Application of nested PCR, whole genome amplification and comparative genomic hybridisation for single cell genetic analysis

by

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To Chiun-Mei, Ping-I, and Mu-Chun

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List of Abbreviations

A	Adenine
ADO	Allele drop-out
AFP	Alphafetoprotein
APC	Adenomatous polyposis coli
ART	Artificial reproductive technique
β HCG	Beta human chorionic gonadotrophin
bp	Base pair
C	Cytosine
°C	Degree of Celsius
CCD	Charge couple device
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CGH	Comparative genomic hybridisation
CISS	Chromosomal in situ suppression
Cot	Concentration over time
CVS	Chorionic villi sampling
DAPI	4'-6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
del	Deletion
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOP-PCR	Degenerate oligonucleotide primed PCR
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
e.g.	exemplia gratia (for example)
EHF PCR system	Expanded High Fidelity PCR system
et al	et alia
FACS	Fluorescent activated cell sorting
fg	Femtogram
FISH	Fluorescence in situ Hybridization
FITC	Fluorescein isothiocyanate
F-PCR	Fluorescent PCR
FR	Fluorescent ratio

G	Guanine
g	Gram
inv	Inversion
ISH	In situ hybridisation
IVF	In vitro fertilisation
kb	Kilobase
LINEs	Long interspersed nuclear elements
LOH	Loss of heterozygosity
l	Microliter
M	Molar
MACS	Magnetic activated cell sorting
Mb	Megabase
M-FISH	Multiplex-FISH
mg	Milligram
ml	Milliliter
mM	Millimolar
µg	Microgram
µg	Microgram
µl	Microliter
ng	Nanogram
NPD	Non-invasive prenatal diagnosis
NRBC	Nucleated red blood cell
NTD	Neural tube disease
OD	Optical density
p	Short arm of chromosome
PBS	Phosphate-buffered Saline
PCR	polymerase chain reaction
PEP	Primer extension preamplification
pg	picogram
PGD	preimplantation genetic diagnosis
PHA	Phytohaemagglutinin
pmol	Picomol
PRINS	Primed in situ labelling
PUBS	Percutaneous umbilical blood sampling
q	Long arm of chromosome
QF-PCR	Quantitative fluorescent PCR
RNase	Ribonuclease
rpm	Revolutions per minute

SDS	Sodium dodecyl sulphate
SINEs	Short Interspersed Nuclear Elements
SKY	Spectral karyotyping
SSC	Saline sodium citrate
T	Thymine
t	Translocation
TBE	Tris-acetate ethylenediaminetetra acetic acid
TRIS	Tris(hydroxymethyl) amniomethane
UV	Ultraviolet
v/v	Volume per volume
WCP	Whole chromosome painting

Declaration

I certify this thesis does not contain material previously published or written by any other person except where referred to in the text and the results of this study has not been submitted for any other degree or diploma.

S Chiang

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As far as it may look like between Taipei and Glasgow, the gap between the scalpel and the pipette is never so wide as we imagine nowadays. However, a Ph.D project can be a winter Ben Nevis which will make great demands on you. The work could really get tough without a lot of support and enthusiasm. I would like to thank people who have given these and friendship as well:

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Summary

Single cell genetic analysis is a necessity in the field of preimplantation genetic diagnosis (PGD), non-invasive prenatal diagnosis and can be applied to isolated tumour cells in the blood. Current techniques used in PGD and non-invasive prenatal diagnosis are mainly based on either the polymerase chain reaction (PCR) or fluorescence in situ hybridisation (FISH). However, the genetic analysis at a specific locus by PCR has the drawback of sacrificing further detection of common chromosomal aberrations if there is only one cell available. The limitation of FISH using specific probes is that it provides information on only one or a few loci at a time.

Comparative genomic hybridisation (CGH), a molecular cytogenetic technique, has the advantage over FISH in permitting a comprehensive analysis of chromosomal imbalances across the whole genome. Combining degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) and CGH techniques, it is possible to study minute quantities of DNA prepared from a very few cells. The aim of this project is to verify the feasibility of applying these techniques to a single cell and investigate the prospect of a novel strategy using whole genome amplification (WGA), nested PCR and CGH to increase the scope and capacity of single cell genetic analysis.

The work started first to prove the amplification power of DOP-PCR from a single cell. Various protocols aiming at whole genome amplification (WGA) have different efficiencies in terms of yield and genomic coverage. DOP-PCR is superior to primer extension preamplification (PEP) in producing more quantity of DNA from a single cell. The amplification power of DOP-PCR from a single cell (5 pg) resulted in 10 µg yield with the bulk of DOP-PCR products between 200-2000 bp. DOP-PCR is designed to faithfully amplify the genome, and provide sufficient DNA template. Thus, numerous specific loci and the imbalance of every chromosome can be assessed in a single cell.

To investigate the feasibility of locus detection from WGA products, two sets of nested PCR aiming at sex determination or CF ΔF508 detection were optimised and tested on a range of DNA sources. The results proved that a strategy of nested PCR could be used to determine the sex and CF status on DOP-PCR-amplified DNA derived from single cells. Nested PCR was also used in the genetic analysis directly on a single cell. In the study of single blastomeres, the sex could be determined in 55.6% of cases (10/18) when nested PCR was used directly on single blastomeres.

The feasibility of single cell CGH in the diagnosis of major chromosome abnormalities such as sex chromosome anomaly, trisomy 18 and 21 was established in this study. To produce successful single cell CGH experiments, the normal reference DNA could be made from either amplified DNA or non-amplified DNA. This study demonstrated that a reliable non-amplified reference DNA could increase the success rate of single cell CGH and help to identify the underlying causes of failed CGH experiments.

For quantitative analysis of CGH experiments, the fluorescence ratio cut-off value was usually set at 1.2 and 0.8 to represent chromosomal gains and chromosomal losses, respectively. However, the fluorescent ratio profiles using stringent cut-off values would reduce the false positives with the risk of failing to detect the true abnormalities. In this study, the average number of false positives was 5 when threshold of 1.2/0.8 was used. Apart from the objective quantitative analysis, single cell CGH diagnosis of trisomy 21, 18 and sex could also be qualitatively verified from CGH images.

After the DOP-PCR/CGH techniques had been proved feasible from a single cell, the sensitivity and reliability of CGH was further tested on 10-20 stained cells scraped from slides in order to simulate the current strategy used in the non-invasive prenatal diagnosis. This part also involved firstly, the correct diagnosis of trisomy 18 and 21 and secondly, several coded samples of known sex were tested for the performance of

single cell CGH. The results showed that chromosomal aberrations such as isochromosome X and segmental aneuploidy could be reliably detected. However, a small deletion at single band level could not be detected by single cell CGH. Thirdly, a manufactured mosaicism designed by mixing trisomy 18 male cells with normal female cells was tested to see if at 50-70 % mosaic level the trisomy could be detected by single cell CGH. The results illustrated that trisomy 18 could not be detected at the 70% mosaic level using the current protocol. This may illustrate that accurate identification of cells of fetal origin is mandatory if current DOP-PCR/CGH techniques are to be applied in the field of non-invasive prenatal diagnosis.

The final part involved using CGH on blastomeres and tested the possibility of employing a shortened protocol for single cell CGH. The preliminary results confirm that chromosome abnormalities may be a common phenomenon in the early embryonic cells. The success of overnight CGH illustrates that further reduction of time is possible. This may allow an expansion of its future application in PGD.

Overall, this study demonstrates that DOP-PCR/nested PCR/CGH has the potential to serve as a powerful supplement to the present genetic analysis from a single cell. Concomitant detailed chromosome analysis and specific locus detection may become feasible in the field of PGD and non-invasive prenatal diagnosis in the near future.

Chapter 1

Introduction

1.1 Structure of human genomic DNA

The composition of the human genome is organised in two forms, a complex nuclear genome and a simple mitochondrial genome. The nuclear genome contributes the great volume of essential genetic information, most of which is decoded to specific polypeptides on cytoplasmic ribosomes. Mitochondrial DNA is in the form of single circular double-stranded DNA, 16569 bp long, which specifies only a very small portion of mitochondrial function.

1.1.1 Nuclear genome constitution

The nuclear genome of a human haploid cell contains about 3 x 10^9 bp of DNA. The great majority of the cellular DNA is never transcribed in any cell and only about 3% of the human genome is coding DNA (Strachan 1992, Figure 1.1).

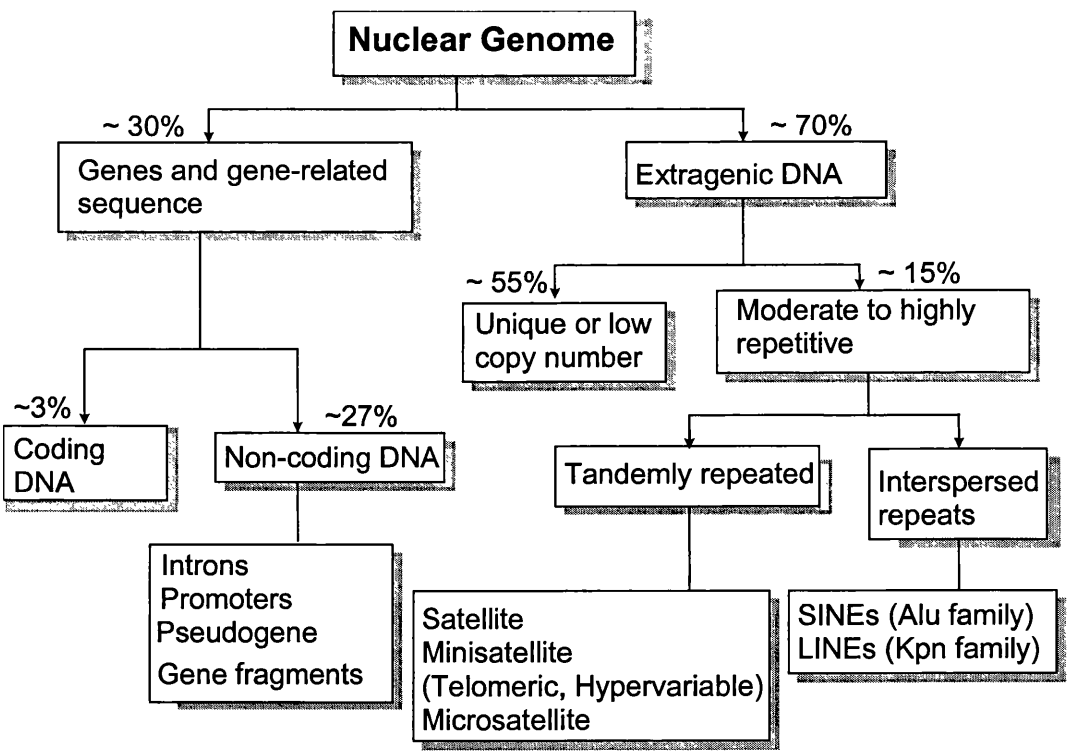


Figure 1.1: Organization of human nuclear genome (Adapted from Bennett 2000)

The recent publication of two draft sequences of the human genome is a landmark achievement in the history of mankind. Consortium and Celera Genomics have completed sequencing 90% of the euchromatic chromosomal regions (International Human Genome Sequencing Consortium 2001; Venter et al, 2001).

Mapping the genome has located each of approximately 100,000 genes on our 24 different chromosomes (Williams and Hayward 2001). In a 400-band metaphase karyogram, on this basis, there are about 200 genes per band. (Strachan 1992) However, the average gene density is dependent on the base composition of the chromosomal region. Chromosomal regions with abundant GC (guanine, cytosine) content are thought to be rich in genes, while those with rich AT (adenine, thymine) contents are deficient in genes. Although on average the human genome has about one gene per 30-60 kb of DNA, certain chromosomal regions such as the centromeres and telomeres are particularly deficient in genes (Strachan 1992).

1.1.2 Repeated sequences

The moderately to highly repetitive sequences can be divided into two types, depending on whether the individual repeat units are dispersed singularly (interspersed repetitive DNA) or clustered together (tandem repeat). There are several families of interspersed repetitive DNA sequences, and the two largest are known as short and long interspersed nuclear elements (SINEs and LINEs, respectively). The most abundant, Alu repeat family, which belongs to SINEs, is primate specific and accounts for 5% of total human DNA. These tandemly repeated sequences may be sub-divided according to the average size of the arrays of repeats into satellite DNA, minisatellite DNA and microsatellite DNA (Bennett 2000).

Human satellite DNA is found particularly in centromeric heterochromatin, which is essential for ensuring disjunction of the chromosomes into daughter cells following cell division at meiosis and mitosis. Minisatellites

can be subdivided into two types: telomeric and hypervariable. Telomeric DNA is required for complete replication of the DNA at the chromosome termini. Hypervariable minisatellite DNA includes those first discovered and termed as 'DNA fingerprinting' (Jeffrey et al 1985).

Microsatellites or STRs (short tandem repeats) are ideal genetic markers, because they are abundant, highly polymorphic, occur evenly throughout the whole human genome, and are small enough to be amplified using PCR. Microsatellite-associated applications are very useful in several fields: (1) Diagnostic testing for trinucleotide repeat associated diseases (2) loss of heterozygous (LOH) analysis (3) linkage analysis (4) paternity test (5) forensic science (Bennett 2000).

1.2 Chromosomes

The basic material of chromosomes consists of DNA complexed with different DNA-binding proteins, notably histones, and acidic proteins (Figure 1.2).

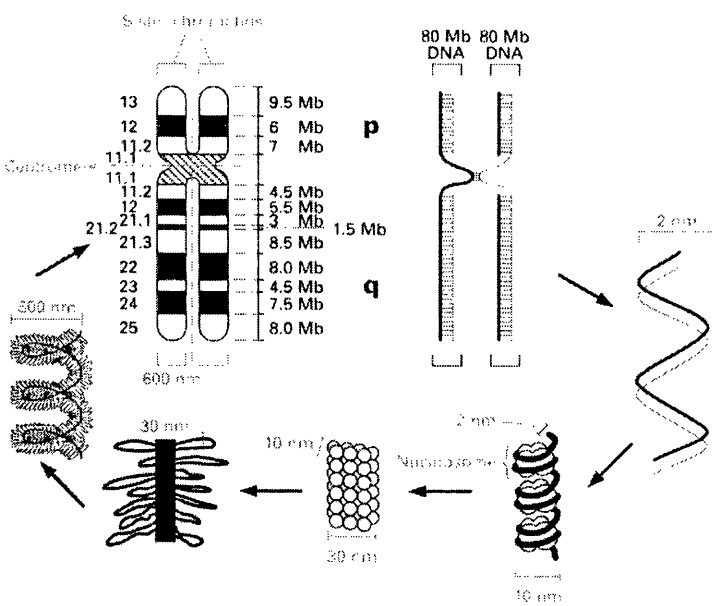


Figure 1.2: From DNA duplex to metaphase chromosome (Human chromosome 17, G-banded, 550-band preparation. Adapted from Strachan 1992)

The DNA content of each chromosome is thought to consist of a single linear double-stranded DNA molecule and an average size chromosome has approximately 130 Mb but can vary between 50 Mb and 250 Mb (Table 1.1).

Chromosome	Amount of DNA (Mb)	Chromosome	Amount of DNA (Mb)
1	250	**13**	110
2	240	**14**	105
3	190	**15**	100
4	180	**16**	85
5	175	**17**	80
6	165	**18**	75
7	155	**19**	70
8	135	**20**	65
9	130	**21**	55
10	130	**22**	60
11	130	**X**	140
12	120	**Y**	60

The DNA content is given for a chromosome prior to entering the S (DNA replication) phase of cell division

Table 1.1: DNA content of human chromosome (Adapted from Strachan 1992)

At the metaphase stage, the chromosomes become more condensed and can be resolved by optical microscopy as structures which are over 1 μm wide and range in length from 2 μm (chromosome 21) to 10 μm (chromosome 1). A variety of treatments cause chromosomes in dividing cells to become visible as a series of alternating dark and light staining bands. The dark bands are generally recognised to contain AT-rich DNA, whereas the light bands contain GC-rich DNA. G-banding provides high quality chromosome analysis with approximately 400 bands per haploid genome, while each of these bands corresponds to around 8 Mb of DNA.

The bulk of the chromatin is euchromatin which uncoils following cell division, becomes light-staining and includes active genes interspersed with non-transcribed DNA sequences. However, certain chromosomal regions continue to remain condensed throughout the life cycle of the cell. They appear as dark-staining areas, termed heterochromatin, and have been presumed to be genetically inactive (Strachan and Read 1997).

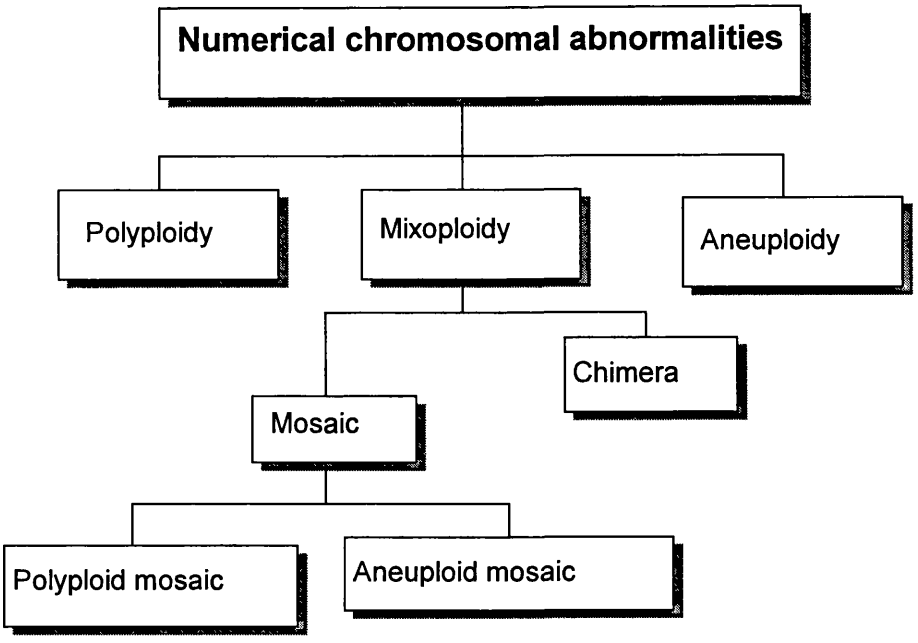
1.3 Chromosome disorders

Chromosomal abnormalities may be present in cells throughout the body, or may be present in a small subset of cell or tissues. Chromosome abnormalities mostly fall into one of two categories: numerical abnormalities and structural abnormalities. Chromosome disorders affect 6 per 1000 newborn infants and two-thirds of these infants will be disabled, and a proportion of the remainder will, in adult life, be at risk of either miscarriage or having chromosomally unbalanced offspring (Tolmie 1995).

1.3.1 Numerical abnormalities

Numerical abnormalities involve a change in the number of chromosomes, without involving chromosome breakage. Three classes of numerical chromosomal abnormalities can be distinguished: polyploidy, aneuploidy, and mixoploidy (Figure 1.3).

The most common numerical chromosomal abnormalities are demonstrated in Table 1.2. Trisomy 21 is the most common aneuploidy found at birth and the majority of cases are the result of non-disjunction occurring during maternal meiosis; only 5% are attributable to mitotic error (Yoon et al 1996). Similarly X chromosome aneuploidy associated with XXX and XXY is mainly the result of maternal meiotic errors, with 9% being attributable to mitotic error (MacDonald et al 1994).



Polyploidy: Extra copies of all chromosomes, e.g. triploidy (3n) or tetraploidy (4n)

Aneuploidy: Loss or gain of only certain chromosomes (e.g. trisomy 21or monosomy X)

Mixoploidy: Two or more cell lines which differ in chromosome number

Mosaic: The different cell lines derive from a single zygote

Chimera: The different cell lines originate from different zygotes

Polyploidy mosaic: e.g. diploidy/triploidy

Aneuploidy mosaic: e.g.normal/trisomy 21

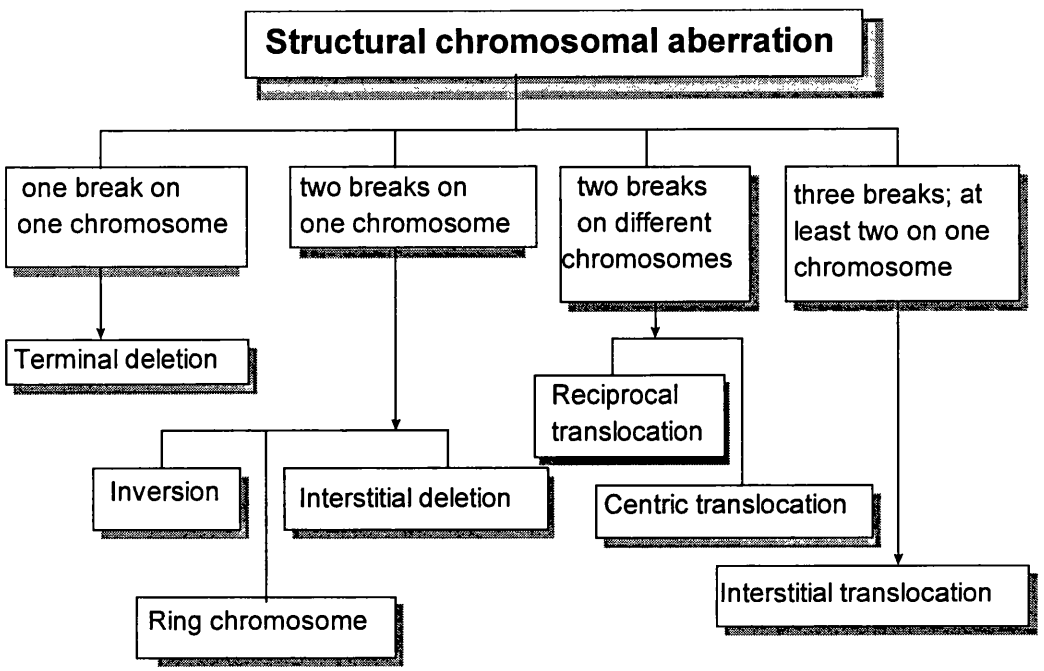
Figure 1.3: Types of numerical chromosomal abnormality (Adapted from Strachan and Reed 1997)

The most common numerical chromosomal aberrations
Trisomy 21 (Down syndrome)
47,XXX
47,XXY (Klinefelter syndrome)
47,XYY
Trisomy 18 (Edwards syndrome)
45,X
Trisomy 13 (Patau syndrome)

Table 1.2: The most common numerical chromosomal aberrations.

1.3.2 Structural abnormalities

Structural chromosomal abnormalities results from chromosome breakage with subsequent reunion in a different configuration and can be balanced, if there is no net gain or loss, or unbalanced, if there is net gain or loss (Figure 1.4).



Terminal deletion: Acentric fragment is lost

Inversion: region between breakpoints is inverted

Ring chromosome: Region between breakpoints forms a circle by fusion between breakpoints

Interstitial deletion: Region between breakpoints is discarded; terminal fragments fuse

Centric translocation: Fusion of centric fragments of two acrocentric chromosomes

Reciprocal translocation: Balanced exchange of acentric fragments

Insertional translocation: Region between two breakpoints on one chromosome is excised and inserts at location of third breakpoint which may be on the same or different chromosome

Figure 1.4: Major types of structural chromosomal aberrations. (Adapted from Strachan and Reed 1997)

In balanced rearrangements, they are usually harmless unless one of the breakpoints involves an important functional gene, whereas unbalanced rearrangements would cause severe physical or mental disability due to the presence of both monosomy and trisomy. If two breaks occur in a single chromosome, one of three outcomes is usually observed: a chromosome inversion, an interstitial deletion, or a ring chromosome (Figure 1.5).

