captured. Figure 3.3a shows a normal male uncultured lymphocyte simultaneously hybridised to biotin labelled DXZ1 and digoxigenin labelled pDP97. Figure 3.3b illustrates the same nucleus exposed by blue and green light. This figure clearly shows binding of green fluorochrome to the probe DXZ1 which has been detected with rhodamine. Figure 3.3c shows the same nucleus exposed by blue and red light. No binding of red fluorochrome to the probe pDP97 which is detected by FITC is observed. A comparison between figures 3.3a and 3.3c shows the colour difference of the red signal specific for chromosome X on two images. By modifying the reagents to monoclonal anti-digoxin (FITC conjugate) and FITC labelled anti-mouse IgG to detect the digoxigenin labelled probes, pure red and green signals were produced and cross reaction was eliminated. Figures 3.3d-f show a normal male uncultured lymphocyte hybridised to biotin labelled DXZ1 and digoxigenin labelled pDP97 and detected with rhodamine-avidin and FITC conjugated anti-digoxin using a three layer detection procedure. Comparison of the three images in figure 3.3d-f clearly shows the specificity of the new detection reagents. Figure 3.3g demonstrates a normal male metaphase spread simultaneously hybridised to probes DXZ1 and pDP97 and shows the localisation of the probes on the specific targets.

The probe sets specific for chromosomes X and Y were also hybridised to a limited number of uncultured amniocytes. The amniocyte preparations were treated with RNase to remove the cellular and nuclear RNA molecules and hybridised to the probe mixture as described earlier. Normal male and female amniotic fluid samples were readily distinguished using this method (Fig. 3.3h).

To simultaneously detect chromosomes X and 18, a combination of biotin labelled DXZ1 and digoxigenin labelled L1.84 was hybridised to uncultured lymphocytes. A high hybridisation and detection efficiency was achieved for both probes using a limited number of uncultured lymphocyte preparations (Fig. 3.3i).

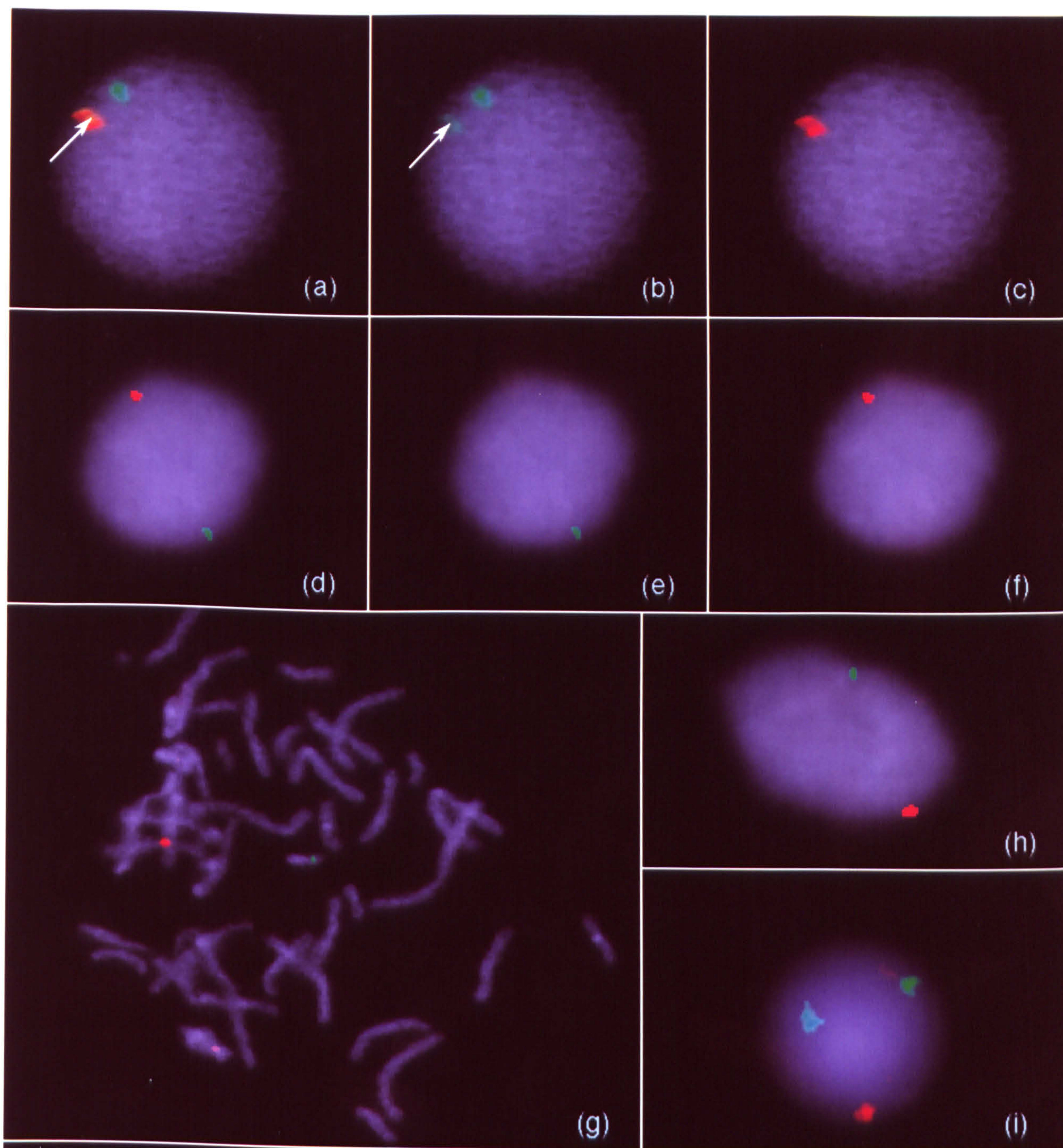


Figure 3.3. Comparison of two detection systems in dual-colour FISH experiments. (a-c) Illustration of the binding of fluorescein anti-sheep antibody to goat anti-avidin (arrowed); (d-f) demonstration of the specificity of the monoclonal anti-digoxin system in detection of digoxigenin-labelled probes (see text for details); (g) a metaphase spread prepared from peripheral blood; (h) an uncultured amniocyte; (i) an uncultured lymphocyte.

In figs. a, d, g and h : ■ X ■ Y In fig. i : ■ X ■ 18

3.3 Development Of Three Colour Ratio-Mixing FISH

Once the reliability of the three alpha satellite DNA probes DXZ1, pDP97 and L1.84 had been established both singly and in pairs using various detection systems, a three colour ratio-mixing FISH approach was developed to detect the three probes simultaneously. In order to optimise the technique, a hybridisation mixture containing biotin labelled DXZ1, digoxigenin labelled L1.84 and a 1:1 ratio mixture of biotin labelled and digoxigenin labelled pDP97 was prepared in a total volume of 12-13 μ l and hybridised to cultured lymphocytes. After treatment of slides with fresh fixative and acetone the probe and the target DNA were denatured individually and allowed to hybridise at 42°C overnight. Post-hybridisation washes were carried out as described for uni-colour and dual colour FISH experiments. Detection of probes in three colour ratio-mixing FISH experiments was carried out as described for dual colour FISH. Biotin labelled probes were detected with rhodamine-avidin (red) and amplified once using biotin labelled anti-avidin and rhodamine-avidin. Digoxigenin labelled probes were detected with FITC labelled monoclonal anti-digoxin (green) and amplified by anti-mouse conjugated with FITC. A third colour (yellow) was produced by detection of the ratio-mixed probe with both rhodamine and FITC. Figure 3.4a shows a normal male uncultured lymphocyte simultaneously hybridised to the probe sets specific for chromosomes X, Y and 18. Figures 3.4b and c show composite pictures from the same cell produced by merging the blue/green and blue/red images respectively. No cross reactivity of green fluorochrome with red fluorochrome was observed in repeated experiments using the above detection reagents. To study the effect of cross reactivity on three colour FISH experiments, the probes were also detected using detection mixtures containing anti-digoxigenin fluorescein fab fragments and anti-sheep conjugated with FITC. Figure 3.4d shows a normal male metaphase spread hybridised to the probe set. The signal specific for chromosome X which was expected to be red appeared as a orange signal. The binding of green fluorochrome to chromosome X is shown in figure 3.4e by an arrow. Comparison of figures 3.4d and 3.4f shows the colour difference of the signal specific for chromosome

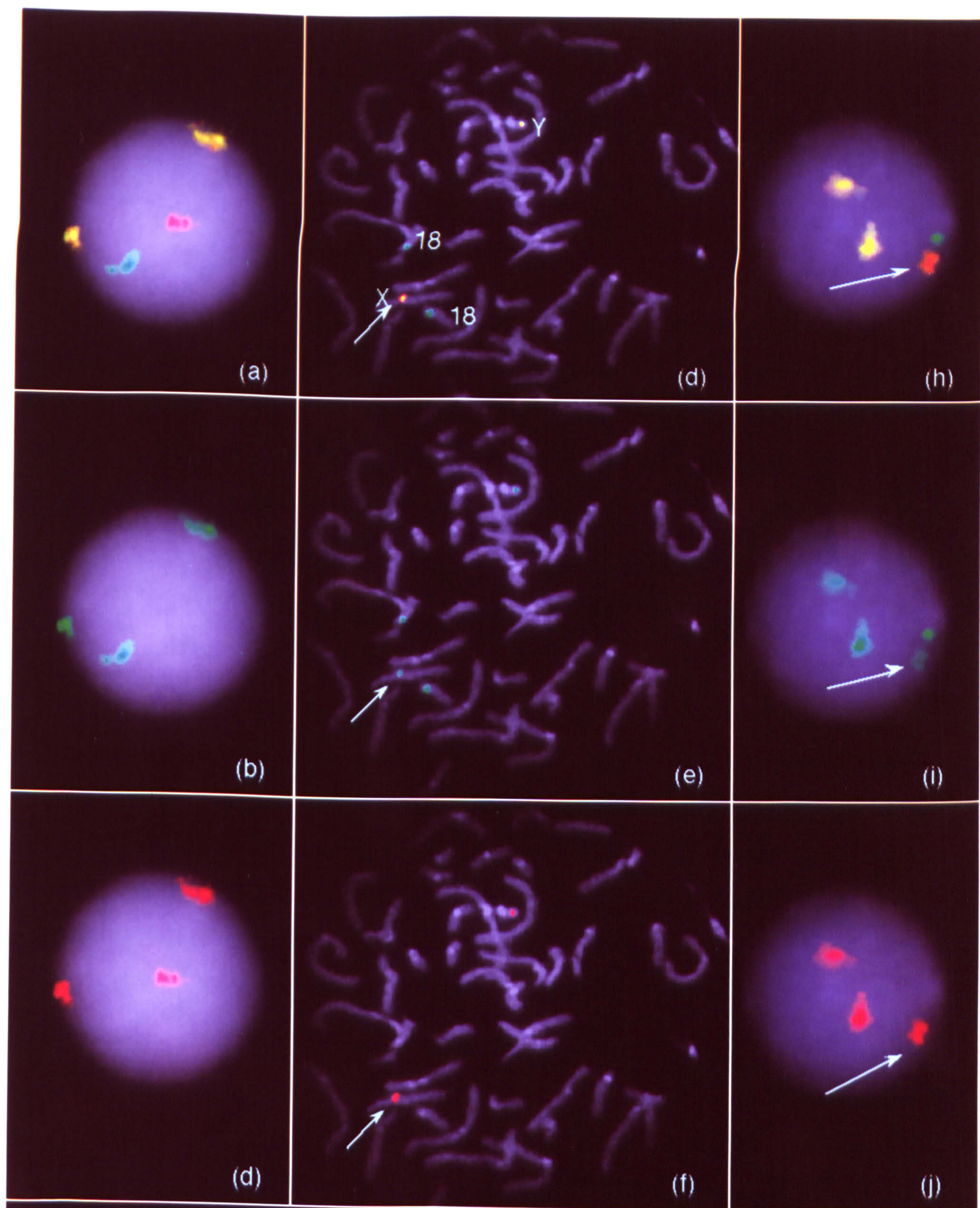


Figure 3.4. Comparison of two detection methods in three colour ratio-mixing FISH experiments using probes DXZ1, pDP97 and L1.84. The arrows show the cross-reactivity of the green fluorochrome with the red signals. See text for details.

In figs. a and h : ■ X ■ Y ■ 18

X in two images. A similar situation has been illustrated in figures 3.4h-j which shows a normal male uncultured lymphocyte hybridised to the probe set. A ratio-mixed L1.84 probe specific for chromosome 18 has been used in this experiment. In spite of cross reactivity the colour difference was enough to be distinguished using the fluorescence microscope of CCD system.

The three colour ratio-mixing FISH allowed simultaneous detection of chromosomes X, Y and 18 in three different colours by employing two haptenisation and detection systems. Analysis of results was carried out using a CCD system and smartcapture programme. All of the probes used in this series of experiments generated strong signals with proposed localisation on the specific target chromosomes.

Similar results were obtained where a 1:1 ratio mixture of biotin labelled and digoxigenin labelled L1.84 was used to detect the copy number of chromosome 18 in a three colour ratio-mixing FISH experiment.

The optimised technique was initially applied on a number of normal and abnormal samples of cultured lymphocytes. Figure 3.5a demonstrates a normal male metaphase spread hybridised with the combined probe set. Figures 3.5b shows a cell from a 47, XXY sample and 3.5c shows a cell from a 47, XY+18 sample hybridised to the probe set. Figure 3.5d shows a 47, XYY nucleus from a cultured lymphocyte, hybridised with the probe set. These results clearly validate the chromosomal specificity of the probe set to be used for simultaneous enumeration of three chromosomes within interphase nuclei.

Experiments were then carried out using an unselected series of uncultured lymphocytes. Twenty uncultured lymphocyte preparations were analysed by scoring 50 nuclei for each sample. All of the probes produced intense signals with little background. However probe L1.84 displayed diffuse signals on some of the nuclei from each sample. Nine samples were correctly detected as normal male and eleven samples were correctly detected as normal female (Fig. 3.5e and f). In normal male uncultured lymphocytes, an average of 93 per cent of the hybridised cells displayed one signal for chromosome X,

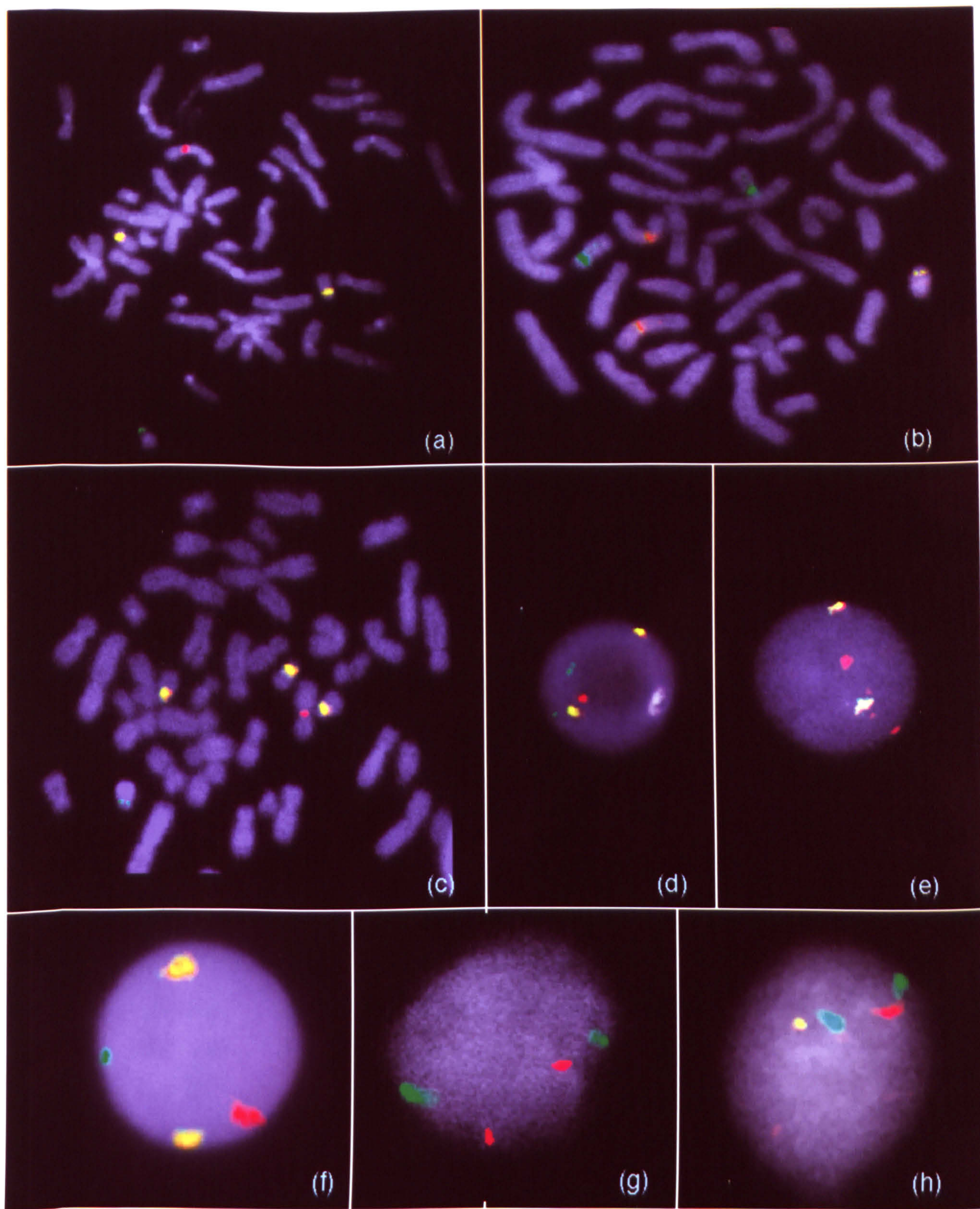
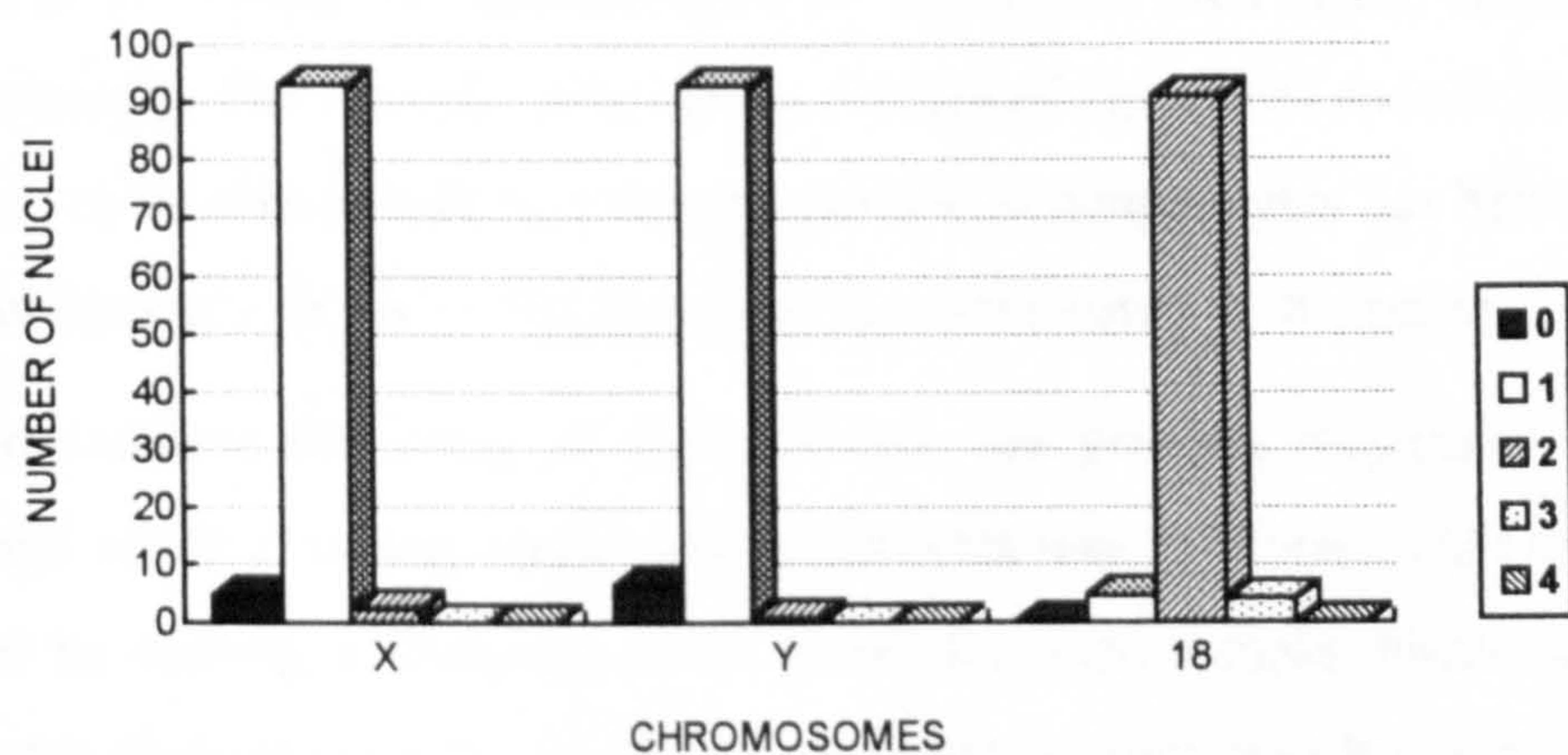


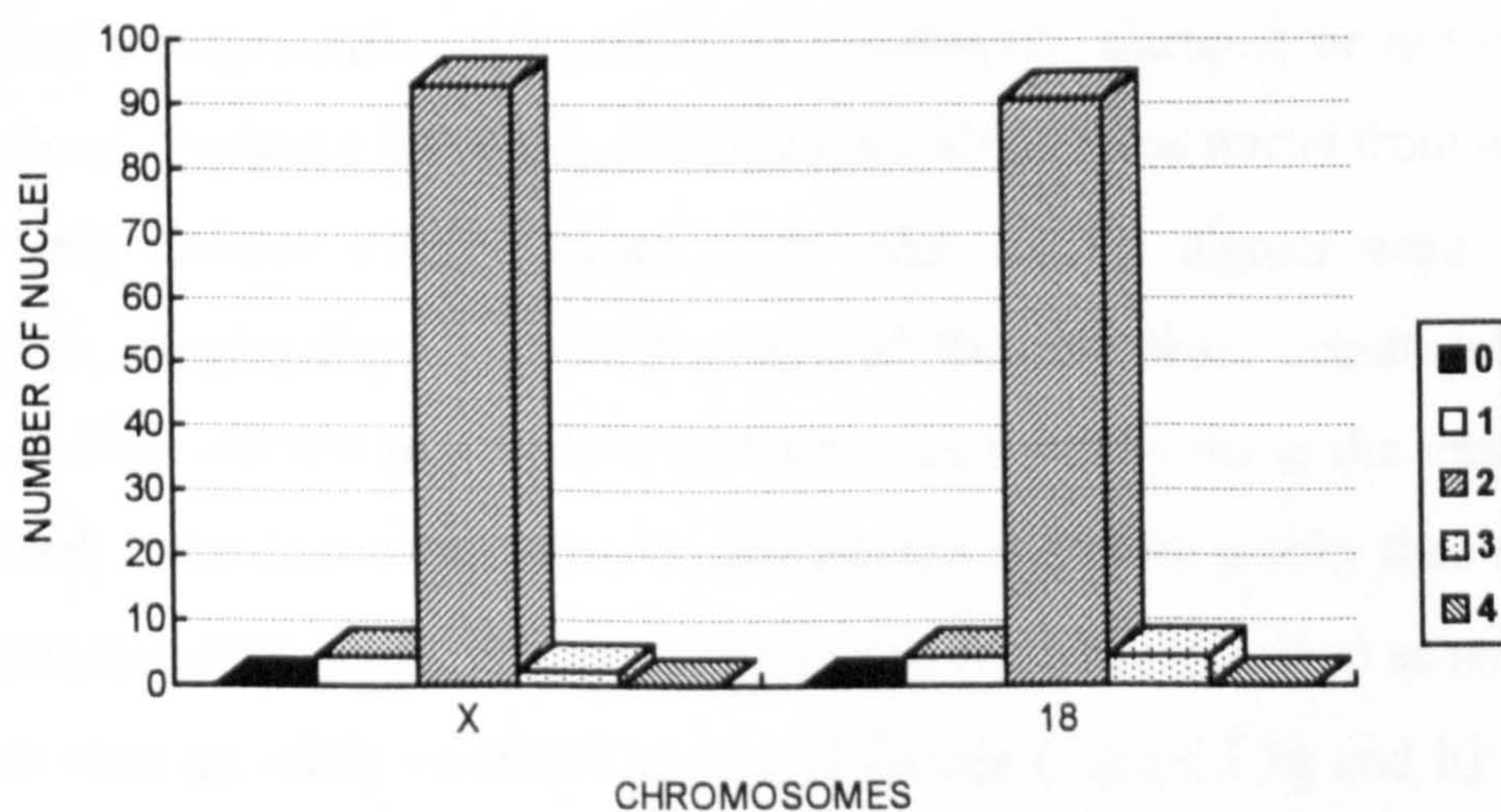
Figure 3.5. Three colour ratio-mixing FISH using probes DXZ1, pDP97 and L1.84. (a-c) Metaphase cells prepared from peripheral blood samples; (d) an interphase nucleus from a cultured lymphocyte sample; (e) and (f) uncultured lymphocytes; (g) and (h) uncultured amniocytes.

In (a, c, d, e and f) : ■ X ■ Y ■ 18

In (b, g and h) ■ X ■ Y ■ 18



(a)



(b)

Figure 3. 6. Distribution of signals specific for chromosomes X, Y and 18 in uncultured male (a) and female (b) lymphocytes using three colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

92.8 per cent showed one signal for chromosome Y and 91 per cent showed two signals for chromosome 18. In normal female lymphocytes, an average of 93.1 per cent and 90.8 per cent of the hybridised cells showed two signals for each of the chromosomes X and 18 respectively. The detection efficiencies of each of the probes specific for chromosomes X, Y and 18 in normal male and female uncultured lymphocytes has been diagrammatically shown in figures 3.6a and b. No abnormal samples existed in this series.