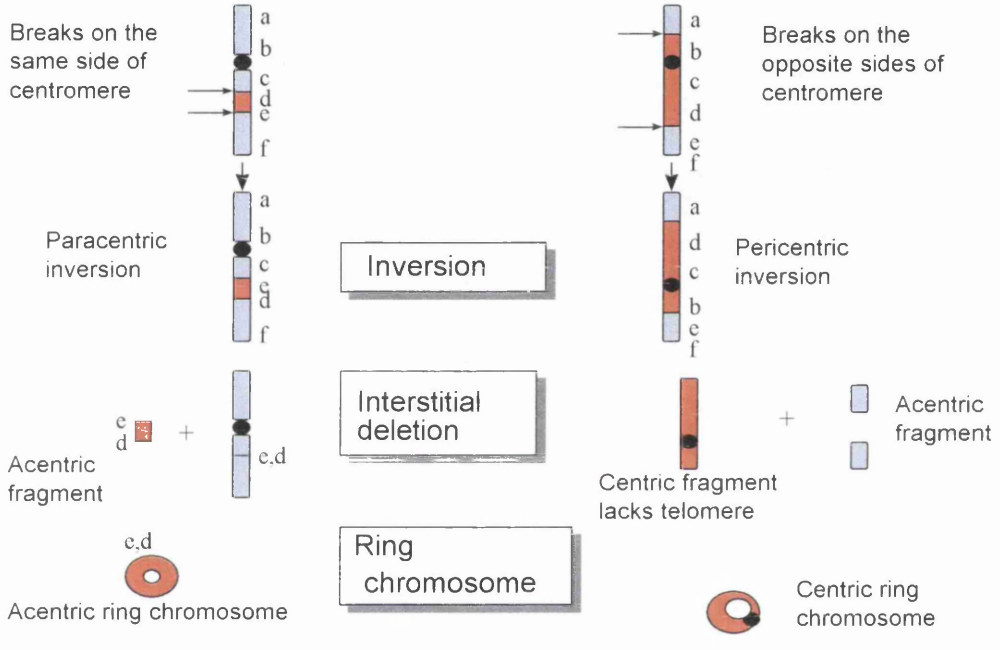


Figure 1.5: Alternative outcomes of two breaks on a single chromosome. (Adapted from Strachen and Reed 1997)

If breaks occur on two or more chromosomes, hybrid chromosomes may be formed by joining segments originally on different chromosomes (chromosome translocation). Three types of translocation are known: a reciprocal translocation, a centric (Robersonian) fusion, or an insertional

translocation. In Robertsonian translocations acrocentric chromosomes fuse at their centromeres. Because acrocentric short arms are mainly composed of repeated sequences and contain no essential genes, balanced carriers are phenotypically normal despite having only 45 chromosomes. In reciprocal translocations, the rearrangement involves two or more chromosomes but the breakpoints are not at their centromeres. A carrier of a balanced reciprocal translocation can produce unbalanced gametes, resulting in zygotes with partial trisomy and partial monosomy for defined chromosomal regions (Figure 1.6).

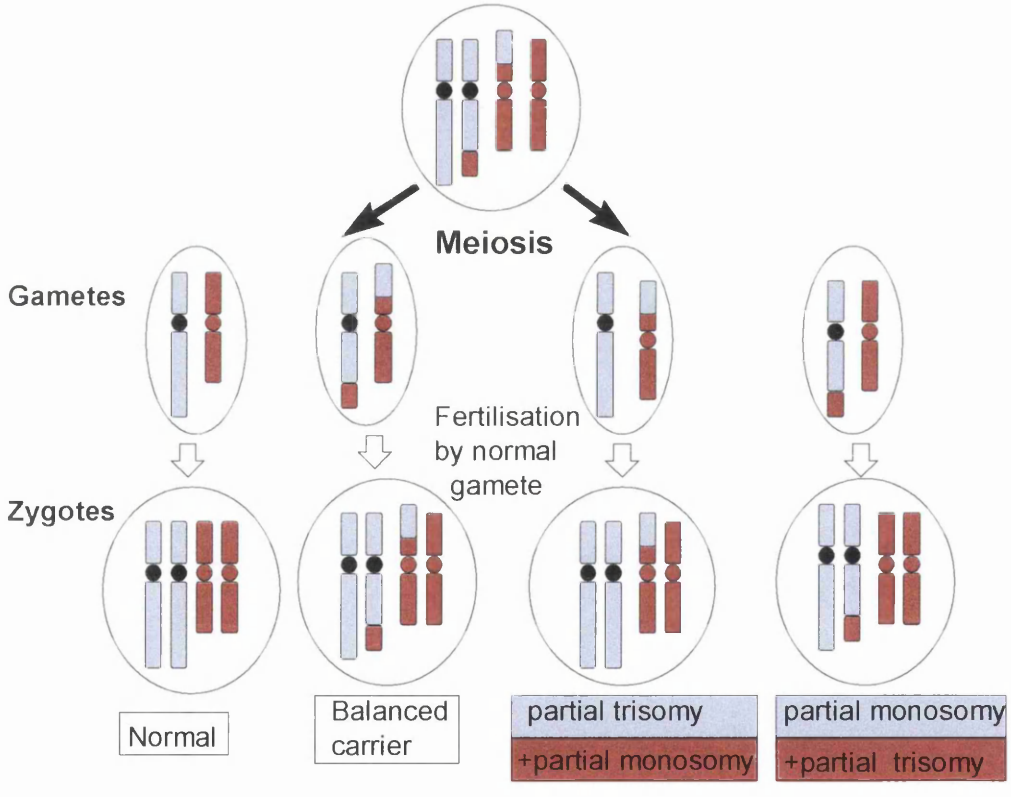


Figure 1.6: Gametogenesis of a balanced reciprocal translocation and the possible outcomes. (Adapted from Strachan and Reed 1997)

1.4 Prenatal diagnosis

The advances in the prenatal diagnosis of birth defects have opened a new era in preventive medicine. With the rapid proliferation of newer techniques in prenatal diagnosis, the list of disorders that can be diagnosed and treated is increasing rapidly. Current practice for detection of fetal abnormalities includes ultrasound and maternal serum screening for NTD/Down syndrome (e.g. test combining the risk of maternal age, AFP, β-HCG, or other markers) (Raghad et al 1999). When genetic abnormality is suspected, fetal tissues can be sampled by the employment of various invasive procedures such as chorionic villi sampling (CVS), amniocentesis, percutaneous umbilical blood sampling (PUBS), or fetal biopsy. Fetal cells obtained can be tested directly with various molecular techniques or processed by culturing the tissues for karyotyping.

1.5 Principal techniques used in prenatal diagnosis

Apart from conventional cytogenetic analysis, molecular laboratory techniques are increasingly important in the evaluation of fetuses at risk of single gene disorders or chromosomal abnormalities. New assays or techniques such as amplification refractory mutation system PCR, fluorescent PCR, heteroduplex analysis and the protein truncation test are now applied in prenatal diagnosis (Van den Veyver and Roa 1998). Recent advances in molecular cytogenetics such as CGH, primed in situ labelling (PRINS), five-colour FISH, the development of new telomeric probes and 24-colour FISH are being evaluated for their roles in the prenatal diagnosis of chromosomal aberrations (Lapierre et al 1998, 2000; Bryndorf et al 1995; Daniely et al 1998, 1999; Divane et al 1994; Lesto et al 1999, 2000, Mohaddes et al 1996; Muller-Navia et al 1996).

1.5.1 Conventional cytogenetics

Conventional cytogenetics involves the examination of a dividing population of cells by blocking cell division at metaphase with an inhibitor of spindle formation (i.e. Colcemid). The development of chromosome

banding techniques allowed identification of individual chromosome pairs and chromosome regions. Human chromosomes are numbered from 1 to 22, X and Y, the classification being based on the size and arm ratios, and banding pattern of the chromosomes.

1.5.1.1 G-banding

The G-banding obtained by digesting the chromosomes with proteolytic enzymes are the most widely used technique in clinical cytogenetic laboratories for routine chromosomal analysis. This technique is described as GTG (G-bands by trypsin using Giemsa). Chromosomes stained by this protocol exhibit light- and dark-stained regions (light and dark bands) along their lengths. The G-band pattern induced by trypsin is very similar to that produced by quinacrine. Although the mechanism of G-banding is not yet completely understood, it is hypothesized that the differences between positive and negative G-bands may be due to the distribution of chromosomal protein and DNA (Verma and Babu 1995).

The G-bands obtained by GTG are far superior in resolution to those using fluorochromes. The permanent nature of these preparations facilitates a thorough microscopic analysis of metaphases (Verma and Babu 1995).

1.5.2 Molecular cytogenetics

Molecular cytogenetics can be described as the microscopic analysis of chromosomes and chromatin in metaphase and interphase using labelled DNA probes. Slides of metaphase chromosomes and interphase nuclei are prepared from cells that grow in suspension culture, such as lymphocyte, or from cells that grow attached to a substrate, such as fibroblasts and amniocytes. DNA in the metaphase chromosomes or interphase nuclei is then hybridised with a probe labelled either non-isotopically with biotin or digoxigenin, or isotopically with ^{3}H. Non-isotopic probes are generally detected by fluorescence or an enzymatic method; ^{3}H-labelled probes are visualised by emulsion autoradiography (Ferguson-Smith 1992).

The emergence of non-isotopic alternatives has greatly improved the application of molecular cytogenetics, particularly in prenatal diagnosis, because rapid results become possible. The methodology depends on the incorporation of a variety of haptens into the nucleotides used for making the probes, which can be detected immunologically by binding the reporter molecule with specific antibodies (indirect detection). The most widely used reporter molecules are biotin and digoxigenin. To build strong fluorescent signals, two layers (sandwich amplification) of fluorescently labelled antibodies can be applied. However, while the signal intensity increases, the background noise also increases. Direct detection involves fluorochromes coupled directly to the nucleotides (e.g. FITC-12-dUTP or Texas Red) or fluorescently labelled reporter molecules (Figure 1.7).

In situ hybridisation (ISH) can be used to determine the chromosomal map and the relative order of genes and DNA sequences within a chromosomal band. It can also be used to detect aneuploidy, gene amplification, and subtle chromosomal rearrangements. New molecular cytogenetic techniques using the same principles as ISH have been developed and evaluated in various fields. These include: CGH, PRINS, SKY, micro-FISH, Multi-FISH, Fiber FISH, and DNA microarrays (Kallioniemi et al 1992; Koch et al 1989; Engelen et al 1998; Macville et al 1997; Schrock et al 1996, 1997; Speicher et al 1996; Florijn et al 1995; Kraus et al 1997; Ried et al 1997; Pinkel et al 1998).

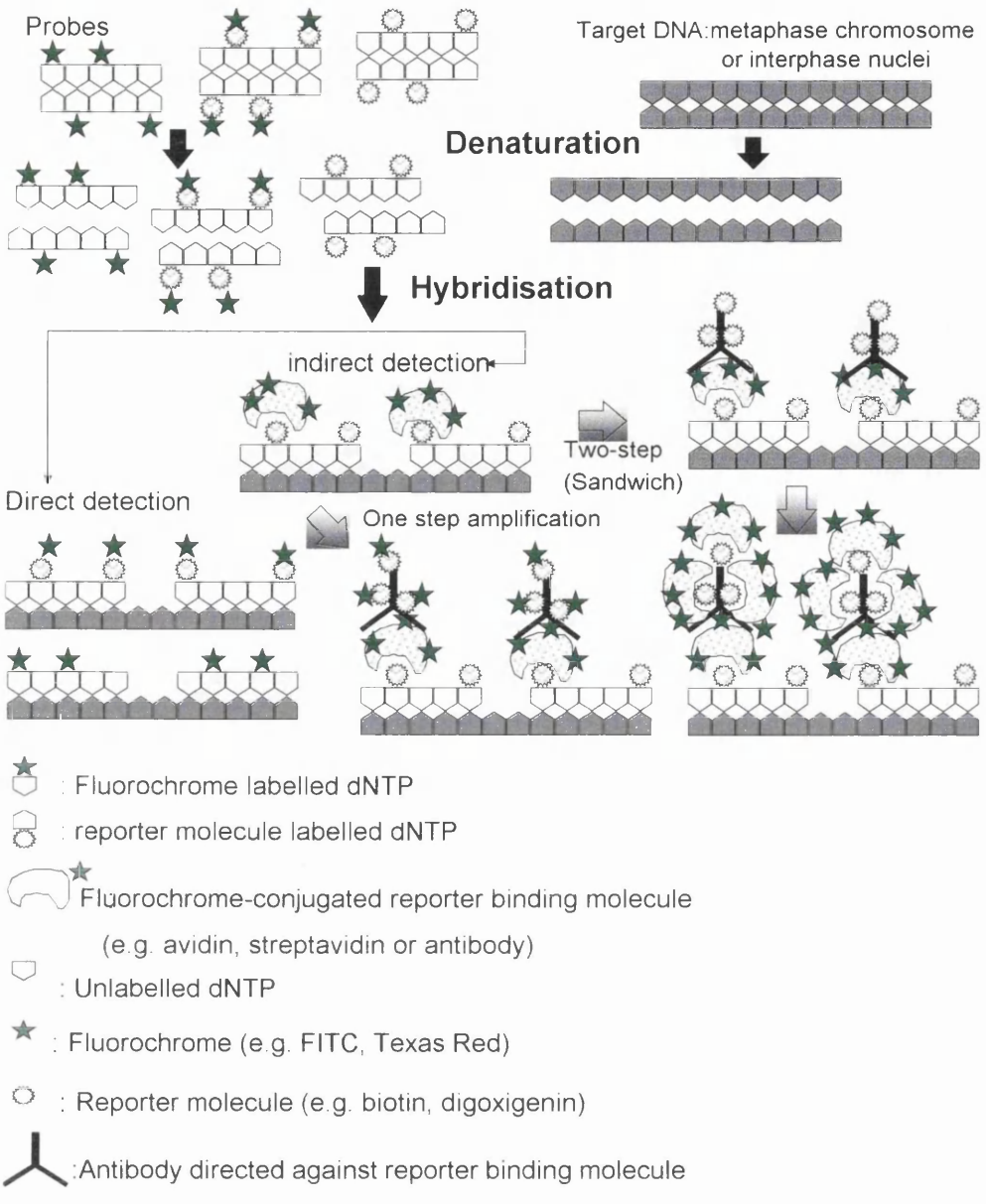


Figure 1.7: Schematic illustration of the detection of fluorescent signals. For direct detection, it uses fluorochrome-conjugated dNTP or fluorescent reporter molecule. For indirect detection, it uses one layer of fluorochrome-conjugated reporter binding molecule. To amplify the signal, the procedure can be either one step or two steps. In the case of two-step amplification, usually the last detection reagent is the same as the one used for the primary detection; this procedure is therefore also referred as 'sandwich' amplification.

1.5.3 Polymerase chain reaction (PCR)

PCR is an enzyme-catalysed biochemical reaction in which small amounts of a specific DNA segment is amplified into large amounts of linear double-stranded DNA. PCR is a powerful technique which can permit rapid amplification of DNA sequence, even when the starting material is from a single cell or badly degraded DNA. As a result it has been extensively employed in areas such as medicine, evolution studies, and forensic science.

In PCR, two primers (short single stranded DNA with 15-30 nucleotides) are used that are complementary to opposite strands of the target DNA to be amplified. After heat mediated denaturation of the template DNA, the primers bind to their respective sequences (annealing) on the template DNA and a DNA polymerase synthesizes a complementary strand in the 5' to 3' direction (extension). Each round of denaturation, annealing, and extension is known as a cycle. After 30 cycles, the products of PCR will include, in addition to the starting DNA, about 10^5-10^6 copies of the specific target sequence, an amount which is easily visualised as a discrete band of a specific size on agarose gel electrophoresis (Baumforth et al 1999).

Like all enzymatic reactions, PCR is not an unlimited process. In most applications, after 20-40 cycles, the reaction enters an 'amplification plateau', a linear phase where additional cycles will not lead to any further increase in amplified product. This plateau effect results from the exhaustion of reagents such as dNTPs and primers. Another limitation of PCR is that it becomes progressively more difficulty to amplify large DNA sequences and the method has largely been confined to amplifying small DNA regions (generally less than 5 kb).

1.6 Genetic analysis on minute amounts of DNA

Genetic analysis performed on scarce DNA, or even to a single cell level, has been used in a number of clinical and research applications. Examples include: (1) preimplantation diagnosis (2) non-invasive prenatal

diagnosis (3) isolated tumor cells from blood or bone marrow (4) forensic science (5) investigation of ancient DNA (6) DNA microdissected from various cytogenetic preparations (Handyside et al 1997; Sekizawa et al 1996; Ohshima et al 1999; Pantel et al 1999; Racila et al 1998; Hummel et al 1999; Viesbach et al 1994; Engelen et al 1998).

1.6.1 Micromanipulation techniques

A number of techniques have been described to retrieve single cells from cell suspensions or slides. A microscope fitted with hydraulic micromanipulators is necessary for preimplantation diagnosis or chromosome microdissection (Cui et al 1994; Hozier et al 1996). Microdissection has become a very popular method for making both whole-chromosome and region-specific painting probes (Meltzer 1992; Guan et al 1994). For genetic analysis of tumour samples, cell heterogeneity is one major problem, as a large variety of cell types usually present in these specimens can mask cell-specific genetic alterations associated with disease. Although cells of interest can now be retrieved by microdissection techniques, it is difficult to isolate single cells from a tissue section without the risk of contamination by the neighbouring cells. However, this can now be overcome by a new laser-assisted microdissection technique (Becker et al 1996, 1997; Aubele et al 1998). The development of laser microbeam based, contamination-free preparation of one single or a few homogeneous cells out of a heterogeneous cell population is an important step towards highly precise molecular analysis of genetic defects, underlying cancer, infection or disease (Schütze et al 1997)

1.6.2 Preimplantation genetic diagnosis (PGD)

PGD is a very early form of prenatal diagnosis aimed at identifying embryos carrying serious genetic diseases before implantation. In current prenatal diagnosis, fetuses at risk are tested on samples of chorionic villi obtained at 10 weeks' gestation, or fetal cells obtained by amniocentesis

at 16 weeks' gestation. It can be as late as 18-22 weeks' gestation when a definite diagnosis is made and this can leave families confronting the social and religious dilemmas regarding termination of pregnancy.

PGD was first developed to diagnose genetic diseases such as sex-linked or autosomal inherited diseases. However, the scope of PGD has been greatly widened and the indications for diagnosis now may include: carriers of balanced translocations, couples with recurrent abortions, aneuploidy screening in aged women, in ICSI (intracytoplasmic sperm injection) candidates with a severe oligoospermia and /or the presence of meiotic anomalies, in patients with recurrent artificial reproductive technique (ART) failures, and in patients in whom a gonadal mosaicism is suspected (Handyside et al 1998).

The clinical application of PGD uses the methods of super-ovulation and in vitro fertilisation (IVF) established for the treatment of infertile couples. Human embryo biopsy has been successfully carried out at the cleavage stages on day 3 post-insemination, at the six- or eight-cell stage, or at the blastocyst stage on days 5 or 6. However, very few embryos reach blastocyst stage and to date there have been no reports on blastocyst biopsy for clinical PGD cycles (Harper and Wells 1999).

In PGD, the time required for genetic analysis is kept to a minimum because the prolonged culture of human embryos tends to reduce viability and the resulting pregnancy rates decline. In most cases, the aim is to diagnose the status of the embryos and transfer a maximum of two unaffected embryos later on the same day as embryo biopsy. Recent advances in the culture of human embryos to the blastocyst stage would allow an additional 2-3 days to perform some more lengthy diagnostic procedures (Gardner et al 1998; Gianaroli et al 1998; Veigh et al 1999).

1.6.2.1 Early cell cleavage and mosaicism

The application of the FISH technique to spare, untransferred embryos after IVF cycles has led to the most interesting findings that 30% of normally developing cleavage stage embryos are chromosomally mosaic

(Delhanty et al 1993, 1997). Using conventional cytogenetic analysis, karyotyping was possible in 39.8% of embryo preparations. The data also show that 30% of apparently normally fertilized embryos carry a major chromosome anomaly including aneuploides, mosaicism, structural anomalies and undetected abnormality of ploidy. (Jamieson et al 1994) More recently a comprehensive analysis of early embryos using CGH technique have demonstrated around 75% chromosomal aberrations in early embryo cells (Voullaire et al 2000, Wells et al 2000).

All blastomeres of monospermic embryos will be abnormal when the chromosome abnormality occurs during the first embryonic division. Mosaicism occurring at the second cleavage in one of the two cells will result in an embryo in which half of the cells are abnormal. Mosaicism occurring at the third cleavage in one of the four cells will result in one fourth of the cells being abnormal (Munne et al 1994a; Figure 1.8).

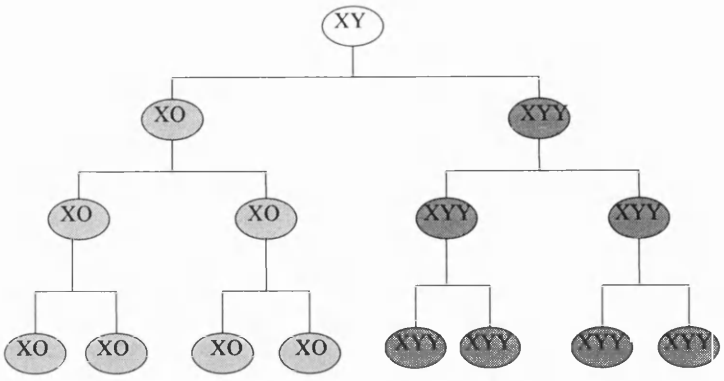
In most dividing cells, a series of checkpoints act to ensure that each phase of the cell cycle is completed before progression to the next. Absence of this checkpoint has been shown in murine oocytes (Le Maire-Adkins et al 1997). It is likely that deficiency of the metaphase-anaphase checkpoint could also be responsible for the chromosome malsegregation seen in human embryos (Wells et al 2000).

The influence of mosaicism needs to be examined, because it could lead to incorrect results in PGD (Munne et al 1994b, Katagiri et al 1996).

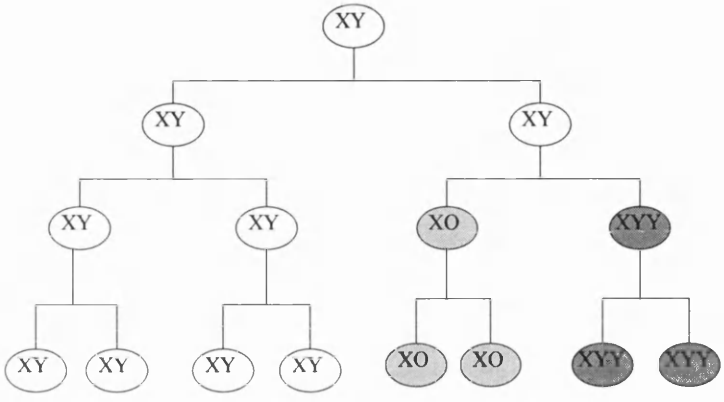
The consequences of chromosomal mosaicism for early human embryonic development are unknown. The frequent occurrence of chromosomal mosaicism may be a contributing factor to the relatively low success rate of IVF (Katagiri et al 1996).

1.6.3 Non-invasive prenatal diagnosis

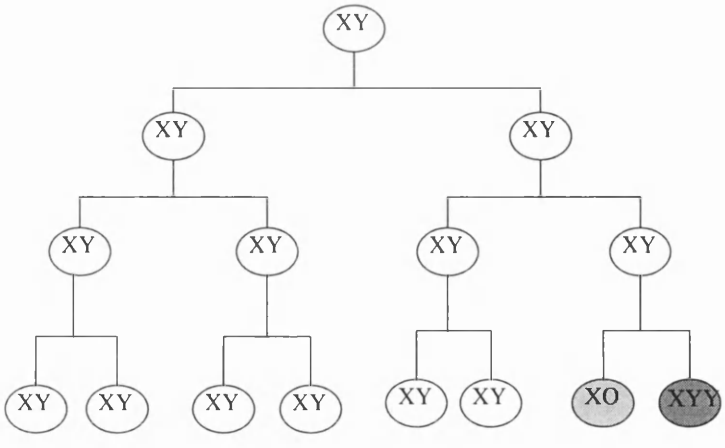
The possible resources for noninvasive prenatal diagnosis include fetal DNA in maternal plasma, exfoliated trophoblasts through cervical canal and fetal cells in maternal blood (Adinolfi 1995; Bianchi 1995; Lo et al 1997, 1999).