To evaluate the utility of the technique for prenatal diagnostic applications, an unselected series of twenty uncultured amniocytes was hybridised with the probe set and analysed by scoring a minimum of 25 nuclei for each sample. Nuclei from uncultured amniocytes showed irregular edges and were smaller than uncultured lymphocytes. Cells observed on the slides presented three different morphological aspects, which were (i) isolated undegenerated nuclei with hybridisation signals; (ii) nuclei covered by cytoplasm, most of them without hybridisation signals or displaying one or two spots (Fig 3.7a); and (iii) degenerated nuclei without a hybridisation signal (Fig 3.7b). A percentage of hybridised undegenerate nuclei displayed overlapped, clumped or out of focus signals (figs 3.7c-e). Probe L1.84 displayed diffuse signals on some nuclei from each sample (Fig 3.7f). Only isolated undegenerated nuclei with distinct signals were scored for this evaluation, ranging from 5 to 50 per cent of the cells from uncultured amniotic fluid samples. Each cell was searched for all signals produced by the probe sets which had been hybridised. Hybridisation signals were less intense and more patchy than signals observed in uncultured lymphocytes. Eleven samples were correctly identified as normal male and 9 samples were correctly identified as normal female (Figure 3.5g and h). In normal male samples an average of 91.9 per cent of the hybridised cells showed one signal for chromosome X, 91.4 per cent showed one signal for chromosome Y, and 89.4 per cent showed two signals for chromosome 18. In normal female uncultured amniocytes 92.7 per cent and 89.5 per cent of the hybridised cells displayed two signals for each of the chromosomes X and 18 respectively. The hybridisation efficiency of each of the probes specific for chromosomes X, Y and 18 hybridised to uncultured amniotic fluid cells are

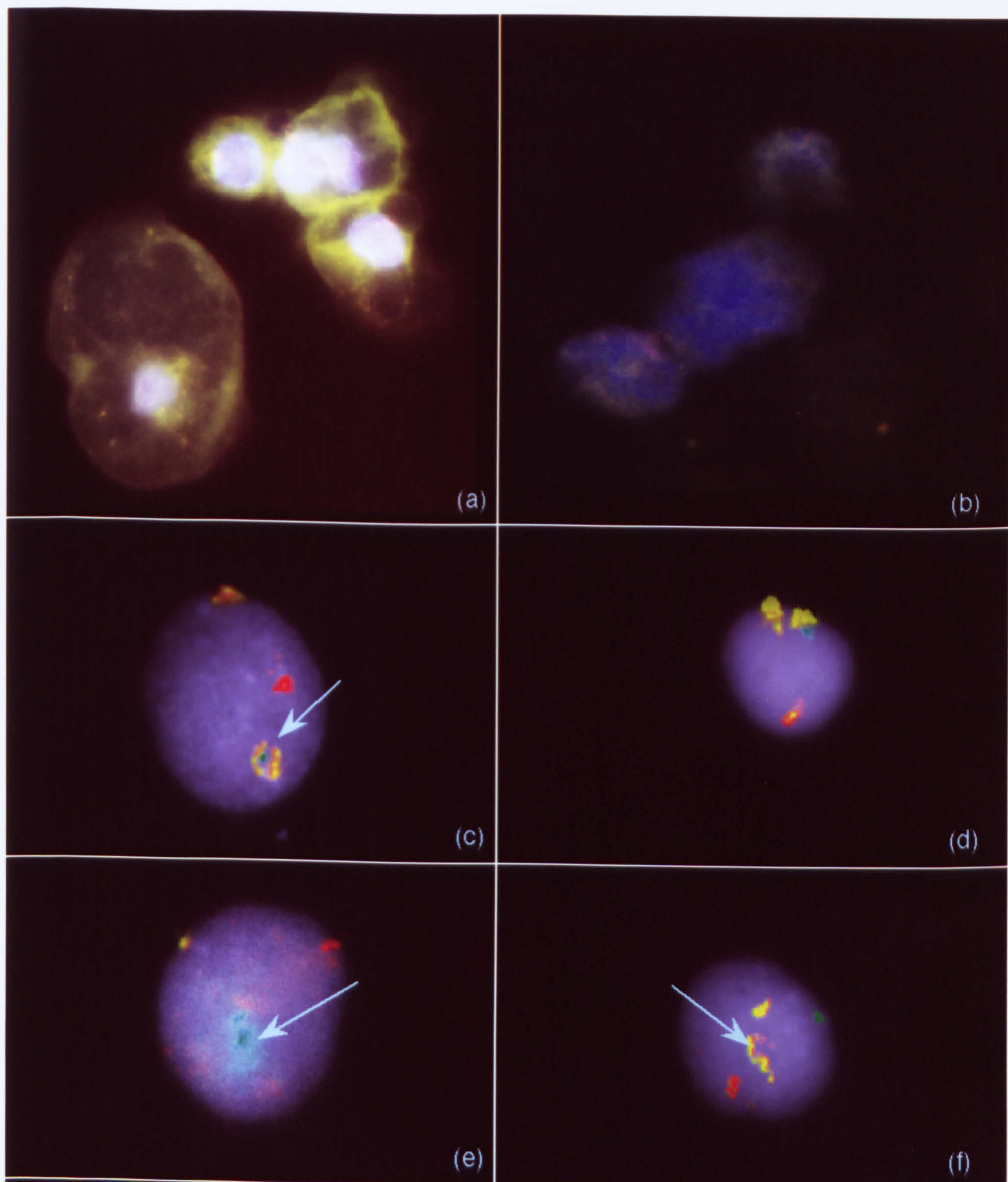


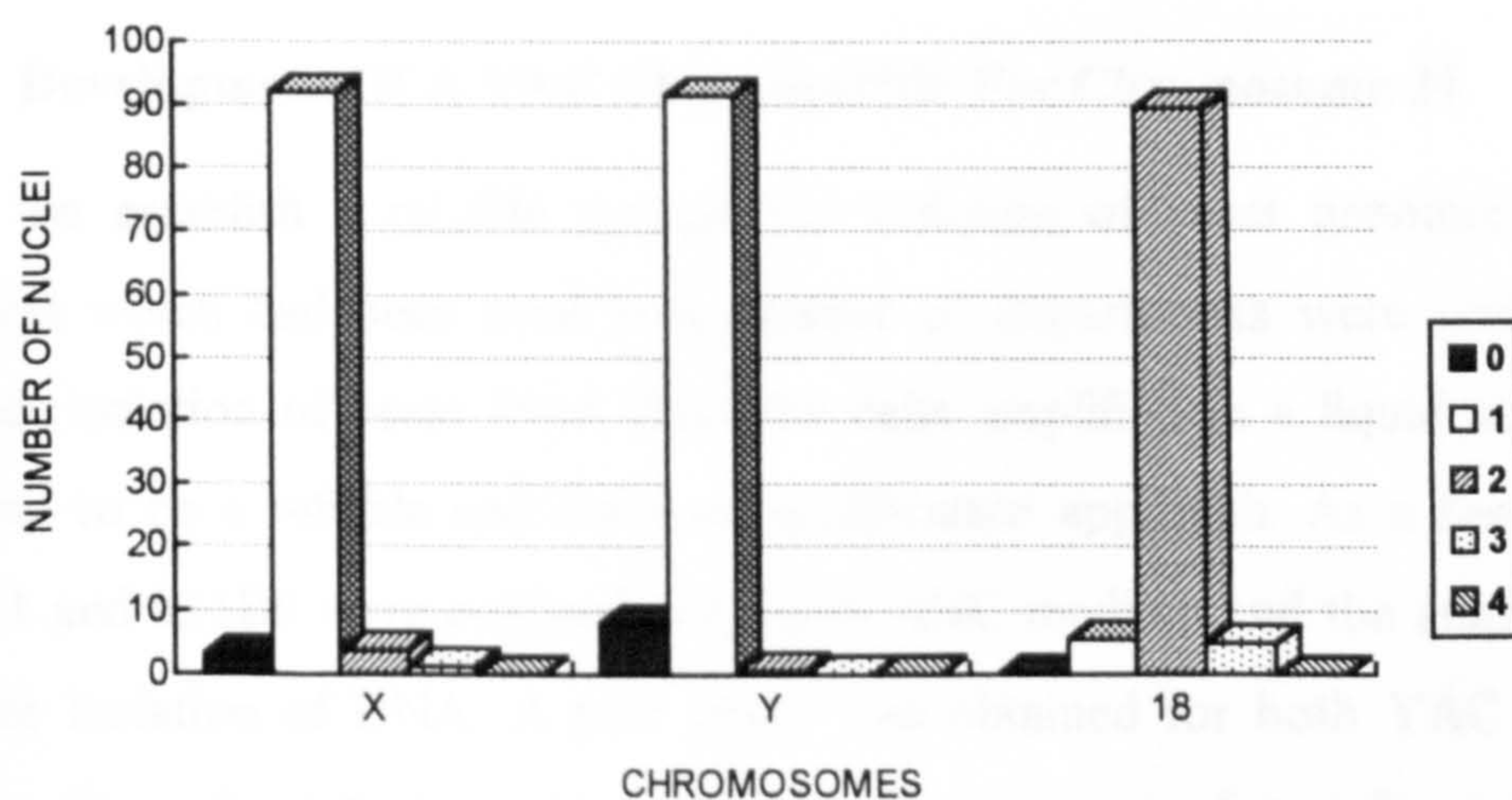
Figure 3.7. Problems encountered in the simultaneous detection of probes specific for chromosomes X, Y and 18 in uncultured amniocytes. (a) Cells covered with cytoplasm; (b) degenerate cells; (c) overlapping signals specific for chromosomes Y and 18 (arrowed); (d) clumped signals; (e) out-of-focus signals (arrowed); and (f) a diffuse signal specific for chromosome 18 (arrowed).

Figs. (a-e)

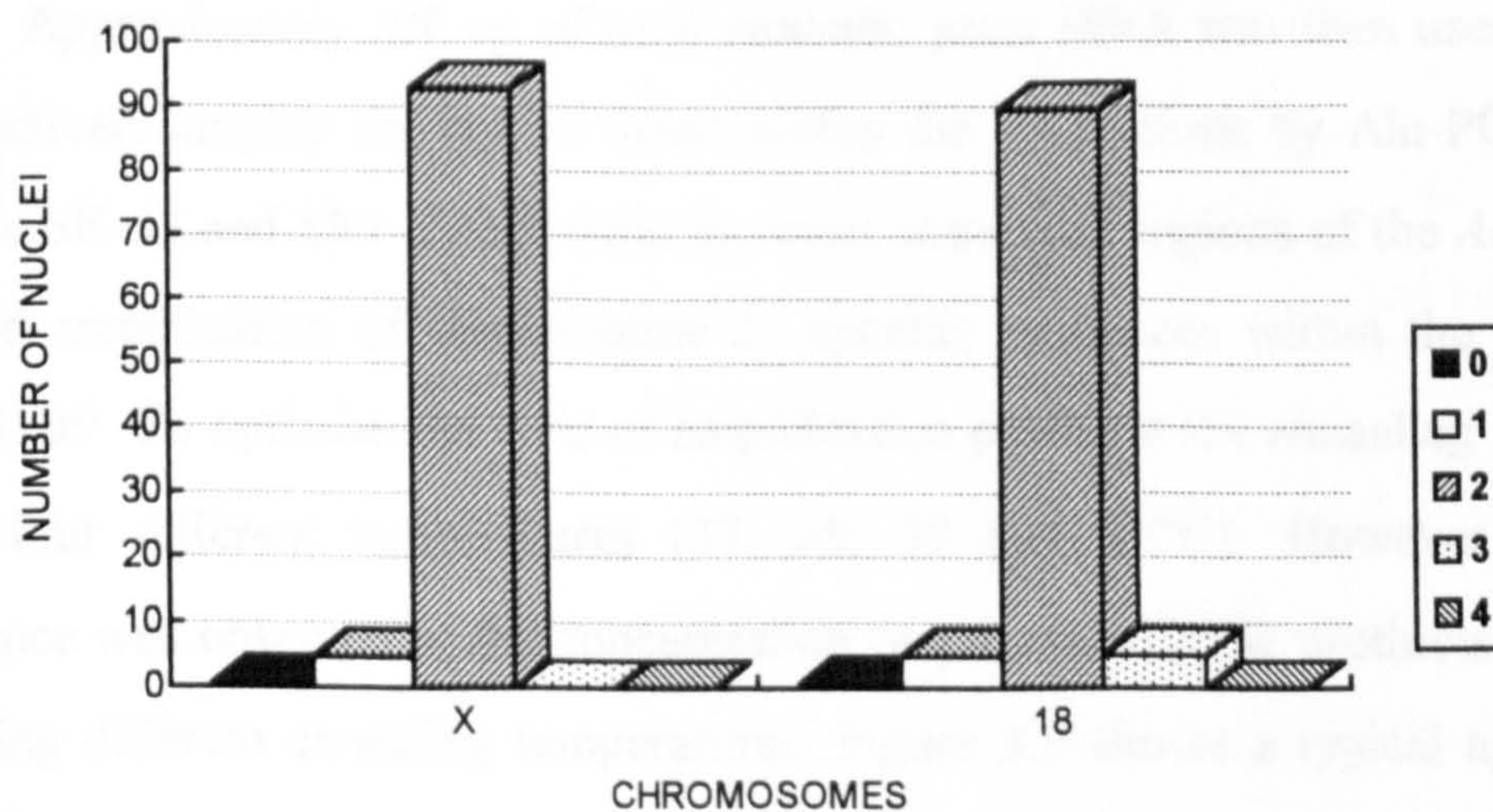
■ X ■ 18 ■ Y

Fig. (f):

■ X ■ Y ■ 18



(a)



(b)

Figure 3.8. Distribution of chromosomes X, Y and 18 specific signals in normal male (a) and female (b) uncultured amniocytes using three colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

diagrammatically illustrated in figure 3.8a and b. No abnormal amniotic fluid samples were present in this series.

3.4 Development Of A YAC Clone Specific For Chromosome 21

To establish a reliable method for isolation of yeast genomic DNA, various protocols which had been used in a number of departments were reviewed. For our purpose, isolation of yeast DNA from the cells amplified in a liquid selective medium appeared to be a reliable and more straightforward approach. As a result YAC clones 745H11 and 831B9 were cultured in a liquid AHC medium and the amplified cells were used for isolation of DNA. A pink pellet was obtained for both YAC clones after to spinning the cells at high speed, indicating the presence of transformed yeast cells by yeast artificial chromosome (YAC). The procedure used for isolation of yeast genomic DNA resulted in a high concentration of purified DNA yield (100-150µg in each reaction).

Approximately 100 ng of total genomic yeast DNA was then used as a template to selectively amplify the human insert within the YAC clone by Alu-PCR. The use of primers BK-33 and SR1 chosen from the most conserved regions of the *Alu* repeat family allowed amplification of chromosome 21 specific sequences within the YACs 745H11 and 831B9. To optimise the yield of amplification products the annealing was carried out using four different temperatures (37, 38, 39 and 40°C). However no significant difference was observed in the concentration or pattern of PCR products on the agarose gel using different annealing temperatures. Figure 3.9 shows a typical agarose gel with Alu-PCR amplified sequences obtained from the two YAC clones. A smear and visible bands ranging from 200bp to 900bp were observed when the PCR products of both YACs were fractionated on agarose gel. The bands and the smear produced by amplified 831B9 YAC were more intense than those produced by the amplified DNA of YAC 745H11. (Figure 3.9).

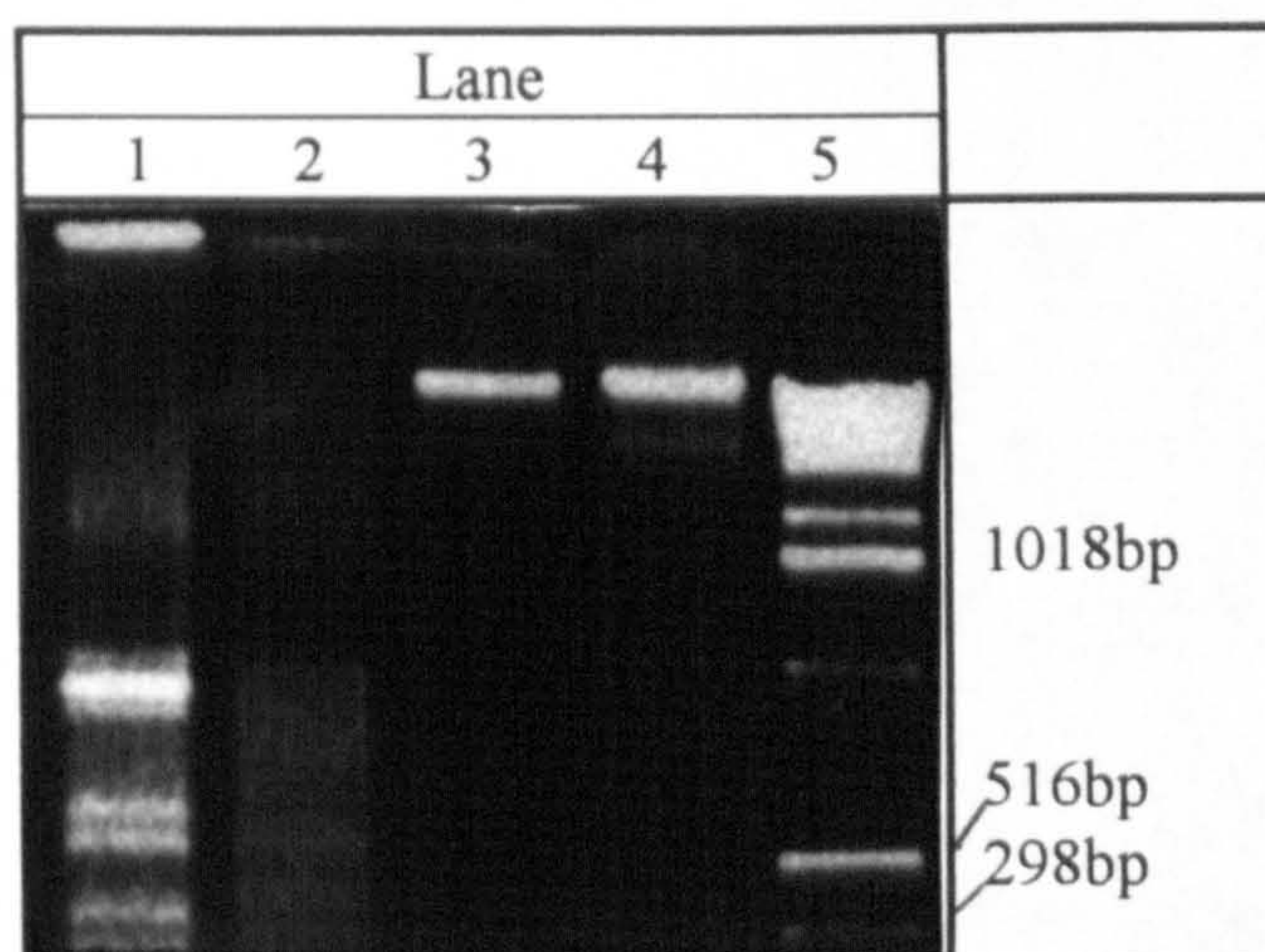


Figure 3.9 Agarose gel electrophoresis of *Alu*-PCR amplified sequences obtained from YAC clones 745H11 and 831B9. Lane 1, PCR amplified YAC 831B9; lane 2, PCR amplified YAC 745H11; lane 3, unamplified YAC 831B9; lane 4, unamplified YAC 745H11 and lane 5, 1Kb ladder.

Amplification by *Alu*-PCR as described above yielded approximately 5 to 7 μg PCR product from 100 ng of template DNA, this is sufficient to allow hybridisation to 50-70 slides. As a minimum of 100 μg yeast genomic DNA could be produced in each DNA isolation reaction, the DNA yield would be enough for 50000 hybridisation experiments. The PCR products were ethanol-precipitated and used for labelling with biotin or digoxigenin by standard nick translation protocol.

The labelled probes were then hybridised to cultured and uncultured lymphocytes using a chromosome *in situ* suppression (CISS) hybridisation protocol. Hybridisation and detection conditions employed in this series of experiments were the same as described for uni-colour FISH with alpha satellite DNA probes. The repetitive sequences such as *Alu* sequences within the probes were suppressed by preannealing the denatured probe DNA with sonicated human placental DNA as competitor. Various concentrations of probe and competitor DNA were investigated to achieve intense signals specific for chromosome 21 with little background. Figure 3.10a demonstrates a cultured lymphocyte from a normal individual and figure 3.10b a cell from an individual with trisomy 21

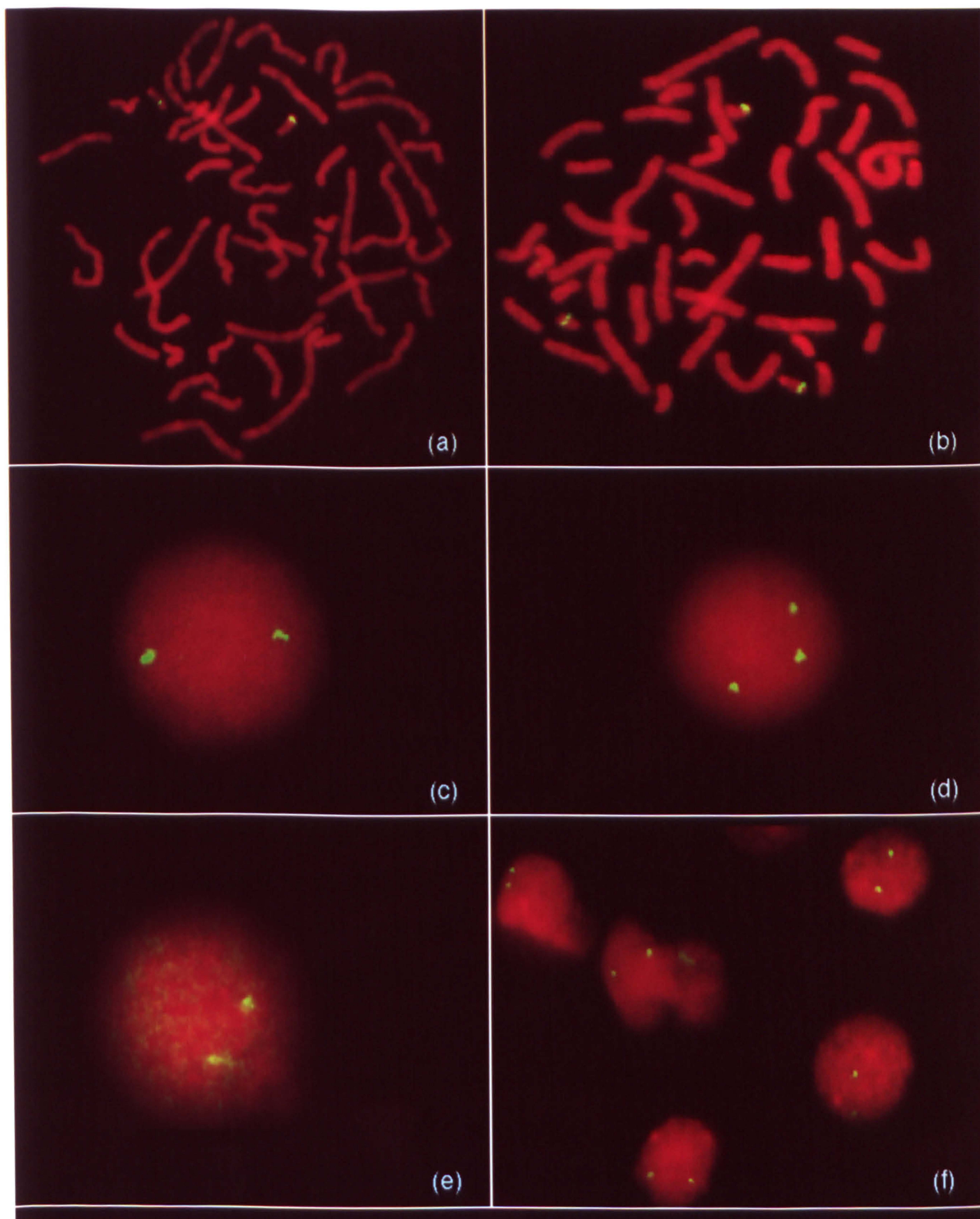


Figure 3.10. Hybridisation of YACs 831B9 and 745H11 to lymphocytes and amniocytes. (a) A normal cultured lymphocyte; (b) a trisomy 21 cultured lymphocyte; (c) and (e) normal uncultured lymphocytes; (d) a trisomy 21 uncultured lymphocyte; and (f) normal uncultured amniocyte. (a), (b), (c), (d) and (f) illustrate signals specific for 831B9 and (e) shows signals specific for 745H11.

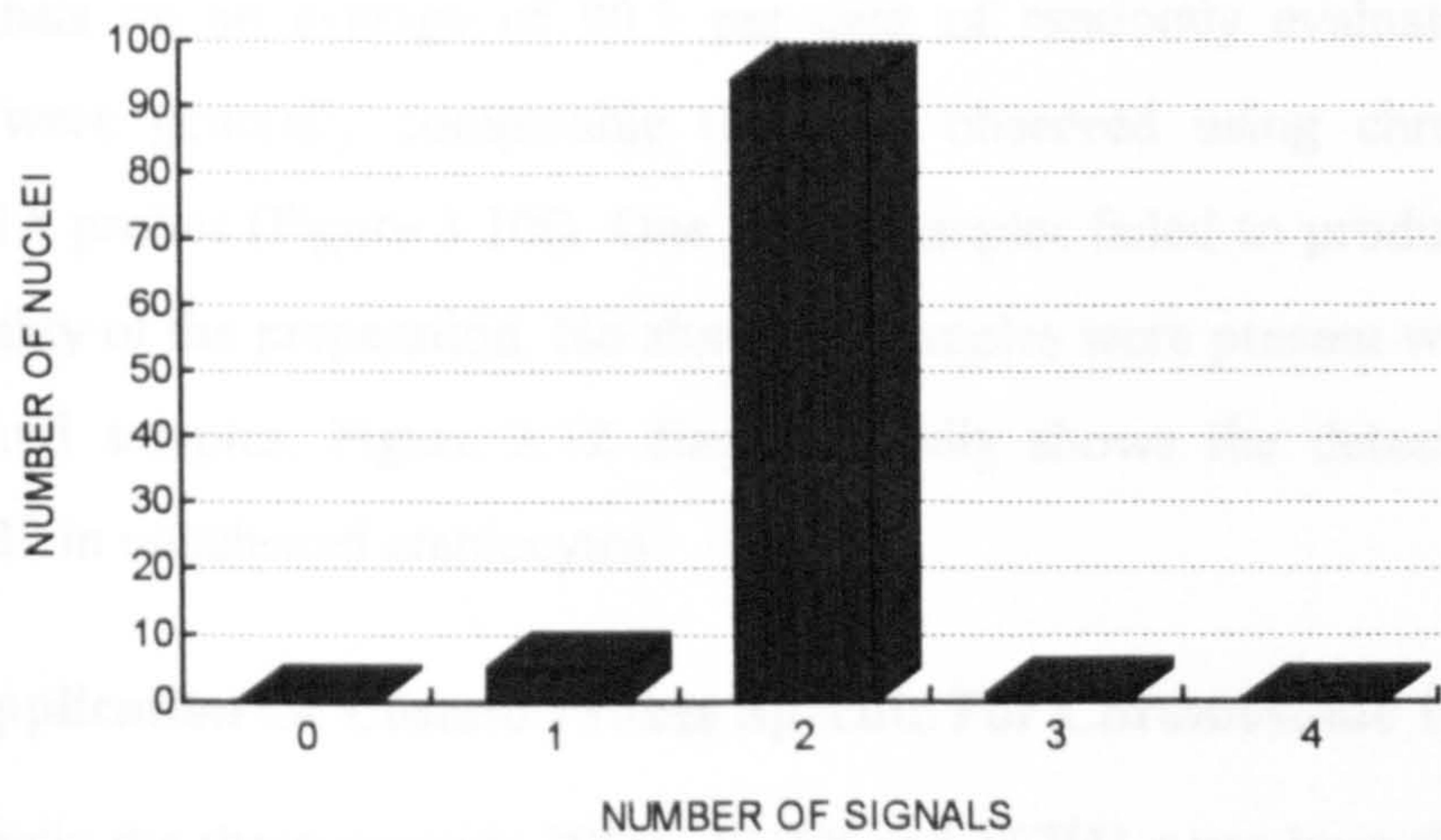
hybridised with probe 831B9. In all experiments carried out using probe 831B9, strong signals were observed on both chromatids of chromosome 21 at the expected locus on the long arm (21q22).

To detect the copy number of chromosome 21 in interphase nuclei, the probe was initially applied to a number of normal uncultured lymphocytes. Two distinct signals with minimum of background were observed in most of the nuclei hybridised to Alu-PCR amplified YAC 831B9. The probe was then hybridised to a series of twenty uncultured lymphocytes and studied in a blind fashion. Seventeen samples were correctly scored as normal displaying two distinct signals specific for chromosome 21 on an average of 94 per cent of the hybridised cells (Figure 3.10c). Three samples showed three signals on an average of 87 per cent of hybridised cells and were correctly identified as trisomy 21 (Figure 3.10d). Figure 3.11a and b diagrammatically illustrates the detection efficiency of probe 831B9 on uncultured normal and abnormal lymphocytes respectively.

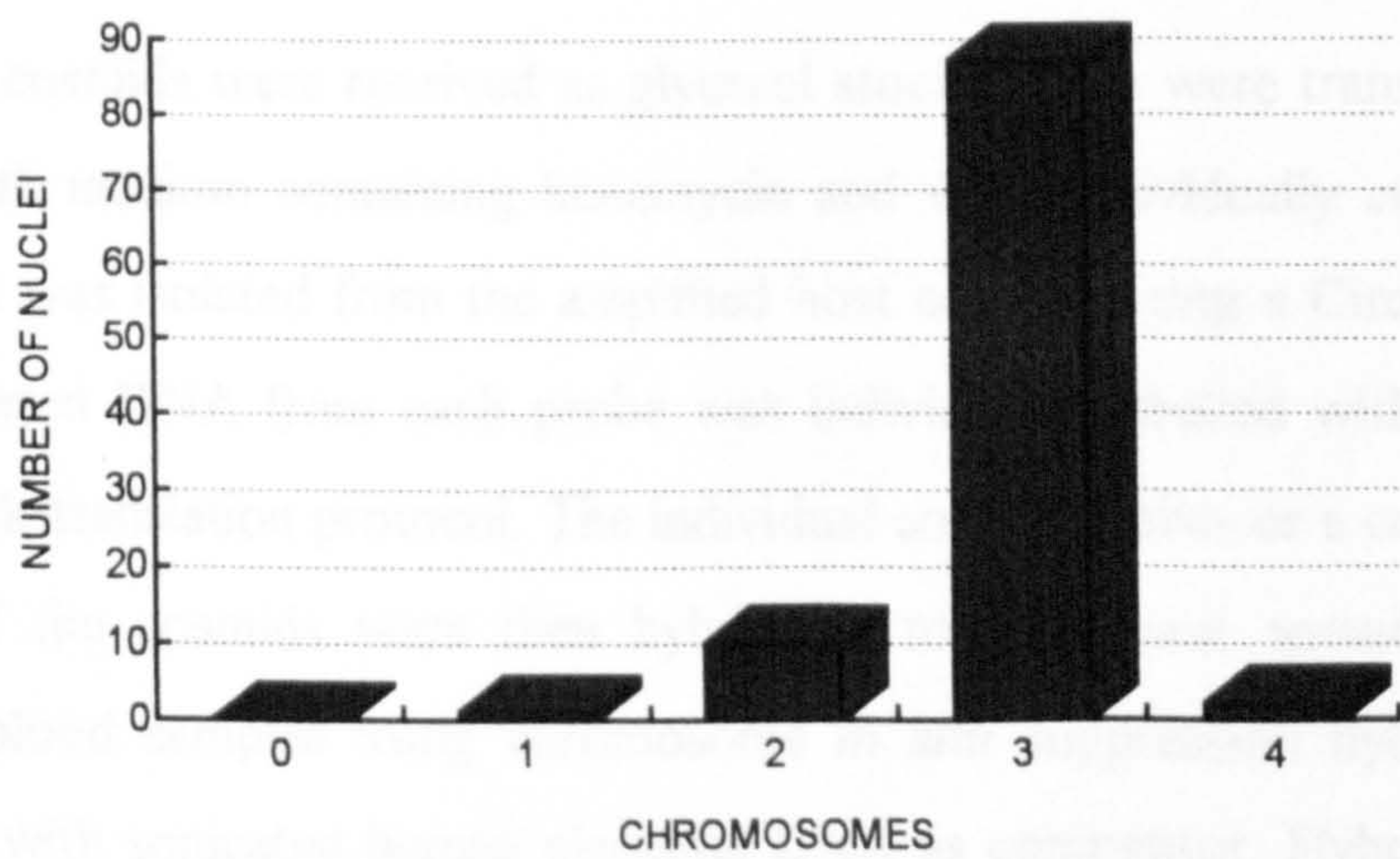
A number of uncultured lymphocytes were also hybridised with Alu-PCR amplified YAC 745H11. When these slides were compared with those hybridised with probe 831B9 using the same stringency conditions, a significantly higher level of background was observed. This led to difficulties in determining the number of true signals present in the interphase nuclei, and the probe was considered unsuitable for this purpose. No further work was undertaken using this probe. Figure 3.10e shows an interphase nucleus hybridised to the probe 745H11.

To compare the efficiency of amplified and unamplified YACs for interphase FISH analysis, the total yeast genomic DNA containing YAC 831B9 was labelled with biotin and hybridised to normal uncultured lymphocytes. No detectable signals and high background were obtained using the unamplified genomic DNA isolated from YAC clone 831B9.

When the procedure had been optimised using cultured and uncultured lymphocytes, the probe 831B9 was hybridised to an unselected series of uncultured amniocytes, to detect the copy number of chromosome 21 in interphase nuclei. Twenty-



(a)



(b)

3. 11. Distribution of signals in (a) normal and (b) trisomy 21 uncultured lymphocytes hybridised with Alu-PCR amplified YAC 831 B9 specific for chromosome 21.

three uncultured amniotic fluid preparations were hybridised with the probe and analysed using a conventional epifluorescence microscope. Twenty-two samples showed two distinct signals on an average of 90.5 per cent of randomly evaluated nuclei. Signal intensities were generally comparable to those observed using chromosome-specific alphoid DNA probes (Figure 3.10f). One of the samples failed to produce a result owing to poor quality of the preparation. No abnormal samples were present within this series of amniotic fluid samples. Figure 3.12 diagrammatically shows the detection efficiency of probe 831B9 in uncultured amniocytes.

3.5 Application Of Cosmid Probes Specific For Chromosome 13

Initially the three cosmids 29G3, 121F8 and 167H1 were investigated individually or in combination to detect the copy number of chromosome 13 in uncultured nuclei. These probes were being used for the first time, and so the conditions of probe DNA preparation and hybridisation had to be optimised to achieve signals suitable for interphase analysis.

The cosmids were received as glycerol stocks which were transferred to a liquid Terrific broth medium containing kanamycin and were individually cultured overnight. Probe DNA was isolated from the amplified host cells by using a Circleprep kit. About 1µg of isolated DNA from each probe was individually labelled with biotin using the standard nick-translation protocol. The individual cosmid probes or a combination of two or three of the cosmids were then hybridised to metaphase spreads prepared from peripheral blood samples using chromosome *in situ* suppression hybridisation (CISS) procedures with sonicated human placental DNA as competitor. Hybridisation of 29G3 cosmid to cultured and uncultured peripheral blood cells resulted in cross reactivity of the labelled probes with other chromosomes. Figure 3.13a shows a metaphase cell hybridised with cosmid 29G3. Strong non-specific signals are observed on the short arm of acrocentric chromosomes. Four to six signals were observed on most of the uncultured lymphocytes hybridised to this probe as illustrated by figure 3.13c. The signals observed

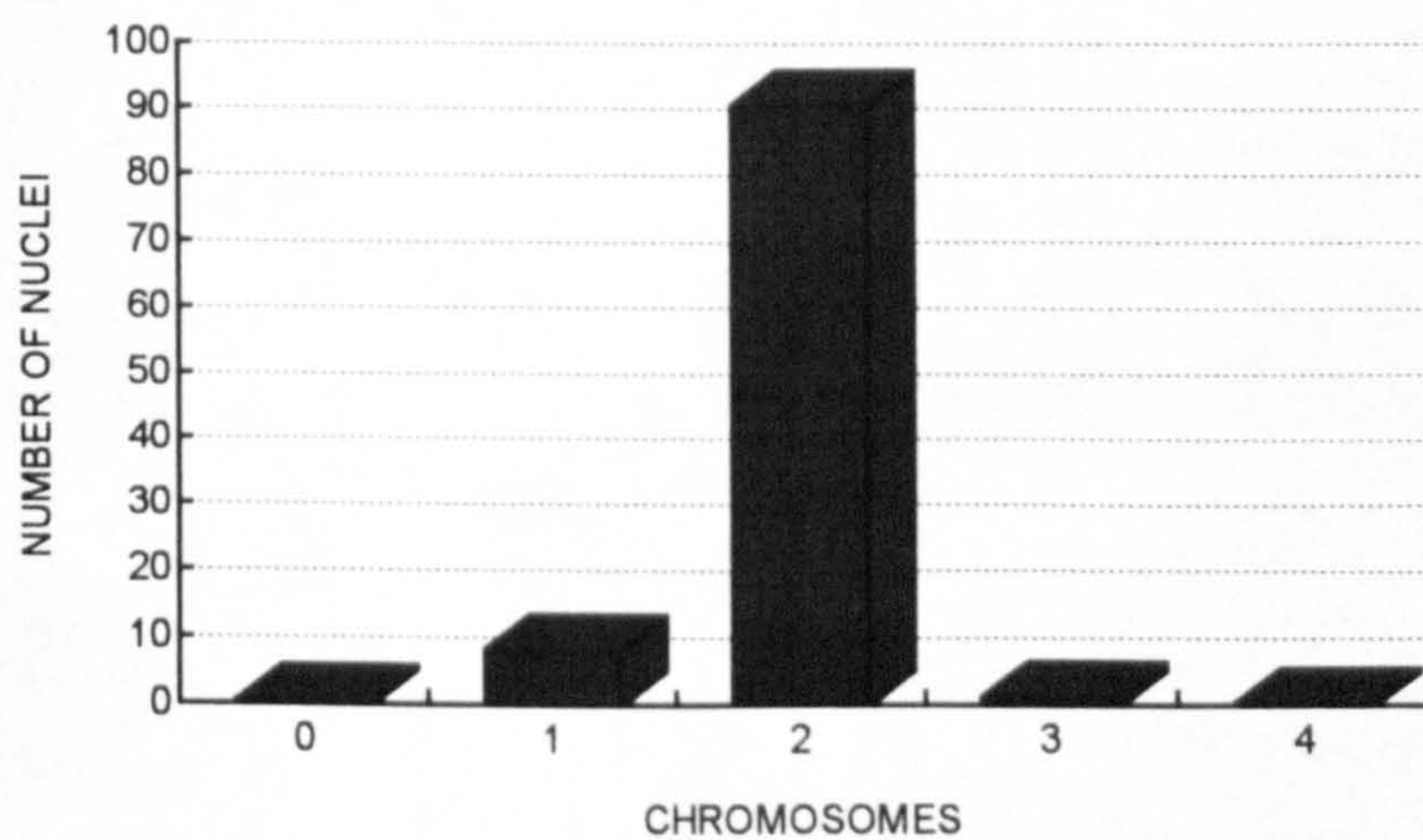


Figure 3.12. Distribution of signals in normal uncultured amniocytes hybridised with Alu-PCR amplified YAC 831 B9 specific for chromosome 21.

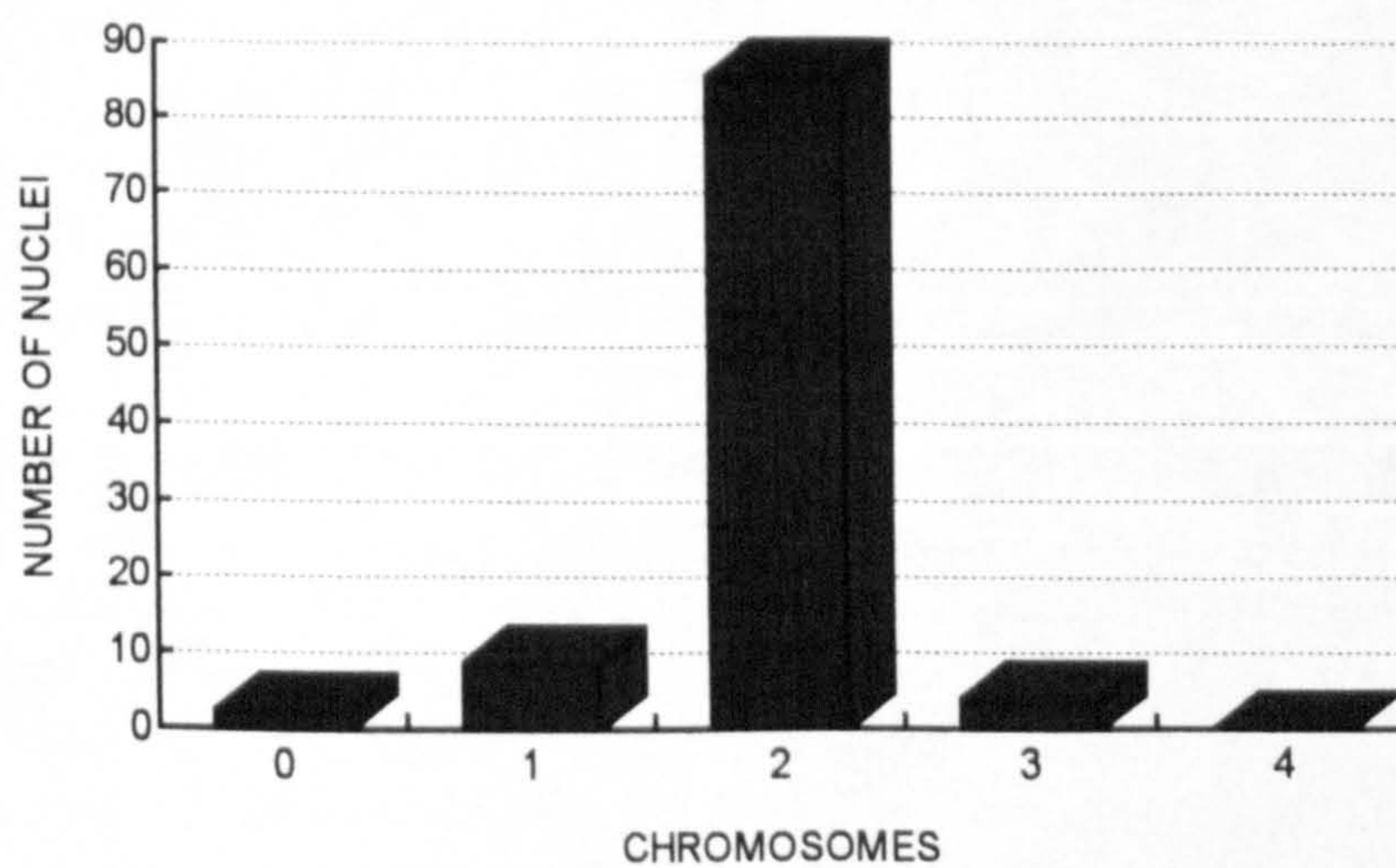


Figure 3. 15. Distribution of signals in uncultured amniocytes hybridised with chromosome 13 specific contig using uni-colour FISH.

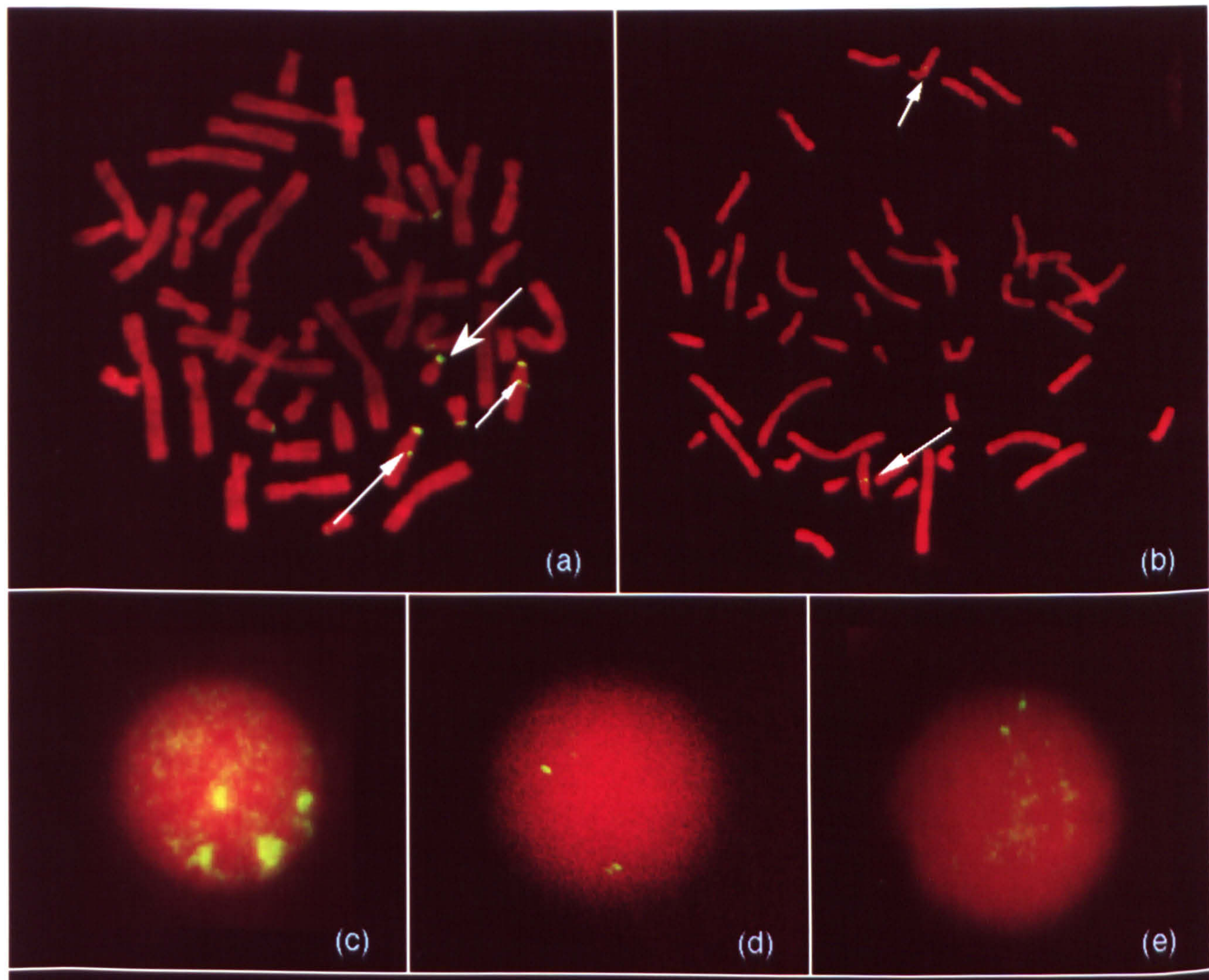


Figure 3.13. Illustration of the cross-hybridisation of one of the chromosome 13-specific cosmids with other chromosomes. (a) A metaphase spread hybridised with cosmid 29G3, showing cross-reactivity with centromeric regions of acrocentric chromosomes. Small arrows show the specific signals and the big arrow shows the cross-hybridisation; (b) a metaphase cell hybridised with both 121F8 and 167H1 cosmids. Specific signals with no cross-hybridisation are observed (small arrows); (c) an interphase nucleus hybridised with 29G3 cosmid showing cross-hybridisation with other chromosomes; (d) an interphase nucleus hybridised with cosmid 121F8; and (e) an interphase nucleus hybridised with cosmid 167H1.

on non-specific sites were stronger than those on specific targets. No cross hybridisation was observed when the cosmids 121F8 and 167H1 were hybridised either individually or in combination with each other to metaphase cells (Figure 3.13b). Hybridisation of uncultured lymphocytes with each of the cosmids 121F8 and 167H1 resulted in two signals on each nucleus (Figs 3.13d and e). However the signals were not strong enough to be easily distinguished from background fluorescence.

To overcome this problem the cosmid probes were individually cultured in agar plates containing Terrific broth. A single colony from each probe was then transferred to a liquid medium and amplified overnight. The probe DNA was then isolated from the amplified cells which had been generated from a single bacterial host cell and labelled with biotin-11-dUTP. Specific signals with no cross hybridisation were obtained when each of the three cosmids or the contig composed of the three cosmids were hybridised to normal cultured or uncultured lymphocytes (Figure 3.14a-d). The hybridisation conditions were optimised using various concentrations of probe and competitor, while the stringency conditions remained the same as used for previous probes. Specific signals with the least background fluorescence were produced using 80 ng of each probe and 5µg of competitor DNA.

Hybridisation with the individual cosmids 29G3, 121F8 and 167H1 showed two signals on an average of 75%, 69% and 71% of the hybridised normal interphase cells respectively (Figure 3.14a-c). Hybridisation with the combination of cosmids 121F8 and 167H1 showed two signals on an average of 79 per cent of hybridised cells (Figure 3.14e). An average of 87 per cent of the hybridised cells displayed two signals when the contig composed of the three cosmids was hybridised (Figure 3.14f). The signals produced by a combination of all three cosmids were intense and could be clearly detected on the long arm of chromosome 13 (13q12) or on uncultured lymphocytes using a conventional epifluorescence microscope. These experiments revealed the suitability of the cosmid contig for analysis of chromosome 13 copy number in interphase nuclei.

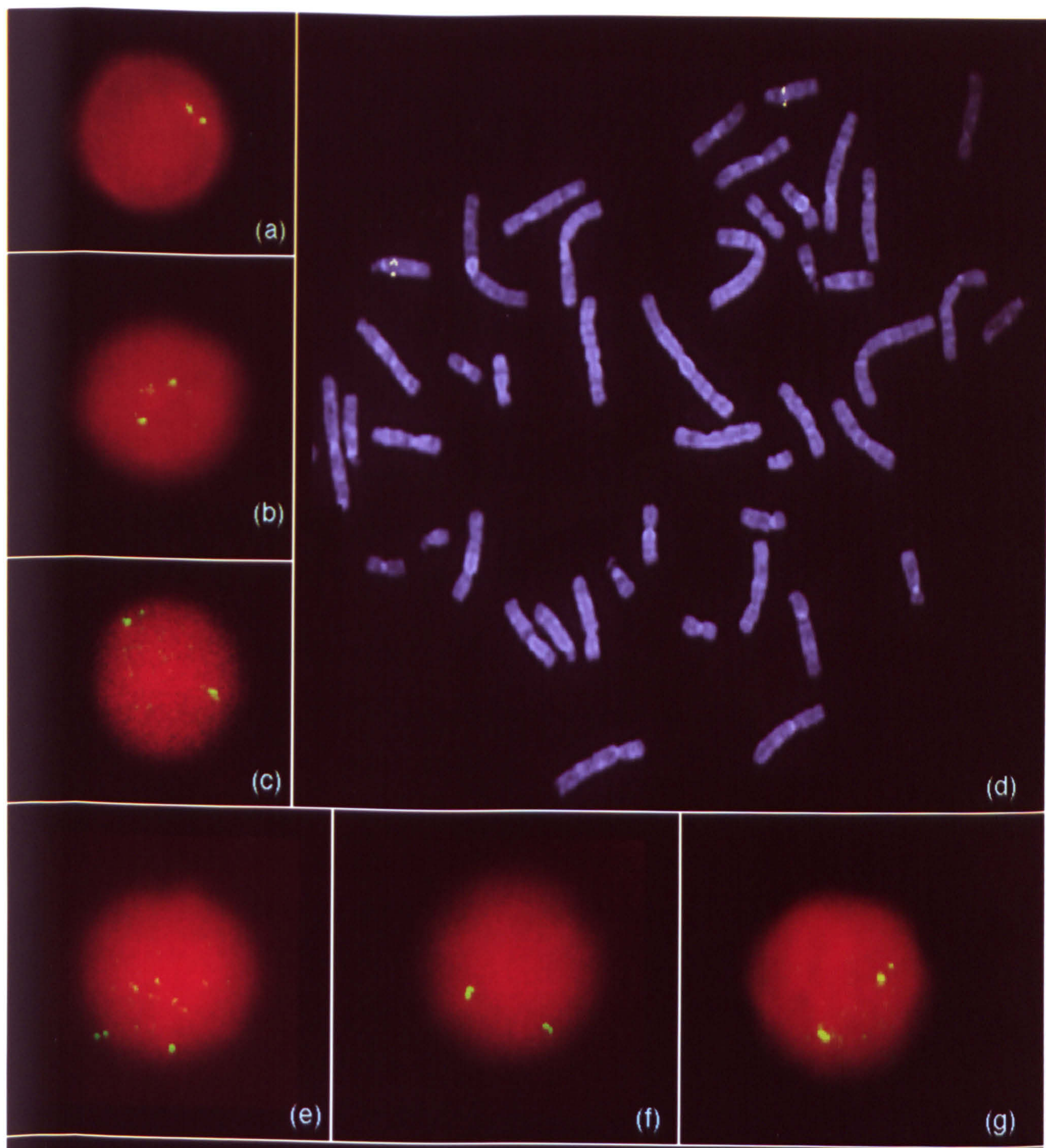


Figure 3. 14. Metaphase and interphase cells hybridised with the cosmids and the cosmid contig specific for chromosome 13. (a-c) Uncultured lymphocytes hybridised with cosmids 29G3, 121F8 and 167H1 respectively; (d) a metaphase spread hybridised with the cosmid contig composed of all three cosmids; (e) an uncultured lymphocyte simultaneously hybridised with cosmids 121F8 and 167H1; (f) an uncultured lymphocyte hybridised with the contig; and (g) an uncultured amniocyte hybridised with the contig.

For prenatal detection of chromosome 13 copy number, an unselected series of fifteen uncultured amniocytes was hybridised with the contig. The analysis was carried out by scoring a minimum of 50 nuclei for each sample. Cells covered by cytoplasm, and degenerate and clumped cells were not counted. Two of the samples could not be analysed as most of the cells were degenerate or were covered by cytoplasm. All the remaining thirteen samples were detected as normal samples and this was subsequently confirmed by the cytogenetic results. Figure 3. 14g shows an uncultured amniocyte displaying two signals specific for chromosome 13. An average of 85.5 per cent of the hybridised cells showed two signals, 2.2 per cent of the cells showed no signal, 8.5 per cent showed one signal and 3.8 per cent showed three signals. The distribution of the signals on normal uncultured amniocytes has been diagrammatically shown in figure 3. 15.

3.6 Five Colour Ratio-Mixing FISH

Development of specific probes for chromosomes 13 and 21 extended the number of simultaneously detectable probes to five. A five colour ratio-mixing FISH approach was then developed using two haptenisation and detection systems. The technique was optimised using normal uncultured lymphocytes. To maintain the morphology of interphase cell preparations the slides were treated with fixative (3:1 methanol and acetic acid) and acetone as described earlier. Each probe was labelled with biotin and digoxigenin individually. The hybridisation mixture was prepared in a total volume of 13-15 μ l using a biotin labelled probe, a digoxigenin labelled probe and a ratio mixture of biotin labelled and digoxigenin labelled of the three remaining probes. As two haptenisation systems were employed, one of the probes was visualised as red signals using rhodamine avidin as a detection system. A second probe was detected as green signals using FITC conjugated monoclonal anti-digoxin. The three remaining probes were visualised as intermediate colours using both detection systems. Various combinations of labelled probes were investigated to achieve a reliable strategy to produce distinguishable

signals to assess the ploidy of the five most important chromosomes with respect to prenatal diagnosis for aneuploidy syndromes (table 3.1).

To optimise the technique of five colour ratio-mixing FISH a biotin labelled contig specific for chromosome 13, a digoxigenin labelled 831B9, and 1:1, 3:1 and 1:3 ratio mixture of biotin labelled and digoxigenin labelled DXZ1, pDP97 and L1.84 respectively, was initially hybridised to normal male uncultured lymphocytes (table 3.1 probe combination 1). Probes specific for chromosomes 13 and 21 were hybridised using CISS hybridisation approach. No competitor DNA was required with alpha satellite DNA probes specific for chromosomes X, Y and 18. The detection procedure employed in five colour ratio-mixing FISH experiments was the same as described for dual colour hybridisation or three colour ratio-mixing FISH. Biotin labelled probes were detected with rhodamine (red) and digoxigenin labelled probes were detected with fluorescein (green). The three differently ratio-mixed probes generated signals with intermediate colours between red and green due to detection with a mixture of rhodamine and FITC.

<i>Probe combination</i>	<i>DXZ1 Biot : Dig</i>	<i>pDP97 Biot : Dig</i>	<i>13contig Biot : Dig</i>	<i>L1.84 Biot : Dig</i>	<i>831B9 Biot : Dig</i>
1	1 : 1	3 : 1	1 : 0	1 : 3	0 : 1
2	1 : 3	1 : 1	1 : 0	3 : 1	0 : 1
3	1 : 3	3 : 1	1 : 0	1 : 1	0 : 1
4	1 : 3	3 : 1	0 : 1	1 : 1	1 : 0

Table 3.1: Various combination labelled probes used to optimise the technique of five colour ratio mixing FISH. Note, Biot = Biotin and Dig = Digoxigenin.

The following strategy was pursued during analysis of the results:

- Cells were focused using DAPI excitation filter and then examined using the triple dichromic emission filter block of the CCD system. Each cell was searched for all designed signals specific for the hybridised probe sets. Three colours: red, green and yellow could be directly distinguished under the microscope. A relatively high

percentage (30-50 per cent) of the hybridised cells displayed clumped or out of focus signals which were not suitable for analysis (Figure 3.16a and b).

- The hybridised cells displaying clear and isolated signals were captured using Smartcapture programme. A number of captured images showed fewer signals (e.g. an image with more than three missing signals specific for two or more hybridised probes) and were excluded from further analysis (Figure 3.16c). The red, green and yellow signals could be readily distinguished on the captured images (Figure 3.16d). However to detect the five hybridised probes distinctly, the colour difference between the signals required to be enhanced using colour ratio software.
- The DAPI image (blue) displaying the pattern of the nucleus, was split from the signal images (green and red) using appropriate software from Smartcapture programme (Figures 3.16e-g). A comparison between green and red images could show the mixing of green and red colours to produce signals with intermediate colours. Biotin labelled probes detected with rhodamine displayed red signals and digoxigenin labelled probe detected with FITC showed green signal. No cross reactivity was observed between the red and green colours in the signals specific for the two latter probes.
- The two red and green images were merged and the background was removed using appropriate software (Figure 3.16h). The original image was kept for further comparison with the new image. In case of data loss while removing the background, the process was repeated with new settings for background remover until a satisfactory result was obtained. The signals were then pseudo-coloured using colour ratio software. Signals specific for chromosomes 13, 21, X, Y and 18 were pseudo-coloured in red, green, pink, orange and white respectively (Figure 3.16i).
- The nucleus and the signal images were finally joined using Script software (Figure 3.16j).