Mitotic error at first cleavage: mosaic with 100% cells abnormal



Mitotic error at second cleavage: mosaic with 50% cells abnormal



Mitotic error at third cleavage: mosaic with 25% cells abnormal

Figure 1.8: Onset of mosaicism in monospermic human embryos. This is exemplified in the figure by a non-disjunction of sex chromosomes occurring at different cleavage stages (Adapted from Munne et al 1994a).

1.6.3.1 Fetal DNA in maternal plasma and serum

Free DNA has been demonstrated in serum in cancer, autoimmune diseases and in pregnancy (Mulcahy et al 1996; Bianchi et al 2000). There are similarities between a growing fetus and a neoplasm because both are immunologically foreign to, and have an extensive vascular interface with, their hosts. Prompted by such reports, Lo demonstrated that fetal DNA is present in maternal plasma and serum. Detection of fetal DNA sequence was possible, in 80% and 70% of cases, with just 10 µl of boiled plasma and serum, respectively (Lo et al 1997). The underlying processes that cause free DNA to be released into the maternal circulation have yet to be explained. Possible mechanisms include cell lysis resulting from immunological and physical damage, and developmentally regulated apotosis of certain fetal tissues (Sekizawa et al 2000).

As well as sex-linked disorders, techniques for fetal-DNA detection in maternal plasma or serum can also be used to detect many paternally inherited DNA sequences that differ from their maternal counterpart (Lo et al 1997). More recently, a proportion of such DNA has been seen in intact fetal cells within maternal plasma and trisomy 21 has been diagnosed using FISH (Poon et al 2000).

1.6.3.2 Fetal cells in maternal circulation

The candidate fetal cells in maternal blood may include trophoblasts, lymphocytes, granulocytes and NRBCs (nucleated red blood cells). Lymphocytes are poor candidates because these cells are known to persist from previous pregnancies. It has also been reported that fetal cells may persist in maternal blood for as decades (Bianchi et al 1996). Granulocytes are largely unexplored as candidate cells. Trophoblasts remain theoretically attractive, but unequivocal proof of a specific monoclonal antibody is still lacking. Moreover, the multinucleated nature can complicate FISH analysis and the phenomenon of confined placental mosaicism is another drawback for its candidacy (Bianchi et al 1996). Despite these limitations, some investigators have successfully detected Y

chromosome sequences by PCR and FISH techniques in trophoblast sprouts physically isolated from maternal blood (Mueller et al 1990, Durrant et al 1996, Mavrou et al 1998).

There are several reasons why NRBCs are the best candidate cells to be used in noninvasive prenatal diagnosis: fetal erythroblasts are nucleated and larger in size when compared with maternal red blood cells. This distinctive morphological difference is used in the separation of fetal nucleated cells from maternal components. Second, the DNA contained by these cells makes it possible to do direct genetic analysis once the cells are isolated. Third, NRBCs possess various cell surface markers, which can be used for isolation. Lastly, NRBCs have a definite life span in the maternal blood (Bianchi et al 1996).

NRBCs are present from at least 10-18 weeks' gestation, with no obvious diminution in their frequency after 16 weeks. It has been estimated that 30% of erythroblasts in maternal blood are of fetal origin, with the frequency of these cells varying through gestation (Shulman et al 1998; Troeger et al 1999). By using a density gradient purification method from 10 ml of maternal blood, 7-95 erythroblasts could be identified between 15-24 weeks gestation (Kuo et al 1999). Based on flow cytometry data, the ratio of fetal NRBCs to maternal blood cells is $1:1x10^7$ to $1:1x10^8$. The separation of fetal NRBCs from maternal blood relies on differences in cell size, volume, and DNA content. Based on these characteristics, fetal cells can be isolated with density gradient centrifugation or lysis of the non-nucleated maternal erythrocytes. The remaining cells can then be purified further using fluorescent activated cell sorting (FACS), magnetic activated cell sorting (MACS), or other techniques (Herzenberg et al 1979; Bianchi 1995).

The presence of fetal cells in maternal blood has been termed as 'microchimerism', and is associated with a number of maternal diseases such as scleroderma (Bianchi 2000).

1.7 Technical developments for single cell genetic analysis

1.7.1 Conventional cytogenetic study of blastomeres

Human embryo cells remain in interphase for most of their 16-hour cell cycle. Only a small proportion of blastomeres, isolated at random from such embryos, can be expected to enter metaphase during the short period available for analysis. Even after overnight culture of isolated blastomeres in the presence of colcemid, only one-third of the cells produce metaphases suitable for karyotyping (Santalo et al 1995). The proportion of successfully karyotyped embryos was reported to be between 23.9-76% (Angell et al 1986; Rougier et al 1993; Jamieson et al 1994; Kalousek et al 1995).

In general, a complete analysis of blastomeres from human embryos between the 2-cell to the blastocyst stage by conventional karyotyping studies is very labour intensive if possible to obtain at all. The main reasons for analytical failure are, lack of chromosomes, cell loss and over-spreading of individual metaphase plates (Jamieson et al 1994). Detailed chromosome analysis is usually not feasible due to sub-optimal metaphase morphology and inconsistent chromosome banding.

1.7.2 Nuclear conversion for blastomere karyotyping

The possibility of applying nuclear transplantation techniques for karyotyping a single blastomere from preimplantation human embryos has been reported recently. In this 'nuclear conversion' technique, human oocytes or abnormally fertilised zygotes were enucleated and the blastomere was injected into the perivitalline space. The results of human blastomeres fusion with enucleated human zygotes showed that 87% of the nuclei could be transformed into analysable chromosomes. If blastomeres were fused with human oocytes, the figure for analysable chromosomes was 55% (Evsikov et al 1999).

When cryopreserved in vitro matured bovine oocytes were fused with human blastomeres, the transferred human nuclei were 'forced' into

metaphase within a few hours (Willadsen et al 1999). In 87 of the hybrid cells, 21 had no trace of human chromatin, while 64 of the remaining 66 (73%) displayed chromosomes suitable for analysis. Furthermore, a relatively simplified and efficient method for obtaining metaphase chromosomes was also proposed, and chromosome plates could be obtained in 91% when human blastomeres were fused with commercial available frozen mouse zygotes (Verlinsky et al 1999).

Interphase nuclei conversion may be of particular help for translocation patients and age related aneuploidy screening. In cases of complicated rearrangements, recent advance in multi-colour FISH such as SKY or mutliplex-FISH could be applied to the metaphase spreads produced from these converted nuclei (Speicher et al 1996; Schrock et al 1996, 1997; Marquez et al 1998).

1.7.3 Single cell molecular diagnosis

The amount of DNA in a single cell is estimated to be around 5 pg (Schaaff et al 1996). Conventional PCR, which applies around 25-30 cycles for amplification, is unable to amplify a single cell to a detectable level on ethidium bromide-stained agarose gel. Two stages of PCR amplification (e.g. nested PCR) involving 55-60 cycles are required for specific locus detection from a single cell. Various researchers for single cell genetic analysis are now focusing on new methods such as fluorescent PCR, multiplex PCR, and whole genome amplification. These methods are being developed in order to speed up the diagnosis, to increase the sensitivity, or to expand the range of diseases that can be detected at the single cell level (Blake et al 1999; Findlay et al 1995, 1998; Kristjansson et al 1994; Harper and Wells, 1999).

To shorten the time for PGD, one stage PCR involving 35-40 cycles is possible if a sensitive detection method is applied. Fluorescent PCR, performed as conventional PCR, is labelled with fluorophores so that they can be detected after excitation by a laser during electrophoresis (Findlay et al 1995). Fluorescent PCR products are 1000 times more detectable

than those achieved using conventional non-radioactive methods (Harper et al 1999; Scherlock et al 1998).

Unlike nested PCR, however, detection of fluorescent PCR products requires the use of an automated sequencer and has high running costs (Findlay et al 1995). With only one cell available, the diagnostic spectrum is usually limited and repeat tests are impossible. The development of multiplex PCR and whole genome amplification has the potential to overcome these disadvantages and maximize information gained from a single cell (Wells et al 1998).

1.7.3.1 Nested PCR

Conventional gel analysis from a single cell is not attainable from one step of 30 cycles of PCR amplification, whereas this could be overcome by using a strategy of nested PCR (Handyside et al 1992). In nested PCR, two sequential amplifications are used. The first one uses a set of primers that yield a large product, which is then used as a template for the second amplification. The second set of primers anneal to the sequences within the initial product. Nested PCR may enhance the specificity and reduce the risk of carry-over contamination. Sensitivity is also increased because two sets of amplification, both in the order of 25-30 cycles, are used (Figure 1.9).

The first clinical application of PGD using a nested PCR strategy for sex determination was reported in a case of a male X-linked disorder (Handyside et al 1990). Using a similar strategy, the first single gene defect to be successfully screened was the common 3 bp ΔF508 deletion in exon 10 of the cystic fibrosis transmembrane regulator (CFTR) gene causing CF and was achieved by heteroduplex analysis (Handyside et al 1992).

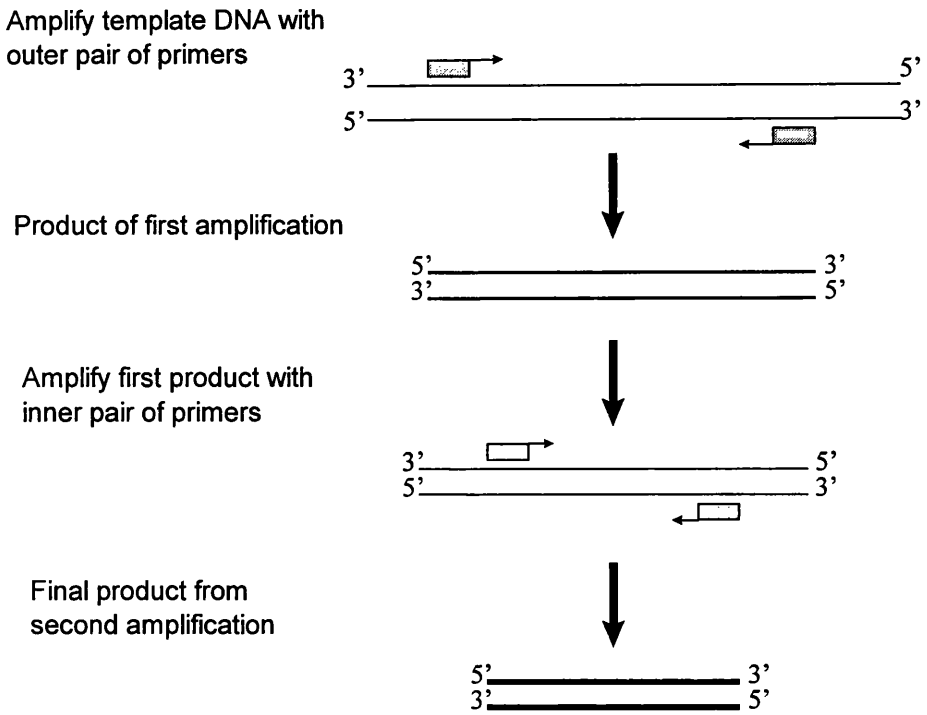


Figure 1.9: Principle of nested PCR. In nested PCR, two separate reactions were applied. The first uses a pair of outer primers yielding a large product, which is then used as a template for second amplification. The second pair of primers anneals to sequences within the initial product, producing a second small product.

1.7.3.2 Multiplex PCR

Multiplex PCR makes use of two or more primer sets within the same reaction mix. This system is useful for the simultaneous detection of a number of different sequences. As interaction between unrelated primers and PCR products may occur, each multiplex PCR system may need tedious optimisation processes.

A quantitative multiplex fluorescent PCR system has been enhanced simultaneously to diagnose sex and cystic fibrosis and determine a reliable and accurate DNA fingerprint in a single cell. Simultaneous diagnosis of Down syndrome and sex could be completed in five hours if a rapid fluorescent PCR system is used (Findlay et al 1995, 1998).

1.7.3.3 Quantitative fluorescent PCR

Fluorescent-based DNA detection technology, first developed for automated sequencing, has been adapted for PCR fragment analysis and applied for genetic screening. Incorporation of fluorescently labelled primers into PCR products enables a laser excitation/detection system to register the DNA fragments as excitation peaks. This detection system is more sensitive than standard gel electrophoresis. The signal intensities of the fluorescent peaks can be compared and used for quantitative PCR analysis. This allows accurate genotyping of samples in which one allele has been dramatically over-amplified relatively to the other (Findlay et al 1995). Such results may resemble allele drop-out (ADO) using less sensitive detection method. The superior sensitivity of fluorescent PCR means that fewer copies of the template are necessary for the product to be detected and fewer cycles are needed. This has the effect of speeding up the diagnosis while reducing the threat of contamination by PCR products. As a consequence of increased sensitivity, a low ADO rate (4%) has been reported due to an ability to distinguish true ADO from preferential amplification of one allele over the other (Findlay et al 1995).

1.7.3.4 Whole genome amplification (WGA)

The term of 'WGA' is used to indicate a PCR method that is aiming to generate a non-specific amplification of whole genomic sequences from a minute quantity of starting DNA. WGA represents a more flexible alternative to multiplex PCR and aims to amplify the entire genome in an unbiased way. Several versions of WGA (e.g. primer extension preamplification (PEP), degenerate oligonucleotide primed PCR (DOP-PCR), Alu-PCR, tagged PCR, and ligation-mediated PCR) have been presented over the past decade for various research purposes: single sperm genotyping, simultaneous linkage and mutation analysis, genotyping for multiple disease genes, identification of genetic changes in small lesions (Zhang et al 1992; Telenius et al 1992a, 1992b; Baldini et al 1991; Grothues et al 1993; Vincent et al 1994; Sanchez-Cespedes et al

1998; Aubele et al 1998; Kallioniemi et al 1996; Klein et al 1999).

PEP uses random pentadecanucleotides as primers and is able to amplify about 80% of a cell's genome to a minimum 30 copies from a single sperm (Zhang et al 1992). This method produces a sufficient number of DNA copies from a single cell to perform multiple loci genetic analysis, or to verify molecular findings by repeat analysis. PEP has been applied successfully in the analysis of sex (ZFX/ZFY), cystic fibrosis (ΔF508), two major mutations responsible for Tay-Sachs disease and a common restriction polymorphism in the gene responsible for haemophilia A (Snabes et al 1994).

Kristjansson applied PEP technology for single-cell analysis of the five most commonly deleted exons of the dystrophin gene in conjunction with ZFX/ZFY analysis (Kristjansson et al 1994). Ao et al also applied PEP for preimplantation genetic diagnosis of familial adenomatous polyposis coli (Ao et al 1998).

Another method of WGA is DOP-PCR which uses primers defining sequences at the 5' end and the 3' end. Between these regions is a random hexamer sequence (Telenius et al 1992a). PCR is performed under low-stringency conditions during the first 5 cycles followed by 28-35 cycles with a more stringent temperature. It has been shown that DOP-PCR produces sufficient DNA for over 100 independent PCR amplifications. These amplified templates have been successfully used in the analysis of CFTR, APC, β-globin and DNA markers at chromosome 21 and 18 (Wells et al 1999).

Using DOP-PCR, efficient amplification was achieved from the genome of all species. This PCR technique had important applications to genome mapping, including chromosome painting and chromosome-specific marker generation (Telenius et al 1992b).

1.7.3.5 Problems related to single cell PCR

For single cell PCR, the principal problems encountered are amplification failure, ADO and contamination. Amplification failure occurred in about

10% of isolated blastomeres, and may have resulted from transfer failure or incomplete lysis (Wells et al 1998).

ADO is a phenomenon where only one of the two alleles present in a cell is amplified to a detectable level. It affects a significant proportion of single cell amplifications and is a problem that is yet to be understood (Avner et al 1994; Rechitsky et al 1996). Improving the lysis method or increasing PCR denaturing temperature may reduce the occurrence of ADO, although such modifications cannot entirely eliminate it (Ray et al 1996; El-Hashemite et al 1997).

Although the exact cause of ADO is not known, it is believed that it may be due to sub-optimal PCR conditions or degradation of target DNA molecules. For genetic analysis, ADO will cause serious misdiagnosis particularly in autosomal dominant conditions or in autosomal recessive compound heterozygotes. Failure to amplify the mutant allele (PCR failure or loss of the chromosome) will lead to transfer of an affected embryo.

PCR is also highly prone to sources of error due to its sensitivity, particularly when the starting DNA is from a single molecule. For this reason, stringent precautions must be taken in order to avoid contamination in PGD. In IVF, many sperm may be embedded in the zona pellucida and may be accidentally transferred along with the blastomeres during embryo biopsy. Maternal cumulus cells are another potential source of contamination. Contamination might also be of laboratory origin (e.g. previously amplified DNA) or external in origin (e.g. operator DNA).

1.7.4 Single cell FISH

In PGD, sexing by PCR has now largely been abandoned in favour of a FISH technique due to the risk of misdiagnosis by PCR (Griffin et al 1994; Munne et al 1994). FISH has an advantage over PCR for sexing in that it detects the X and the Y chromosomes simultaneously, as a result of which not only the sex of the embryo can be determined but also whether there is aneuploidy of the sex chromosomes (Harper et al 1995). An additional advantage is that the risk of contamination with irrelevant cellular matter is

minimal with FISH, as the nucleus is viewed at all stages of the process, from initial spreading to final analysis.

Most PGD have been performed for age-related aneuploidy, with most groups using FISH probes to detect chromosomes X, Y, 18, 13 and 21 which together account for 95 % of all postnatal chromosome abnormalities. More recently this approach has been questioned and modified to include a re-probing stage with additional probes for chromosomal 14,15 and 22 (Munne et al 1998; Bahce et al 1999).

Interphase FISH is not as efficient as metaphase FISH and the addition of every extra probe and re-probing step decreases the hybridisation efficiency further. Therefore FISH is a compromise between the number of chromosomes examined and the efficiency of the FISH procedure. Overlapping signals and hybridisation failure can affect all FISH protocols and so strict criteria have to be followed (Bischoff et al 1998).

1.7.5 Five-colour FISH for single cells

FISH with 5 fluorescent DNA probes has been reported for analysis of five chromosomes in a single blastomere or fetal cells isolated from maternal blood (Munne et al 1996; Bischoff et al 1998). However, simultaneous analysis of more than the 5 chromosomes is limited by several causes:

(1) lack of alpha-satellite specific probes for some chromosomes
(2) limited number of fluorochromes or fluorophore-labelled probes for visual detection with spectrally well-separated emissions
(3) lack of excitation and emission filters for visualising both primary and mixed colours concurrently
(4) difficulties in counting resulting from spatial relationships when assessing more than 8 hybridisation signals in a single cell.

Because of the limited number of fluorescent colours of DNA probes, the number of chromosomes that can be examined simultaneously in each blastomere is limited to a maximum of five (Bischoff et al 1998).

1.7.6 Repeated FISH on the same blastomere

In order to increase the number of chromosomes examined in each blastomere, some reported a repeated FISH procedure in which six or more chromosomes can be analysed per blastomere of an embryo. Based on the initial number of nuclei, the percentages of nuclear loss and presence of signals were 3 and 92% after the first FISH; the percentages were 7 and 87% respectively after the second FISH, and were 13 and 78% respectively after the third FISH (Liu et al 1998). The rate of nuclei with signals decreased in the second and third FISH procedures. This might be due to repeated washing and denaturation procedures, which would affect the hybridisation between DNA probes and target sequences. The loss of blastomere nuclei might also occur in the FISH or repeated FISH procedures (Liu et al 1998).

1.7.7 Primed in situ labelling (PRINS)

The PRINS method is an alternative to conventional FISH for chromosomal detection based on the use of chromosome-specific oligonucleotide primers. In this method, chromosomal identification is performed by primer extension by Taq DNA polymerase in the presence of labelled nucleotides (Koch et al 1989, 1996). Pellestor has however defined specific primers for several chromosomes and established a dual-colour PRINS protocol (Pellestor et al 1996 a, b). The PRINS reaction is performed on a programmable temperature cycler, with specific labelling obtained in less than 2 hours.

It has been demonstrated that the PRINS technique is more specific and considerably faster than classical FISH using alpha-satellite DNA sequences for chromosome identification (Gosden et al 1994). Diagnostic use of centromeric repeat probes is hampered by the lack of specificity as there is a high degree of homology between certain chromosomes in their satellite DNA sequences (Ried et al 1992).

1.7.8 Multicolour FISH for single cells

A genome-wide screening for chromosomal aberrations has become possible with the recent developments of colour karyotyping. This has led to the development of 24-colour FISH, a process which allows the visualisation of the 22 human autosomes and the 2 sex chromosomes in a single hybridisation. The ability to conduct FISH experiments in multiple colours permits complex chromosomal abnormalities to be analysed in a single hybridisation.

Two strategies are available for producing 24-colour chromosome paint. In ratio labelling, paints may be generated from a number of fluorochromes mixed together in different proportions. Due to difficulties in accurately recreating the dye ratios of the paint, this strategy is unsuitable for mass production. Combinatorial labeling, however involves the construction of a multiple-coloured probe set through mixing, in equal proportions, of differentially labelled chromosome specific paints. The number of permutations following this strategy is given by the formula 2^n-1 where n represents the number of dyes. Paint sets can easily be reproduced and software programmed to automatically classify the resultant FISH signals, making combinatorial labelling the method of choice for reproduction of the 24-colour chromosome paint (Verma and Babu 1995).

Two systems currently in use for the imaging of 24-colour FISH slides are multiplex-FISH (M-FISH) and spectral karyotyping (SKY) (Speicher et al 1997; Schrock et al 1996). The M-FISH system is based on a series of narrow bandpass filters selected to correspond with excitation and emission characteristics of the incorporated fluorochromes of the paint. SKY utilises a Sagnac scanning interferometer to measure the spectra of the emitted fluorescent signals of the hybridised probe. It employs 24 chromosome-specific painting probes. Each probe is labelled with different proportions of five separate fluorochromes and observed by spectral imaging, providing a different colour for each human chromosome.

SKY FISH has already been employed to examine chromosomes from oocytes and polar bodies. However, a significant number of metaphases

will be unsuitable for analysis due to poor chromosome morphology, extensively overlapping chromosomes, and loss of chromosomes from the spread (Fung et al 1998; Marquez et al 1998).

1.7.9 DNA microarrays

A rapidly evolving technique for molecular analysis is the DNA chip which provides the potential to simultaneously detect thousands of possible sequence variants in previously defined genes (Solinas-Toldo et al 1997; Pinkel et al 1998, Snijders et al 2000). This technique accommodates high throughput, integrated data acquisition and computation. Microchip technology is mainly suited for large-scale screening including single base polymorphism, gene expression and pathogen detection, but may become relevant also in preimplantation genetic diagnosis (Harper et al 1999, Bredbacka 2001).

1.8 Concomitant molecular/cytogenetic diagnosis for single cells

The information provided from single cell genetic analysis is usually limited due to the scarcity of cells available. To increase the scope of single cell analysis, techniques have been refined to the demand of certain clinical conditions which require multi-purpose diagnosis at both molecular and cytogenetic level. These include: cell recycling and WGA/CGH.

1.8.1 Cell recycling

A 'cell recycling' procedure combines the two powerful techniques of PCR and FISH on a single fixed cell (Thornhill et al 1996). In this procedure, the initial PCR analysis takes place on a single cell fixed to a miniature glass slide, thus allowing subsequent FISH analysis of that cell (Rchitschy et al 1996; Figure 1.10).

Using single blastomeres from mouse embryos as a model system, cell cycling procedures detected a single-copy mouse beta-hemoglobin gene sequence at efficiency of 70% and sex constitution at an efficiency of 74%

in the same single cell (Thornhill et al 1994). Theoretically, the number of detected genes can be further increased if WGA is to be coupled with multiplex PCR (He et al 1999).