A relatively higher background was observed when the results obtained from this series of experiments were compared to those of three colour FISH. Various conditions

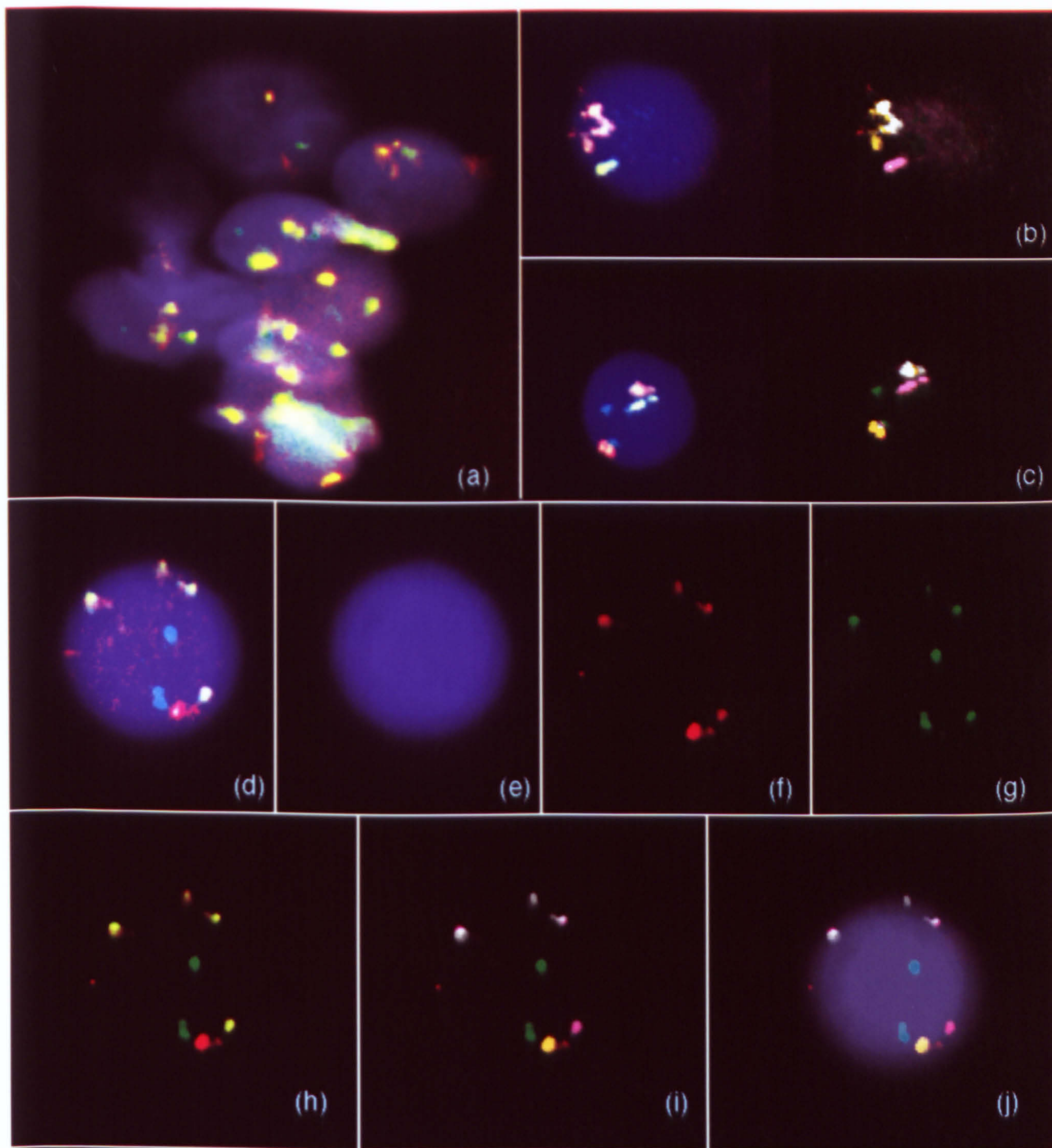


Figure 3.16. Five-colour ratio-mixing FISH using a combination of three cosmids specific for chromosome 13. (a) Clumped cells; (b, left) clumped signals; (b, right) pseudo-coloured signals in the same cell; (c) an uncultured lymphocyte displaying 5 signals out of the expected 8; (d) an uncultured lymphocyte hybridised with the probe mixture; (e) the DAPI image showing only the nucleus; (f) the rhodamine image; (g) the FITC image; (h) the merged rhodamine and FITC images; (i) pseudo-coloured signals; and (j) the final image after merging the nucleus and signals image. The figures (f-h) show the signal images after removing the background.

In fig. (j):

X
 Y
 21
 18
 13

of post-hybridisation washing were investigated and the concentrations of probe and competitor DNA were altered to decrease the background. However, the background which was predominantly caused by the contig probe specific for chromosome 13 still remained high. Signals produced by the contig were not intensive enough to be visualised under the microscope and it was not possible to distinguish true signals from background fluorescence when the signals were analysed on captured images (Figure 3.16d). The results were analysed in a limited number of normal uncultured lymphocytes by counting a minimum of 25 nuclei for each sample. Sixty five to 71 per cent of the hybridised cells which were suitable for analysis displayed two red signals specific for chromosome 13. Chromosome 21 specific YAC clone produced intensive signals in 81-86 per cent of the hybridised cells analysed and could be clearly visualised under the fluorescence microscope. The signals produced by alpha satellite DNA probes specific for chromosomes X, Y and 18 were strong and could be distinguished using the computer software. However some parts of the pseudocoloured signals specific for chromosomes X and 18 displayed the same colour indicating insufficient colour difference between these signals (Figure 3.16 i-j). Probes DXZ1 and pDP97 produced strong and distinct signals (pseudocoloured in pink and orange respectively) in 81-84 per cent and 80-85 per cent of the analysed cells respectively. The signals produced by probe L1.84 were diffuse on some cells from each sample and the detection efficiency was lower compared to the other two centromeric probes used (range 78-81 per cent).

Other combinations of labelled probes were investigated to establish a reliable method of five colour ratio-mixing FISH. Similar results were obtained when the probe combinations 2 and 3 (table 3.1) were used.

A relatively lower background was observed when the combination 4 was used (Figure 3.17a). Signals specific for chromosomes 13 and 21 (green and red respectively) could be visualised on some of the nuclei hybridised to the probe sets using a fluorescence microscope. However the intensity of the signals specific for chromosome 13 was lower than that of other signals (Figure 3.17a). The colour difference between

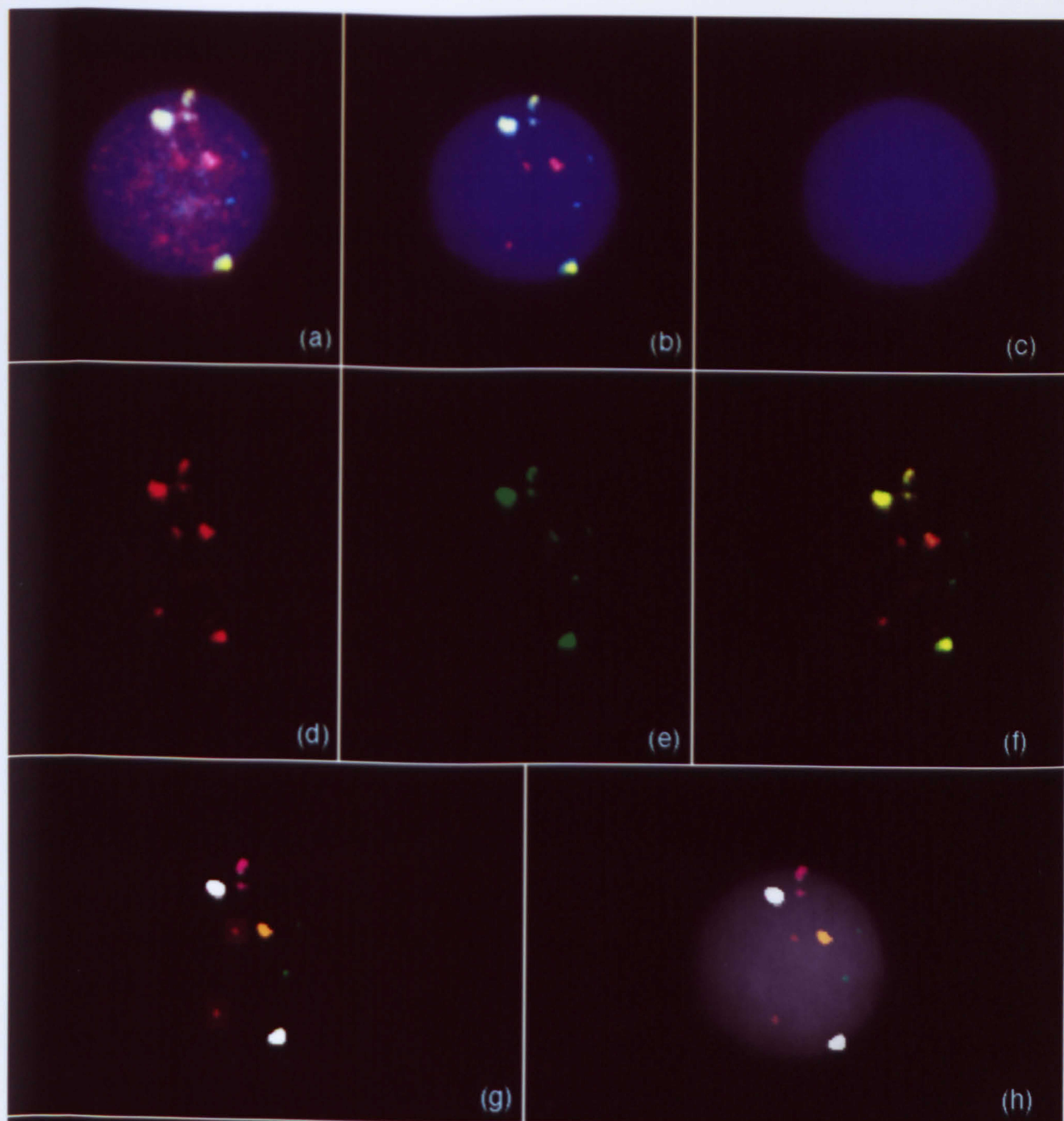


Figure 3.17. An uncultured lymphocyte hybridised with probe mixture using five-colour ratio-mixing FISH. In this experiment, biotin-labelled YAC 831B9 and digoxigenin-labelled cosmids had been used to detect chromosomes 13 and 21 respectively. (a) The original image; (b) the original image after removal of background; (c) the DAPI image showing only the nucleus; (d) the rhodamine image; (e) the FITC image; (f) the resulting signals image produced by merging rhodamine and FITC images; (g) the pseudo-coloured signals image using computer software; and (h) the final image produced by merging DAPI and pseudo-coloured signals images. ■X ■Y ■13 ■18 ■21

various signals was enough to be distinguished using appropriate software (Figure 3.17b-h). An average of 71.5 per cent, 86 per cent and 87.5 per cent of the limited number of normal uncultured lymphocytes hybridised with this probe combination displayed two signals specific for chromosomes 13, 18 and 21 respectively. The average percentage of the cells displaying one signal specific for each of the chromosomes X and Y were 90 per cent and 90.5 per cent respectively when normal male uncultured lymphocytes hybridised to the probe combination. As a result in comparison with the other strategies of probe ratio-mixing which were investigated this method proved to be more reliable.

To test the reliability of the technique for prenatal diagnostic applications the probe set was hybridised to three uncultured amniotic fluid samples. In the samples used most of the cells (more than 80 per cent) were degenerate, and did not hybridise to the probe sets (Figure 3.18a). A considerable percentage of the remaining cells were covered by cytoplasm, which caused unacceptable background and were unsuitable for signal analysis (Figure 3.18b). As the uncultured amniotic fluid cells are smaller than uncultured lymphocytes, the percentage of the cells displaying overlapping and clumped signals was higher than that observed when uncultured lymphocytes were hybridised to the probe mixture (Figure 3.18c and d). One of the samples failed to produce a result due to poor quality of the preparation. Twenty five nuclei were analysed in each of the remaining two samples by searching for the signals specific for all hybridised probe sets. The fetal sex and the number of autosomes 13, 18 and 21 was correctly identified in both samples (Figure 3.18e and f). However a low percentage of the cells showed two signals specific for chromosome 13 (16 cells in the first sample and 17 cells in the second sample within the 25 cells which were analysed for each hybridisation area) compared to the other probes used. Chromosome 21 specific YAC clone produced distinct red signals on an average of the 85.5 per cent of the cells. Probes specific for chromosomes 18, X and Y generated strong signals on most of the hybridised cells (84.5 per cent, 88.5 per cent and 88 per cent respectively) and the colour difference between the signals allowed the signals to be distinguished by appropriate computer software.

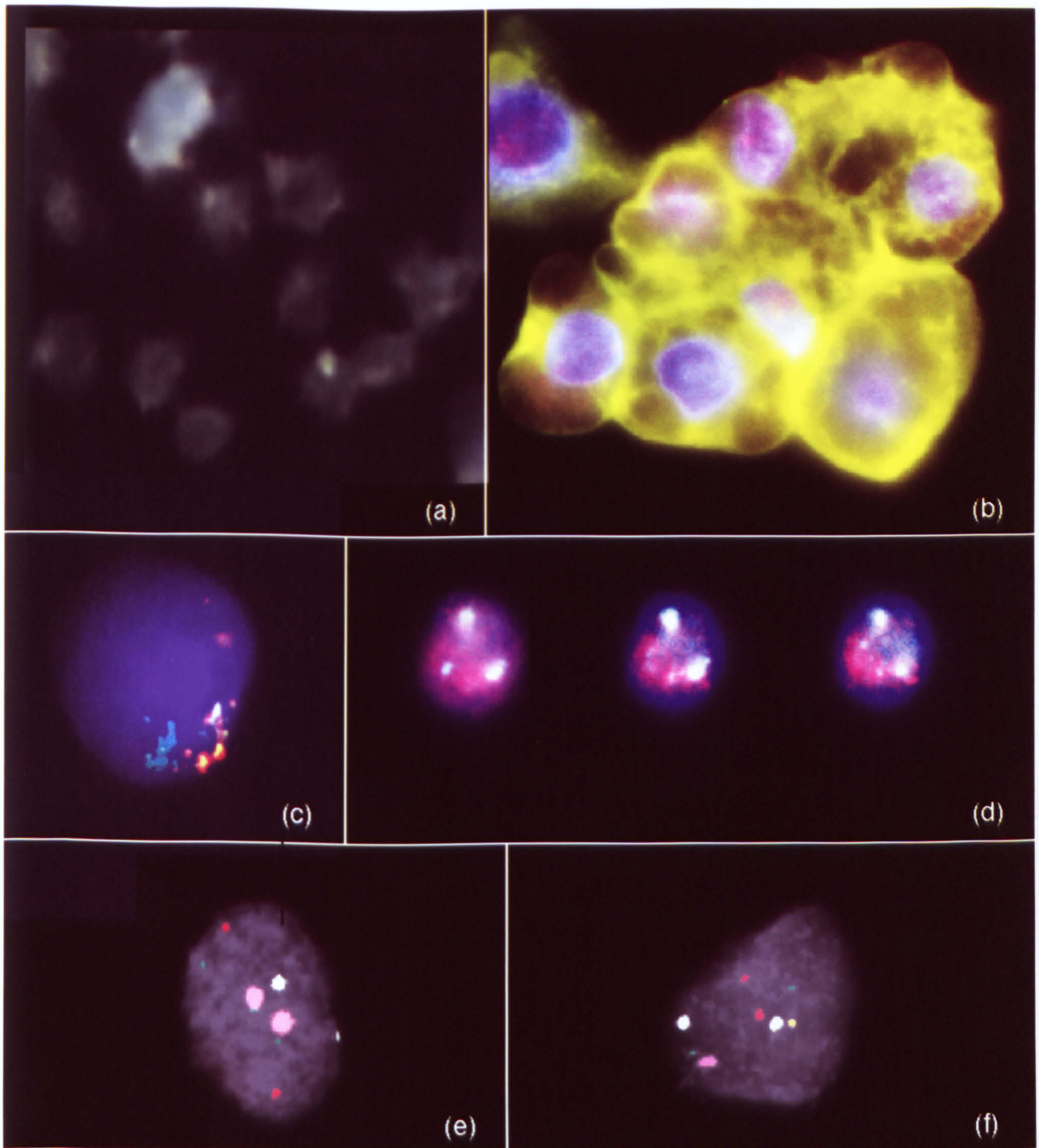


Figure 3.18. Uncultured amniotic fluid preparations simultaneously hybridised with the probes specific for chromosomes X, Y, 13, 18 and 21 using five-colour ratio-mixing FISH. (a) Degenerate cells; (b) amniocytes covered by cytoplasm; (c) clumped nuclei in an amniotic fluid cell; (d) a small amniocyte showing signals on different focal planes; (e) and (f) uncultured female and male amniocytes respectively hybridised with the combined probe set.

In figures (e) and (f): pink X yellow Y blue 13 white 18 red 21

3.7 Development Of A YAC Clone Specific For Chromosome 13

Because the cosmid contig for chromosome 13 proved unsatisfactory for five colour FISH, the YAC 744D11 was obtained and developed as an alternative probe to study the copy number of chromosome 13 in interphase nuclei. The yeast cells containing YAC were cultured in AHC medium and the genomic DNA was isolated from amplified yeast cells according to the procedures described for YAC clones specific for chromosome 21. A high concentration of yeast genomic DNA was achieved (0.9-1.2 mg/ml) using the procedure described earlier. The chromosome 13 specific sequences within the YAC 744D11 were then selectively amplified by Alu-PCR using 100 ng of isolated DNA as template. The primers and conditions used for the Alu-PCR reaction were the same as those used for amplification of chromosome 21 specific sequences within the YACs 745H11 and 831B9. The result of PCR reaction was analysed by fractionation of PCR product on a 1.3 per cent agarose gel in 1×TBE buffer. Figure 3.19 shows an agarose gel with *Alu*-PCR sequences obtained from YAC clone 744D11.

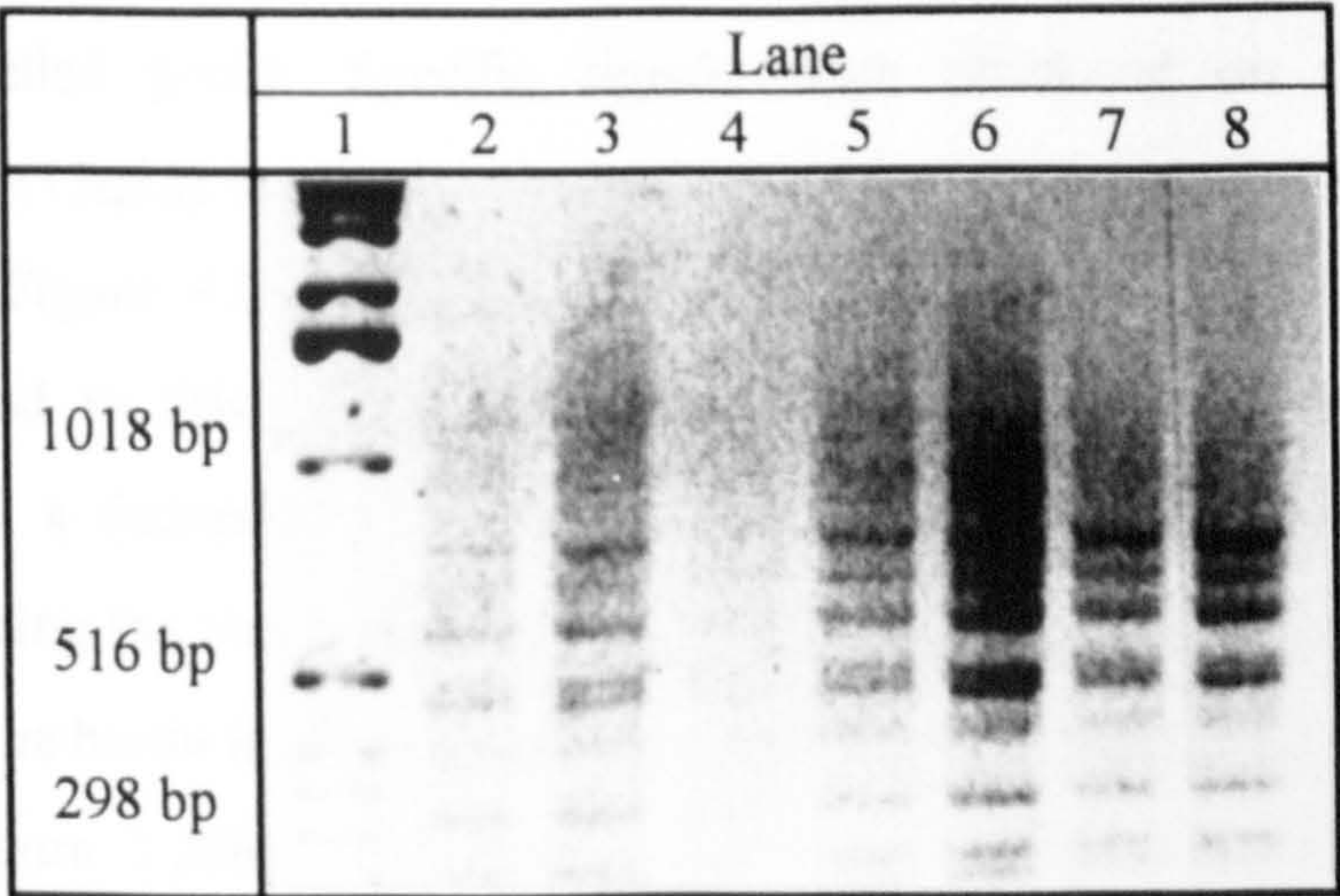


Figure 3.19. Agarose gel electrophoresis of Alu-PCR amplified sequences obtained from YAC 744D11. Lane 1, 1Kb ladder; lanes 2-8, Alu-PCR amplified YAC 744D11.

A smear and visible bands ranging from 150 bp to 900 bp were observed when the gel was visualised on a UV light box. The size and intensity of the individual bands and the smear were highly reproducible. PCR products were ethanol precipitated,

resuspended in deionised water and used for nick translation with biotin-11-dUTP or digoxigenin-11-dUTP.

The labelled probe was initially hybridised to normal metaphase preparations from peripheral blood using CISS hybridisation protocols as described earlier. The biotin labelled YAC 744D11 was detected with avidin conjugated with FITC. The intensity of signals was enhanced using biotin labelled anti-avidin as a second layer and fluorescein conjugated avidin as a third layer. Digoxigenin labelled probe was detected with antidigoxigenin fluorescein fab fragments prepared in sheep. The intensity of signals was enhanced using fluorescein anti-sheep as a second layer. Monoclonal anti-digoxin prepared in mouse was also used as an alternative system to detect the digoxigenin labelled probe. The enhancement of the signals was carried out using fluorescein conjugated anti-mouse anti-body.

To produce specific signals and a minimum of background fluorescence, various concentrations of probe and competitor DNA were investigated using biotin labelled or digoxigenin labelled probe. Specific signals were produced on the long arm of chromosome 13 (13q12) in all of the metaphase cells hybridised with the biotin labelled YAC 744D11. Figure 3.20a shows a metaphase cell from cultured peripheral blood sample hybridised to this probe. In comparison with the cosmid contig specific for chromosome 13, a decreased background fluorescence and greater intensity of signals was produced using the biotin labelled YAC 744D11. Distinct and intensive signals were observed when the biotin labelled probe was hybridised to a number of normal uncultured lymphocytes (figure 3.20b). The signal intensity was comparable to that produced by YAC 831B9 specific for chromosome 21, although the signals produced by 744D11 YAC were smaller in size than those of 831B9 YAC. A minimum of 50 nuclei were analysed for each hybridisation. More than 86 per cent of the hybridised cells displayed two signals specific for chromosome 13.

A limited number of uncultured peripheral blood samples were hybridised with the biotin labelled and digoxigenin labelled probe and analysed in a blind fashion. An average

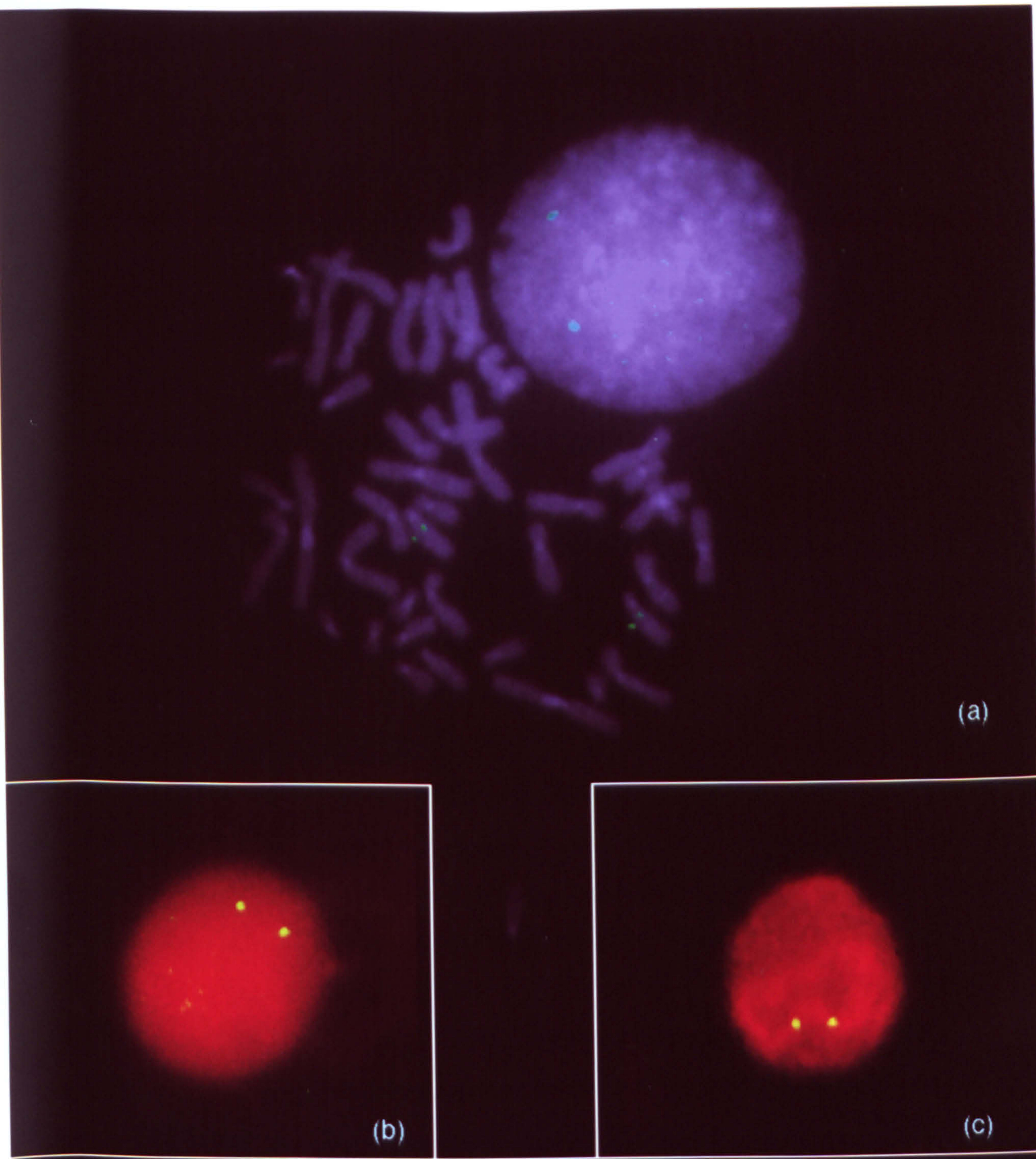


Figure 3.20. Metaphase and interphase cells hybridised with Alu-PCR amplified YAC 744D11 specific for chromosome 13. (a) A metaphase spread prepared from a peripheral blood sample; (b) an uncultured lymphocyte; and (c) an uncultured amniocyte.

of 90.5 per cent of the hybridised cells displayed two signals specific for chromosome 13. The digoxigenin labelled probe was also hybridised to a number of uncultured amniocytes. The signals observed on hybridised cells were intense and could be readily detected by direct microscopy (figure 3.20c). In the limited number of samples that were analysed, an average of 89 per cent of the cells displayed two signals specific for chromosome 13.

3.8 Five Colour Ratio-Mixing FISH Using YAC 744D11 As A Probe Specific For Chromosome 13

When conditions for using the PCR product of YAC 744D11 had been optimised by uni-colour FISH, it was substituted for the cosmid contig in five colour work. In this series of experiments a digoxigenin labelled YAC, 744D11 and a biotin labelled YAC, 831B9 were used to detect the chromosomes 13 and 21 respectively. The chromosomes X, Y and 18 were detected by 1:3, 3:1 and 1:1 ratio mixture of biotin labelled and digoxigenin labelled DXZ1, pDP97 and L1.84 probes respectively. This strategy of probe ratio-mixing allowed the five probe sets to be distinguished clearly using appropriate computer software. The method of hybridisation, post-hybridisation washing and detection used in this study was the same as described earlier. The technique was optimised using a series of normal uncultured lymphocytes. Figure 3. 21a shows a male uncultured peripheral blood cell hybridised with the combined probe set. The signals produced by digoxigenin labelled 744D11 were intense and could be visualised under the microscope. YAC 831B9 produced red signals which were greater in size than those of 744D11. The alpha satellite DNA probes specific for chromosomes X, Y and 18 produced signals with intermediate colours between green and red. The colour difference between the signals was sufficient to distinguish all the signals clearly using the colour ratio software, and to produce qualified images. In spite of the improved signals obtained for chromosome 13 a high percentage of hybridised cells remained unsuitable for analysis for the reasons discussed earlier. To localise each of the probes on specific targets the combined probe sets were hybridised to a number of metaphase cells. Figure 3.21b

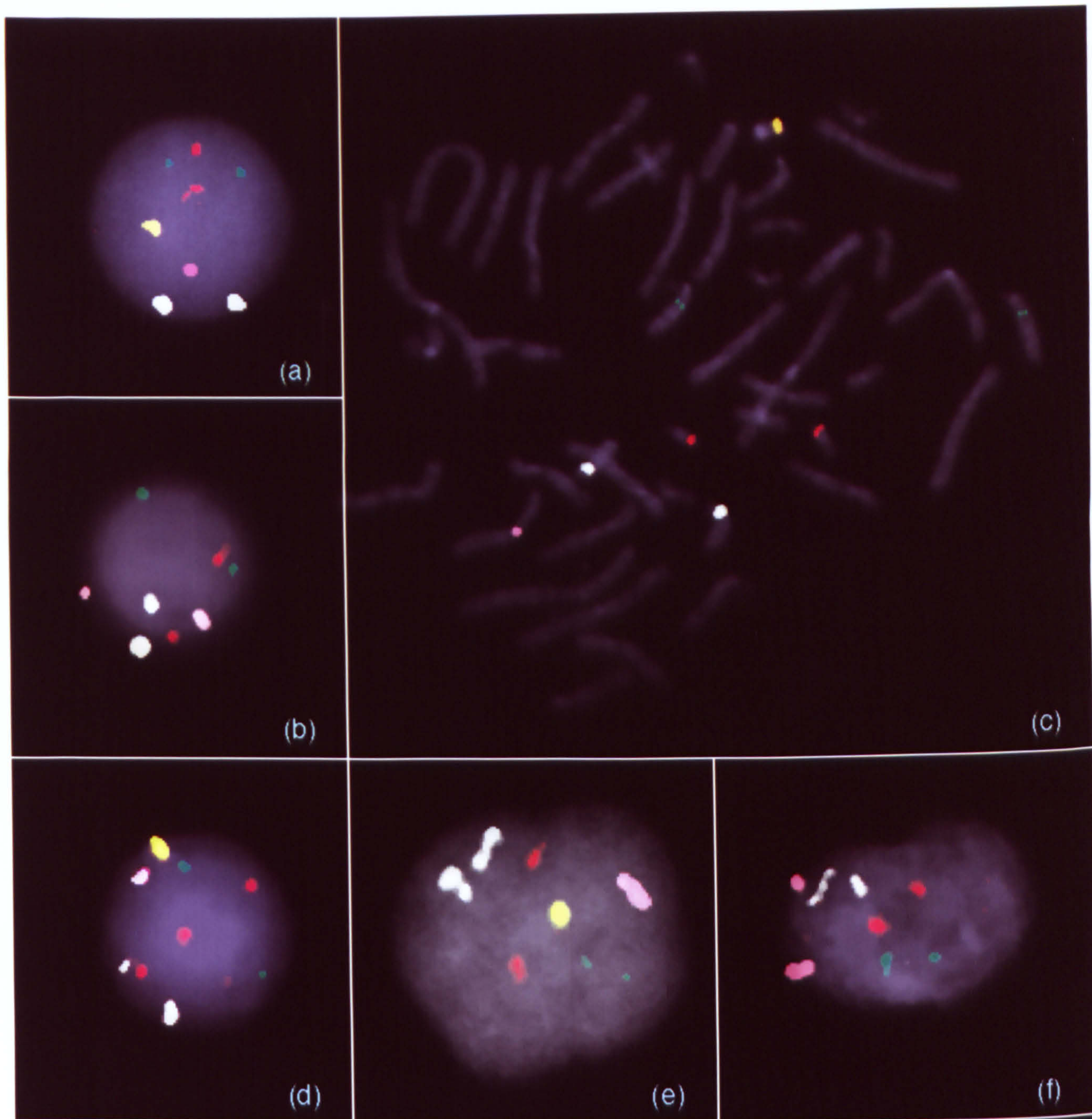


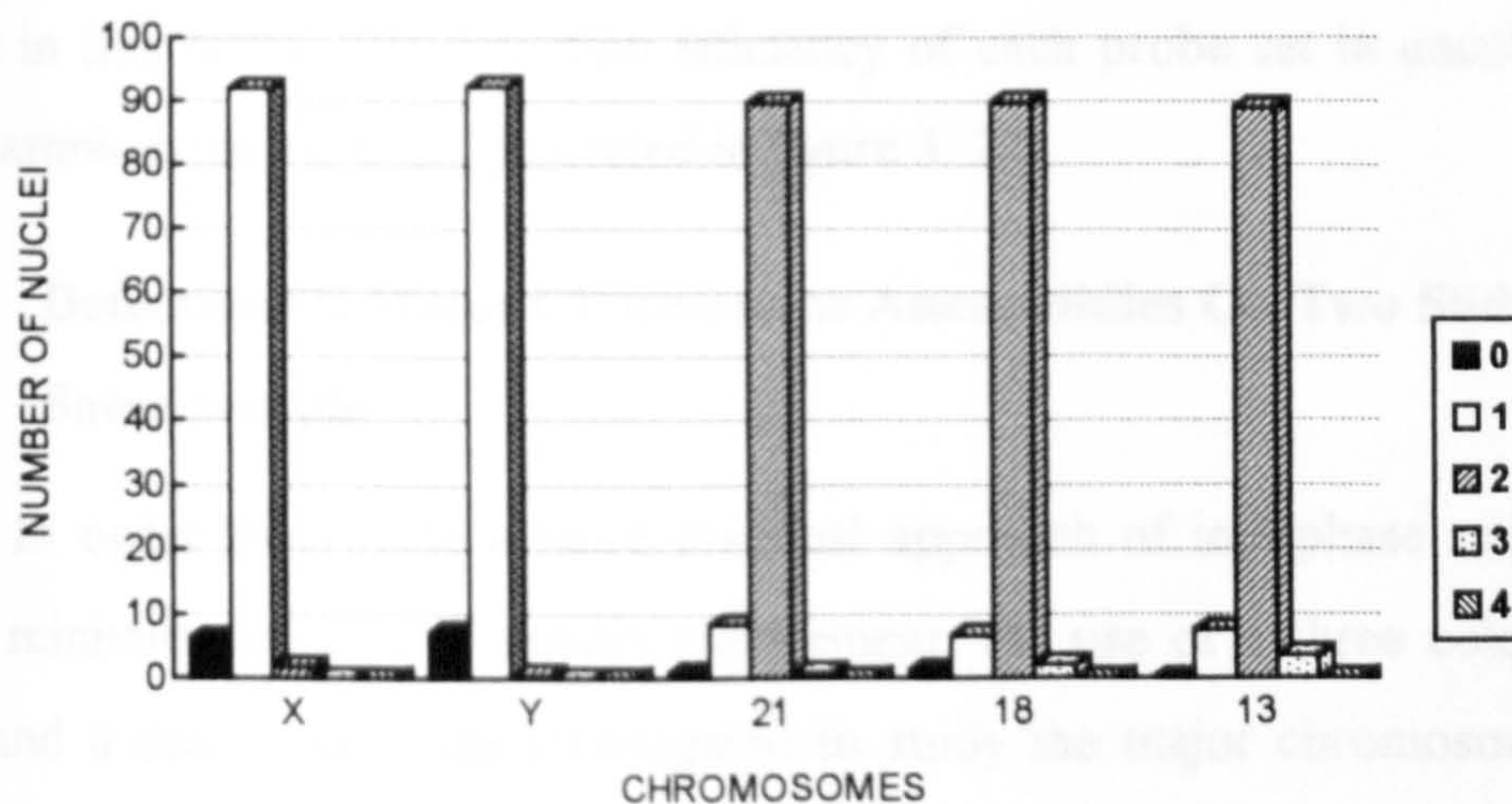
Figure 3.21. Simultaneous detection of chromosomes X, Y, 13, 18 and 21 using five-colour ratio-mixing FISH. Chromosomes X, Y and 18 have been detected with alphoid DNA repeats DXZ1, pDP97 and L1.84 respectively. Chromosomes 13 and 21 have been detected with Alu-PCR amplified YAC clones 744D11 and 831B9 respectively. (a) A normal uncultured male lymphocyte; (b) a normal uncultured female lymphocyte; (c) a normal metaphase spread prepared from peripheral blood; (d) a trisomy 21 uncultured male lymphocyte; (e) a normal uncultured male amniocyte; and (f) a normal uncultured female amniocyte. In all figs.

■ X
 ■ Y
 ■ 13
 ■ 18
 ■ 21

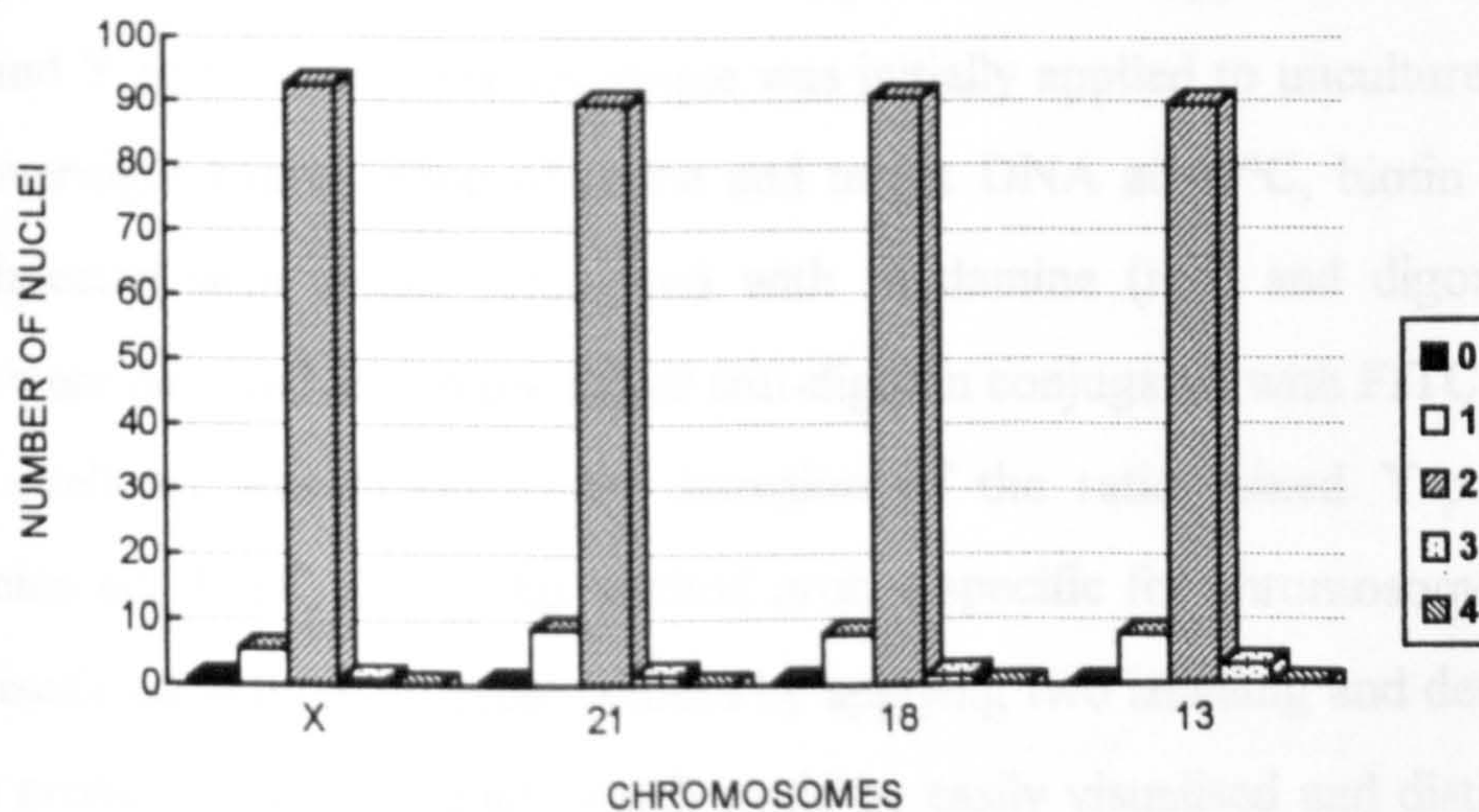
illustrates a peripheral blood metaphase cell hybridised with probe mixture. Specific green and red signals are observed on the long arm of chromosomes 13 (13q12) and 21 (21q22) respectively. The signals specific for chromosomes X, Y and 18 can be visualised in intermediate colours between red and green. These experiments clearly showed the chromosomal location of each probe in the five colour ratio-mixing FISH experiment.

Twenty uncultured lymphocyte specimens were then hybridised with the combined probe sets and analysed in a blind fashion. Twenty five nuclei were scored for each sample. Ten samples were correctly identified as normal male (Fig. 3.21a) and 8 samples were correctly identified as normal female (Fig. 3.21c). In normal male samples an average of 92 per cent of the hybridised cells displayed one signal specific for chromosome X, 92.4 per cent showed one signal for chromosome Y, 88.9 per cent showed two signals for chromosome 13, 89.8 per cent showed two signals for chromosome 18 and 89.5 per cent showed two signals for chromosome 21. In normal female samples the average percentage of the hybridised cells which displayed two signals for each of the chromosomes X, 13, 18 and 21 was 92.5 per cent 89.5 per cent, 90.5 per cent and 89.5 per cent respectively. Two trisomy 21 male samples were detected displaying three chromosome 21 specific signals on an average of 80 per cent of the hybridised cells (Fig. 3. 21d). Figure 3. 22 diagrammatically shows the detection efficiency of each probe set in five colour ratio-mixing FISH experiments on uncultured male and female lymphocytes.

The technique was then applied to 27 unselected and uncultured amniotic fluid preparations. For each sample 25 nuclei were analysed. The nuclei covered by cytoplasm, overlapping nuclei and nuclei displaying clumped signals were excluded from counting. Thirteen samples were correctly scored as normal male (Fig 3. 21e). In these samples an average of 91.7 per cent of the cells showed one signal for chromosome X, 92 per cent showed one signal for chromosome Y, 88.9 per cent showed two signals for chromosome 13, 88.3 per cent showed two signals for chromosome 18 and 85.5 per cent showed two signals for chromosome 21. Nine samples were correctly scored as normal female (Fig



(a)



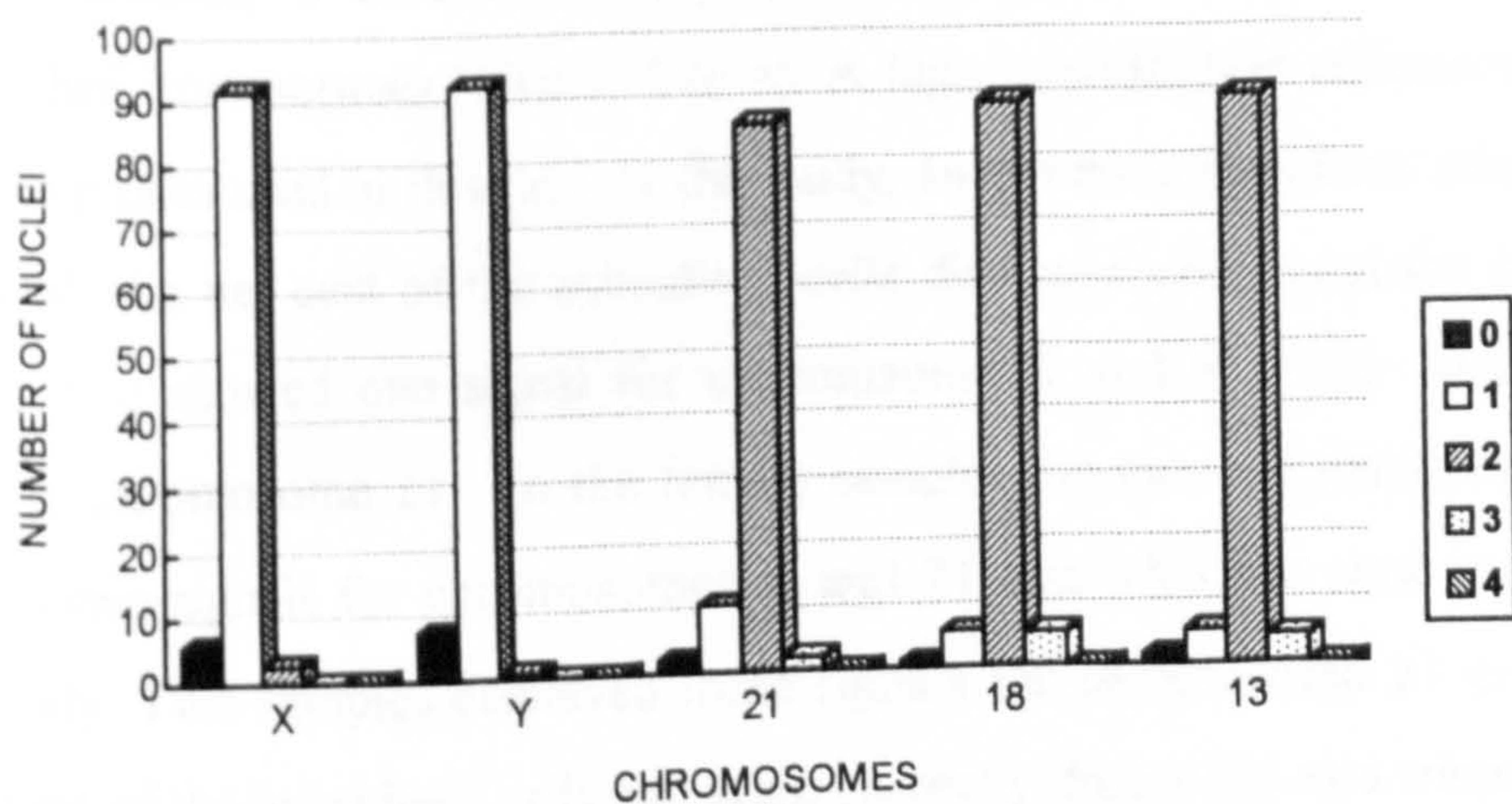
(b)

Figure 3. 22. Diagrammatic illustration of detection efficiency of probe sets specific for chromosomes X, Y, 21, 18 and 13, simultaneously hybridised to uncultured male (a) and female (b) lymphocytes using five colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

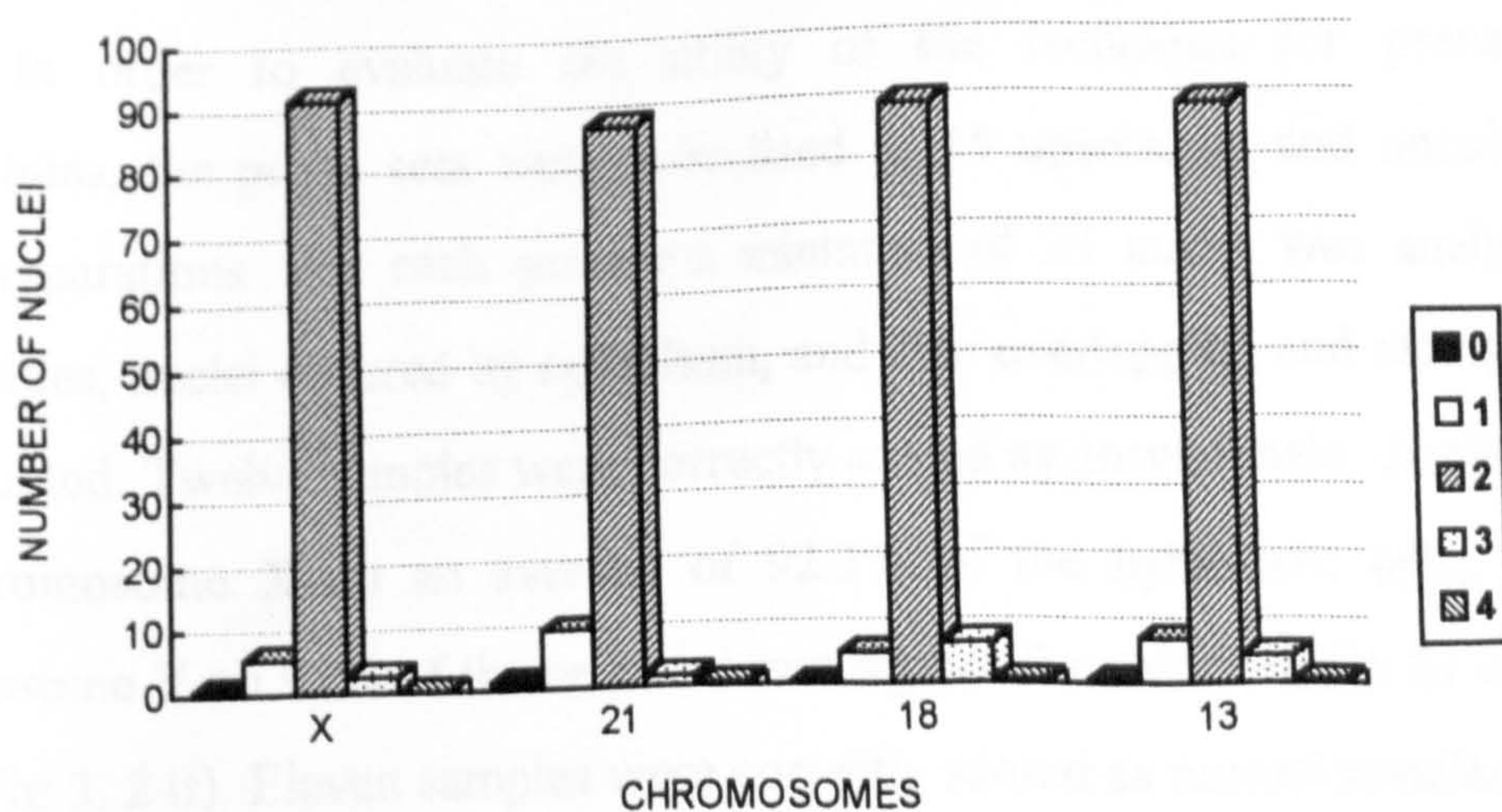
3.21f). The average percentage of the cells in normal female samples displaying two signals for each of the chromosomes X, 13, 18 and 21 was 91.5 per cent, 88 per cent, 88.4 per cent and 86.6 per cent respectively. The remaining five samples failed to produce a result due to the poor quality of the preparations. No abnormal cases were present in this series. The detection efficiency of each probe set in uncultured male and female amniocytes has been illustrated in figure 3. 23.

3.9 Detection Of Major Chromosome Aneuploidies On Two Slides From The Same Sample

In order to provide a more practical approach of interphase ratio-mixing FISH with a minimum volume of sample requirement, the use of a three colour ratio-mixing FISH and a dual colour was investigated to study the major chromosome aneuploidies using two slides from the same sample. A three colour ratio-mixing FISH had already been established to study the copy number of chromosomes X, Y and 18 in uncultured lymphocytes and amniocytes. This study was continued using biotin labelled DXZ1, digoxigenin labelled YAC 831B9 and a 1:1 ratio mixture of biotin labelled and digoxigenin labelled pDP97 to simultaneously detect the copy number of chromosomes X, 21 and Y respectively. The technique was initially applied to uncultured lymphocytes. After overnight hybridisation of probe and target DNA at 42°C, biotin labelled probes were detected with avidin conjugated with rhodamine (red) and digoxigenin labelled probes were detected with monoclonal anti-digoxin conjugated with FITC (green), a third colour (yellow) was produced by detection of the ratio mixed Y-probe with both rhodamine and FITC. Using this method probes specific for chromosomes X, Y and 21 were visualised in three different colours by applying two labelling and detection systems. All the probes produced signals which could be easily visualised and distinguished under the fluorescence microscope of the CCD system. Figure 3.24a illustrates an uncultured male lymphocyte hybridised to the probe set. To evaluate the hybridisation and detection efficiency for each of the probes the technique was applied to a number of normal uncultured lymphocytes. The results were mainly analysed by fluorescence microscopy of



(a)

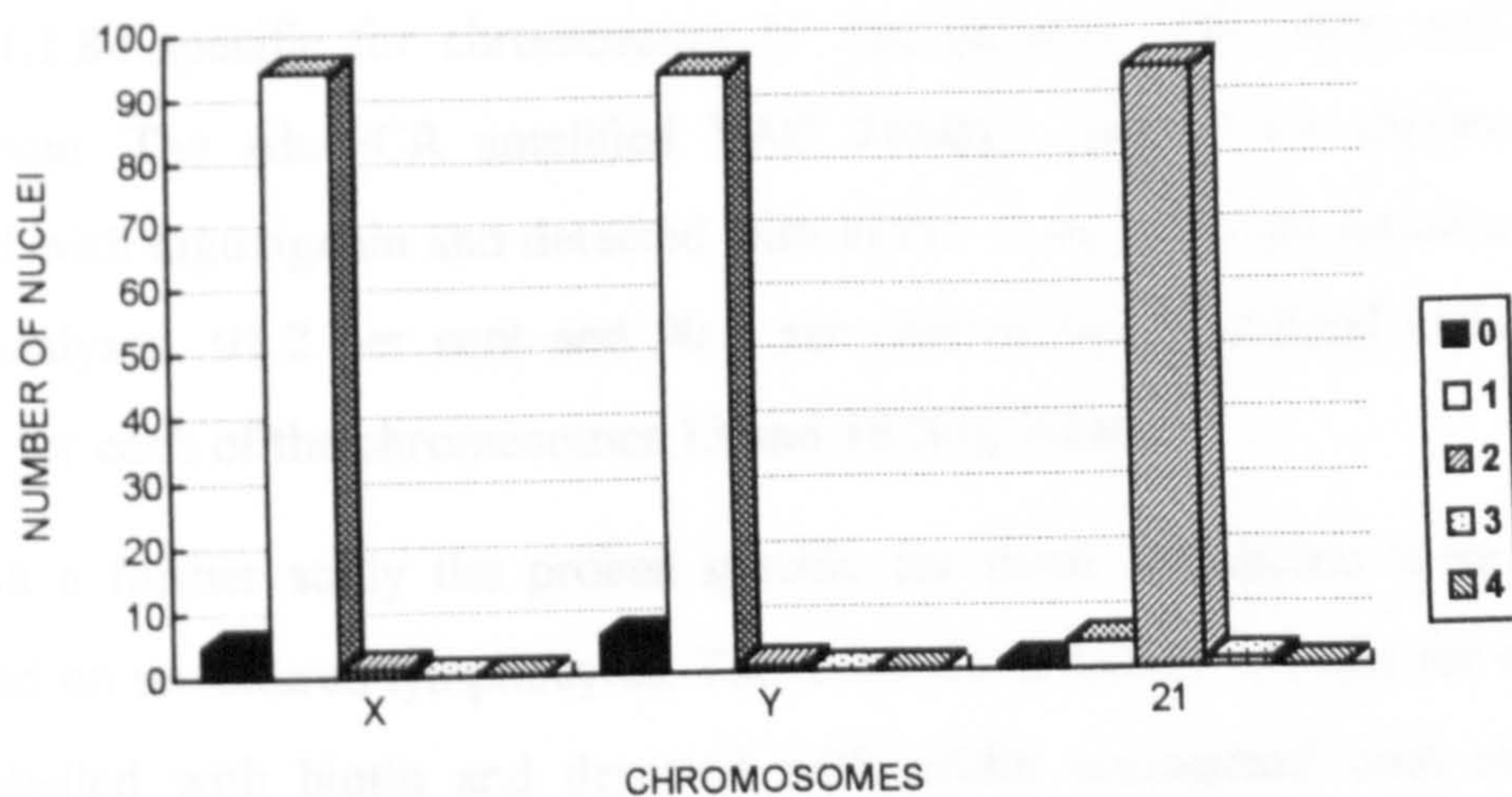


(b)