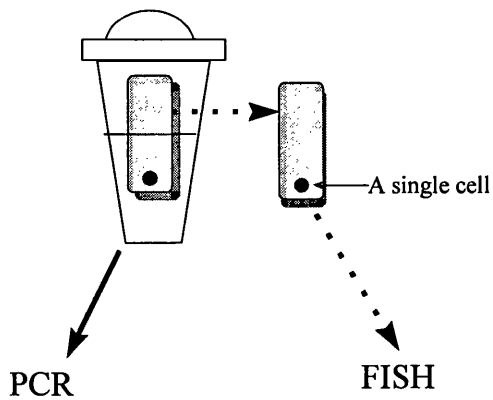


Figure 1.10: Schematic illustration of cell recycling on a single cell. A single cell is fixed and marked on a miniature glass slide in a PCR tube. After PCR amplification reaction, this single cell is analysed by FISH in order to reveal more information.

1.8.2 WGA and CGH

The study of combining WGA and CGH for diverse diagnostic purposes from a single cell was first reported in 1999 (Wells et al 1999). Using this strategy allows the possibility of analysing a variety of mutations from amplified DOP-PCR products. Meanwhile, fluorescent labelling of DOP-PCR products could be applied for CGH for detailed chromosome analysis.

Apart from DOP-PCR/CGH, Klein used the strategy of ligation-mediated PCR/CGH to detect the LOH and mutations from a micrometastatic cancer cell in the bone marrow (Klein et al 1999).

1.9 Comparative genomic hybridisation (CGH)

CGH is a molecular cytogenetic approach with the potential of detecting chromosomal imbalances in previously inaccessible specimens as only DNA (and not dividing cells) is required for the procedure. Therefore, the study of genetic alterations in tumour tissues was greatly enhanced as previously the cytogenetic analysis of solid tumour was usually hampered by the difficulties involved with cell culturing and interpretation (Kallioniemi et al 1992).

CGH allows the detection of chromosomal gains and losses across the entire genome and provides a relatively rapid screening method that can point at specific chromosomal regions. This may help to understand the pathogenesis or progression of the tumors. With more specific molecular biological techniques such as FISH, LOH analysis and sequencing, the information revealed by the results of CGH can lead to the identification of oncogenes or tumor suppressor genes in these regions (Forozan et al 1997).

1.10 Principles of the CGH technique

In CGH, the green-labelled genomic DNA from tissue samples (test DNA) is simultaneously co-hybridised with red-labelled DNA from normal tissue (control DNA) on to normal metaphase slides. The green and red labelled DNA fragments compete for hybridisation to their locus of origin on the metaphase chromosomes. As a result, a general background staining of two colours over all the chromosomes is obtained. Chromosomal sequences present in additional copies within the test DNA will result in a higher green staining at the corresponding chromosomal targeted sequences, as compared with the control DNA, while losses of chromosomal sequences will result in higher red staining of the corresponding target chromosomes. Using dedicated computer software for quantitative analysis, the green to red fluorescence ratio is measured along the chromosomal axis and represents gain (ratio>1) or loss (ratio<1) of the test sample DNA at that specific locus (Figure 1.11).

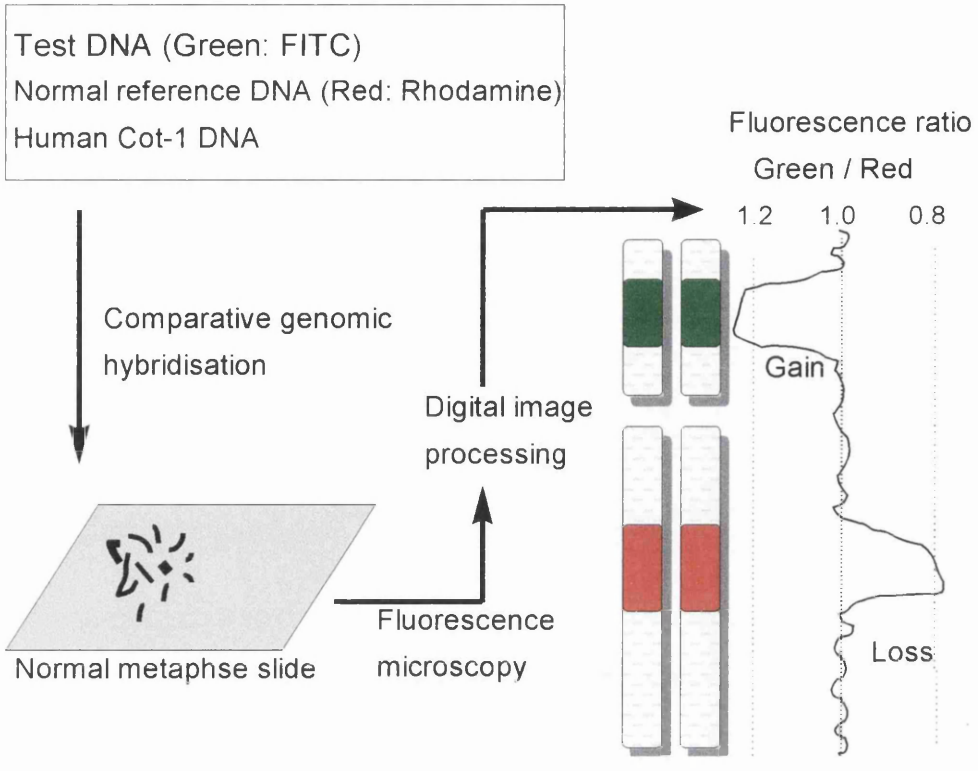


Figure 1.11: Schematic illustration of CGH. Green labelled test DNA, red labelled normal reference DNA and human Cot-1 DNA are co-hybridised to a normal metaphase slide. Images of fluorescence signals are captured and the green to red ratio are digitally quantified for each chromosomal locus along the chromosomal axis.

1.10.1 Metaphase slides for CGH

Metaphase slides are prepared according to standard protocols using PHA-stimulated peripheral blood from normal individuals. The selection of high-quality slides is crucial for CGH experiments. Ideal preparations should have little cytoplasm, minimal overlapping, low cell density coupled with a high mitotic index. Furthermore, the chromosomes should be of adequate length and contain no separated chromatids. Under phase microscope, these chromosomes should appear relatively dark, because shiny and faded chromosomes would result in poor hybridisation (Kallioniemi et al 1996).

It is recommended that several batches of slides are prepared at a time, and slides should be tested with known positive and negative control DNA samples that have previously worked well in CGH experiments (Kallioniemi et al 1994). Despite careful selection, the only certain criterion for slide quality is an actual CGH experiment (Kallioniemi et al 1996).

1.10.2 Extraction of DNA for CGH

It is most important to prepare high quality DNA from specimens to be tested. DNA extracted from fresh or frozen tissues is usually of high molecular weight and will be of the best quality for labelling purposes. In the case of tumour specimens, contamination of the tumour DNA with normal stromal and inflammatory cells is an inevitable problem. Chromosomal aberrations could go undetected if the proportion of malignant cells were less than 50%.

DNA from archived, formalin-fixed samples is almost always degraded and cross-linked. During the labelling processes by nick translation, the DNA becomes even shorter and might be too small for optimal CGH results. Recently, improved methods for extracting DNA from formalin fixation and paraffin-embedded tissues have been described; however, fresh samples are always preferable, because some archival tissues never yield DNA that is suitable for CGH analysis (Weiss et al 1999).

1.10.3 Labelling of probes

The labelling of genomic DNA for CGH analysis falls into two categories. One involves a flurochrome-conjugated molecule such as FITC-dUTP or rhodamine-dUTP for direct detection after hybridisation. Another involves a hapten biotin-14-dUTP or digoxingenin-11-dUTP and is detected with avidin-FITC or anti-digoxigenin rhodamine. Compared with the indirect method, directly conjugated DNA probes provide improved hybridisation quality and speed, although the signals are weaker.

Nick translation is the most popular method used to incorporate biotin, digoxigenin or flurochromes into probe DNA. This enzyme-mediated

reaction involves DNase I and DNA polymerase I in the presence of dNTPs, with the dTTP being partially reduced by hapten-labelled (or flurochromes-labelled) dUTP. The DNase I produces single strand nicks in the double-stranded DNA of the probe to expose a free 3'-OH. The 5' to 3' exonuclease activity of DNA polymerase I deletes a mononucleotide at the 5' side of the nick, and catalyses the incorporation of new dNTPs at 3'OH end of nick. By manipulating the DNase I concentration and reaction time, optimal probe size can be achieved, which is important in relation to the accessibility of the probe to the target sequences. The digested fragments of both test and reference DNA should be in the same range of lengths and within the limits of 500-2000 bp. The size distribution can be checked by electrophoresis on agarose gels stained with ethidium bromide (Weiss et al 1999).

1.10.4 Blocking

When interspersed repetitive sequences (IRS) are present in high-complexity probes, these will hybridise to the ubiquitous complementary sequences throughout the genome. Signals caused by IRS within a given probe can be suppressed effectively by preannealing with unlabelled genomic or Cot-1 DNA to the labelled probe before hybridising the probe to the target DNA (Lichter et al 1988). This permits only the unique probe sequences to bind to their complementary target during the subsequent ISH proceedings.

Short repetitive sequences occur throughout the whole genome, but are particularly abundant at all centromeres, telomeres, and some specific regions such as chromosome arms 1p, 16p, and chromosomes 19, 22 (Weiss et al 1999). The lengths of these regions are very variable between individuals and this can interfere with CGH analysis. Therefore, unlabelled human Cot-1 DNA is added to suppress the binding of labelled repetitive sequences from the genome of both the test and reference probes to the centromeric and repetitive regions of the chromosomes (Weiss et al 1999). Cot-1 DNA is prepared from human placental DNA which, with a size from

50-100 bp, is rich in repetitive DNA sequences. In CGH analysis, true variations in the fluorescence signals in the regions of interest can only be analysed after the repeated sequences are blocked. Inadequate suppression of the repetitive sequences can interfere with the true signal intensity, and lead to a reduced amplitude of the fluorescence ratio; hence true chromosomal gains and losses may go undetected (Weiss et al 1999).

1.10.5 Pre-hybridisation

The CGH protocol is similar to the method described for FISH with painting probes. Equal amounts of test and reference DNA (100ng-1μg) are pooled together with 100 times the same amount of human Cot-1 DNA, precipitated, and re-suspended in hybridisation. Formamide, in the buffer solution, decreases the melting temperature of the DNA, while dextran sulfate increases the effective probe concentration (Weiss et al 1999).
In practice, opposite sex test and reference DNAs are used. The resulting uneven fluorescence ratio for the X chromosome functions as an extra control of the quality of the CGH experiments. However, additional sex matching should be taken into consideration when analysing the X chromosome (Weiss et al 1999).

1.10.6 Denaturation of metaphase slides and probes

Hybridisation between single-stranded DNA molecules forms the basis of the use of DNA probes to detect specific sequences in tissue samples. The specificity of the annealing reaction can be adjusted by altering the temperature and/or pH. In CGH, the chromosomes require sufficient denaturation to permit accessibility of labelled probes, while still retaining the chromosomal morphology for identification purposes. The probe and the normal metaphase slides are denatured separately, and this is carried out at 70-76°C for 2-10 minutes in 70% formamide/2XSSC solution. To produce optimal CGH results, it is important to test each slide batch at various denaturation conditions (Weiss et al 1999).

1.10.7 In situ hybridisation and DAPI staining

The hybridisation takes place under a coverslip in a humid incubator at 37-40°C for 2-4 days. The exact time of hybridisation is not critical for optimal results. Hybridisation over 3 days is acceptable as long as the hybridisation mix does not dry out. Overnight hybridisation has been found not to be as homogeneous as when carried out for 2-3 days. After hybridisation, the slides are washed and counterstained with DAPI in an antifade solution. DAPI staining provides a banding pattern similar to that of quinacrine banding for chromosomal identification and karyotyping. The optimal concentration of DAPI in antifade may vary with slide batches or with the microscope used. Staining should be bright enough to permit easy band detection but not so bright as to obscure the bands (Weiss et al 1999).

1.10.8 Hardware and software requirements for CGH anaylsis

To complete both qualitative and quantitative analysis of CGH experiments, hardware for image acquisition and software for fluorescence ratio measurement are required (Du Manoir et al 1995a; Piper et al 1995).

1.10.8.1 Image capture

Fluorescent molecules absorb light and release energy in the form of light and heat. Because some energy is lost as heat, the average wavelength of emitted fluorescence is longer than the average excitation wavelength for any given fluorochrome.

Visualisation of fluorescence signals is carried out with a fluorescence microscope equipped with a mercury lamp, appropriate excitation and emission filter sets and camera. The acquisition of images is carried out firstly by scanning the entire slide for the best metaphase spreads. The metaphases are selected based on the following criteria: homogeneous hybridisation signals across each chromosome, similar chromosome length, low background noise, adequate suppression over centromeres

and heterochromatic regions, proper DAPI banding for chromosome identification. For each sample, around ten metaphases are captured for digital processing.

Selection of proper filters is important since cross-talk between different fluorochromes can occur which will bias CGH ratio measurements. Filter sets for the often used DAPI/TRITC/FITC fluorochromes combination are commercially available now, and optical shift becomes negligible if either a computer-controlled filter wheel or microscopes with an automatic filter change are used for image acquisition. CGH images are captured by a cooled CCD (charged coupled device) camera, which although not necessary, has advantages including digital readout, high spatial resolution, high sensitivity, low noise and high dynamic range (Du Manoir S et al 1995b). Some CGH systems used a simpler and cheaper video-rated camera which is sufficient for CGH analysis (Du Manoir et al 1995a).

1.10.8.2 Image processing

Visual analysis of CGH is limited by the subjectivity and poor sensitivity of the human eye in detecting small colour intensity or intensity ratio differences. In practice, only high-level amplification can be reliably evaluated without digital processing. For detailed CGH copy-number karyogram analysis, the captured images must be software-processed.

Current dedicated CGH software should enable the following steps to be carried out (Piper et al 1995):

(1) background removal

(2) segmentation and removal of non-chromosomal objects

(3) normalization of the FITC: rhodamine ratio for the whole metaphase

(4) interactive karyotyping

(5) interactively position the centromeres correctly and scale the chromosomes to a standard length.

After processing, the total fluorescence for green and red is measured for each separate image and equalised to a fluorescent ratio (FR) of 1.0. This allows subsequent integration of data from several different metaphase

images and identification of chromosomal regions showing abnormal FR for that test sample. The average FR of several selected metaphases are depicted along the idiograms of corresponding chromosomes, presenting chromosomes that are more green (chromosome gains) or red (losses) (Fig 1.12).

The interpretation of the ratio profiles, at a locus or an entire chromosome arm, can be done using fixed or statistical thresholds. Fixed limits of 1.15-1.25 and 0.75-0.85 can be used to identify chromosome gains and losses respectively, while others use the 95% confidence interval (CI) limits of the ratio profile. In the latter, deviations from normal are interpreted when the 95% CI of the fluorescence ratio does not contain 1.0. Fixed thresholds do not take account the variation of ratio values between different experiments, different metaphases of the same slide and even different chromosome regions. Calculation of statistical confidence limits may improve the interpretation of CGH profiles (Moore et al 1997; Kirchhoff et al 1998).

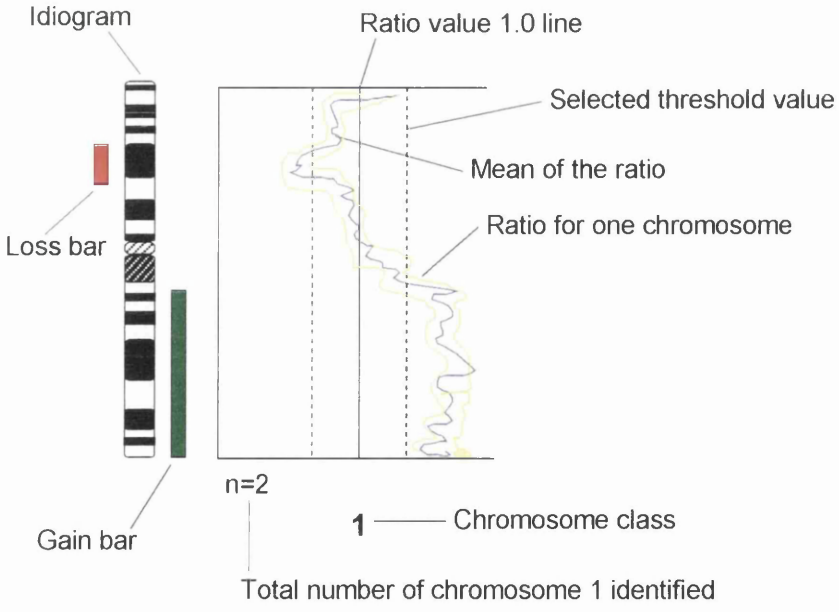


Figure 1.12: Ratio profile with selected displayed options

1.10.9 Limitations of CGH

There are varying estimates regarding the smallest size of low-copy-number gains or losses that can be detected reliably by CGH. According to simulation experiments, the total amount of DNA of increased or decreased copy number smaller than 2Mb is considered as unlikely to be detected (Piper et al 1995; Parente et al 1997). Based on CGH experiments, this technique can detect genetic imbalances with estimated size of 10-20 Mb (Kalliomiemi et al 1994; Bentz et al 1995).

CGH cannot however detect the following genetic aberrations (Kalliomiemi et al 1994): (1) balanced structural aberrations such as balanced translocations, inversions (2) small intragenic rearrangements and point mutations (3) ploidy changes (4) pericentromeric and heterochromatic regions (5) low level mosaicism (>50% normal cell contamination).

1.11 Applications of CGH

1.11.1 Application for cancer cytogenetics

By using techniques such as microdissection and whole genome amplification, CGH can be applied to minute quantities of DNA. This has allowed studies using archival formalin fixed and paraffin-embedded materials whereas previously only DNA from fresh and frozen tumors materials could be used (Hermsen et al 1996; Kuukasjarvi et al 1997; Ozcan et al 2000).

CGH is a powerful tool to identify chromosomal aberrations in precancerous conditions and to study tumour progression and clonal evolution (Forozan et al 1997). The interpretation of CGH could also be used as a supplement to tumour staging and prognosis prediction (Forozan et al 1997; Yutaka et al 2000). Heselmeyer et al found that gain of 3q was consistently detected when severely dysplastic uterine cervical cells progressed into invasive cancer (Heselmeyer et al 1996).

Combining laser capture microdissection and DOP-PCR/CGH, small samples (consisting 3-40 cells) out of routinely processed and Papanicolaou-stained cervical smears could be accurately analysed

(Aubele et al 1998). This may help in the understanding of the progression of cervical cancer, especially for low grade lesions, and lead to future revised classification of high and low grade cervical intraepithelial neoplasm (CIN) lesions (Aubele et al 1998).

1.11.2 Application for early embryo development

Although a high incidence of mosaicism is acknowledged in early embryo cells using a FISH technique, the comprehensive study of every chromosome requires the use of CGH or multicolour FISH (Delhanty et al 1997). Using DNA amplified from a single blastomere, CGH is able to provide comprehensive analysis of the diploid set of human chromosomes. This will shed light on the mechanisms underlying the unprecedented levels of chromosomal mosacisms reported in human embryos at the cleavage stage (Wells et al 1998).

Recently, two WGA methods (i.e. DOP-PCR and ligation-mediated PCR) used in three different centres have reported reliable single cell CGH (Wells et al 1999, 2000; Vollaire et al 1999, 2000). The data demonstrate 75% chromosomal aberrations in early embryo cells (Voullaire et al 2000; Wells et al 2000).

1.11.3 Application for clinical cytogenetics

CGH was developed initially for cancer studies; however, it has been used as a supplementary diagnostic tool more recently for a number of clinical cytogenetic applications such as detection of euchromatic involvement in marker chromosomes, characterization of complicated unbalanced chromosome rearrangements, cryptic telomeric translocation screening and studies of aneuploidies in spontaneous abortion and stillbirth (Du Manoir et al 1993; Bryndorf et al 1995; Ghaffari et al 1998a, 1998b; Daniely et al 1998, 1999).

1.12 Aims of the project

The overall aim of the present study was to develop the genetic analytical techniques using nested PCR, whole genome amplification (WGA) and comparative genomic hybridisation (CGH) on a single cell. Specific aims included:

1. Optimisation of two sets of nested PCR for the diagnosis of sex and cystic fibrosis.
2. Establishment of a single cell manipulative model and of contamination control.
3. Whole genome amplification from a single cell.
4. Optimisation of single cell CGH.
5. Application of single cell CGH on 10-20 stained cells.
6. Application of the DOP-PCR/CGH technique on coded samples.
7. Investigation of the potential of multiple-purpose diagnosis using nested PCR/DOP-PCR/CGH.
8. Investigation of the feasibility of a short protocol for single cell CGH.
9. Application of nested PCR and CGH on single blastomeres.

Chapter 2

Materials and Methods

2.1 Samples

The single cells used in this study included amniocytes, buccal cells, and lymphocytes. Amniocytes was supplied by the Clinical Cytogenetics Laboratory of the Medical Genetics Department, Yorkhill, NHS Trust. Cultured amniocytes had been treated with trypsin and the cell suspensions were used for retrieval of a single cell. The remaining cells were stored in a tube and frozen in a -60°C freezer. Frozen cells might be thawed and retrieved again, if the initial experiment failed. The karyotypes of amniocytes included: normal males, females, and common aneuploidies (e.g. trisomy 18, 21 and sex chromosome aberrations).

At an early stage of this study involving the single cell retrieval technique, buccal cells prepared from mouthwashes were used. These cells received several washes in PBS solution in order to remove the debris.

Lymphocytes, which had been treated with fixative (i.e. 3 methanol/1 acetic acid), were also used for the retrieval of a single cell. These cells were stored in the fixative at 4°C for periods of one week to several weeks. The sources of stained cells were from both amniocytes and lymphocytes. These cells were microdissected from G-banded slides. After the procedure, the slides were stored in a sealed box in a -60°C freezer for recycling use if further experiments were indicated. The karyotypes of these samples included: normal males, females, and common numerical aneuploidies (e.g. trisomy 18, 21) To test the reliability of CGH, a number of samples were coded for use in 'blind' testing.

Blastomeres were supplied by in vitro fertilisation unit of Glasgow Royal Infirmary. These samples were received frozen and subsequent experiments were carried out within 1-4 week(s). Each PCR tube (250 µl) contained 1-5 blastomeres in 10 µl lysis buffer. Some tubes contained only Tyrode acid solution or water and were used as negative controls.

2.2 Single cell manipulation

The micropipettes were hand-prepared from Pasteur pipettes using a burner. By applying proper forces, the micropipettes were produced with

appropriate shape and diameter. For single cell transfer, 30-50 micropipettes were made each time.

Before a single cell manipulation, several droplets containing 50 µl of PBS solution were put on a sterilised Petri-dish to perform serial dilution of the amniocytes and wash off any lysed DNA (Figure 2.1). Single cell suspension was then transferred to a sterilised Petri-dish by using heat-pulled glass micropipettes which were newly prepared. For each transfer, a new micropipette was employed to prevent the residual cells or lysed DNA coating the walls of the micropipettes and carrying over into the next manoeuvre.

The presence of a single amniocyte could be confirmed under an inverted microscope. To eliminate any lysed DNA, this single cell received a further wash. It was then transferred to a PCR tube (250 µl) containing 10 µl lysis buffer. Two µl of the final washing solution was used as a negative control to ensure that there was no extraneous DNA contamination.

2.3 Microdissection of stained cells

G-banded slides, which had never been covered and sealed, were used for microdissection. Clusters of 10-20 stained interphase cells were scraped using a sterile needle (Microlance®, 23G, Becton Dickinson, Spain). The scraped cell clumps were checked under a microscope and then carefully transferred to a PCR tube containing 10 µl of lysis buffer. The tip of the needle was inspected again to make sure there were no remaining cells. To increase the success rate, 2-3 transfers were carried out for each case with a change to a new needle for each transfer. By using a new needle to touch an area without cells, a sham microdissection was used as a negative control.

To test the tolerance of cell contamination in single cell CGH, a series of manufactured mosaicism (50-70%) was made. For a manufactured mosaic at the 70% level, the stained cells were prepared from 14 male trisomy 18 cells and 6 normal female cells.