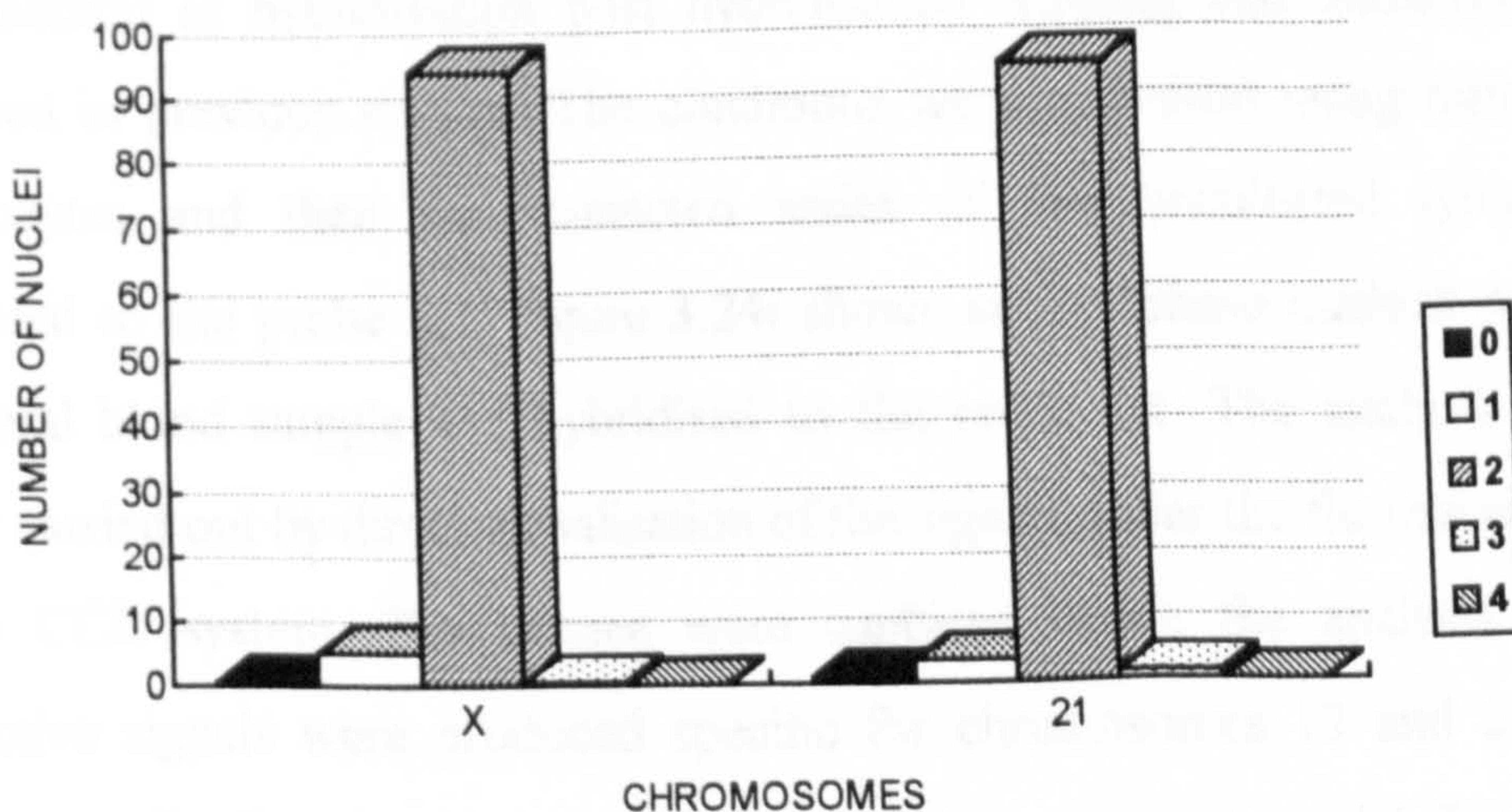
Figure 3. 23. Distribution of signals specific for chromosomes X, Y, 21, 18 and 13 in normal male (a) and female (b) uncultured amniocytes using five colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

CCD system. The images were captured where required. Twenty uncultured lymphocyte specimens were then hybridised with the combined probe sets and studied in a blind fashion. Analysis of the results was carried out by scoring 50 nuclei for each sample. Nine samples were normal male and 8 samples were normal female (figure 3. 24a and b). Three abnormal samples, a trisomy 21 male, a trisomy 21 female and an XYY were distinguished from normals (Figs 3. 24c-e). A high hybridisation efficiency was achieved for all the probes used in this part of the study. In the male peripheral blood samples, an average of 94.1 per cent of the hybridised cells displayed one signal for chromosome X, 93.3 per cent showed one signal for chromosome Y and 93.7 per cent displayed two signals for chromosome 21. In the female samples the average percentage of cells that displayed two signals for chromosomes X and 21 was 93.9 per cent and 94.4 per cent respectively. Two samples displayed three signals for chromosome 21 on an average of 84 per cent of the hybridised cells and were correctly diagnosed as a trisomy 21 male and female. A third sample was identified as XYY, as 92 per cent of the hybridised cells displayed one signal for chromosome X and 88 per cent displayed two signals for each of the chromosomes Y and 21. The detection efficiency of each probe used in this part of the study has been diagrammatically shown in figure 3. 25.

In order to evaluate the utility of the technique for prenatal diagnostic applications, the probe sets were hybridised to 25 unselected and uncultured amniotic fluid preparations. For each sample a minimum of 25 nuclei was analysed. To avoid ambiguities, nuclei covered by cytoplasm, and also overlapping and clumped nuclei were not counted. Twelve samples were correctly scored as normal male, displaying one signal for chromosome X on an average of 92.3% of the hybridised cells, one signal for chromosome Y on 92% of the cells and two signals for chromosome 21 on 89.3% of the cells (Fig 3. 24f). Eleven samples were correctly scored as normal females displaying two signals for each of the chromosomes X and 21 on 91.3% and 89.4% of the hybridised cells respectively (Fig 3. 24g). One of the samples failed to produce a result due to the poor quality of the preparation. No abnormal cases were present in this series. Figure



(a)



(b)

Figure 3.25. Distribution of the signals specific for chromosomes X, Y and 21 in normal male (a) and female (b) uncultured lymphocytes using three colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

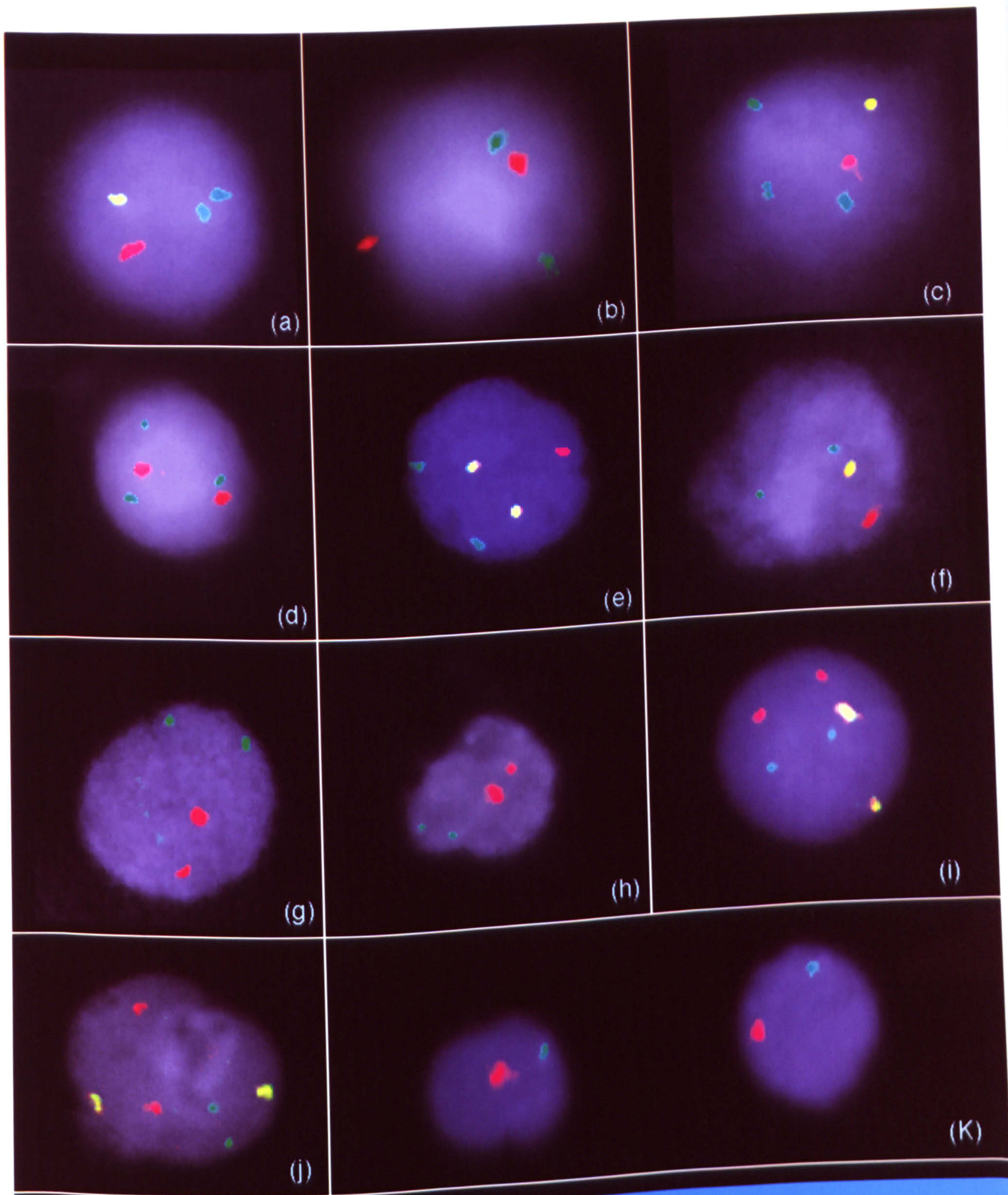


Figure 3.24. Three-colour ratio-mixing FISH using uncultured lymphocytes and amniocytes. (a) A normal male lymphocyte; (b) a normal female lymphocyte; (c) a normal female lymphocyte; (d) a trisomy 21 male lymphocyte; (e) an XYY male lymphocyte; (f) a normal male amniocyte; (g) a normal female amniocyte; (h) a normal female amniocyte; (i) a normal lymphocyte and (j and k) normal amniocytes.

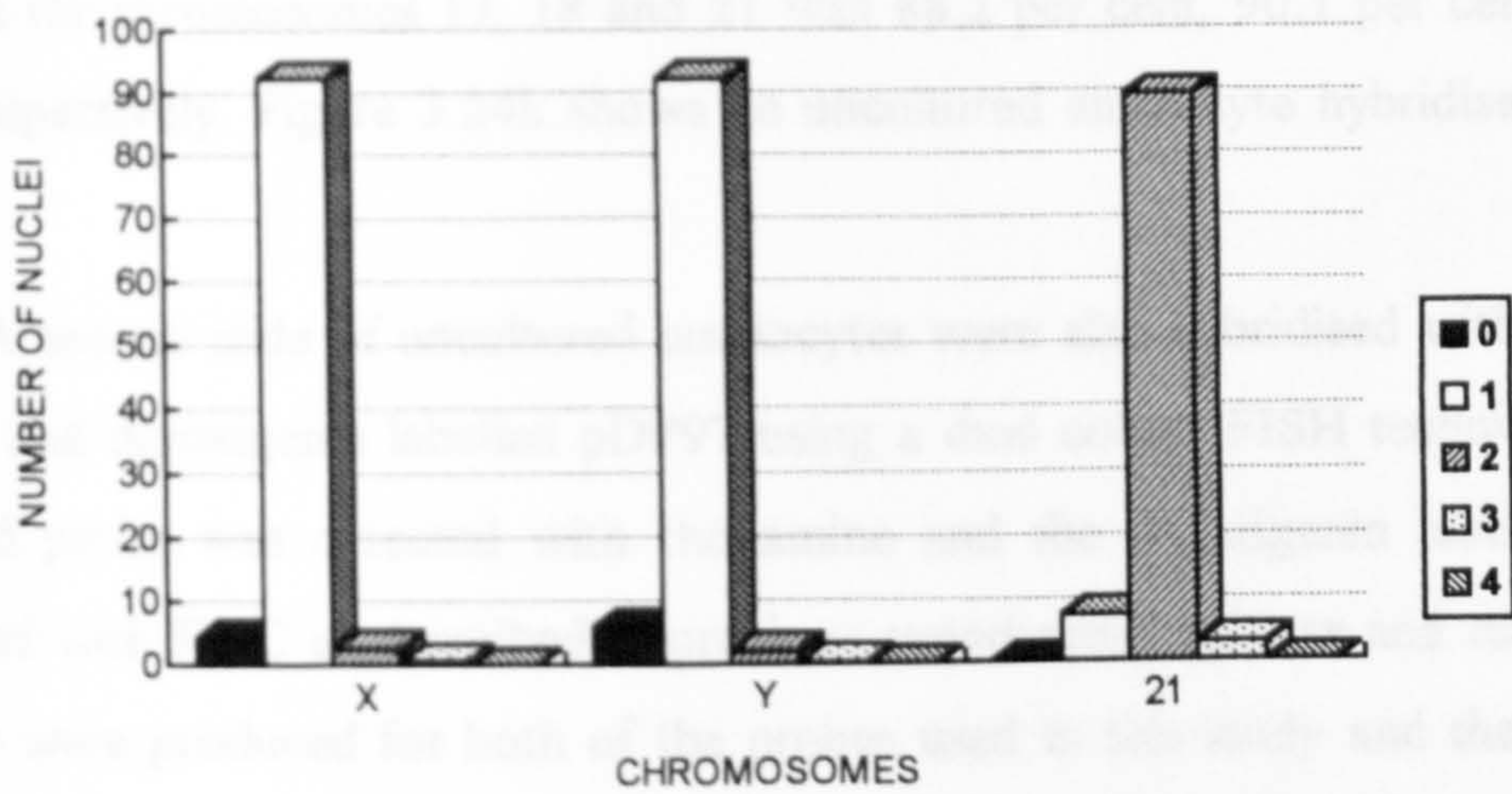
In figures a-g: ■ X ■ Y ■ 21 In h: ■ 18 ■ 13 In i-j: ■ 21 ■ 18 ■ 13

In k: ■ X ■ Y

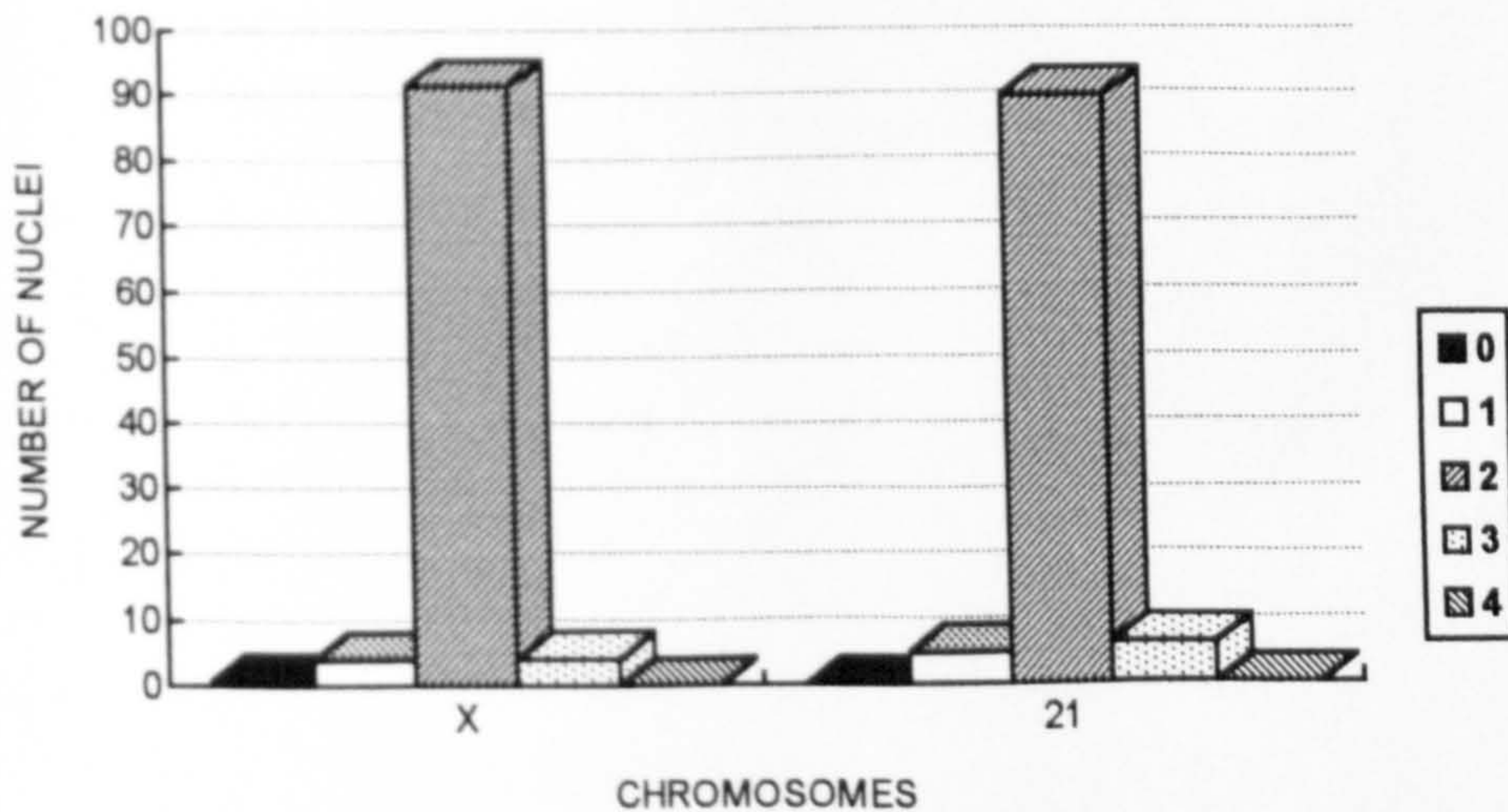
3.26 shows the detection efficiency of the probes in three colour ratio-mixing FISH experiments using uncultured amniocytes.

Where possible a second slide of the uncultured lymphocyte and amniocyte preparations which had been used for three colour FISH, was hybridised with probes specific for chromosomes 18 and 13 using a dual colour FISH approach. The centromeric probe L1.84 specific for chromosome 18 was labelled with biotin and detected with rhodamine. The Alu-PCR amplified YAC 744D11 specific for chromosome 13 was labelled with digoxigenin and detected with FITC. A series of ten uncultured amniocytes were analysed. 91.2 per cent and 90.5 per cent of the hybridised cells displayed two signals for each of the chromosomes 13 and 18 (Fig 3.24h).

In a further study the probes specific for three autosomes were simultaneously detected on uncultured lymphocytes. The YAC clone 831B9 specific for chromosome 21 was labelled with biotin and detected with avidin conjugated with rhodamine (red). Chromosome 13 specific YAC 744D11 was labelled with digoxigenin and detected with monoclonal anti-digoxin conjugated with FITC (green). A 1:1 ratio mixture of biotin labelled and digoxigenin labelled L1.84 probe was used and detected with both rhodamine and FITC which resulted in yellow signals specific for chromosomes 18. The same method of hybridisation post hybridisation washing and detection was used as described in previous studies. The conditions were optimised using normal uncultured lymphocytes and then an unselected series of five uncultured lymphocytes were hybridised to the probe set. Figure 3.24i shows an interphase nucleus prepared from a peripheral blood sample and hybridised to the probe set. The analysis of signals was mainly carried out by direct visualisation of the signals under the fluorescence microscope of the CCD system. The images were captured where the analysis was in doubt. Distinctive signals were produced specific for chromosomes 13 and 21, however the signals specific for chromosome 18 were diffuse on some nuclei from each sample. Within the five uncultured lymphocytes which hybridised to the probe set an average of 88.5 per cent, 90.1 per cent and 93.2 per cent of the hybridised cells displayed two



(a)



(b)

Figure 3.26. Distribution of signals specific for chromosomes X, Y and 21 in normal male (a) and female (b) uncultured lymphocytes using three-colour ratio-mixing FISH. The boxes with the shaded squares to the right of each diagram indicate the number of each signal detected.

signals specific for chromosomes 13, 18 and 21 respectively. The probe mixture was then hybridised to six unselected, uncultured amniocyte preparations. Isolated amniotic fluid cells displaying distinct signals were selected for analysis. Intensive signals specific for each of the probes used in this part of the study could be distinguished under the fluorescence microscope of the CCD system. The detection efficiency of the probes specific for chromosomes 13, 18 and 21 was 88.2 per cent, 90.1 per cent and 88.9 per cent respectively. Figure 3.24k shows an uncultured amniocyte hybridised to the probe set.

A second slide of uncultured amniocytes were also hybridised with biotin labelled DXZ1 and digoxigenin labelled pDP97 using a dual colour FISH technique. The biotin labelled probe was detected with rhodamine and the digoxigenin labelled probe was detected with FITC as described in previous experiments. Intense and readily detectable signals were produced for both of the probes used in this study and the fetal sex were correctly detected in all six samples used for dual-colour FISH analysis. Figure 3. 24k shows uncultured male amniocytes displaying a red signal specific for chromosome X and a green signal specific for chromosome Y.

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4. DISCUSSION

4.1 Design Of Experiments

4.1.1 Background At The Start Of The Project

At the time this project was started various groups were investigating FISH for prenatal purpose and several reports using different approaches had been published. Kuo *et al* (1991) used chromosome specific libraries for detection of numerical aberrations involving chromosomes 13, 18 and 21 in metaphase and interphase amniocytes. This study proved to be successful where the metaphase spreads were used. However, the signal analysis on interphase nuclei from cultured amniocytes resulted in decreased detection efficiency for each probe due to domain coalescence caused by the juxtaposition of two or more target chromosomes. This problem was reported to be worse in uncultured amniocytes.

Zahed *et al.* (1992) investigated alphoid repeats and found satisfactory detection of chromosomes 18, X and Y. However the probe L1.26 did not prove to be suitable for detection of chromosomes 13 and 21 as the probe did not distinguish between the two chromosomes. Bryndorf *et al.* (1992) used a chromosome 21 specific YAC clone for rapid prenatal diagnosis of trisomy 21 on uncultured amniocytes. The authors reported satisfactory results using this probe, although the technique needed to be further evaluated and improved. A cosmid contig developed by Zheng *et al.* (1992) also gave promising results, as four trisomy 21 cases in the 49 uncultured amniotic fluid samples were successfully diagnosed in a blind analysis. One sample could not be analysed in this study as it contained only a few degenerate and squamous cells, which are unsuitable for *in situ* hybridisation.

Following this initial feasibility study Spathas *et al.* (1993) used the same probe in a prospective blind study to evaluate the application of FISH in clinical practice. Within 240 uncultured amniotic fluid samples analysed in this study, they could correctly identify 236 samples as normal for chromosome 21 and three samples as trisomy 21, while one abnormal case involving a chromosome 21;21 translocation was falsely scored as normal.

Klinger *et al.* (1992) constructed cosmid probes derived from specific subregions of chromosomes 13, 18, 21 and X and used these in combination with alpha satellite DNA probe pDP97 specific for chromosome Y, for prenatal FISH analysis. Using these probe sets in a large prospective study the authors were able to detect the major chromosomal aneuploidies in uncultured amniotic fluid samples. However from a total of 526 samples, only 117 cases could be analysed using all five probe sets and the remaining fluid samples were insufficient for analysis of all five chromosomes.

In addition to the uni-colour FISH, a number of research groups were attempting to develop the technique of multi-colour FISH for simultaneous detection of numerical and structural chromosome abnormalities. In prenatal aneuploidy detection, the method can overcome the problem of sample volume limitation which is always encountered in diagnostic laboratories as most of the amniotic fluid sample is required for standard cytogenetic techniques and it is unlikely that there would be sufficient material to allow detection of the major chromosome aneuploidies in the same sample by uni-colour FISH. However the number of haptenisation and detection systems was limited and the use of more than two labelling systems results in prolonged detection procedures. To overcome these limitations the combinatorial haptenisation and detection systems had been developed.

The report of Ried *et al.* (1992a) described the most comprehensive study. This group used combinatorial labelling and a digital imaging system to simultaneously detect five probes representing the three most common autosomal aneuploidies (chromosomes 13, 18 and 21) and the sex chromosomes. They employed three reporter molecules, biotin (for chromosome 13), digoxigenin (for chromosome 18) and DNP (for chromosome 21). Probes specific for sex chromosomes were labelled with a combination of two reporter molecules. The biotinylated probe sequences were detected with ultralite 680TM conjugated to streptavidin (infrared fluorescence), digoxigenin labelled probes were detected with rhodamine (red fluorescence) while DNP labelled sequences were visualised using a rat anti-body against DNP and a secondary fluorescein conjugated anti-

body against rat IgG (green fluorescence). Finally scoring of the signals was carried out by acquiring separate images using a CCD and pseudocolouring. Using this approach Ried *et al.* were able to identify the five tagged chromosomes unambiguously. Although this was not a full-scale clinical trial, the results could demonstrate the possibility of simultaneous detection of major chromosome aneuploidies in interphase nuclei from a variety of clinical specimens including uncultured amniocytes. The technique is obviously a good system but technically very demanding. The use of three reporters resulted in a complicated detection procedure and the need for a CCD camera for signal analysis meant that this was not a method available to the majority of diagnostic cytogenetic laboratories.

The major objective of the present project was to find the most reliable, straightforward and practical approach to detection of the clinically most important aneuploidies which could be employed in diagnostic cytogenetic labs which did not necessarily have access to sophisticated equipment.

4.1.2 Choice Of Probe

The accuracy, reproducibility and reliability of analysis by FISH depends on a number of factors. However the factor which can most significantly affect the hybridisation and detection efficiency is the specificity, size and complexity of the probes used.

Chromosome specific library probes can be used for detection of numerical and structural abnormalities of chromosomes in metaphase cells, but they produce large and diffuse signals on interphase nuclei and overlap of hybridisation domains are common (Kuo *et al.*, 1991; Zheng *et al.*, 1992). They are thus not suitable for accurate detection of numerical abnormalities in interphase cells.

Centromere repeat probes are well suited for analysis of aneuploidies in interphase nuclei, as they generate strong and distinct signals. However, there are difficulties with use of these probes for some chromosomes. In general, the significant polymorphisms

that characterise most of the repetitive sequences influence the signal size and can lead to misdiagnosis. A repeat probe which hybridises to both chromosomes 13 and 21 is available (Devilee *et al.*, 1986), but it is difficult to use for interphase analysis as it does not distinguish between the chromosomes and twice the number of signals are usually produced. Furthermore the centromeric position of the probe does not allow identification of Robertsonian translocations.

Cosmids and YAC clones have large inserts and can be used as alternatives to repeat probes. The use of total yeast genomic DNA or YAC DNA isolated from pulsed-field gels results in higher background and decreased hybridisation efficiency due to cross hybridisation of yeast ribosomal repeat sequences with human DNA (Zheng *et al.*, 1992). In the present project we thus used centromeric probes for chromosomes 18, X and Y and looked for alternatives for the probe L1.26 specific for chromosomes 13 and 21.

4.1.3 Simultaneous detection of multiple targets

Multi-colour FISH has the ability to detect more than two targets on the same slide, but the number of haptenisation and detection systems are limited and the use of more than two labelling systems results in prolonged detection procedures. Combinatorial labelling of probes with two or more different reporters (ratio-labelling) can increase the number of distinguishable targets relative to the number of available fluorochromes. With two haptens (for example, biotin and digoxigenin) and two fluorophores (rhodamine and FITC), three different targets can be simultaneously visualised, assuming that an equimolar mixture of reporters is used for the third hapten. Using different ratios of the two haptenisation and detection systems it seems to be possible to detect up to five different targets in a single hybridisation experiment. The number of different targets can be further increased when three labelling and detection systems are used. Ried *et al.* (1992b) reported the simultaneous detection of seven DNA probes on metaphase chromosomes and interphase cells using a combinatorial labelling scheme with up to three reporter molecules. In a similar study Dauwerse *et al.* (1992b), have simultaneously detected up to 12 distinctly painted chromosomes in a single metaphase cell by three

haptens and detection systems. Using a conventional epifluorescence microscope it would be difficult to distinguish between the signals generated by differently labelled probes, specifically when extra colours are generated by using different ratios of the same reporters. This restriction has been considerably overcome with digital imaging systems, such as cooled CCD camera. Using this system the images under the microscope can be captured and the signals can be pseudocoloured using appropriate software. In the presence of a reliable technique of ratio-labelling FISH, this system allows unambiguous analysis of the signals generated by the differently labelled probes.

The above aim can also be achieved if a probe which is individually labelled with two or more reporters, is mixed with different ratios (ratio-mixing). Although, no differences have been observed between the results of ratio-mixing and ratio-labelling (Dauwerse *et al.*, 1992b), ratio-mixing provides for more flexibility and safeguards against failure of one specific component in a labelling reaction.

Direct fluorophore labelling of probes is advantageous for diagnostic procedures since it obviates the immunological detection steps which are time consuming. The major limitation of directly labelled probes is their decreased sensitivity of detection compared to indirectly labelled probes.

In the present study we developed a ratio-mixing FISH approach using two haptens and detection systems to produce up to five different colours, and applied it for simultaneous detection of major chromosome aneuploidies in uncultured lymphocytes and amniocytes.

4.2 Detection Of Chromosomes X, Y and 18

4.2.1 Optimisation Of Each Probe Individually

Centromere repeat probes produce distinct and intense signals in interphase nuclei when labelled and hybridised with sufficient stringency (Cremer *et al.*, 1986; Poddighe *et al.*, 1991; Christensen *et al.*, 1992; Cacheux *et al.*, 1994). Although there are limitations with

the use of the repetitive probes, they have proved to be useful for enumeration of chromosomes 1, 18, X and Y in uncultured amniotic fluid cells and chorionic villus cells (Rao *et al.*, 1993; Bryndorf *et al.*, 1994). Based on the above knowledge, the alpha satellite DNA probes DXZ1, pDP97 and L1.84 were employed to enumerate the copy number of chromosomes X, Y and 18 in cultured and uncultured lymphocytes.

The use of Circle prep kit for isolation of probe DNA resulted in a highly concentrated yield of DNA. However the time required for DNA isolation was about 3.5 - 4 hours. The Insta prep kit produced pure probe DNA within 15 minutes, but the amount of the isolated DNA was considerably less than of Circle prep kit. The isolated plasmid DNA containing the human insert was directly used for labelling and no isolation of probe DNA from plasmid DNA was carried out. The standard nick translation protocol was used for biotin labelling. For digoxigenin labelling the nick translation method was compared to random primed labelling. In these experiments nick translation appeared to be more efficient than random primed labelling.

The labelled probes were hybridised to cultured and uncultured cells prepared from peripheral blood. A comparison between the two methods of denaturation and hybridisation used in this study (part three, results) showed that a superior quality of chromosomal and nuclear morphology is produced and intensive signals on a decreased background fluorescence are obtained when the probe and target DNA are denatured individually. This seemed to result from the more moderate temperature of denaturation (65-70°C) which was required when the target DNA was denatured in 70 per cent formamide. Treatment of the slides with fresh fixative and acetone before denaturation further improved the morphology of cells. All of the biotin labelled probes used in this part of study generated strong hybridisation signals. More than 95 per cent of nuclei showed the expected number of signals when hybridised to the specific probe used in each experiment, indicating a high hybridisation efficiency for each probe. The signals produced by pDP97 were characteristically smaller than those generated by the other two probes. Probe L1.84 was observed to produce diffuse signals on some uncultured nuclei.

Although no problem was encountered in analysis where the signals were well separated in the nucleus, cells where the signals were close together were not scored. A similar intensity of signals was observed when the biotin labelled probes were detected with avidin conjugated with rhodamine.

Digoxigenin labelled DXZ1, pDP97 and L1.84 produced distinct and intense signals on both metaphase and interphase nuclei prepared from peripheral blood. The background fluorescence on the cells hybridised with digoxigenin labelled probes was significantly less than that in the cells hybridised with biotin labelled probes. This is thought to be because digoxigenin occurs naturally only in digitalis plants, and in other biological material the anti-digoxigenin antibody can bind specifically only to the digoxigenin labelled probe. These results indicate that centromeric probes produce reproducible results and are in agreement with the previous studies (Zahed *et al.*, 1992; Christensen *et al.*, 1992; Cacheux *et al.* 1994). As the major purpose of this part of the project was optimisation of the hybridisation and detection conditions and finding of an efficient concentration for each of the alpha satellite DNA probes specific for chromosomes X, Y and 18 to be employed in further studies, no hybridisation of these probes to uncultured amniocytes was carried out.

4.2.2 Dual Colour FISH

In order to optimise a method for simultaneous detection of differently labelled probes and investigate the reliability of the probes when visualised in a single hybridisation experiment a dual colour FISH approach was employed to detect simultaneously the probes specific for chromosomes X and Y or X and 18 in cultured and uncultured lymphocytes. During the development of the method attempts were made to avoid adding any unnecessary steps, as this would result in complex procedures with no significant increase in quality.

The use of sheep anti-digoxigenin for detection of digoxigenin labelled probe resulted in binding of green fluorochrome with red signals, generated by biotin labelled

probe. This appeared to be because of the cross reactivity of fluorescein anti-sheep with anti-avidin raised in goat which was used to enhance the intensity of green and red signals. In spite of the cross-reactivity the signals could be clearly distinguished and analysed. However, this could be problematic in ratio-mixing FISH experiments where the same reporters are used to produce more than two targets. As a result it was decided to replace the sheep anti-digoxigenin with monoclonal anti-digoxin raised in mouse. Using this method the digoxigenin signals were enhanced using FITC conjugated anti-mouse. No cross-reactivity was observed using the new detection mixtures. A number of cultured and uncultured lymphocytes were hybridised to biotin labelled DXZ1 and digoxigenin labelled pDP97 when the method was optimised. The signals were analysed using the fluorescence microscope of CCD system. No capturing of images was required as the signals could be readily distinguished under the microscope when the triple emission filter block was used in combination with filter wheel. The same result was obtained when a digoxigenin labelled L1.84 was used instead of biotin labelled L1.84.

The dual colour detection method optimised in this part of the study is applicable to any multi-colour FISH experiment which uses only biotin labelled and digoxigenin labelled probes for simultaneous visualisation of two or more targets. This study also revealed the reliability and sensitivity of the aforementioned probes in a multi-colour FISH experiment.

4.2.3 Three Colour Ratio-Mixing FISH

To minimise the number of reporter molecules required and provide a simple method of detection to visualise the three probe sets in a single hybridisation experiment, a 1:1 ratio mixture of probe pDP97 was used to visualise the chromosome Y specific probe in an intermediate colour (yellow).

This study showed that biotin-11-dUTP and digoxigenin-11-dUTP and subsequent detection of the labelled probes with rhodamine and FITC can be readily used to produce three different colours. The biotin labelled probes were detected with

rhodamine-avidin and the digoxigenin labelled probes were detected with monoclonal anti-digoxin conjugated with fluorescein as discussed earlier for dual colour FISH. However, to study the effect of cross-reactivity of fluorescence anti-sheep with goat anti-avidin on three colour ratio-mixing FISH, a number of hybridised cells were detected using the combined detection system including sheep anti-digoxigenin and fluorescein anti-sheep. A comparison between two detection systems revealed an obvious colour difference between the chromosome X specific red signals obtained using the two procedures. This clearly indicated that sheep anti-digoxigenin cannot be reliably used in a multi-colour ratio-mixing FISH experiment, as the cross reactivity of anti-sheep antibody with goat anti-avidin can change the ratio of colours and may lead to incorrect diagnosis.

The signals could be distinguished under the fluorescence microscope of CCD system, however the images were captured where required. The time required for signal analysis in uncultured lymphocytes was in the range of 30-60 minutes depending on the quality of cell preparation and the efficiency of hybridisation and detection procedures. The detection efficiency of each probe analysed in uncultured lymphocytes was in the range of 88-96 per cent for chromosome X, 86-96 per cent for chromosome Y and 86-94 per cent for chromosome 18.

Uncultured amniocytes hybridised with the same probe combination required 60-90 minutes for analysis, as only nuclei displaying good morphology and isolated signals were selected for this evaluation. For uncultured amniotic fluid cells, the detection efficiency of probes specific for chromosomes X, Y and 18 hybridised to uncultured amniocytes were in the range of 78-94 per cent 78-96 per cent and 80-96 per cent respectively which is comparable with those obtained in uni-colour FISH experiments using the same probes individually.

4.3 Development Of Probes Specific For Chromosomes 21 And 13

At the time the present trial was started it had been shown that the centromeric probe L1.26 was unreliable for detection of aneuploidy of chromosomes 13 and 21 in

uncultured nuclei (Zahed *et al.* 1992). Further work in this department using a contig consisting of two overlapping chromosome 21 cosmids to detect the aneuploidy of chromosome 21 in uncultured amniocytes had also been proved to be unreliable. For these reasons we developed the Alu-PCR YAC probes 745H11 and 831B9 to enumerate the chromosome 21 on uncultured lymphocytes and amniocytes. As the highly repeated Alu sequences are specific for the human genome, using the Alu-PCR approach, the human inserts within the YAC were selectively amplified and used as a specific probe for chromosome 21.

Hybridisation of cultured and uncultured lymphocytes with both biotin labelled YACs revealed that the signals produced by YAC 831B9 are larger and more intense than those of 745H11. The background fluorescence on the slides hybridised with YAC 831B9 was also less than on the slides hybridised with the other YAC. Analysis of twenty uncultured lymphocytes and twenty-three uncultured lymphocytes revealed a high hybridisation and detection efficiency on both cell types. The detection efficiency of the probe in uncultured lymphocytes was in the range of 88-96 per cent and in uncultured amniocytes was in the range of 86-94 per cent of the hybridised cells. The signal intensity was comparable to those of alpha satellite DNA probes. These results compare favorably with the similar study reported by Romana *et al.* (1993). Uncultured lymphocytes and amniocytes hybridised with digoxigenin labelled 831B9 displayed intensive signals with less background compared to the biotin labelled probe.

For chromosome 13 three cosmids (Fisher *et al.* 1994), were initially obtained and developed for interphase FISH purposes. One of the cosmids (29G3) showed cross-hybridisation with short arms of the other acrocentric chromosomes. This problem was thought to originate in the presence of bacterial host cells carrying other DNA sequences in the same glycerol stock. Isolation of a single colony of bacterial cells originating from a single cell and amplification of the isolated cells in a liquid medium was a possible solution for this difficulty. However, there was a possibility of picking a colony from the other cell type. Using this method specific signals were produced on the long arm of

chromosome 13. The signals generated by cosmid 29G3 were stronger than those produced by the two other cosmids. A greater intensity of signals was obtained when a combination of the three overlapping cosmids was used to enumerate the chromosome 13 on interphase nuclei. However, the signal intensity was less than that produced by YAC clone 831B9 and the background fluorescence was higher. Using a combination of the three cosmids, a range of 87-91 per cent of uncultured lymphocytes showed two signals specific for chromosome 13. The detection efficiency was in the range of 82-88 per cent when the uncultured amniocytes were hybridised with the contig. Hybridisation of a number of interphase cell preparations from peripheral blood and amniotic fluid samples with digoxigenin labelled contig resulted in signals with decreased background fluorescence, however the intensity of the signals was less compared to the biotin labelled probe. This series of experiments revealed that the contig can be used for detection of chromosome 13 aneuploidy on uncultured lymphocytes and amniocytes, providing the uncultured amniocytes are of a high quality.

As an alternative probe, the Alu-PCR YAC probe 744D11 (Fischer *et al.* 1994) was developed and used as a specific probe to detect the copy number of chromosome 13 on uncultured nuclei. Hybridisation of a limited number of uncultured lymphocytes revealed a greater intensity, less fluorescence background and higher detection efficiency of the YAC 831B9 than cosmid contig. The major objective of the present study in development of the probes specific for chromosomes 13 and 21 and optimisation of the hybridisation and detection conditions in uni-colour FISH, was to be used in combination with the probes specific for chromosomes X, Y and 18 in a five colour FISH approach. For this reason effort was made to use the same stringency conditions as had been previously used for centromeric repeat probes. The only difference was that, the chromosomes 13 and 21 specific probes were hybridised according to chromosome *in situ* suppression hybridisation procedure using sonicated human placental DNA as competitor DNA, whereas no competitor DNA was used in hybridisation of alpha satellite DNA probes to their specific targets.

4.4 Five Colour Ratio-Mixing FISH

The technique of five colour ratio-mixing FISH which has been presented in this study has the advantage of smaller sample requirement and the use of a minimum number of probe labelling and detection systems. This study has shown that biotin-11-dUTP and digoxigenin-11-dUTP and subsequent detection of the labelled probes with rhodamine and FITC can be readily used to produce up to five different colours. Compared to the method reported by Ried *et al.* (1992a) the technique is simple as only two haptenisation and detection systems has been used instead of three. The post-hybridisation washing and detection steps that we used in this method are the same as those currently used for uni-colour FISH in this department; the only difference being the detection solutions. As a result the method does not load additional procedures to the routine laboratory work and compares favorably to the procedure described by Divane *et al.* (1994).

The probes could be directly labelled with fluorophores (Divane *et al.* 1994), however it does not seem to be an advantage in a multicolour FISH approach, where a directly labelled probe would be used in combination with probes that are indirectly labelled with haptens.

The probes which were initially used consisted of alpha satellite DNA probes specific for the sex chromosomes and the Alu-PCR product of YAC 831B9 for chromosome 21 and the cosmid contig for chromosome 13. The alpha satellite DNA probes generated strong hybridisation signals with intermediate colours between red and green. The intensity of chromosome 21 specific signals (red) were comparable to those produced by alpha satellite DNA probes, where the cosmid contig specific for chromosome 13 produced hybridisation signals with less intensity and caused increased background fluorescence on hybridisation areas. The colour difference between the different signals could be clearly distinguished using the colour-ratio software. However, the number of hybridised cells in normal uncultured lymphocytes and amniocytes which displayed the normal number of signals for chromosome 13 were lower than those of the other probes.

In a further trial the Alu-PCR YAC, 744D11 was developed as described earlier and used for enumeration of chromosome 13 in five colour experiments. The signals produced by this probe were stronger than those produced by cosmid contig. A high hybridisation and detection efficiency was achieved when the combined probe set was hybridised to uncultured lymphocytes using the optimised method of five colour ratio-mixing FISH. In normal samples the detection efficiency of each of the chromosomes X, Y, 21 and 18 was in the range of 84-96 per cent. This range was slightly lower for chromosome 13, as 80-92 per cent of the hybridised cells showed two signals. Diagnosis of two trisomy 21 male samples with a detection efficiency of 80 per cent showed that the technique has the ability to detect the major chromosome aneuploidies on uncultured lymphocytes.

From 27 uncultured amniotic fluid preparations hybridised with combined probe set twenty-two samples were correctly identified as normal male and female samples. An acceptable detection efficiency was achieved for each probe. The detection efficiency of probes specific for chromosomes X and Y was in the range of 84-92 per cent and 88-92 per cent respectively. Probes specific for chromosomes 13, 18 and 21 were detected with an efficiency in the range of 80-92 percent. Five samples were uninformative due to poor quality of the amniotic fluid preparations. One sample displayed a green/black pellet. In this preparation more than 99 percent of the cells had not hybridised to the combined probe set as the cells were covered by a green layer. The remaining four samples were cloudy on arrival and the cell preparations obtained from these samples were poor.

4.5 Difficulties Of Prenatal Detection Of Aneuploidy By Interphase FISH

Factors which influence the hybridisation and detection efficiency in a FISH experiment include sample fixation, cell permeability and contamination of the amniotic fluid sample with maternal blood (Lichter *et al.* 1991; Christensen *et al.* 1993; Winsor *et al.* 1996). These parameters may vary with cell type as has been demonstrated in earlier studies (Cremer *et al.* 1986; Klinger *et al.* 1990). In uncultured lymphocytes the majority (70-90 per cent) of nuclei displayed a superior quality of morphology and showed isolated and

clear signals which were suitable for scoring. However, cells from uncultured amniotic fluid preparations were found to be much more variable in quality. Most of the cells were degenerate squamous epithelial cells, which are rarely accessible for the probes and cover large parts of the hybridisation area when samples are hypotonised and fixed. A high proportion of nuclei were covered by cytoplasm which caused unacceptable background signals and significantly reduced the permeability of the cells for probes. Many preparations also contained clumps of cells with few single nuclei available for study. On average greater than 70 per cent of nuclei were rejected and in some cases the figure could be as high as 90 per cent. The method of uncultured amniotic fluid preparation on the slide that we used in the present study could overcome part of these limitations as few hybridisable cells were lost during cell preparation and some of the large squamous cells were washed away when fixative was dropped on the slide. However signal analysis appeared to be more time-consuming compared to the uncultured lymphocytes.

The presence of maternal cells in uncultured amniotic fluid may result in misinterpretation of prenatal diagnosis. Christensen *et al.* (1993) assessed 18 macroscopically blood-stained amniotic fluids from male fetuses using dual colour FISH with X and Y specific probes and calculated that based on their criteria, trisomy 21 would probably have been misdiagnosed as normal in two samples. Similar studies have been reported by other workers (Rebello *et al.* 1994; Nuss *et al.* 1994). For this reason in order to minimise the risk of maternal cell contamination, Ward *et al.* (1993) excluded amniotic fluid samples with visible blood from their study. In our study four slightly blood-stained samples were present within the samples hybridised using a five colour ratio-mixing FISH approach, however, no microscopic evidence of maternal cell contamination was observed. Two of the four blood-stained samples were correctly identified as normal male and the two others were correctly identified as normal female samples.

A number of nuclei were occasionally observed displaying signals located on the outside of the uncultured nuclei. This might suggest that the counterstain does not diffuse homogenously in some nuclei and it is more aggregated in the centre than edges of a

given nucleus. Other reasons have been considered by other workers. Christensen *et al.* (1992) reported that this phenomenon is only rarely seen in nuclei from cultured amniotic fluid cells and suggest that the nuclear membrane might be more fragile and / or heterochromatin more uncondensed in uncultured cells than in cultured cells.

4.6 Specific Difficulties Of Five Colour Ratio-Mixing FISH

The ideal nucleus for scoring in an interphase FISH experiment is one where the signals are clearly separated from one another and where they lie as nearly as possible in the same focal plane. In the five colour experiments described here, a high proportion of nuclei failed to meet these criteria because of the presence of overlapping or clumped signals or because the focal planes varied to such an extent that not all signals could be captured. Thus the hybridised cells had to be carefully searched for nuclei with signals suitable for analysis and as this could only be achieved by image capture and pseudocolouring, the selection of appropriate nuclei was very time-consuming. The time required to analyse a preparation of uncultured lymphocytes ranged from 110-160 minutes depending on the quality of cell preparation and efficiency of hybridisation and detection. With uncultured amniotic fluid cell preparations where the nuclei are significantly smaller, time for analysis ranged from 2-3 hours per slide. This is a long time when processing a large number of amniotic fluid samples per week.

In the series of amniotic fluid samples which were hybridised to the combined probe set 18.5 per cent of cases (five samples of 27 samples) failed to produce a result. This would be unacceptably high in diagnostic circumstances. This problem was highlighted by Divane *et al.* (1994) who worked on a retrospective series of amniotic fluid samples and were able to select for study only samples which displayed good nuclear morphology. They pointed out that in prospective series a proportion of cases would be unsuitable for analysis. The largest series so far reported, that of Ward *et al.* (1993) covering 4500, specimens found that in 9.8 per cent of samples no result could be achieved. These two problems would make automatic signal counting more difficult.

Combination of all the above factors meant that although the technique is reproducible as a diagnostic test it appeared to be time consuming and impractical.

4.7 A Practical Strategy For Detection Of Major Chromosome Aneuploidies Using A Minimum Volume Of Sample

The five colour ratio-mixing FISH approach developed in the present study for simultaneous detection of five clinically important aneuploidies, produced reliable results using uncultured lymphocytes and amniocytes. However the limitations of the technique as described above hampered its use as a routine diagnostic test.

To provide a simple, straightforward and practical strategy for prenatal detection of major chromosome aneuploidies using a smaller volume of amniotic fluid sample than uni-colour FISH a combination of three colour ratio-mixing FISH and a dual colour was employed. The simultaneous detection of chromosomes X, Y and 18 had already been studied in uncultured lymphocytes and amniocytes. Following this study the probes specific for chromosomes X, Y and 21 were simultaneously hybridised to a series of 20 uncultured lymphocytes and 25 uncultured amniocytes using a three colour ratio-mixing FISH and the results were compared to those of previous experiments. Simultaneous analysis of chromosomes X, Y and 21 was easily and reliably carried out by counting the red, yellow and green signals on uncultured lymphocytes and amniocytes. All of the probes produced strong and distinct signals and the percentage of the cells displaying overlapped and clumped signals was less than that observed in similar experiments using probes specific for chromosomes X, Y and 18. This significantly increased the percentage of the cells suitable for analysis. The colour difference between the signals could be clearly distinguished under the fluorescence microscope of the CCD system. This significantly reduced the time required for signal analysis as the images did not require to be captured using computer software. Compared to the experiments using specific probes for chromosomes X, Y and 18 a higher hybridisation and detection efficiency was achieved for the probe sets used in this strategy. (Tables 4.1 and 4.2).

<i>FISH approach</i>	<i>X</i>	<i>Y</i>	<i>13</i>	<i>18</i>	<i>21</i>
Dual colour (13/18)			92.1	91.7	
Three colour on male samples (X/Y/18)	93	92.8		91	
Three colour on female samples (X/Y/18)	93.1			90.8	
Three colour on male samples (X/Y/21)	94.1	93.3			93.7
Three colour on female samples (X/Y/21)	93.9				94.4
Three colour (13/18/21)			88.5	90.1	93.2
Five colour on male samples	92	92.4	88.9	89.9	89.5
Five colour on female samples	92.5		89.5	90.5	89.5

Table 4.1 Detection efficiency (given as percentages) of the probes specific for chromosomes X, Y, 13, 18 and 21 in various multi-colour FISH experiments using normal uncultured lymphocytes.

Simultaneous analysis of the three autosomes on uncultured lymphocytes and amniocytes was reliably carried out using a three colour ratio-mixing FISH approach. This strategy would also be useful if the pregnant woman did not want to choose termination of pregnancy because of sex chromosome abnormalities. However, the signal analysis appeared to be more time-consuming than the strategy of using the combination of probes specific for chromosomes X, Y and 21, as six signals had to be simultaneously analysed instead of four. As a result the combination of probes specific for chromosomes X, Y and 21 was regarded as a more reliable and straightforward strategy than the two others studied during this evaluation.

<i>FISH approach</i>	<i>X</i>	<i>Y</i>	<i>13</i>	<i>18</i>	<i>21</i>
Dual colour (13/18)			91.2	90.5	
Three colour on male samples (X/Y/18)	91.9	91.4		89.4	
Three colour on female samples (X/Y/18)	92.7			89.5	
Three colour on male samples (X/Y/21)	92.3	92			89.3
Three colour on female samples (X/Y/21)	91.3				89.4
Three colour (13/18/21)			88.2	90.1	88.9
Five colour on male samples	91.7	92	88.9	88.3	85.5
Five colour on female samples	91.5		88	88.4	86.6

Table 4.2. Detection efficiency (given as percentages) of probes specific for chromosomes X, Y, 13, 18 and 21 in various multi-colour FISH experiments using normal uncultured amniocytes.

The results also indicate that the copy number of chromosomes 13 and 18 can be readily detected by dual colour FISH in uncultured lymphocytes and amniocytes. Although the probe L1.84 produced diffuse signals on some of the hybridised cells, no problems were encountered in analysis, since only two colours had to be distinguished and the signals produced by Alu-PCR amplified YAC 744D11 specific for chromosome 13 were clear and intense. In comparison to the other probe combinations, a higher hybridisation and detection efficiency was obtained for both probes specific for chromosomes 13 and 18 when simultaneously hybridised to uncultured lymphocytes and amniocytes. On the other hand it could be an advantage in the prenatal diagnostic situation to have the probe for the chromosome X in one combination and that for the chromosome Y in the other. This would provide some measure of check that the slides were from the same sample.

4.8 Advantages Of Three Colour Ratio-Mixing FISH OVER The Five Colour Ratio-Mixing FISH

The strategy of detecting major chromosome aneuploidies on two slides from the same sample has several advantages over the five colour ratio-mixing FISH. The technique is more accurate, reliable and straightforward, as only one intermediate colour is produced using two labelling and detection systems. This approach can be simply employed in most of the laboratories equipped for FISH, as it does not require the use of digital imaging system which is not available for many diagnostic laboratories. The time required for signal analysis is significantly less (20-50 minutes) than that needed for five colour FISH. The percentage of suitable cells for signal analysis is significantly higher than those hybridised to a combination of five different probes. This decreases the number of samples which fail to produce a result due to an inadequate number of cells for signal analysis. Finally compared to five colour ratio-mixing FISH, the detection efficiency of the probes is higher. The detection efficiency of five clinically more important chromosomes in normal uncultured lymphocytes and amniocytes using five colour, dual colour and three colour FISH has been compared in tables 4.1 and 4.2.

A significant increase is observed in detection efficiency of chromosome 21 when probes specific for chromosomes X, Y and 21 are simultaneously detected in uncultured nuclei from peripheral blood and amniotic fluid samples. This probe combination may be of major importance in prenatal diagnosis of trisomy 21, as about 50 per cent of the false results arising from maternal cell contamination in macroscopically blood stained samples, can be excluded using two strong controls to distinguish the male fetal cells from female contaminating maternal cells.

4.9 Conclusion

Rapid clinical diagnosis of the common fetal aneuploidies is now possible by fluorescence *in situ* hybridisation. This can significantly reduce the anxiety of the pregnant women who are waiting for results. The accuracy of FISH for aneuploidy analysis has been greatly increased by development of suitable probes and reliable hybridisation and detection methods.

The present study provides a rapid and reproducible method of FISH for diagnosis of major chromosome aneuploidies in uncultured lymphocytes and amniocytes. Our results indicate that the alpha satellite DNA probes can be reliably used for enumeration of chromosomes X, Y and 18 on interphase nuclei. The Alu-PCR product of YAC 831B9 proved to be a suitable probe for prenatal diagnosis of trisomy 21 using uncultured amniocytes. Both cosmid contig and Alu-PCR product of YAC 744D11 produced reliable results in detection of chromosome 13 copy number in uncultured lymphocytes and amniocytes, however the signals produced by YAC was more distinct and intensive. More experiments are required to prove the suitability and reproducibility of these probes for prenatal diagnosis of trisomy 13 using uncultured amniocytes.

The present study reveal that the five most clinically important aneuploidies can be readily and reliably detected on two slides from the same sample using a ratio-mixing FISH to detect the chromosomes X, Y and 21 and a dual colour to detect the chromosomes 13 and 18. At present this approach appears to be a more practical way for

prenatal detection of major chromosome aneuploidies than simultaneous detection of five chromosomes in a single hybridisation area. However, technical advances in clinical sample preparation, defined probe sets and automation of signal counting can extend the diagnostic limits of five colour ratio-mixing FISH, providing a powerful technique for rapid detection of multiple chromosomal abnormalities in a single hybridisation experiment.