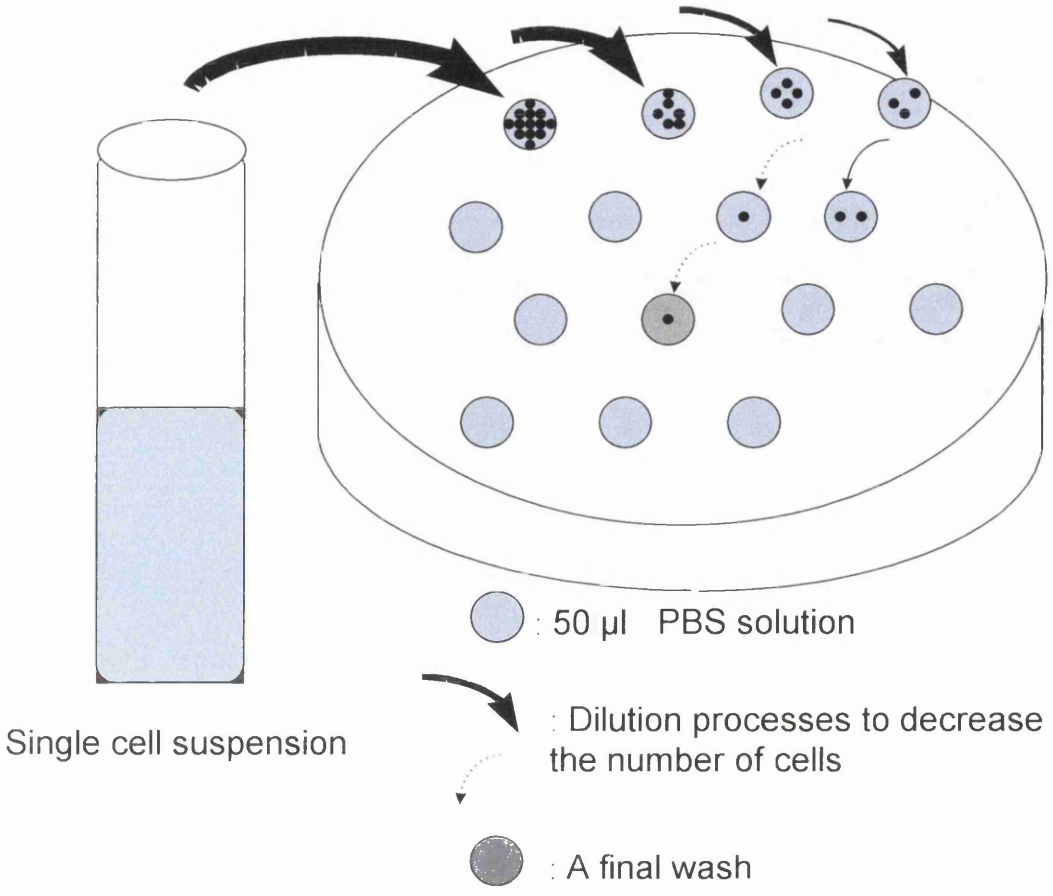


Figure 2.1: Schematic illustration of single cell retrieval technique. A series of washes were carried out to remove the lysed DNA and decrease the number of cells. Single cell retrieval was eventually confirmed under inverted microscope. After retrieval of a single cell, the remaining PBS washing solution was used as a negative control to demonstrate the absence of extraneous DNA.

2.4 Lysis methods for single cells

Reagents:

For single amniocyte	
	20 mM Tris-HCl, pH 9.3
	3 mM $MgCl_2$
	100 mM KCl, pH 8.3
	0.5 mg/ml proteinase K
For stained cells	
	100 mM Tris-HCl, pH 7.5
	1 mg/ml of proteinase K

Procedure:

1. In the case of fresh cells, incubate at 55°C for 1 hour in a PCR cycler (OMN-E, Hybaid). For stained cells, the time is increased to 1-2 days.
2. Incubate at 95°C for 10 minutes to inactivate proteinase K.

2.5 Primer synthesis and de-protection

The primers were synthesised by a Synthesizer PCR-MATE (Applied Biosystem) and required de-protection processes:

1. Connect a polypropylene syringe containing 1 ml of ammonium hydroxide (40 mM) to the end of the column (primer).
2. Attach an empty syringe to the other end.
3. Gently push the ammonium hydroxide through the column; consequently the barrel of the syringe was displaced.
4. Push ammonium hydroxide back and forth through the column, 2-3 times, for 2-3 hours. The column should be filled with liquid, which will decolourise from yellow to white.
5. Withdraw the ammonium hydroxide and transfer into a small Nunc tube.

6. Add ammonium hydroxide up to 2 ml, tighten the cap and incubate in a 55°C water bath overnight.
7. Store at -70°C.

To work with primers, 200 µl of primer stock was transferred to a 500 µl PCR tube and left in a fume hood overnight.

2.6 Extraction of genomic DNA

Reagents:

RBC lysis solution
- 155 mM Ammonium Chloride
- 10 mM Potassium Hydrogen Carbonate
- 1 mM EDTA

Cell lysis solution
- 25 mM EDTA
- 2% SDS

Proteinase K (20 mg/ml, Gibco BRL)

Protein precipitation
- 10 M Ammonium Acetate

Isopropranol

Ethanol (70% v/v)

TE buffer
- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA

Procedure:

1. Add 3 volumes cold RBC lysis solution to one volume of whole blood.
2. Invert the sample several times to mix and keep at room temperature for 20 minutes before centrifuging at 2000 g for 7 minutes.
3. Discard the supernatant, leaving visible white cell pellet and 10-20

µl of residual fluid.

4. Re-suspend cell pellet by vortexing. Add one volume of cell lysis solution, and vortex again.
5. Add 1/3 volume of protein precipitation solution. Vortex and centrifuge at 3000 g for 20 minutes.
6. Transfer supernatant to clean tube containing 1 volume isopropanol and mix thoroughly to form DNA precipitate.
7. Spool out DNA using sealed Pasteur pipette and wash in 70% ethanol.
8. Dry the DNA pellet and dissolve in an appropriate volume of TE buffer or distilled water.

2.6.1 Measurement of DNA concentration

The concentration of the DNA was measured by optic density at A_{260} nm in a spectrophotometer. 5 µl of DNA were added to a tube containing 995 µl dH_2O, and mixed well by vortexing. The concentration was measured by following the formula below:

Concentration = OD A_{260} x 200 (dilution factor) x 50 ng/µl

50: a constant corresponding to 50 µg DNA per ml at 260 nm per 1 unit OD.

Once the concentration of DNA was determined by spectrophotometry, a series of DNA dilutions were prepared by adding distilled water to the extracted genomic DNA. The diluting process was repeated to a single cell level which was estimated to be around 2-7 pg. Instead of using a single cell, the diluted DNA was applied in order to test and optimise the PCR protocols. The concentration of diluted DNA, together with the sex and other information, were noted and the samples stored in separate containers from other reagents at -60°C.

2.7 Primer extension preamplification (PEP)

Reagents:

10X PCR buffer (Boehringer Mannheim)

25 mM $MgCl_2$

500 mM KCl

100 mM Tris HCl (pH 8.3)

1 mg/ml Gelatin

10X nucleotide mixture (Boehringer Mannheim)

2 mM of each dNTP (dATP, dGTP, dCTP, dTTP)

10X primer (4 mM): random 15-mer oligonucleotides

Distilled water

Taq polymerase (5 U/μl, Boehringer Mannheim)

Procedure:

PEP on individually isolated cells was carried out as the method described previously (Zhang et al 1992).

Thermal cycling conditions:

50 cycles	92°C	1 minute
	37°C	2 minutes
	(37°C-55°C transition at rate of 10 sec/°C)	
	55°C	4 minutes

A short PEP protocol was carried out as the method described by Sermon (Sermon et al 1996):

Thermal cycling conditions:

50 cycles	92°C	15 seconds
	37°C	30 seconds
	(37°C-55°C transition at rate of 6.6 sec/°C)	
	55°C	2 minutes

2.8 DOP-PCR

DOP-PCR on individually isolated cells was performed as previously reported (Telenius et al 1992a). The master mix of the PCR reaction was added to a final volume of 50 µl in a tube containing 2 µM of degenerate primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 U Taq enzyme (Boehringer Mannheim) and overlaid with 50 µl mineral oil.

Reagents:

10X PCR buffer with 15 mM $MgCl_2$ (Boehringer Mannheim)

10X nucleotide mixture (Boehringer Mannheim)

10X 6MW primer 20 µM

5'-CCGACTCGAGNNNNNNATGTGG-3'

DNA polymerase 1U/µl (Boehringer Mannheim)

Distilled water

Expanded™ High Fidelity (EHF) PCR System

Polymerase mix 0.8 µl (2.7U/µl, Boehringer Mannheim)

10X EHF buffer (15-40 mM $MgCl_2$)

Procedure:

The amount of reagents added was as follows:

DNA samples (single cell in lysis buffer)	12 µl
10X PCR buffer	5 µl
10X dNTP	5 µl
DOP primer	5 µl
Taq polymerase	2.5 µl
Distilled water	20.5 µl

Thermal cycling conditions:

1 cycle	95°C	5 minutes
8 cycles	94°C	1 minute
	30°C	1 minute
	30°C-72°C transition in 3 minutes	
	72°C	3 minutes
28 cycles	94°C	1 minute
	56°C	1 minute
	72°C (with an increment of 1sec/cycle)	3 minutes
1 cycle	72°C	10 minutes

2.8.1 PCR labelling

To prepare probes for CGH, 5 µl of the first DOP-PCR amplified DNA was transferred to a second tube where a PCR identical to the first except for reduction of the dTTP concentration to 0.15 mM, 4 mM $MgCl_2$ and inclusion of 2 µl of Flurorescent-11-dUTP (Amersham) was carried out.

Thermal cycling conditions:

20 cycles	95°C	1 minute
	56°C	1.5 minutes
	72°C	3 minutes
1 cycle	72°C	10 minutes

2.9 Nested PCR for sex determination

Reagents

10X PCR buffer with 15 mM $MgCl_2$ (Boehringer Mannheim)
10X nucleotide mixture (Boehringer Mannheim)
10X first round PCR primer sequences (1 µM)
AMG 3: 5'-CTT CCC AGT TTA AGC TCT GAT G-3'
AMG 4: 5'-CCT TGC TCA TAT TAT ACT TGA C-3'
Second round PCR primer sequences (4 µM)
AMG 5: 5'-CTC AGG GAG GTT CCA TGA-3'
AMG 6: 5'-TGA GAA AAC CAG GGT TCC-3'
Distilled water

Procedure:

1. Nested PCR was performed as previously reported (Schaaff et al 1996). For the first round of PCR amplification, the master mix of the reaction was added to a final volume of 25 µl in a tube containing 0.1 µM of first round PCR primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 U Taq enzyme (Boehringer Mannheim).
2. Both first and second round PCR were carried out in an oil-free environment, which required a hot-lid thermal cycler (OMN-E, Hybaid). Alternately, commercial Ready-To-Go™ PCR Bead (Amersham phamacia biotech) could be used for the PCR amplification. In this method, the final volume was 25 µl and only primers and template DNA were added as the beads contained all other reagents which were required in a PCR reaction.

The reagents used in a Ready-To-Go™ PCR tube contained:

DNA template	5 µl (first round)
	1 µl (second round)
Primers	2.5 µl
Distilled water	17.5 µl (first round)
	21.5 µl (second round)
Ready-To-Go™ PCR Bead	1 tube

The amount of reagents added in the first round of nested PCR:

DNA samples (DOP-PCR products)	5 µl
10x PCR buffer	2.5 µl
10x dNTP	2.5 µl
First round PCR primer	2.5 µl
Taq polymerase	1.5 µl
Distilled water	11 µl

Thermal cycling conditions of first round PCR:

1 cycle	95°C	5 minutes
25 cycles	94°C	30 seconds
	52°C	1 minute
	72°C	1 minute
1 cycle	72°C	10 minutes

The amount of reagents added in the second round of nested PCR:

	DNA template (first round PCR product)	1 µl
	10X PCR buffer	2.5 µl
	10X dNTPs	2.5 µl
	Second round PCR primers	2.5 µl
	Taq polymerase	1.5 µl
	Distilled water	15 µl

Thermal cycling conditions of second PCR:

1 cycle	95°C	5 minutes
30 cycles	94°C	30 seconds
	56°C	30 seconds
	72°C	1 minute
1 cycle	72°C	10 minutes

2.10 CF ΔF508 mutation detection

For detection of CF mutation ΔF508, 5µl of DOP-PCR products were added to two different ΔF508 mutation detection methods.

The first one was modified from a method used in the Clinical Molecular Genetic Laboratory in Yorkhill NHS Trust.

Reagents:

2 mM dNTPs	2.5 µl
10X PCR buffer (25 mM $MgCl_2$)	2.5 µl
Primer 1 (10 µM)	1.5 µl
5'-GTTTTCCTGGATTATGCCTGGCAC-3'	
Primer 2 (10 µM)	1.5 µl
5'-GTTGGCATGCTTTGATGACGCTTC-3'	
Template DNA	5 µl
Taq polymerase (1U/µl)	1.5 µl
Distilled water	Up to 25 µl

Thermal cycling conditions:

1 cycle	94°C	2 minutes 30 seconds
	58°C	30 seconds
	72°C	30 seconds
40 cycles	91°C	30 seconds
	58°C	30 seconds
	72°C	30 seconds
1 cycle	72°C	10 minutes

A strategy of nested PCR was used as an alternative approach, which had been described previously (Scobie et al 1996).

Reagents

2 mM dNTPs

10X PCR buffer with 15 mM $MgCl_2$

Taq polymerase (1 U/µl)

10X first round PCR primer sequences (1 µM)

Primer for Exon 10

5'-AAGTGAATCCTGAGCGTGATTTGATAATGA -3'

5'-CACAGTAGCTTACCCATAGAGGAAACATAA-3'

10X second round PCR primer sequences (4 µM)

Common: 5'-GACTTCACTTCTAATGATGATTATGGGAGA-3'

Normal: 5'-GTATCTATATTCATCATAGGAAACACCAC-3'

Mutated: 5'-GTATCTATATTCATCATAGGAAACACCATT-3'

Tube N: contained common and normal primers

Tube M: contained common and mutant primers

Distilled water

Procedure:

1. For detection of ΔF508, 5 µl of DOP-PCR product was added to a PCR bead tube containing 0.1 µM external primers (Exon 10).
2. For secondary PCR, 1 µl of first PCR product was transferred to tube N (containing a pair of common primer and normal primer, each at 0.1 µM) and tube M (containing a pair of common and mutant primer, each at 0.4 µM).

Thermal cycling conditions of first round PCR:

25 cycles	94°C	1 minute
	55°C	30 seconds
	72°C	30 seconds
One cycle	72°C	10 minutes

Thermal cycling conditions of second round PCR:

30 cycles	94 °C	1 minute
	60 °C	30 seconds
	72 °C	30 seconds

2.11 Prevention and monitoring of contamination

A number of guidelines were employed to monitor and prevent contamination:

1. All reagents and PCR tubes were sterilised.
2. Ultraviolet irradiation of the master mix for 15-20 minutes prior to PCR amplification.
3. Used barrier tips throughout whole experiments (except analysing PCR products) to prevent aerosol contamination
4. Master mix was made with a new set of micropipettes which were never in contact with genomic DNA.
5. The PCR products were analysed with separate micropipettes in a separate location.
6. The master mix was prepared in a safety cabinet and the working area was cleaned with 70% ethanol before preparation. If contamination was suspected, the whole batch of master mix for PCR was discarded.
7. The DNA samples and reagents were kept in separate places.
8. Lab coat, gloves, and mask were worn whenever single cells were manipulated.

2.12 Purification of PCR products

To remove the excess of degenerate primers, dNTPs, and salts, further purification of DOP-PCR products was carried out by using a 'High Pure PCR Product purification Kit' (Boehringer Mannheim).

Reagents:

Binding buffer	
	3 M guanidine-thiocyanate
	10 mM Tris-HCl
	5% ethanol (v/v), pH 6.6 (25 °C)
Wash buffer	
	20 mM NaCl
	2 mM Tris-HCl, pH 7.5 (25 °C)
Elution buffer	
	10 mM Tris-HCl
	1 mM EDTA, pH 8.5 (25 °C)
High Pure Filter tube	
Collection tube	

Procedure:

1. Add 500 µl binding buffer to a 50 µl PCR reaction and mix well.
2. Combine the High Pure filter tube and the collection tube and transfer sample to the upper reservoir.
3. Centrifuge for 30 seconds at maximum speed.
4. Discard the flow through and combine the filter tube again with the same collection tube.
5. Add 500 µl wash buffer to the upper reservoir and centrifuge for 30 seconds at maximum speed.
6. Discard the wash buffer flow through and combine the filter tube again with the same collection tube. Add 200 µl wash buffer, and centrifuge as step 3.
7. Discard the collection tube and insert the filter tube in a clean 1.5 ml reaction tube.
8. Use 25-50 µl elution buffer or redistilled water for the elution of DNA. Add elution buffer or water to the filter tube and centrifuge at maximum speed for 30 seconds.

2.13 Analysis of PCR products

2.13.1 Agarose gel analysis

Reagents

Loading mix

0.25% bromophenol blue

0.25% xylene cyanol

40% sucrose

Agarose gel

1.5% (0.75 g/50 ml TBE buffer)

10XTBE buffer (pH 8.3)

108 g Tris

55 g boric acid

9.3 g EDTA

Up to 1000 ml distilled water

Ethidium bromide (10 mg/ml)

Procedure:

1. Weigh out 0.75 g of agarose and add to 50 µl TBE buffer.
2. Dissolve by heating in a microwave oven.
3. Allow to cool to 70°C, then add 1 µl of ethidium bromide to the dissolved agarose solution inside the fume hood.
4. Pour the gel onto the gel-casting unit.
5. Mix 5 µl of reaction products with 1 µl loading mix and load on the gel.
6. Run the gel at 15 V/cm for 30 minutes and take photographs under ultraviolet illumination (UVP Imagestor 5000).

2.13.2 Polyacrylamide gel analysis

Reagents:

Distilled water	30 ml
40% stock acrylamide	12.5 ml
5XTBE	7.5 ml
10% APS (Ammonium persulphate)	250 µl
Temed	50 µl

Procedure:

1. Assemble the glass plates and spacers (1mm) for pouring the gel on gel forming tray. The glass plate must be free of grease spots to prevent air bubbles from forming in the gel.
2. Prepare gel mix.
3. Add the mixture to gel set-up and immediately insert the appropriate comb.
4. Allow the acrylamide to polymerise for 60 minutes at room temperature.
5. Carefully remove the comb. Immediately rinse out the wells with water.
6. Attach the gel to the gel tank. The notch plate should face inward toward the buffer reservoir.
7. Fill the reservoirs of gel tank with 1XTBE.
8. Add 2 µl of loading mix to each sample. Load 8 µl of each sample and a molecular weight marker
9. Run the gel until the marker dyes have migrated the desired distance.
10. Gently dismantle the gel and submerge the gel in ethidium bromide solution for 30-45 minutes. Photograph the gel under ultraviolet illumination.

2.13.3 Silver staining of polyacrylamide gels

Reagents:

Solution 1: 10% ethanol + 0.5% acetic acid
Solution 2: 0.1% silver nitrate
Solution 3: 1.5% NaOH + 0.1% formaldehyde
Solution 4: 0.75% Na_2CO_3

Procedure:

1. Assemble a mini-polyacrylamide gel cast (1 mm thick) using a modified RunOne system (Embi Tec, San Diego, USA).
2. Pour the mixture (10% polyacrylamide gel was made by adding 3 ml 40% acrylamide, 8.9 ml 1XTBE buffer, 12 µl Temed and 120 µl APS) into the cast.
3. Allow the acrylamide to polymerise for 60 minutes at room temperature.
4. Add 2 µl of loading mix to each sample. Load 5 µl of each sample and a molecular weight marker.
5. Run the gel until the marker dyes have migrated the desired distance.
6. Submerge the gel in solution 1 for 5 minutes on a shaker.
7. Repeat step 6.
8. Submerge the gel in solution 2 for 15 minutes.
9. Remove the gel and rinse quickly with two changes of distilled water.
10. Submerge the gel in solution 3 for 20 minutes.
11. Submerge the gel in solution 4 for 10 minutes.
12. Remove the gel and seal in a plastic bag. Photograph the gel immediately.

2.14 Culture techniques and chromosome preparation from peripheral blood

2.14.1 Peripheral blood culture

Reagents:

Iscoves medium (Imperial Lab Prod.)	100 ml
Heparin (5000 units/ml, LEO Laboratory)	1 ml
Glutamine (Gibco BRL)	1 ml
PHA (Gibco BRL)	2 ml
Penicillin/Streptomycin (100 U/ml and 100 µg/ml, respectively, Gibco BRL)	1 ml

Procedure:

1. Add 0.5 ml of heparinised whole blood to 4.5 ml of culture medium using aseptic technique.
2. Mix the contents of each culture tube by gently inverting several times.
3. Incubate for 72 hours with tubes tilted at 45 degree in a humidified 37°C incubator.

2.14.2 Harvesting

Reagents:

Colcemid (Gibco BRL)	10 µg/ml
Hypotonic solution	75 mM KCl
Fixative solution	3 methanol/1 acetic acid

Procedure:

1. Initiate harvest by adding 0.1 ml Colcemid. Mix by gently shaking the tube and incubate at 37°C for 45 minutes.

2. Centrifuge the tube at 1200 rpm for 7 minutes. Discard the supernatant using pipette into a container of 10% Chloros.
3. Add 10 ml of 75 mM KCl (pre-warmed at 37°C) and gently re-suspend pellet.
4. Incubate the tube at 37°C for 10 minutes.
5. Centrifuge the tube at 1200 rpm for 7 minutes. Then discard the supernatant into a container of 10% Chloros.
6. Re-suspend the pellet by adding 10 ml of fresh fixative.
7. Centrifuge the tubes at 1200 rpm for 7 minutes and remove the supernatant.
8. Re-suspend the pellet in 10 ml of fixative. Centrifuge the tube at 1200 rpm for 7 minutes and remove the supernatant.
9. Re-suspend the pellet by adding 5 ml of fresh fixative and place the tube in the freezer (+4°C) for a minimum 30 minutes.
10. Centrifuge the tube at 1200 rpm fro 7 minutes and remove the supernatant.
11. Re-suspended the pellet in 1 ml of fresh, cold fixative to produce a light milky suspension.
12. Prepare slides and analyse chromosome spreads

2.14.3 Slide preparation

1. Clean microscope slides by soaking in Decon® overnight and rinsing with running water for at least 2 hours. Store the clean slides in 70% ethanol.
2. Remove the slides from ethanol and rinse with running water until the ethanol has gone and a thin, uniform film of water covers the slides. Keep the slides in water at +4°C at least for 30 minutes.
3. Centrifuge the tubes containing the cell suspension at 1200 rpm for 8 minutes and discard the supernatant, re-suspend the pellets in 0.5-1 ml of fresh cold fixative solution. Place 3-4 drops of the cell suspension onto cold grease-free, wet slides.

4. For G-banding, place the slides on a hotplate to dry and evaluate under a light microscope (phase contrast). For CGH, allow the slides to air dry.

2.14.4 G-banding

Reagents:

Trypsin solution		
	Difco trypsin (1:250)	1.2 g
	Sorenson's Buffer	1000 ml
Sorenson's Buffer		
	Distilled water	1 liter
	Disodium dihydrogen phosphate	9.48 g
	Potassium dihydrogen phosphate	9.08 g
Staining solution		
	Leishmans powder	3 g
	Methanol	Up to 2 liters
Filter this solution before use. For staining purpose dilute this solution with buffer (pH 6.8) in the ratio 1 part stain to 3 parts buffer.		

Procedure:

1. Age the slides by placing them in a dry oven at 80°C for two hours.
2. Immerse the slides in trypsin solution for 10-15 seconds.
3. Rinse with saline and stain with Leishmans solution for two minutes.
4. Place the slides on the hotplate to dry.

2.15 Comparative Genomic Hybridisation

To validate the feasibility of CGH in analysing a single cell, the CGH experiments were first carried out by using samples from single amniocytes of numerical aneuploidy (e.g. trisomy 18 and trisomy 21) while the reference DNA was from normal individuals of the opposite sex. Further experiments were carried using 10-20 stained cells scraped from

G-banded slides. To test the sensitivity and reliability of single cell CGH, a number of samples were coded in order to be analysed blind (the sex was known).

To rule out the metaphase factor, two hybridisation reactions were performed simultaneously on the same slide. On one area the CGH was carried out using probes which had previously worked well. To test the efficiency of probes prepared from single cells, some experiments used one-colour hybridisation prior to the real 2-colour CGH experiment.

Throughout the study period, CGH experiments were carried out using various sources of test and reference DNA. The test and reference DNAs were prepared from either non-amplified DNA (i.e. total extracted DNA) or amplified DNA (i.e. whole genome amplification from single cells) (Table 2.1).

Test DNA	**Labelling method**	**Reference DNA**	**Labelling method**
4Total genomic DNA	Nick translation	Total genomic DNA	Nick translation
Total genomic DNA	Vysis	Total genomic DNA	Vysis
*MPE 600	Vysis	Total genomic DNA	Vysis
Amplified DNA (1 cell)	PCR or nick translation	Amplified DNA (one cell)	PCR or nick translation
Amplified DNA (1 cell)	PCR	Amplified DNA (10-20 cells)	PCR
Amplified DNA (1 cell)	PCR	Total genomic DNA	Vysis
Amplified DNA (10-20 stained cells)	PCR	Amplified DNA (10-20 cells)	PCR
Amplified DNA (10-20 stained cells)	PCR	Total genomic DNA	Vysis
Amplified DNA (1 blastomere)	PCR	Total genomic DNA	Vysis

Table 2.1: The combination of test and reference DNA used in CGH experiments. Labelling methods were noted to the right of the test DNA and reference DNA respectively.

The painting probes were labelled by either the method of nick translation or PCR. Commercial kits were also employed as positive or negative controls. The SpectrumGreen MPE 600 (Vysis) was used as a positive CGH control to verify the hybridisation conditions. Negative controls were from SpectrumGreen-labelled or SpectrumRed-labelled normal human genomic DNA. The majority of the metaphases were prepared using commercial slides except during the early stages of the study.

2.15.1 Metaphase slides

Metaphase spreads were made as described in 2.14.3. Wet slides were left at room temperature to dry and then aged for 2 days. These slides were stored at -70°C if not used immediately. Usually, 60-100 metaphase slides were made at a time and several slides from each batch were checked for the hybridisation quality of metaphases. If the quality of the slides was not suitable, the entire batch was discarded. Commercial slides (Vysis, Downers Grove, Illinois) were also employed in this study. These slides were kept at room temperature during transportation; however, they were stored at -60°C once they were received from a supplier. Because of the laborious nature of testing and selecting suitable metaphases, homemade metaphase slides were gradually substituted with commercial slides. These commercial slides were prepared using standard cytogenetic slide preparation methods and have been optimised for use in CGH experiments. They were manufactured from phytohemagglutinin (PHA) stimulated lymphocytes derived from a karyotypically normal male donor. The chromosome length was estimated to be at the 400-550-band level. Both homemade and commercial slides required detailed scrutiny and the criteria for inclusion included: high mitotic index, minimal overlapping of the chromosomes, and little evidence of cellular debris around the nuclei.

2.15.2 Pre-treatment of slides for CGH

In the early stage of this study, the metaphase slides were pre-treated by washing in PBS solution for five minutes at room temperature, followed by dehydration through an ethanol series (70%, 90%, 100% for 5 minutes each) and then air-drying. The slides were then treated with 100 µg/ml RNase in 2XSSC and incubated for 1 hour at 37°C in a humidified chamber. The RNase was eliminated with two washes in 2XSSC at room temperature, each lasting 5 minutes. Slides were then washed in proteinase K buffer (10X proteinase K buffer: 20 mM $CaCl_2$; 200 mM Tris-HCl, pH 7.5) at 37 °C for 5 minutes, before a 5 minute treatment again at 37 °C with proteinase K (50 ng/ml in proteinase K buffer). Following a brief immersion in PBS/1% $MgCl_2$, the slides were fixed with paraformaldehyde (1% paraformaldehyde; 1% w/v $MgCl_2$ in PBS) for 10 minutes at room temperature. After this the slides were washed in PBS, followed by dehydration through an ethanol series as before, and then air-drying.
Pre-treatment of slides was not necessary if the slides had little cytoplasm remaining on them.

2.15.3 Nick translation

Reagents:

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1 µg of DNA in 36 µl distilled water

5 µl 10X A4 mixture (0.2 mM dATP, dGTP, dCTP in 500 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM β-mercaptoethanol, 100 µg/ml bovine serum albumin)

2 µl Texas Red-5-dUTP (Dupont, Boston, MA) or Rhodamine-4-dUTP (Amersham) for reference DNA

2 µl FITC-12-dUTP (Dupont, Boston, MA) or Fluorescein-11-dUTP (Amersham) for test DNA

5 µl DNA Polymerase/DNase (Gibco BRL, Gaithersburgh, MD)

1 µl DNA Polymerase I (Promega, Madison, WI)

Procedure:

1. Mix all the reagents (50 µl) in a 1.5 ml micro-centrifuge tube.
2. Incubate 50 minutes-3 hours at 15°C.
3. Stop the reaction by 10 minutes incubation at 75°C. The distribution of probe size is checked on a 1% agarose gel, and the incubation time and DNase concentration are adjusted in order to obtain the ideal size of probes (500-2000 bp).

2.15.4 Preparation for Hybridisation

Materials and Reagents

Metaphase slides
Labelled test DNA
Labelled reference DNA or 1 µl SpectrumRed total human genomic DNA (200 ng/µl, Vysis)
Human Cot-1 DNA (1 µg/µl, BRL Life Science)
3M sodium acetate (pH 5.2)
100% cold ethanol
Ethanol series (70%, 90%, 100%)
Deionised formamide
Hybridisation buffer (50% formamide, 10% dextran sulfate)
Denaturation solution (70% formamide, 2XSSC pH 7.0)
Coplin jars
Rubber cement
Water bath

Procedure:

Preparation of target metaphase slides:

1. Check the areas of the slides under phase contrast microscope.
2. Mark the appropriate areas with a diamond pen.

3. Denature metaphase slides for 3-5 minutes at 72-75°C in a denaturation solution (70% formamide/2XSSC).
4. Submerge slides into 70% ice-cold ethanol.
5. Dehydrate slides in a series of ethanol (70%, 90%, 100%), one minute each.
6. Allow slides to air dry.

Preparation of reaction mix for comparative genomic hybridisation:

1. Mix 10 µl of PCR labelled test DNA, 200-800 ng reference DNA (or 1 µl SpectrumRed total human genomic DNA), 10 µg Human Cot-1 DNA (Gibco BRL) in a 1.5 ml reaction tube.
2. Add 1/10 volume of 3 M sodium acetate (pH 5.2), mixed, then add 2.5 volume of 100% ethanol for precipitation of DNA.
3. Centrifuge for 30 minutes at maximum speed.
4. Discard the supernatant and vacuum dry for 15-25 minutes.
5. Re-suspend the pellet in 10 µl hybridisation buffer.
6. Denature the probe mix for 5 minutes at 75°C immediately before use.
7. Apply the probe mix on denatured slides.
8. Cover with a glass coverslip (22x22 mm). The edges are sealed with rubber cement to prevent evaporation.
9. Incubate for 48-72 hours in a 37°C moist chamber.

2.15.5 Post-hybridisation wash

Reagents

Washing solution (50% formamide/2XSSC)	50 ml
2XSSC	50 ml
4XSSCT (4XSSC containing 0.05% Tween 20)	50 ml
4,6-diamidino-2-phenylindole-dihydrochloride (DAPI)	1 µg/ml
Antifade (Citifluor, Chem. Lab., Canterbury, U.K.)	
Ethanol series (70%, 90%, 100%)	

Procedure:

1. Pre-warm washing solution in a glass Coplin jar at 45°C.
2. Remove the coverslip carefully.
3. Immerse the slides in pre-warm 50% formamide/2XSSC for 5 minutes.
4. Immerse the slides in pre-warm 2XSSC for 5 minutes.
5. Repeat step 4.
6. Immerse the slides in pre-warm 4XSSCT for 5 minutes.
7. Dehydrate the slides through a series of ethanol (70%, 90%, 100%), one minute each.
8. Allow the slides to air dry in darkness.
9. Apply 10 µl of the antifade solution containing 0.2 µM DAPI. Cover with a glass coverslip and seal with nail polish.
10. Evaluate slides with an epifluorescence microscope.

2.15.6 Chromosome identification with DAPI

Quips CGH/Karyotyper allowed automatic classification and pairing of metaphase chromosomes to generate a karyotype with 80-90% accuracy. The DAPI counterstain could be enhanced to bring out the band structure and thus help in identification of the chromosomes. The DAPI chromosome identification was based on that posted at website http://amba.charite.de (Institute of pathology, University Hospital Charite, Berlin) In general, the chromosome identification was made by following categories and characteristics:

Chromosome 1-3: easy to identify

*Chromosome 1: end of p arm lighter than the rest of the chromosome, centromeric heterochromatin like an isosceles triangle with the base directed towards the q arm.

*Chromosome 2: 2nd largest chromosome, submetacentric.

*Chromosome 3: 3rd largest chromosome, metacentric.

Chromosome 4-5: same size, difficult to identify

*Chromosome 4: darker than 5.

*Chromosome 5: with light area at the end of the q arm.

Chromosome 6, 7, X: difficult to identify

*Chromosome 6: darker q arm, lighter p arm with band/dots at the end .

*Chromosome 7: distinct band visible, one at the p arm, two at the q arm slightly pronounced centromere.

*Chromosome X: particularly difficult to distinguish from chromosome 7, X chromosome usually showed less intense fluorescence signal in the FITC image if the test DNA was from a male (two chromosome 7 compared to one X chromosome).

Chromosome 8-12: are more or less of the same size, difficult to identify.

*Chromosome 9: easy to identify, (look for suppression in the FITC image); Intense centromeric heterochromatin; p arm of chromosome 9 is longer than that of chromosome 8 and 10.

*Chromosome 8: particularly difficult to distinguish from chromosome 10, chromosome 8 usually darker than chromosome 10.

*Chromosome 10: shows three bands on the q arm, in particular a subcentromeric band on the long arm compared to 2 bands on the q arm of chromosome 8.

*Chromosome 11: showed two bright dark bands on the q arm and p arm.

*Chromosome 12: shows only a distinct dark band on the q arm. The p arm is shorter than that of chromosome 11.

Chromosome 13-15: same size, difficult to identify.

*Chromosome 13: dark zone covering about 2/3 of the q arm.

*Chromosome 14: has a distinct band on the q arm followed by a light zone before telomeric end (chromosome 15 may have a band too; however, there is usually no light zone distinguishable before the telomeric end)

*Chromosome 15 shows a more prominent centromere (look for suppression in the FITC image), the dark zone of chromosome 15 extends more telomeric than that on chromosome 14

Chromosome 16-18: usually easy to identify.

*Chromosome 16: distinct centromeric heterochromatin

*Chromosome 17: p arm is longer than that of chromosome 18, distinct band visible on the q arm and mostly on the q arm.

*Chromosome 18:darker than chromosome 17. There are usually two distinct bands visible on the q arm.

Chromosome 19-20: difficult to identify.

*Chromosome 19: more pronounced centromeric heterochromatin (look for suppression in the FITC image)

*Chromosome 20: has a dark band on the p arm

Chromosome 21-22: difficult to differentiate.

*Chromosome 21: has a more pronounced dark band on the q arm compared to chromosome 22. The band takes the shape of a triangle compared to more distinct, rectangular-shaped staining pattern for chromosome 22

Chromosome Y: easy to identify. Carries a lot of heterochromatin.

2.15.7 A short CGH protocol

The procedures of the short protocol were the same as previously described in the CGH method except for the hybridisation time and the amount of probe. For overnight CGH, 20-40 µl of amplified DNA was used, while the hybridisation time was around 12 hours. The CGH experiments were performed in the late afternoon, and hybridisation was carried out overnight. Post-hybridisation wash was performed the next morning and CGH analysis could be completed before noon.

2.15.8 Digital image analysis

CGH results were viewed through an epifluorescence microscope (Zeiss, Axioplan, Germany) equipped with a 50-100 Watt mercury lamp. Metaphases spreads were examined with 100X oil-immersion lens and appropriate filter set for the fluorochromes used. Differences in relative intensity of the two fluorochromes could be visualised by using a dual-band-pass filter that transmitted red and green fluorescence simultaneously, along with a filter for the DAPI counterstain.

For image acquisition, separate digitised grey level images of red, green, and blue were captured with a CCD camera (Photometrics) controlled by SmartCapture™ version 2.1 software (Digital Scientific, Cambridge, U.K.) and analysed with Quips CGH software (Vysis) on a computer (Macintosh). Average green to red fluorescence ratios along the length of each chromosome were calculated from 5-10 metaphase spreads with high intensity and uniform hybridisation. Chromosome regions with fluorescence ratio (FR, green-to-red ratio) above 1.2 were considered to be over-represented (gains), whereas regions with a ratio less than 0.8 were considered to be under-represented (losses) These values could be manipulated using various thresholds depending on the internal control of X chromosomes in each experiment.

2.15.9 Quips CGH analysis system

The Quips CGH analysis software system is a suite of programmes, which provides comprehensive patient management and semi-automatic image analysis for CGH studies. Each programme in the suite can be used independently. In this system, usually the Quips CGH/Karyotyper and Quips Interpreter are employed. Quips CGH/Karyotyper programme is used to edit and analyse a metaphase image to generate a karyotype and CGH ratio profiles. Quips Interpreter is used to edit and analyse ratio profile data from several metaphases in a CGH experiment. Quips CGH analysis system contains features of Quips Karyotyping and uses multicolour images from the microscope obtained from Quips SmartCapture FISH. The sequence of analysis and features of this system are presented as follows:

1. Correct background automatically.
2. Normalise fluorescent intensity automatically.
3. Enhance DAPI banding automatically.
4. Display metaphase as individual colour plane or composite colour.
5. Display karyotype as individual colour plane or composite colour.

6. Compute chromosome symmetry axis and CGH ratio profiles automatically.
7. Optionally include chromosomes in karyotype but not in profile analysis.
8. Display ideograms with karyotype and ratio profiles.
9. Display individual or average ratio profiles.
10. Compute regions of DNA copy number gains or losses.

Chapter 3

Results

3.1 Single cell manipulation

Retrieving a single amniocyte required a series of cell dilution processes using a PBS solution. Under inverted microscopy, it was extremely difficult and unreliable to isolate a single cell without first using this cell dilution process. For each cell manoeuvre, frequent changes of the micropipette were mandatory as this would prevent any cell carry-over. A single cell could be secured when it was seen to pass over the tip of the micropipette. After a single cell was transferred, the remaining PBS solution was used as a negative control to ensure that no DNA remained in the washing solution. During the study period, several cell types including fresh, or frozen amniocytes, fixative-treated lymphocytes and buccal cells were manipulated. The characteristic features of various cell types are listed in Table 3.1.

	Fresh amniocytes	**Frozen amniocytes**	**Fixative-treated lymphocytes**	**Buccal cells**
Cell debris	Mild	Moderate	Mild	Much
Cell identification	Relatively easy	Relatively difficult	Relatively difficult	Easy
Cell size	Medium	Medium	Small	Large
Cell division	Common	No	No	No
Stored solution	Trypsin-treated culture medium	Trypsin-treated culture medium	3 methanol / 1 acetic acid	Water
Cell number	Numerous	Few	Numerous	Numerous
Cell manipulation	Relatively Difficult	Difficult	Difficult	Relatively easy

Table 3.1: Various cell types and their characteristic features under 100X inverted microscope.

Fresh samples proved the best candidates for single cell manipulation, whereas micropipetting a single cell from fixative-treated lymphocytes was technically more difficult. Fixative interfered with surface tension of the PBS solution and made single cell manipulation difficult. In addition, the presence of any remaining fixative was detrimental to the PCR reaction. After several failed PCR amplifications, single cell manipulation of fixative-treated lymphocytes was abandoned.

Amniocytes from patients with a chromosome abnormality were frozen in cases when cell transfer or PCR amplification failed. The frozen sample was subsequently thawed at room temperature and used for cell recovery when repeat experimentation was indicated. However, cell number noticeably decreased and an increase in debris was noted after the freezing and thawing processes. Debris might mimic amniocytes under microscopy, and thus careful differentiation was required.

Fresh amniocytes had a reflective surface and particles in the cytoplasm, while nuclei were less obvious under inverted microscopy. Debris usually manifested with an irregular surface and could be differentiated by careful adjustment of focus. Amniocytes had the tendency to adhere to the Petri-dish surface if manipulative procedures were lengthened. Under such circumstances, cell manipulation became difficult because amniocytes which adhered to surface were resistant to aspiration by micropipettes.

Buccal cells were relatively larger in size. Besides the characteristic features of a large cytoplasm to nucleus ratio the clear nucleus made cell identification easier. However, the disadvantage of using mouthwashes was their plentiful cell debris, which might increase the chance of contamination for PCR amplification. It was also difficult to obtain mouthwash samples from certain chromosome abnormalities such as trisomy 18. Consequently single buccal cell isolation was abandoned when experiments were carried out to test the reliability of single cell CGH in predicting various chromosome anomalies.

To increase the success rate of single cell transfer, two to four single cells were transferred each time because single cells might be lost or might adhere to the PCR tubes during cell transfer (Figure 3.1).

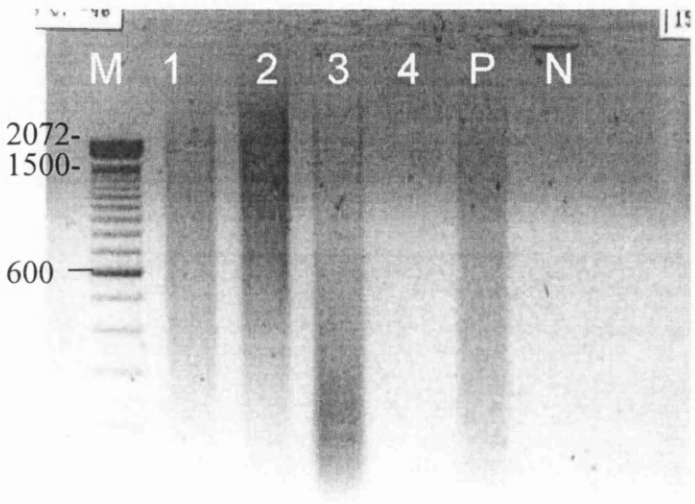


Figure 3.1: DOP-PCR amplification from single cells. Lanes 1-3: single amniocyte transfers. Lane 4: transfer failure. P: from around 20 cells. N: No DNA control. The smear strength may show a discrepancy among single cells after 30 cycles of DOP-PCR. M: 100 bp DNA ladder.

3.2 The problems of contamination

At the beginning of this study when PCR amplification was carried out to determine the sex of samples, the reaction employed 1μg DNA. The experiments used routine laboratory techniques, and the results did not show any predilection for contamination. However, this situation was

greatly challenged when the whole genome amplification protocol was used. In DOP-PCR experiments, it was not uncommon to see a negative control in which only distilled water was added resulting in a smear following agarose gel analysis. An increased rate of contamination was also noted when the number of DOP-PCR cycles was more than 40 (Figure 3.2). The condition was exacerbated when two stages of DOP-PCR involving 70 cycles were adopted. For two stages of DOP-PCR, 5 µl of the first DOP-PCR product was added to the secondary DOP-PCR amplification. All experiments using two stages of DOP-PCR amplification resulted in false amplifications from negative controls. The smear strength of negative control was indistinguishable from that of minute DNA (Figure 3.3).

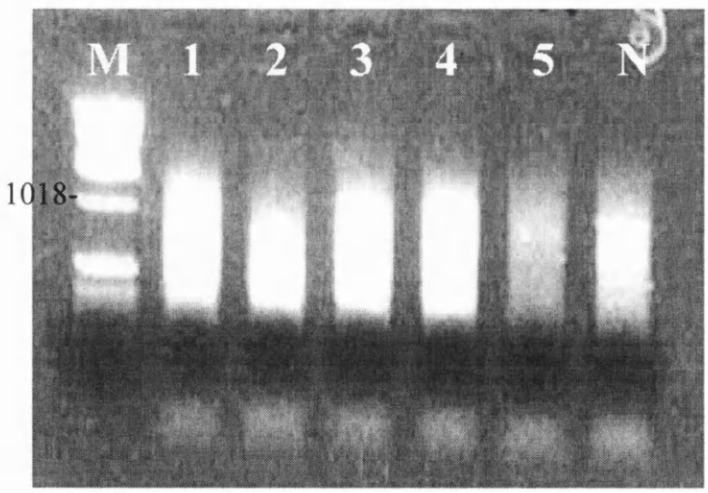


Figure 3.2: DOP-PCR amplification from a series of diluted genomic DNA. PCR cycles including 5 cycles of low stringency and 40 cycles of high stringency were used. Lane 1: 190 pg, Lane 2: 94 pg, Lane 3: 38 pg, Lane 4: 19 pg, Lane 5: 9 pg, Lane N: negative control. M: 1 kb DNA ladder.

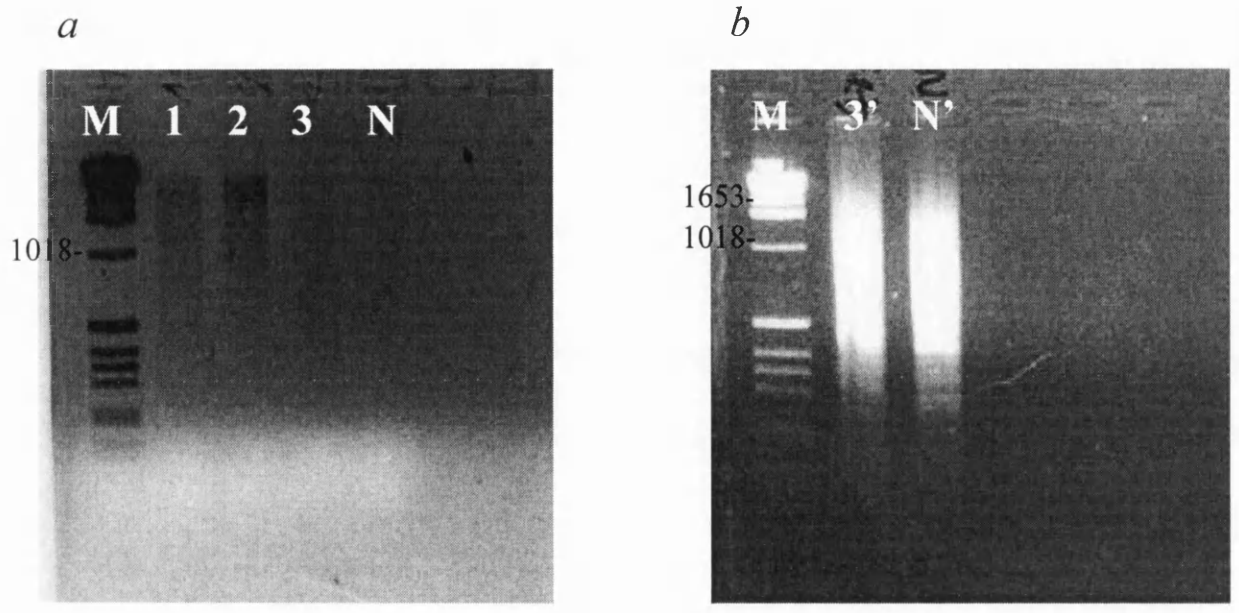


Figure 3.3: Two-stage DOP-PCR protocol. *a:* The robust DOP-PCR protocol could amplify diluted DNA down to femtogram level, which was equivalent to one chromosome band. In lanes 1-3, template DNA was from 1.6 pg, 950 fg, and 475 fg, respectively. Lane N: negative control. Lane 3 and N were unable to produce smear from the first round DOP-PCR. *b:* Two-stage DOP-PCR protocol showed a strong smear on lane 3' and N' derived from lane 3 and lane N, respectively. In cases of similar smear strength, it is not possible to differentiate true amplification of minute DNA from contamination. M: 1kb DNA ladder

To avoid false amplification, the longer DOP-PCR protocol (> 40 cycles) was abandoned and stringent precautions against contamination were adopted. All containers, water, PCR tubes, pipettes were contaminated to some degree with DNA. These were autoclaved and UV-irradiated before they were used to make a PCR reaction mix. Old pipettes were plagued with aerosol contamination because numerous DNA analyses had been

carried out previously. Dismantling these pipettes and cleaning the interior sides of barrels with 100% ethanol still did not completely eliminate the contamination.