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APPENDIX

Appendix 1: Shopping List

1. Bacto-Tryptone (Difco; Cat. No. 0127-17-9)
2. Bacto-Yeast Extract (Difco; Cat. No. 0123-17-3)
3. Bacto- Agar (Difco; Cat. No. 0140-01)
4. Yeast nitrogen base W/O (Difco; Cat. No. 0919-15-3)
5. Acid hydrolysed casein (Difco; Cat. No. 0230-01-1)
6. Adenine hemi sulfate (Sima; Cat. No. A 3159)
7. Sodium dodecyl sulphate (Sigma; Cat. No. L 4632)
8. Sorbitol (Sigma; Cat. No. S 1876)
9. Zymolyase[®]20-T (ICN Biomedical, INC; Cat. No. 32-092)
10. Ultrapure dNTP set, 2'-deoxynucleoside 5'-triphosphate (Pharmacia Biotech; Cat. No. 27-2035-0)
11. 10X PCR Buffer (Perkin Elmer, Cat. No. E0068)
12. AmpliTaq[®]DNA polymerase (Perkin Elmer; Cat. No. E0247)
13. Ethidium bromide, 10mg/ml (Sigma; E-1510)
14. 1Kb DNA Ladder (Gibco BRL, Cat. No. 15615-016)
15. Glycogen (Boehringer Mannheim, Cat. No. 901 393)
16. Isocove's Modified Dulbecco's Medium (Gibco BRL; Cat. No. 21980-32)
17. Heparin sodium, 5 000 units/ml (LEO Laboratories)
18. Thymidine (Sigma; T-5018)
19. Phytohaemagglutinin, lyophilised (Gibco BRL; Cat No. 10576-015)
20. Penicillin/streptomycin (Gibco BRL; Cat No. 15140-114)
21. 2-deoxycytidine (Sigma D-3897)
22. Colcemid[®] solution (10µg/ml), liquid (Gibco BRL 15212-012)
23. Biotin-11-dUTP (Sigma; Cat. No. B6780)
24. Digoxigenin DNA labelling Mixture, 10X conc. (Boehringer Mannheim; 1277 065)

25. Sonicated human placental DNA (Cambio Ltd; Cat. No. 1066-D)
26. Deoxyribonucleic Acid, Native 1gm (Pharmacia Biotech; Cat. No. 27-4564-01)
27. Tween 20 (BDH; BDH 66368)
28. Human AB serum (Sigma; S-7148)
29. RNase, DNase free, 100 mg (sigma; Cat. No. R-4875)
30. Fluorescein Avidin DCS, 0.2mg/ml (Vector Laboratories; ;Cat. No. A2011)
31. Biotinylated Antiavidin D (Affinity purified) 2mg/ml (Vector Laboratories; Cat. No. BA0300)
32. Anti-digoxigenin-fluorescein Fab fragments 200 µg (Boehringer Mnnheim; Cat. No. 1207 741)
33. Fluorescein Antisheep Ig (H+L) Affinity purified 1.5mg/ml (Vector Laboratories; Cat. No. FI-6000)
34. Rhodamine Avidin DCS (Cell sorting grade) 0.2mg/ml (Vector Laboratories; Cat. No. A2012)
35. Monoclonal Anti-digoxin, FITC conjugate (Sigma Immunochemicals; F-3523)
36. Anti-Mouse IgG (whole molecule), Affinity isolated sheep antibody (Sigma Immunochemicals; Cat. No. F-3008)
37. Citifluor, Glycerol/PBS solution (Citifluor UK Chem Lab; Cat. No. AF1)
38. DAPI (4',6'-Diamidino-2-phenylindole) 1mg/ml (Sigma Chemical Co; Cat. No. D-9542)
39. Propidium Iodide 95 - 98% (TLC) 10mg/ml (Sigma Chemical Co; Cat. No. P4170)
40. Alpha satellite DNA Probe for chromosome X Locus DXZ1 Biotin labelled 0.5µg/50µl (Oncor; Cat. No. P5060-B.5)
41. Alpha satellite DNA Probe for chromosome X Locus DXZ1 Digoxigenin labelled 0.5µg/50µl (Oncor; Cat. No. P5060-DG.5)
42. 3-Aminopropyltriethoxysilane (APES) (Sigma; A-3648)

1. Publications And Meeting Presentations :

2. Mohaddes, S. M., Boyd E., Connor, J. M. "Application of ratio-labelling FISH for simultaneous detection of chromosomes X, Y and 18 in uncultured lymphocytes and amniocytes". Presented at the annual conference of the Association of Clinical Cytogeneticists, Cardiff, July, 1994. *Clinical Cytogenetics Bulletin*, Vol. 2, No. 8, PP: 46.
3. Mohaddes, S. M., Morris, A., Boyd E., Connor, J. M., "Detection of chromosome 21 copy number by FISH using Alu-PCR amplified YAC clones in uncultured lymphocytes and amniocytes". Presented at British Medical Geneticists Conference, York, September, 1995.
4. Mohaddes, S. M., Boyd, E., Connor J. M., "Simultaneous detection of probe sets for chromosomes 13, 18, 21, X and Y in uncultured lymphocytes and amniocytes by Ratio-Mixing FISH". Presented at British Medical Geneticists Conference, York, September, 1994.
5. Mohaddes, S. M., Boyd, E., Morris, A. Morrison, N., and Connor, J. M. (1996). A practical strategy for detection of chromosome aneuploidies using ratio-mixing fluorescence *in situ* hybridisation. *Molecular And Cellular Probes*, 10, 147-154.

PAGE

NUMBERING

AS ORIGINAL

A practical strategy for detection of major chromosome aneuploidies using ratio-mixing fluorescence *in situ* hybridization

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We describe the use of ratio-mixing FISH to visualize simultaneously probe sets specific for chromosomes 13, 18 and 21 as well as both sex chromosomes in uncultured lymphocytes and amniocytes. This method has the advantage of a smaller sample requirement than uni-colour FISH and potential for analysis of a larger number of chromosome aneuploidies using a minimum number of different probe haptenization and detection systems. An unselected series of uncultured lymphocytes and amniocytes was used to investigate the reliability of ratio-mixing FISH for diagnostic applications. The results indicate that the five-colour ratio-mixing FISH is a reliable technique and can be used for simultaneous detection of major aneuploidies. However, as a diagnostic approach, the strategy of using a three-colour ratio-mixing FISH and a dual colour to detect the five clinically important aneuploidies on two slides from the same sample, appears to be simpler and more practical.

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KEYWORDS: fluorescence *in situ* hybridization, ratio mixing, aneuploidy, uncultured amniocytes.

INTRODUCTION

Rapid detection of chromosome aneuploidies is of major importance in prenatal diagnosis. It has been shown that fluorescence *in situ* hybridization (FISH) with chromosome specific probes can detect the number of copies of a particular chromosome present in interphase nuclei.^{1–7} The major advantage of this technique over current chromosome analysis is that there is no requirement for cell culture and hence the results can be available in 48 h. The technique has important applications for the prenatal detection of aneuploidy if it can be shown to be reliable in the analysis of uncultured amniotic fluid cells.

The potential of FISH has been greatly enhanced by simultaneous detection of multiple probes each labelled with a distinct hapten.^{8–11} The number of

sequences which can be simultaneously detected in a single hybridization experiment can be further increased by ratio-labelling or ratio-mixing approaches.^{12–14} Digital imaging systems, like the cooled CCD camera with powerful image processing capabilities, can considerably improve the overall detection sensitivity and exploit the full potential of multicolour FISH in clinical cytogenetics.

However, for a technique to be acceptable as a diagnostic test it needs to be reliable and practical. We used five-colour ratio-mixing FISH to detect the copy number of five clinically important chromosomes in a single hybridization area. A combination of a three-colour ratio-mixing FISH and a dual colour was also employed to detect the major

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aneuploidies on two slides from the same sample. This preliminary study on uncultured lymphocytes and amniocytes indicates that both approaches are reliable and can be used for simultaneous detection of the major chromosome aneuploidies in a single hybridization experiment. However, the strategy of detecting probe sets specific for chromosomes X, Y and 21 by three-colour ratio-mixing FISH and a dual colour to detect probes specific for chromosomes 13 and 18 seems to be more straightforward and practical for diagnostic purposes.

MATERIALS AND METHODS

Slide preparation

Uncultured lymphocytes were prepared using 1–2 ml of peripheral blood. The blood samples were incubated with hypotonic solution (75 mM KCl) for 10 min. The hypotonic solution was discarded and the lymphocytes were fixed in freshly prepared fixative (3:1 methanol:acetic acid). The amniocytes were prepared as described by Klinger *et al.*² Briefly, uncultured amniocytes in PBS were dispensed on to 3-aminopropyl triethoxy silane-coated slides at 37°C (35 µl vol/slide), two volumes of d.H₂O pre warmed at 37°C were added and incubated at 37°C for 15 min. The hypotonic solution was carefully decanted and replaced by 100 µl of 30% fixative (3:1 methanol:acetic acid) and 70% 75 mM KCl for 5 min at room temperature. This solution was carefully decanted and fresh 3:1 fix was dropped on to the slide from a height of 60 cm. Excess fix was decanted and slides were dried for 5 min at 60°C, dehydrated through alcohol series (50%, 70%, 90% and 100%), air dried and stored at –20°C until used.

Probes

The probes used in this study consisted of α -satellite DNA probes DXZ1, pDP97 and L1.84 specific for the centromeric regions of chromosomes X, Y and 18, respectively, and the YAC clones 744D11 specific for chromosome 13 and 831B9 specific for chromosome 21. The biotin labelled and digoxigenin labelled DXZ1 was obtained commercially (Oncor). Probes pDP97 and L1.84 were labelled with either biotin-11-dUTP or digoxigenin-11-dUTP. The 13 and 21 specific sequences within the YACs 744D11 and 831B9 were amplified by Alu-PCR using two Alu primers: BK-33 (5'-CTGGGATTACAGGCGTGAGC-

3') priming to the 5' end of the Alu consensus sequences (nt positions 15-34), and SR1 (5'-CCACTGCACTCCAGCCTGGG-3') according to Lengauer *et al.*¹⁵ The PCR products were ethanol precipitated and used for labelling. The Alu-PCR amplified YACs 831B9 and 744D11 were labelled with biotin and digoxigenin, respectively. Standard nick translation protocol was used for both biotin and digoxigenin labelling.

In situ hybridization and detection

Hybridization procedure was basically according to Carter *et al.*¹⁶ In dual colour FISH experiments using chromosomes 13 and 18 specific probes, for each slide 150 ng of digoxigenin labelled 744D11 YAC and 5 µg of human placental DNA were resuspended in 5 µl of hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulphate and 5 µg of salmon sperm DNA) and mixed with 5 µl of hybridization mixture containing 0.5 ng of biotin labelled L1.84 immediately before hybridization.

Three-colour ratio-mixing FISH

In experiments using probe sets specific for chromosomes X, Y and 21, for each slide 100 ng of digoxigenin labelled 831B9 YAC and 5 µg of human placental DNA were resuspended in 5 µl of hybridization buffer and mixed with 5 µl of hybridization mixture containing 0.5 ng of biotin labelled DXZ1 and 50 ng each of biotin labelled and digoxigenin labelled pDP97 (1:1 ratio mixing) prior to hybridization.

In experiments using alpha satellite DNA probes specific for chromosomes X, Y and 18, for each slide 0.5 ng of biotin labelled DXZ1, 5 ng of digoxigenin labelled L1.84 and 50 ng each of biotin labelled and digoxigenin labelled pDP97 were dissolved in 12 µl of hybridization buffer.

Five-colour ratio-mixing FISH

For each slide 2 ng of DXZ1, 10 ng of L1.84 and 120 ng of pDP97 were resuspended in 7 µl of hybridization buffer and mixed with 7 µl of hybridization mixture containing 100 ng of 831B9 YAC and 150 ng of 744D11 YAC immediately before hybridization. The mixing ratio of the probes was as shown in Table 1. All the probes were denatured at 65°C for 10 min and pre-annealed for 30–60 min at 37°C. The slides

Table 1. Mixing ratio of the probes, detection system and the pseudocolours used for simultaneous five-colour detection.

Probe	Biotin	Digoxigenin	Detection	Pseudocolour
DXZ1	1	3	FITC + Rhodamine	Pink
pDP97	3	1	FITC + Rhodamine	Yellow
744D11	—	1	FITC	Green
L1.84	1	1	FITC + Rhodamine	White
831B9	1	—	Rhodamine	Red

were placed in fresh fix (3:1 methanol:acetic acid) for one h and allowed to dry. Slides of uncultured amniocytes were treated with RNase A ($100 \mu\text{g ml}^{-1}$) at 37°C for 20–30 min. The target DNA was denatured by immersing slides in 70% formamide/ $2 \times \text{SSC}$ for 2 min at 65°C . Slides were quenched immediately in cold 70% ethanol and dehydrated in an ethanol series (50%, 70%, 90% and 100%). The two individually pre-annealed probe mixtures were mixed and applied to slides and allowed to hybridize at 42°C overnight.

Detection

After hybridization, the coverslips were removed by rinsing in $2 \times \text{SSC}$ at 42°C . The slides were washed twice in 50% formamide/ $2 \times \text{SSC}$ at 42°C for 5 min, twice in $2 \times \text{SSC}$ at 42°C for 5 min and blocked in wash-A solution ($4 \times \text{SSC}$, 0.05% Tween 20 and 50 mg ml^{-1} dried skimmed milk) for 30 min at 37°C . Immunocytochemical detection was carried out as follows: slides were incubated with 1:200 dilution of rhodamine avidin in wash-A for 15 min at 37°C . Slides were washed three times in wash-A for 5 min at 42°C and then incubated with a mixture of biotinylated goat anti-avidin (1:200 dilution) and FITC-(fluorescein isothiocyanate) conjugated monoclonal anti-digoxin prepared in mouse (1:200 dilution) in wash-A for 15 min at room temperature, followed by two washes in wash-A at 42°C for 5 min. A further 15 min incubation was performed with a mixture of rhodamine avidin (1:200 dilution) and FITC-conjugated anti-mouse IgG (1:200 dilution) in wash-A at room temperature. The slides were then washed twice at 42°C in wash-A for 5 min, twice in $4 \times \text{SSC}$, 0.05% Tween-20 for 5 min and dehydrated through an ethanol series (50%, 70%, 90% and 100%). After air drying the slides were counterstained with $0.4 \mu\text{g ml}^{-1}$ DAPI in mounting medium AF1 (Citifluor Ltd). The signals in dual colour and three colour experiments were analysed by either a conventional fluorescence microscope using a dual band pass filter or a CCD imaging system. In five-colour experiments the images

were captured using a CCD system and pseudo-coloured according to the label ratio using the Smart-capture software (Table 1). All the images were printed using a CCD imaging system equipped with a Mitsubishi colour printer.

RESULTS

In order to optimize the technique of three-colour ratio-mixing FISH for interphase analysis, the method was initially applied to uncultured lymphocytes using X-, Y- and 21-specific probes. After overnight hybridization of probe and target DNA at 42°C , biotin labelled probes were detected with rhodamine (red) and digoxigenin labelled probes were detected with FITC (green), a third colour (yellow) was produced by detection of the ratio mixed Y-probe with both rhodamine and FITC. Using this method we could visualize X-, Y- and 21-specific probes in three different colours by applying only two labelling molecules. The three colours could be easily detected by a conventional fluorescence microscope equipped with a dual band pass filter.

To evaluate the hybridization and detection efficiency for each of the probes the technique was applied to a number of normal uncultured lymphocytes. Twenty uncultured lymphocyte specimens were then hybridized with the combined probe sets and studied in a blind fashion. Analysis of the results was carried out by scoring 50 nuclei for each sample. Nine samples were normal male and eight samples were normal female. Three abnormal samples, a trisomy 21 male, a trisomy 21 female and an XYY were readily distinguished from normals (Fig. 1a–d). A high hybridization efficiency was achieved for all the probes used in this study. In the male peripheral blood samples, an average of 94.1% of the hybridized cells displayed one signal for chromosome X, 93.3% showed one signal for chromosome Y and 93.7% displayed two signals for chromosome 21 (Fig. 1a). In the female samples the average percentage of cells that displayed two signals for chromosomes X and

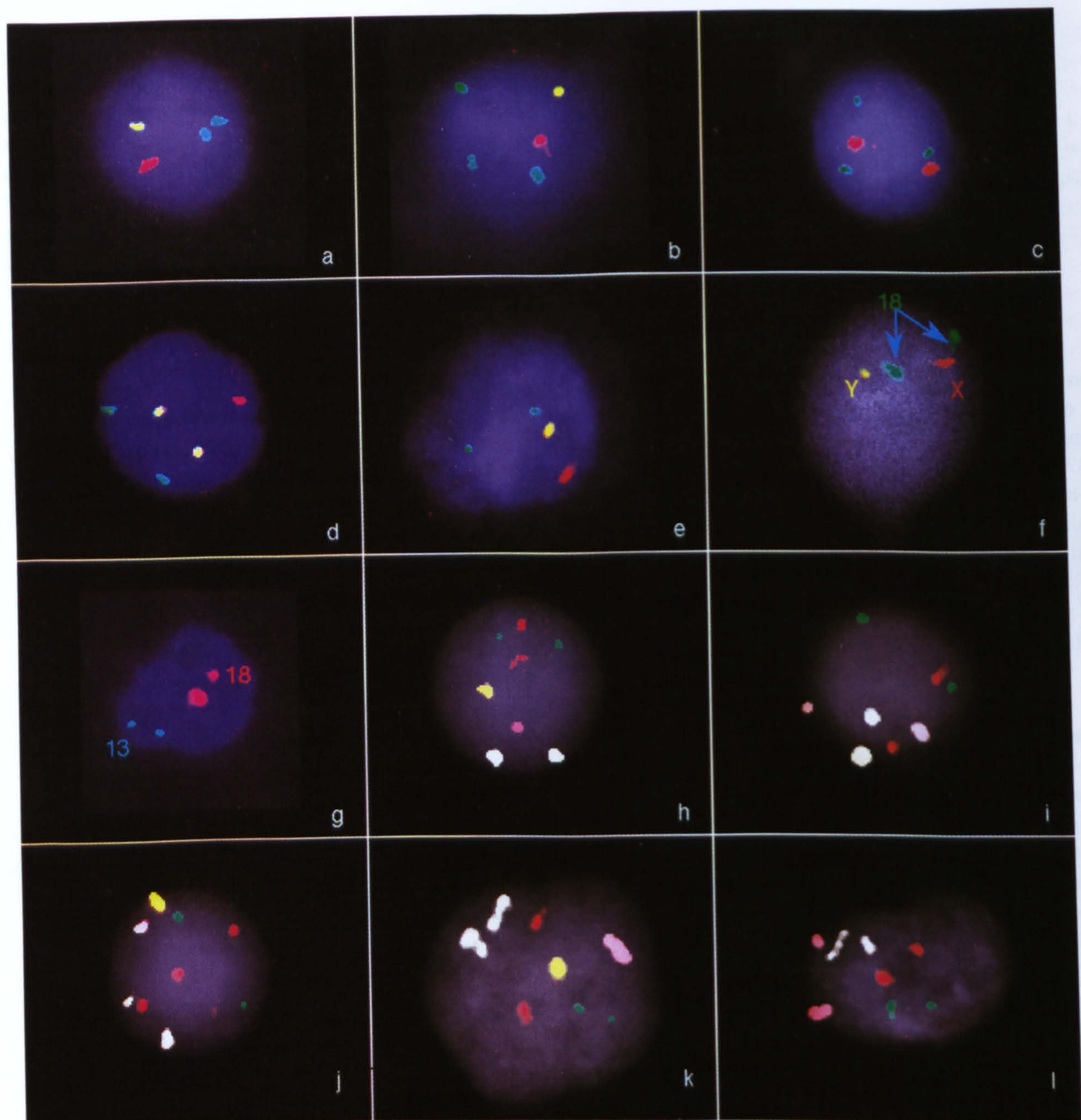


Fig 1. Simultaneous detection of chromosomes 13, 18, 21, X and Y using Ratio-Mixing FISH. a-d, h, i and j are uncultured lymphocytes and e, f, g, k and l are uncultured amniocytes. The colour of signals specific for each chromosome in three-colour and five-colour Ratio-Mixing FISH are illustrated below.

In three colour FISH (a-e): ● X ● Y ● 21

In five colour FISH (h-l): ■ X ■ Y ■ 13 ■ 18 ■ 21

21 was 93.9% and 94.4%, respectively. Two samples displayed three signals for chromosome 21 on an average of 84% of the hybridized cells and were correctly diagnosed as a trisomy 21 male and female (Fig. 1b and c). A third sample was identified as XYY, as 92% of the hybridized cells displayed one signal for chromosome X and 88% displayed two signals for each of the chromosomes Y and 21 (Fig. 1d).

In order to evaluate the utility of the technique for prenatal diagnostic applications, the probe sets were hybridized to 25 unselected and uncultured amniotic fluid preparations. For each sample a minimum of 25 nuclei were analysed. To avoid ambiguities, nuclei covered by cytoplasm, and also overlapping and clumped nuclei were not counted. Twelve samples were correctly scored as normal male, displaying one

signal for chromosome X on an average of 92.3% of the hybridized cells, one signal for chromosome Y on 92% of the cells and two signals for chromosome 21 on 89.3% of the cells (Fig. 1e). Eleven samples were correctly scored as normal females displaying two signals for each of the chromosomes X and 21 on 91.3% and 89.4% of the hybridized cells, respectively. One of the samples failed to produce a result due to the poor quality of the preparation. No abnormal cases were present in this series.

In a further study, probes specific for the centromeric regions of chromosomes X, Y and 18 were simultaneously detected on an unselected series of uncultured lymphocytes and amniocytes. Twenty uncultured lymphocytes were analysed by scoring 50 nuclei for each sample. Nine samples were correctly detected as normal male and eleven samples were correctly detected as normal female. In normal male uncultured lymphocytes, an average of 93% of the hybridized cells displayed one signal for chromosome X, 92.8% showed one signal for chromosome Y and 91% showed two signals for chromosome 18. In normal female lymphocytes, an average of 93.1% and 90.8% of the hybridized cells showed two signals for each of the chromosomes X and 18, respectively. Twenty uncultured amniocytes were also hybridized to the probe sets and analysed by scoring a minimum of 25 nuclei for each sample. Eleven samples were correctly identified as normal male and nine samples were correctly identified as normal female. In normal male samples an average of 91.9% of the hybridized cells showed one signal for chromosome X, 91.4% showed one signal for chromosome Y, and 89.4% showed two signals for chromosome 18 (Fig. 1f). In normal female uncultured amniocytes 92.7% and 89.5% of the hybridized cells displayed two signals for each of the chromosomes X and 18, respectively. No abnormal blood or amniotic fluid samples were identified in this series.

Dual colour FISH was also applied on a limited number of uncultured lymphocytes and amniocytes to detect chromosomes 18 and 13 on a single hybridization area. The centromeric probe L1.84 specific for chromosome 18 was labelled with biotin and detected with rhodamine. The Alu-PCR amplified YAC, 744D11, specific for chromosome 13 was labelled with digoxigenin and detected with FITC. A series of ten uncultured amniocytes was analysed. 91.2% and 90.5% of the hybridized cells displayed two signals for each of the chromosomes 13 and 18 (Fig. 1g).

To extend the number of detectable targets to five, the technique of five-colour ratio-mixing FISH was initially optimized on normal uncultured lymphocytes. We used two haptenization and detection

systems (biotin-rhodamine and digoxigenin-FITC) to simultaneously visualize the specific probes for chromosomes 13, 18, 21, X and Y in a single hybridization experiment. Twenty uncultured lymphocyte specimens were then hybridized with the combined probe sets and analysed in a blind fashion. Twenty five nuclei were scored for each sample. Ten samples were correctly identified as normal male (Fig. 1h) and eight samples were correctly identified as normal female (Fig. 1i). In normal male samples an average of 92% of the hybridized cells displayed one signal specific for chromosome X, 92.4% showed one signal for chromosome Y, 88.9% showed two signals for chromosome 13, 89.8% showed two signals for chromosome 18, and 89.5% showed two signals for chromosome 21. In normal female samples the average percentage of the hybridized cells which displayed two signals for each of the chromosomes X, 13, 18 and 21 was 92.5%, 89.5%, 90.5% and 89.5%, respectively. Two trisomy 21 male samples were detected displaying three chromosome 21 specific signals on an average of 80% of the hybridized cells (Fig. 1j).

The technique was then applied to 27 unselected and uncultured amniotic fluid preparations. For each sample 25 nuclei were analysed. The nuclei covered by cytoplasm, overlapping nuclei and nuclei displaying clumped signals were excluded from counting. Thirteen samples were correctly scored as normal male (Fig. 1k). In these samples an average of 91.7% of the cells showed one signal for chromosome X, 92% showed one signal for chromosome Y, 88.9% showed two signals for chromosome 13, 88.3% showed two signals for chromosome 18 and 85.5% showed two signals for chromosome 21. Nine samples were correctly scored as normal females (Fig. 1l). The average percentage of the cells in normal female samples displaying two signals for each of the chromosomes X, 13, 18 and 21 was 91.5%, 88%, 88.4% and 86.6%, respectively. The remaining five samples failed to produce a result due to the poor quality of the preparations. No abnormal cases were present in this series.

DISCUSSION

In a diagnostic laboratory the volume of the sample which can be provided for FISH analysis is always limited, as most of the amniotic fluid sample is required for standard cytogenetic techniques and it is unlikely that there would be sufficient material to allow detection of the major chromosome aneuploidies in the same sample by uni-colour FISH. Multi-colour FISH has the ability to detect more than

two targets on the same slide, but the number of haptenization and detection systems is limited and the use of more than two labelling systems results in prolonged detection procedures. To overcome these limitations the authors developed the technique of ratio-mixing FISH which has the advantage of smaller sample requirement and the use of a minimum number of probe labelling and detection systems. A method for simultaneous analysis of the probe sets specific for chromosomes X, Y, 13, 18 and 21 on a single hybridization area has been presented. This method has also been compared to the strategy of detecting the above probe sets on two slides from the same sample to provide a practical approach for diagnostic aims.

Although no differences have been observed between the results of ratio-mixing and ratio-labelling,⁹ ratio-mixing seems to provide for more flexibility and safeguards against failure of one specific component in a labelling reaction. It is shown that biotin-11-dUTP and digoxigenin-11-dUTP and subsequent detection of the labelled probes with rhodamine and FITC can be readily used to produce up to five different colours. The post hybridization washing and detection steps that we used in this method are the same as those currently used for uni-colour FISH in this department; the only difference being the detection solutions.

Direct fluorophore labelling of probes is advantageous for diagnostic procedures since it obviates the immunological detection steps; however, it is subject to decreased sensitivity of detection compared to indirectly labelled probes.¹⁰ Furthermore, it does not seem to be an advantage in a multicolour FISH approach, where a directly labelled probe would be used in combination with probes that are indirectly labelled with haptens.

All of the probes used in these experiments generated strong hybridization signals. Each of the probes specific for the sex chromosomes and the chromosome 18 identified a centromeric repeat on the respective chromosome. Centromere repeat probes are well suited for interphase analysis of aneuploidies, because of their superior hybridization signal capabilities.¹⁷⁻¹⁹ However, the signal size is sensitive to pericentromeric heteromorphisms. This appeared to be problematic in both three-colour and five-colour ratio-mixing FISH, using chromosome 18 specific probe L1.84. This probe produced diffuse signals in some nuclei on both blood and amniotic fluid preparations. Although a repeat probe which hybridizes to both chromosomes 13 and 21 is available,¹⁷ it is difficult to use for interphase analysis as it does not distinguish between the two chromosomes. In addition, the centromeric position of the probes does

not allow the identification of Robertsonian translocations, thus limiting the detection of Down's syndrome. For these reasons the Alu-PCR products of YACs 744D11 and 831B9 specific for chromosome 13 and 21, respectively, were used. The use of total yeast genomic DNA or YAC DNA isolated from pulsed-field gels results in higher background and decreased hybridization efficiency due to cross-hybridization of yeast genomic DNA with human DNA.³ As the highly repeated Alu sequences are specific for the human genome, using the Alu-PCR approach, the human inserts within the YACs were selectively amplified and used as specific probes for chromosomes 13 and 21. The signals produced by the amplified DNA probes were clear and intense and the hybridization efficiencies were high.

The results obtained from five colour ratio-mixing FISH experiments indicate that the technique is reliable and can be used for simultaneous detection of major aneuploidies in uncultured lymphocytes and amniocytes. However, a superior quality of amniocyte preparations were needed using this method, as 18.5% of the samples failed to produce a result due to poor quality of preparations. A major disadvantage to a routine diagnostic laboratory is the prolonged time required for signal analysis.

Simultaneous analysis of the chromosomes X, Y and 21 was easily and reliably carried out by three-colour FISH. In comparison to five-colour FISH the time required for signal analysis was short, as the three colours could be readily distinguished under the microscope. The detection efficiency for each of the probes was higher and hence the percentage of the failed experiments was lower (4%) compared to the five-colour FISH.

In three-colour ratio-mixing FISH experiments using specific probes for chromosomes X, Y and 18, overlapping and clumped signals were common since all the probes used were specific for the centromeric regions of target chromosomes producing large signals. As a result, the combination of probes specific for chromosomes X, Y and 21 was regarded as more reliable for three-colour FISH.

The results also indicate that the copy number of chromosomes 13 and 18 can be readily detected by dual-colour FISH in uncultured lymphocytes and amniocytes. Although the probe L1.84 produced diffuse signals on some of the hybridized cells, no problems were encountered in analysis, since only two colours had to be distinguished and the signals produced by the amplified DNA probe specific for chromosome 13 were clear and intense (Fig. 1g).

These studies reveal that the five most clinically important aneuploidies can be readily and reliably detected on two slides from the same sample using

a ratio-mixing FISH to detect the chromosomes X, Y and 21 and a dual colour to detect the chromosomes 13 and 18. At present this approach appears to be a more practical way for prenatal detection of major chromosome aneuploidies than simultaneous detection of five chromosomes in a single hybridization area. However technical advances in clinical sample preparation, defined probe sets and automation of signal counting can extend the diagnostic limits of five-colour ratio-mixing FISH, providing a powerful technique for rapid detection of multiple chromosomal abnormalities in a single hybridization experiment. Simultaneous five-colour detection will almost certainly be necessary, if non-invasive methods of fetal sampling are successfully developed.

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