Contamination was only reliably avoided when a series of new pipettes, which would measure 1-1000 µl, were utilised and frequently UV-irradiated/or autoclaved before preparing the reagents. These pipettes were strictly employed only for reagent preparation and never contacted solutions, which might contain DNA. Laboratory autoclaved yellow tips were not suitable for single cell DOP-PCR as the results showed high rates of contamination. Tips with barriers could effectively decrease the aerosol contamination. This would prevent the PCR preparation and the interior of the pipettes from carry-over contamination. Another set of pipettes for PCR products analysis were prepared and kept entirely separate from DNA-free pipettes. These pipettes were also used for preparing CGH mixtures, which contained DNA.

Despite all these precautions, contamination would still occur. However, this could be kept to a minimal degree and carefully monitored through the use of negative controls. When each single cell was transferred, a respective negative control from the last 50 µl droplet of PBS washing solution afforded the best indicator for monitoring contamination. Successful transfers of single cells were revealed by the apparent discrepancy of smear strength between single cell transfers and negative controls. (Figure 3.4).

3.3 Microdissection of stained cells from slides

Cell clusters of 10-20 stained cells, not in metaphase, could be easily demarcated under the inverted microscope. Areas containing greatly scattered cells were avoided, as this would require more manoeuvres and the genuine number of cells retrieved might be in doubt. Compared to single cell manipulation from cultured amniocytes, this procedure was relatively easy and less labour-intensive. A 'sham' microdissection, which was performed by placing the needle to an area without any cells, was

used as a negative control for each slide. Results of DOP-PCR from stained cells usually revealed a similar smear strength to those obtained from a single cell. However, the size distribution was usually smaller than those obtained from a single cell (Figure 3.5).

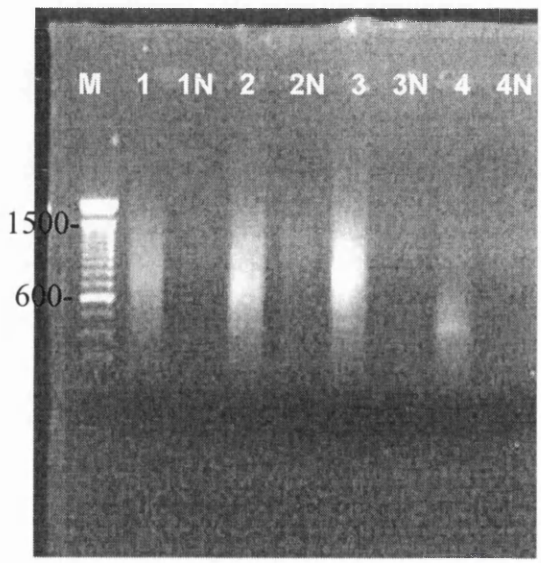


Figure 3.4: The distribution of single cell DOP-PCR products (lanes 1, 2, 3, 4) and the respective negative controls (lanes 1N, 2N, 3N, 4N). M: 100 bp DNA ladder.

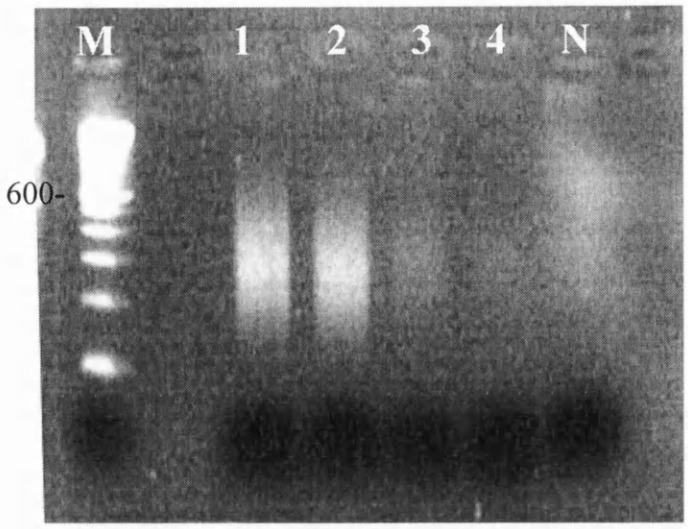


Figure 3.5: DOP-PCR products from microdissected stained cells. The majority of DOP-PCR fragments were less than 800 bp. In lanes 3-4, the smear was very faint and could not be differentiated from the negative control. M: 100 bp DNA ladder.

3.4 Optimisation of whole genome amplification (WGA)

Two WGA protocols (i.e. PEP and DOP-PCR) were tested in this study (Zhang et al 1992; Telenius et al 1992). For DOP-PCR amplification, the work started by testing several parameters including 2-10 µM primer, 1.25-4 U Taq polymerase per 25-50 µl reaction volume, and 2-4 mM $MgCl_2$ on diluted DNA. The PCR conditions included 5-8 cycles of low stringent annealing temperature, and 28-40 cycles of high annealing temperature. The purpose of DOP-PCR optimisation was to obtain a rapid and effective protocol, which would amplify sufficient template from a single cell. The results showed that an obvious smear could be obtained by using any combination of these parameters. Nevertheless, the lower concentration

was preferred in order to avoid the high remaining concentration of degenerate primer which could interfere with subsequent specific locus detection.

PEP, another PCR technique aimed at whole genome amplification, applied 37°C and 10 s/°C ramp temperature to 55°C as the annealing temperature. With this slow ramp rate, it took 10 hours to complete 50 cycles of PEP protocol. A short protocol took around 5-6 hours to complete (Sermon et al 1996). Both PEP protocols did not produce apparent smear on agarose gel analysis after 50 cycles.

3.4.1 Primary DOP-PCR

A series of extracted DNA, which was diluted to picogram level, was tested to validate the performance of DOP-PCR from minute DNA quantities. The results showed that, with diminution of DNA concentration, the smear strength of DOP-PCR products also correspondingly decreased (Figure 3.6). For DOP-PCR cycling conditions, a combination of low stringency (i.e. 5-8 cycles) and high stringency (i.e. 28-40 cycles) was tested for its performance in amplifying minute DNA. All forms of recombinant cycling conditions could produce a visible smear on agarose gel analysis despite the increased tendency for contamination with prolonged cycles. Low stringency conditions were empirically set at 5-8 cycles in order to increase random-priming sites throughout the whole genome. To prevent a lengthy process, high stringency conditions were set at 28-30 cycles. The time requirement for this protocol would then be around 4 hours and 15 minutes.

Preferential amplification occurred occasionally in the DOP-PCR reaction (Figure 3.7). This phenomenon was manifested by the presence of one or more bands amongst the background smear. However, subsequent CGH experiments were not precluded in this case, because it did not necessarily represent contamination. Successful single cell CGH experiments were achieved from these DOP-PCR products and the results did not show over-representation of certain chromosomal regions.

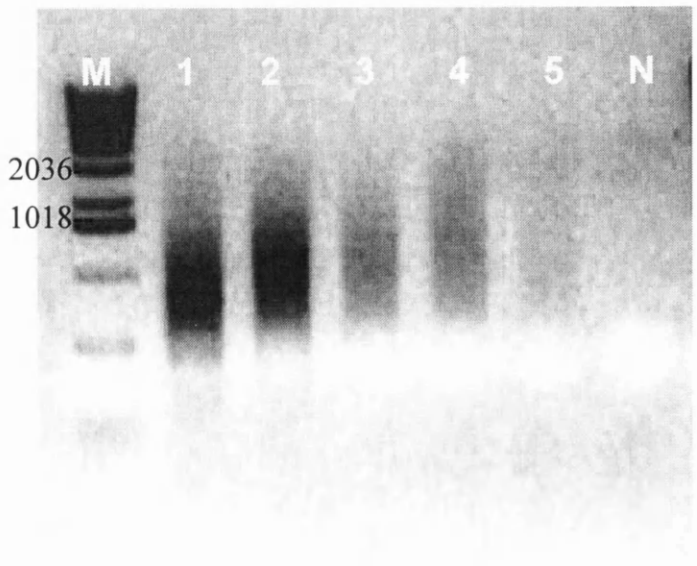


Figure 3.6: DOP-PCR from a series of diluted genomic DNA. Lane 1:760 pg, Lane 2: 500 pg, Lane 3: 380 pg, Lane 4: 250 pg, Lane 5:190 pg, Lane N: negative control. M: 1kb DNA ladder.

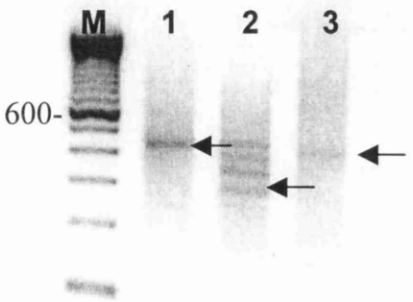


Figure 3.7: Preferential amplification of DOP-PCR from single cells. Lanes 1-3: This phenomenon was manifested by the presence of one or more bands amongst the background smear (indicated by arrows). M: 100 bp DNA ladder.

The low stringency annealing temperature of 30°C is the characteristic feature of DOP-PCR. The PCR conditions included a 'ramp' (i.e. 3 min transition from 30°C to 70°C) for lower stringency conditions while 'increment' (i.e. addition of 1 sec/cycle for elongation step) was applied in high stringency conditions (Telenius et al 1992b). Various experiments revealed that DOP-PCR without ramp and increment could still efficiently produce a smear, whereas the time requirement was decreased by half an hour. It took 6 hours and 42 minutes to complete 48 cycles of DOP-PCR; however, the time requirement was decreased to 4 hours and 20 minutes for 35 cycles of DOP-PCR (Table 3.2).

	DOP-PCR	**DOP-PCR**	**PEP***	**Short PEP****
Cycle	35	48	50	
Low stringent Cycle	5	8		
High stringent Cycle	30	40		
Total Time	4 h 20 m	6 h 42 m	10 h	5 h 30 m

* Zhang et al 1992

** Sermon et al 1996

Table 3.2: Time requirements for various protocols of whole genome amplification.

The oil-free cycler, which used a hot lid to prevent evaporation of PCR mix, caused another problem in the DOP-PCR reaction. The heat accumulated by the hot lid would decrease the efficiency of the cooling system of the PCR cycler, particularly in a warm room where there was heat or on a hot summer day. The temperature would then be raised to several degrees above 30°C. Failure to lower the temperature to 30°C

would ruin the DOP-PCR reaction. Moving the cycler into a cold room (4°C) or avoiding using the hot lid solved this problem. An oil-free PCR reaction was a better choice for PCR labelling because further comparative genomic hybridisation might be interfered with by the presence of mineral oil.

Repeated DOP-PCR experiments using 5 cycles of low stringency conditions and 30 cycles of high stringency conditions produced an obvious smear from diluted DNA at the picogram level. This protocol was then adopted to test on single cells in order to avoid two-stage DOP-PCR or extended DOP-PCR cycles (i.e. over 40 cycles).

3.4.2 DOP-PCR reaction from single cells and stained cells

The size distribution of DOP-PCR products from a single cell was usually smaller than those amplified from diluted DNA; however, variation did occur. The majority of DOP-PCR results usually ranged from 200-2000 bp, with the bulk of the smear less than 1500 bp. Nevertheless, wide-range amplification products, with the size of products more than 2000 bp, could occur. DOP-PCR products from stained cells usually resulted in shorter fragments, with the majority of the smear less than 1000 bp (Figure 3.8).

DOP-PCR tended to amplify shorter sequences more efficiently than longer ones. The majority of the DOP-PCR smear concentrated distally rather than proximally on agarose gel analysis. Occasionally, the distribution of DOP-PCR fragments was less than 1000 bp. Further CGH experiments would not be considered if the distribution of fragments were less than 600 bp. Short DOP-PCR fragments were very unlikely to produce optimal CGH results. DOP-PCR amplification from a single cell would yield 10 µg template in 50 µl reaction volume. This was similar to the results from a positive control from 1 ng diluted DNA.

The Expand™ High Fidelity PCR system, using mix of Taq polymerase and Pwo polymerase, is especially optimised to amplify DNA fragments up to 5-12 Kb from human genomic DNA. However, this PCR system did not produce a wide fragment distribution if compared with conventional Taq

polymerase throughout the study period. Moreover, the yield appeared to diminish when Expand™ Fidelity PCR system was used for DOP-PCR amplification from single cells (Figure 3.9).

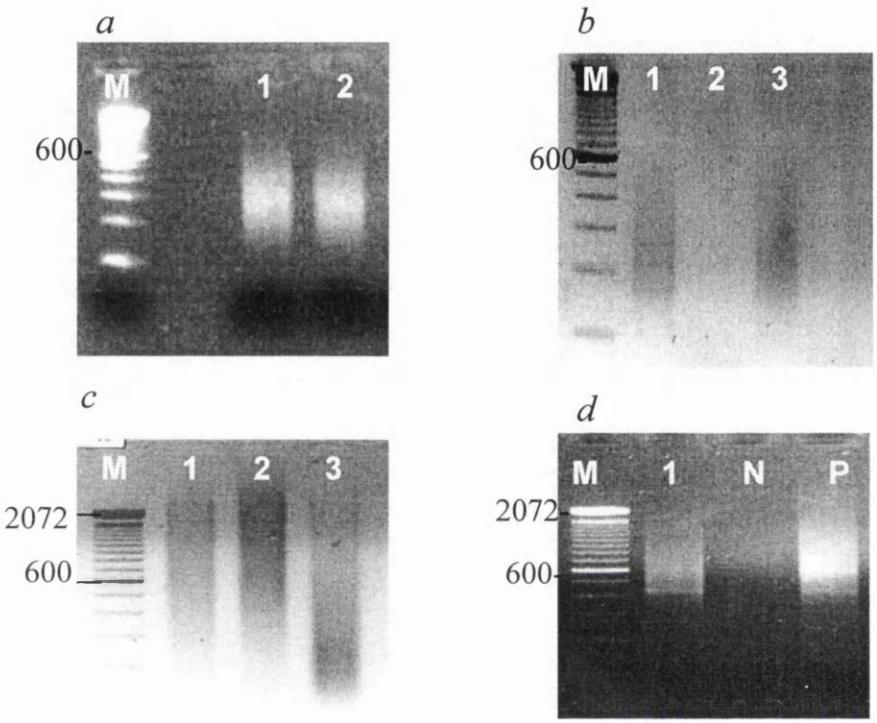


Figure 3.8: The variation of DOP-PCR amplification from single cells. *a-b* DOP-PCR from microdissected single cells. The fragment distribution was less than 1000 bp with the majority of products less than 800 bp. *c-d* The DOP-PCR from a single cell. The majority of fragments ranged from 200 bp to 2000 bp. P: positive control from 10-20 cells. N: Negative control. M: 100 bp DNA ladder.

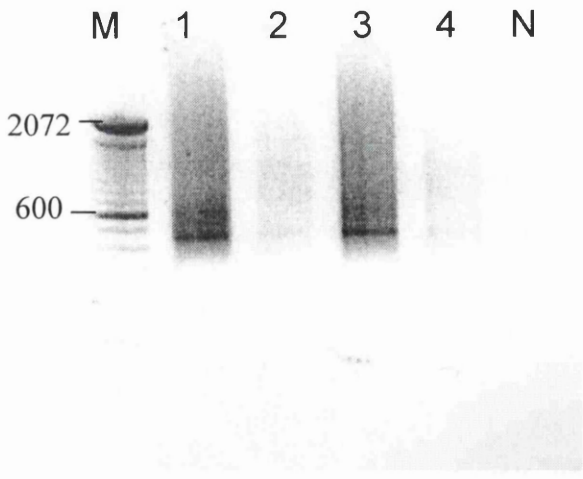


Figure 3.9: Comparison of DOP-PCR using Taq enzyme and Expand™ High Fidelity PCR system on single cells. Lanes 1, 3: DOP-PCR amplification from 20 cells by Taq polymerase. Lanes 2, 4: by Expand™ High Fidelity PCR system. N: negative control. M: 100 bp ladder.

3.4.3 Secondary DOP-PCR for probe labelling

After primary DOP-PCR from a single cell, 5 µl of the product was transferred into a secondary DOP-PCR amplification for probe labelling. Twenty cycles of PCR labelling would yield 5-10 µg of painting probe. The size distribution usually ranged from 200 to 1500 bp. However, the majority of the fluorochrome was not incorporated into the probe and accumulated distally in a free form over the smear trail (Figure 3.10). The molecular size of Fluorescein-11-dUTP (Green) is smaller than that of Rhodamine-4-dUTP (Red), because green fluorescent dye ran further than its red counterpart. Expand™ High Fidelity PCR system could increase probe size distribution; however, Taq polymerase also had similar effects if 4 mM magnesium concentration was used (Figure 3.11). Fluorescence-conjugated nucleotides ranging from 1-2.5 µl were investigated for efficient labelling. The results demonstrated that 2 µl of fluorescence-conjugated

nucleotides could produce consistent and reliable CGH results. A higher concentration would cause waste while lower amounts failed to generate consistent results.

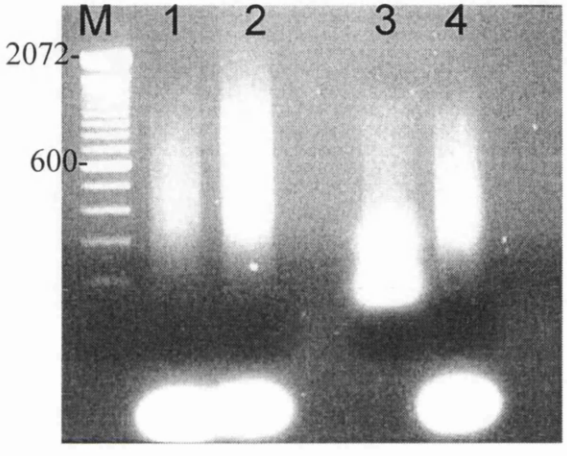


Figure 3.10: Probe labelled by secondary DOP-PCR. Lanes 1, 2, 4: labelled with fluorescein-11-dUTP (green). Lane 3: labelled with rhodamine-4-dUTP (red). The size distribution was usually less than 1500 bp. The molecular size of fluorescein-11-dUTP was less than rhodamine-4-dUTP. M: 100 bp DNA ladder.

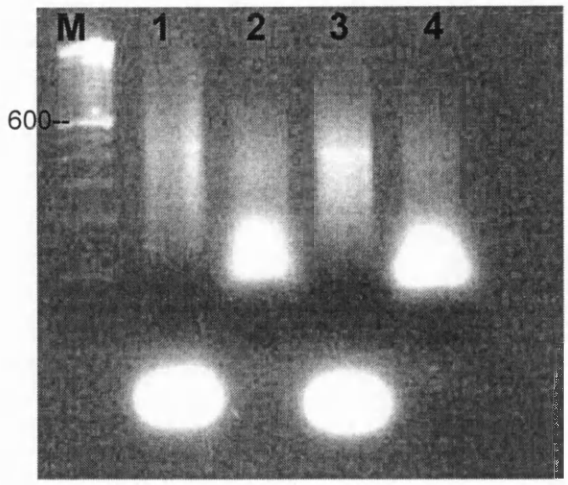


Figure 3.11: Size distribution of the probes made using Expand™ High Fidelity PCR system and Taq polymerase. Lanes 1-2: labelled using Taq polymerase and 4 mM $MgCl_2$. Lanes 3-4: labelled with Expand™ High Fidelity PCR system. M: 100 bp DNA ladder.

3.5 Optimisation of nested PCR for sex determination

The primers designed for sex determination led to primary amplification of 555 and 371 bp for the X and Y chromosomes, respectively (Schaaf et al 1996). The second amplification resulted in one 323 bp band for female cases, and two bands of 323 bp and 142 bp for male cases (Figure 3.12).

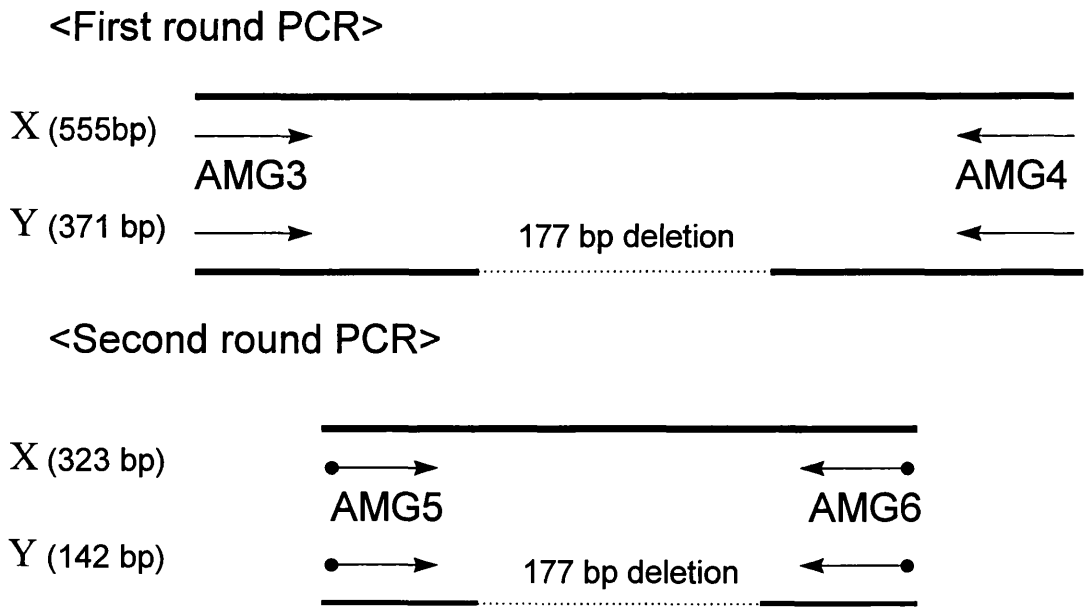


Figure 3.12: Nested PCR strategy for sex determination. Homologous primers for both sex chromosomes were designed by simultaneous amplification of AMGX and AMGY. Both pairs of primers were aligned to encompass a 177-bp deletion in AMGY. Fragments of different sizes for X and Y (323 bp and 142 bp, respectively) could be resolved by simple agarose gel analysis.

A series of diluted DNAs was tested in order to determine the lowest concentration, which could yield detectable products on agarose gel after 25 cycles of the first round PCR. The outer primers flanking the 555 bp sequence could be amplified from 1 μg, and 100 ng DNA template but not 10 ng DNA (Figure 3.13). At first the primer concentration for nested PCR of the amelogenin locus was performed as in the original protocol. When both outer and inner primer concentrations were set at 400 nM, the results demonstrated some extra bands on agarose gel analysis. These unwanted bands were located at the position of 400+ bp and 200+bp (Figure 3.14).

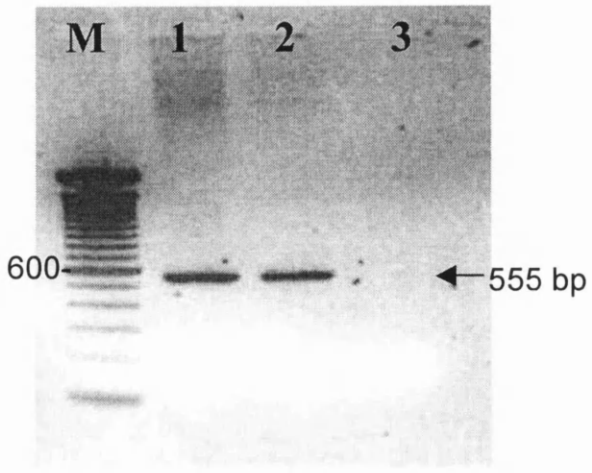


Figure 3.13: Minimal DNA requirements for the first round PCR in sex determination. The reaction was tested with a series of female diluted DNA. Lane 1: 1 µg, lane 2: 100 ng, lane 3: 10 ng. M: 100 bp DNA ladder.

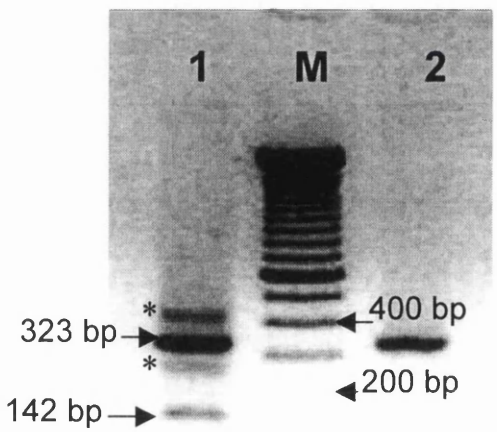


Figure 3.14: Extra bands produced in the nested PCR. Sex was determined by the presence of an X band (323 bp) and a Y band (142 bp). Unwanted bands (*) were noted when 400 nM outer primer was used. Lane 1: Male with unwanted bands at the position of 400+ bp and 200+ bp. Lane 2: Female band at 323 bp. M: 100 bp DNA marker.

To achieve final products of 323 bp for AMGX and 142 bp for AMGY, various concentrations of the outer primer were tested, while the inner primer concentration was set at 400 nM. The results showed that, with decreasing outer primer concentration, the targeted final products of 323 bp for AMGX became more obvious (Fig 3.15). In other experiments, the annealing temperature of the second round PCR was elevated from 56°C to 60-62°C; however, this did not completely prevent the occurrence of unwanted bands.

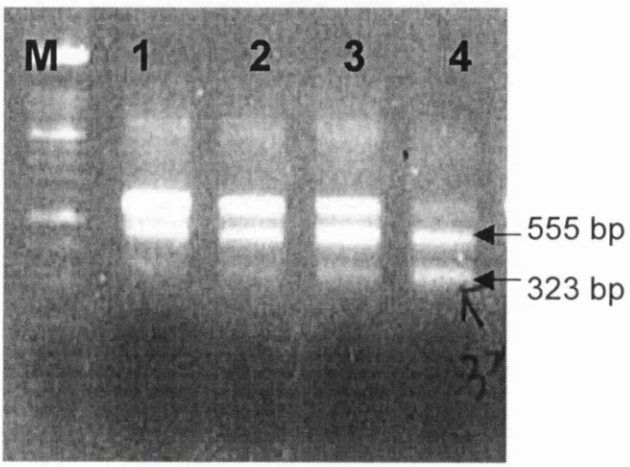


Figure 3.15: The effects of various concentrations of outer primer in the nested PCR reaction. From lane 1 to lane 4, the concentration of outer primer was gradually diluted from 2 μM to 200 nM. The targeted fragment became evident when lower concentration of outer primer was used. M: 100 bp DNA ladder.

To determine the optimal primer concentrations for the nested PCR reaction, subsequent experiments applied a series of outer and inner primer concentrations in testing the template DNA at the picogram level. The experiments set the concentration of outer primer concentration at 2 μM, 400 nM, 200 nM, 133 nM, and 100 nM, respectively. The inner primer concentration was set at 400 nM, and 800 nM. The results showed that targeted band patterns (i.e. 323 bp for AMGX and 142 bp for AMGY) could be obtained when AMG-3/AMG-4 primers were set at 100 nM. The inner primer, when set at 400, or 800 nM, had similar results (Figure 3.16). It was therefore decided that 100 nM and 400 nM were the optimal concentration for outer and inner primers, respectively.

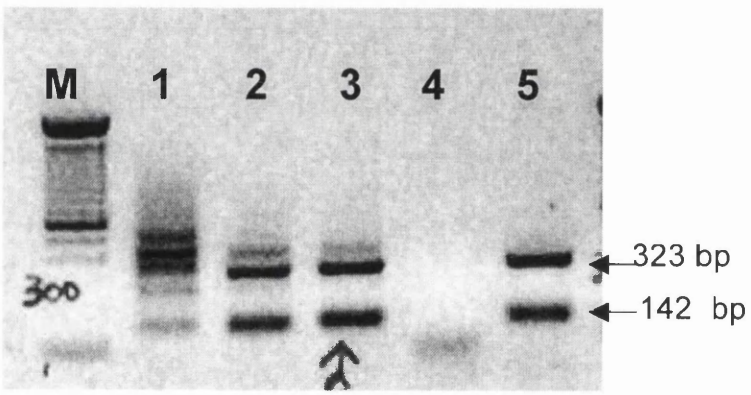


Figure 3.16: Optimisation of nested PCR at the amelogenin locus using various concentrations of outer and inner primers. From lanes 1-5, the outer primer concentration was progressively diluted to 100 nM. Lane 4 showed failed amplification. Unwanted bands were apparent when primer concentration was high (lane 1). Extra band at 400+ bp became less obvious from lane 2 to lane 5. M: 100 bp DNA ladder.

Throughout the study period, it was a general finding that the Y band was usually fainter than X band. For those band patterns recognised as male, some extra bands might occur: a dim band slightly above 323 bp, a band of 200+ bp, or extra bands above 400 bp. Occasionally male cases would manifest as a single Y band of 142 bp (Figure 3.17). Apart from 323 bp for AMGX, some dim extra bands were simultaneously amplified, at the position of 400+ bp, for recognised female cases. However, sex could not be determined for some band patterns as they lacked apparent targeted bands. A pattern, which involved simultaneous amplification of a band of 200+ bp and 323 bp, was equivocal because both males and females could display a similar pattern.

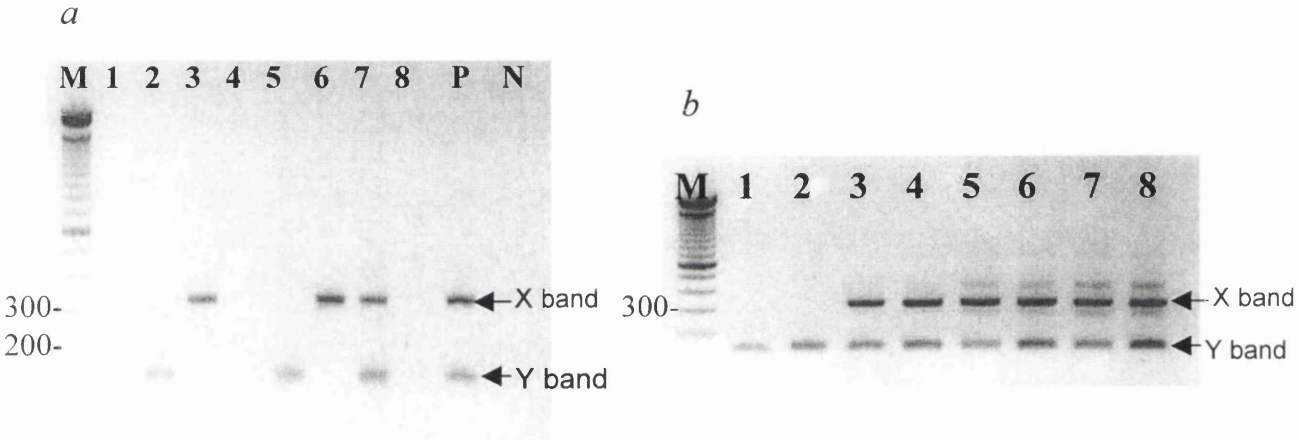


Figure 3.17: The phenomenon of a single Y band. Sex determination from DOP-PCR products derived from 10-20 microdissected stained cells. *a:* In lane 2 and 5, only a single Y band was amplified. The X band was not simultaneously amplified. P: positive control from 5 ng male diluted DNA. N: negative control. This phenomenon may be due to DNA damage, preferential amplification or incomplete lysis. *b:* Various band patterns from male cases. The Y band was usually fainter than X band. However, it may be similar to X band (lane 8). Some extra bands were noted in lane 5-8. In lanes 1-2, only Y bands were produced. M:100 bp DNA ladder.

3.6 Sex determination for DOP-PCR-derived DNA from a single cell

To validate the feasibility of sex determination for DOP-PCR-amplified DNA, 60 single cells were DOP-PCR amplified and subsequently investigated with nested PCR on the amelogenin locus. The distribution of band patterns from both sexes was depicted in figures 3.18-3.19.

Of 60 cases, 26 (43.3%, 26/60) failed to produce PCR products, while five cases resulted in band patterns, which could not be recognised. Identifiable patterns at the amelogenin locus for DOP-PCR-amplified DNA was seen in 48.3% (29/60). However, one male cell was interpreted to be female while another female cell was interpreted to be male, and resulted in a 6.9% (2/29) misdiagnosis rate among those identifiable band patterns. Among 9 single male cells, which had distinguishable band patterns, 4 produced only Y bands. Contamination occurred when a female cell was diagnosed as a male and this represented 3.4% (1/29) among those with recognisable band patterns.

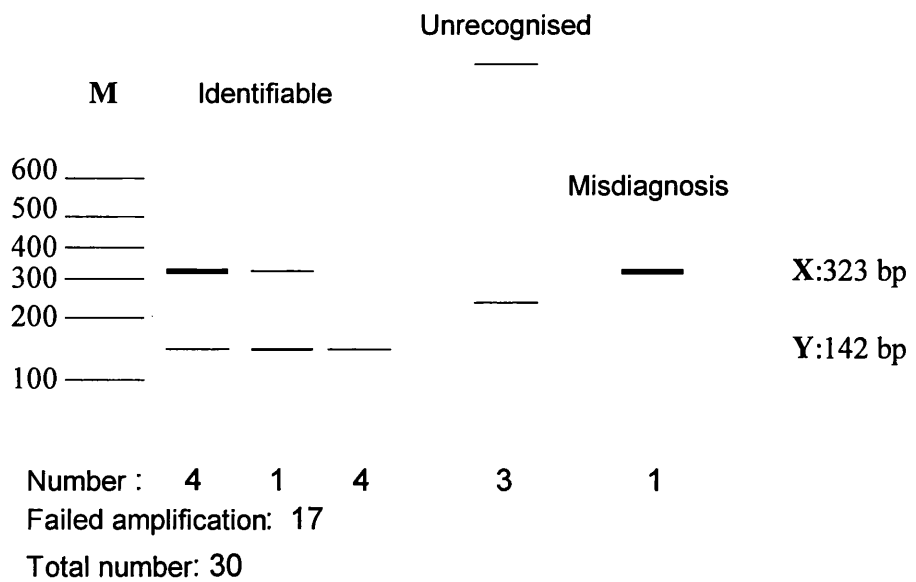


Figure 3.18: The distribution of male band patterns when sexing on DOP-PCR-derived DNA prepared from a single cell.

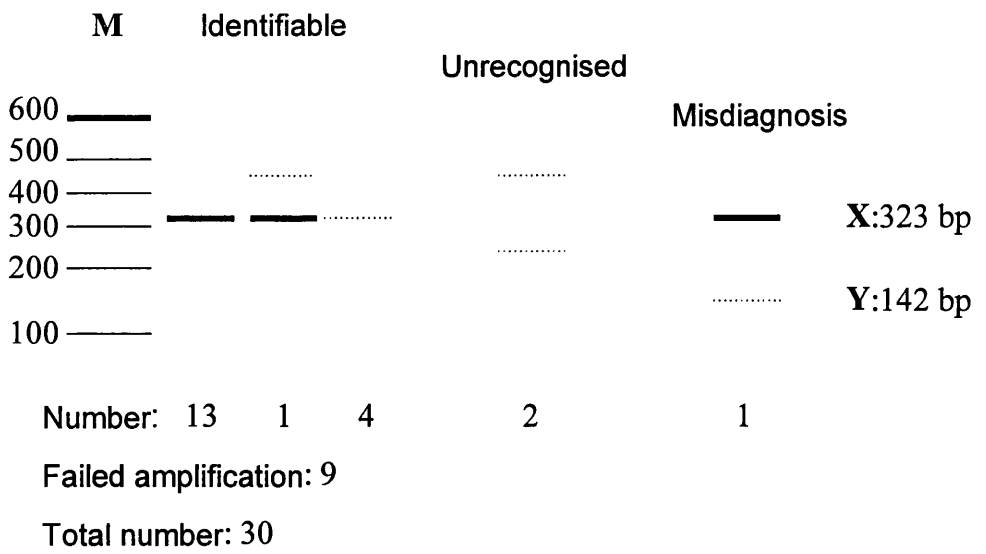


Figure 3.19: The distribution of female band patterns when sexing on DOP-PCR-derived DNA prepared from a single cell.

3.7 Sex determination for DOP-PCR-derived DNA from multiple single cells

Forty-nine cases of DOP-PCR-derived DNA, which were amplified from starting DNA templates of 2-100 cells, were also tested by nested PCR on the amelogenin locus. Fifteen cases were from males and 34 cases were from females. Six cases (12%, 6/49) showed no band on agarose gel analysis, while eight cases produced bands which could not be identified. 43 cases displayed clear bands (71.4%, 35/49). The distribution of band patterns from each sex was shown respectively in figures 3.20-3.21. Thirty-three cases (94%, 33/35) resulted in a correct diagnosis, while two cases (6%, 2/35) were misdiagnosed.

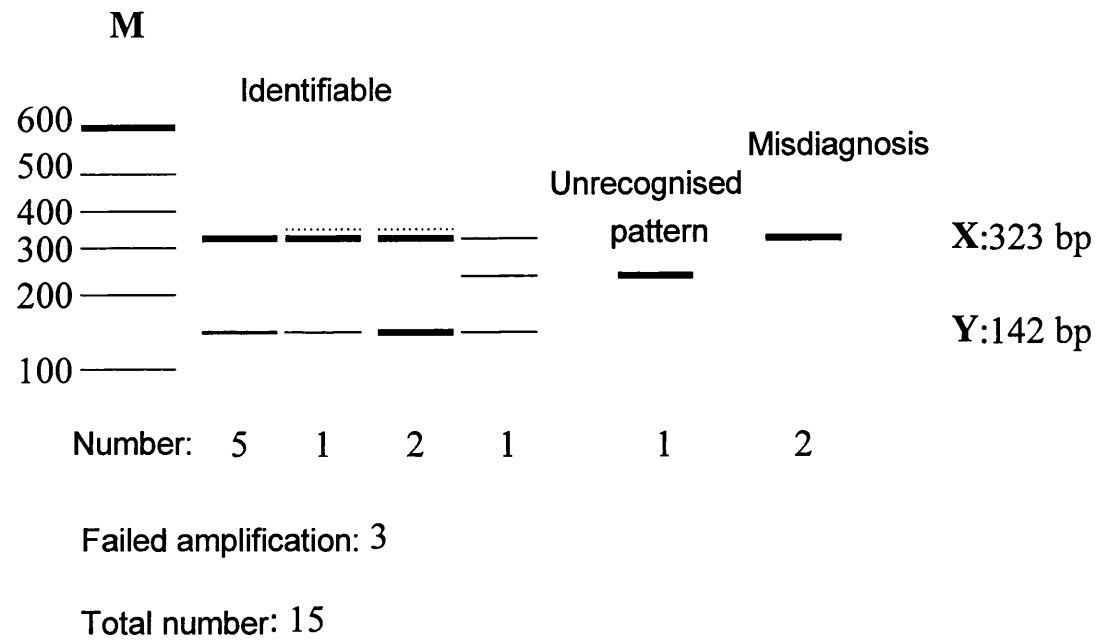


Figure 3.20: The distribution of band patterns when sexing on DOP-PCR-derived DNA prepared from 2-100 male cells.

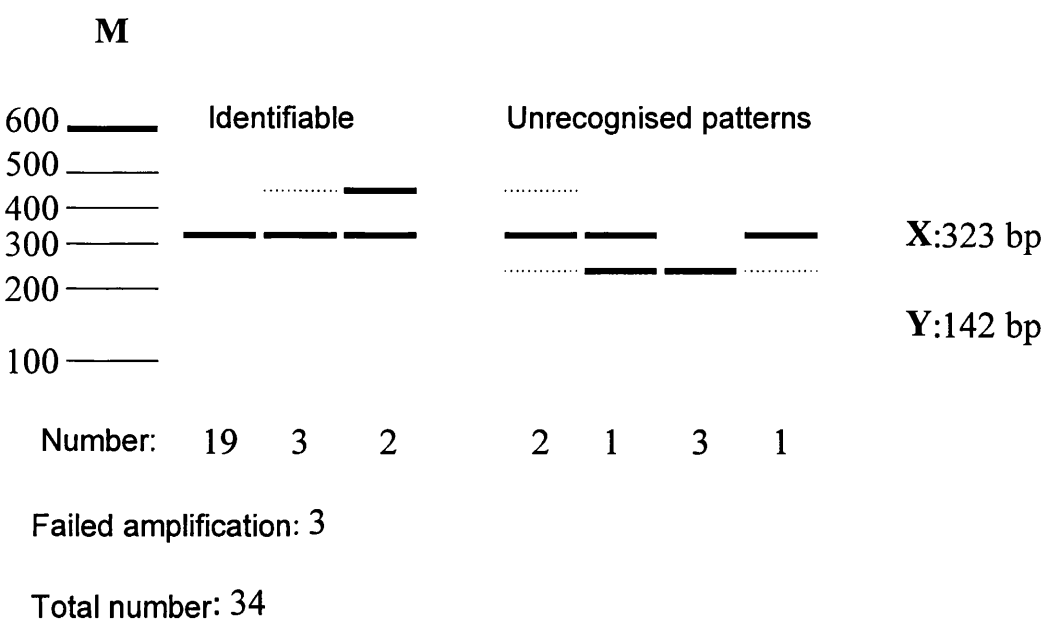


Figure 3.21: The distribution of band patterns when sexing on DOP-PCR-derived DNA prepared from 2-100 female cells.

3.8 Sex determination for blastomeres by nested PCR

Three batches of blastomeres, which contained 1-6 cells in 200 µl PCR tubes, were analysed with the above-mentioned strategy for sex determination. Among 28 cases for sex determination, 18 cases involved nested PCR directly on blastomeres, while 10 cases involved nested PCR on DOP-PCR-derived DNA from single blastomeres. Sex could be determined in 55.6% (10/18) of cases involving nested PCR directly on blastomeres. For sex determination of a single blastomere, 30% (3/10) of cases could be determined.

For another group involving nested PCR on DOP-PCR-derived DNA, sex could be determined in 40% (4/10) of cases. For sex determination of a single blastomere, 37.5% (3/8) of cases could be determined; however, this include 3 cases resulting in only a single Y band (Table 3.3; Figure 3.22).

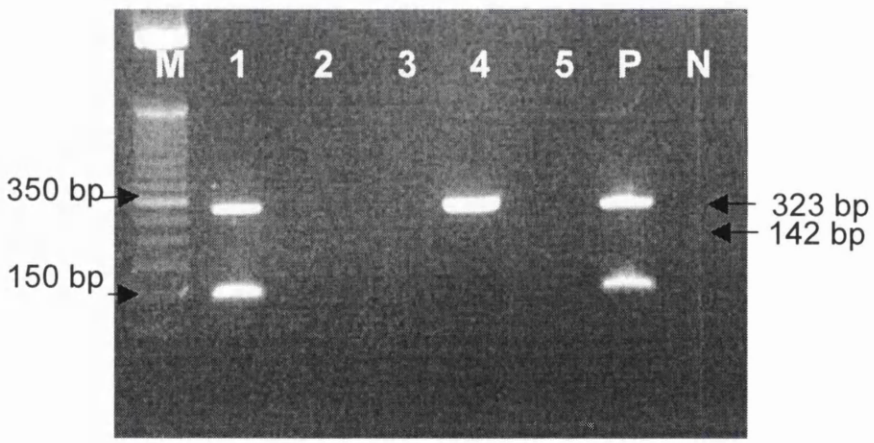


Figure 3.22: Sex determination of a single blastomere. Lane 1: shows a male embryo. Lanes 2, 3, and 5: failed to determine the sex. Lane 4: shows a female embryo. Lane P: positive control from 5 ng male DNA. Lane N: negative control. M: 50 bp DNA ladder.

	DOP-PCR	Sexing on DOP-PCR-derived DNA	Sexing on single Cells
Embryo I-13			
1 cell	x		
1 cell			x
1 cell			x
1 cell			x
Embryo I-9			
1 cell	x	x	
5 cell	x	x	
Embryo I-11			
1 cell			Female
1 cell			x
Rest of embryo 11			Female
Embryo I-12			
1 cell			x
1 cell			x
Embryo I-6			
1 cell			
Rest of embryo 6	+	Female	
Embryo II-11			
1 cell	+	A Y band only	
1 cell		A Y band only	
6 cells			Male
Embryo II-6			
1 cell	-	A Y band only	
Rest of embryo 6			Male
Embryo II-5			
1 cell			Male
1 cell	+-	x	
2 cells			x
4 cells			Male
Embryo II-10			
1 cell			x
1 cell	++	x	
1 cell			Female
1 cell	+-	x	
1 cell	+-	x	
Embryo III-3			
5 cells			Male
Embryo III-5			
5 cells			Female
Embryo III-4			
3 cells			Male

x: no PCR products, +-: faint DOP-PCR smear, +: moderate DOP-PCR smear, ++: strong DOP-PCR smear

Table 3.3: Results of sex determination from single blastomeres. Nested PCR for simultaneous amplification of AMGX and AMGY was applied on single blastomeres, or DOP-PCR-amplified DNA derived from single blastomeres.

3.9 Cystic fibrosis diagnosis for DOP-PCR-derived DNA from single cells

To investigate ΔF508 mutation for DOP-PCR-derived DNA from single amniocytes, two protocols were employed. One involved 40 cycles of PCR on DOP-PCR-amplified DNA, and the results were analysed on a 10% polyacrylamide gel. A normal individual would result in an 88 bp band; an affected individual would display an 85 bp band, while a heterozygous individual would reveal 85 and 88 bp bands plus a heteroduplex doublet. For 10 cases involving 1-20 single amniocytes, clear bands were produced (Figure 3.23).

Apart from analysis on a standard polyacrylamide gel, the results could also be analysed on a simplified polyacrylamide gel (5x8x0.1 cm). This simplified gel was relatively easy to prepare and analysis time was shortened to 2 hour, compared to 4 ½ hours for a standard polyacrylamide gel (Figure 3.24).

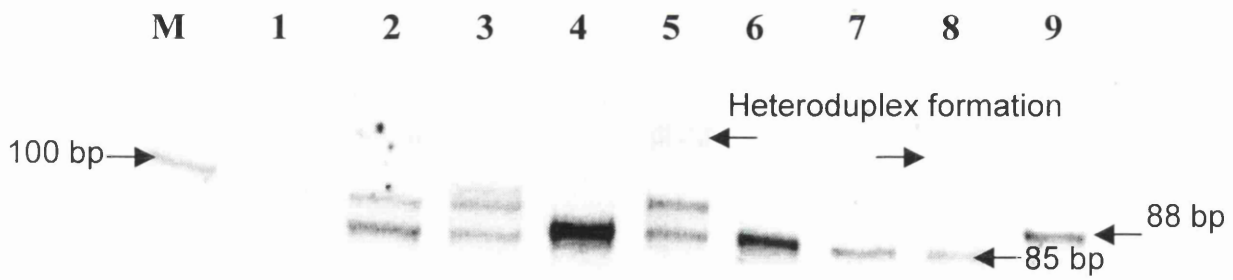


Figure 3.23: Mutation detection of ΔF508 from DOP-PCR-amplified DNA. The normal pattern was shown as an 88 bp band (lane 9). The heterozygous patterns were shown as 85 bp, and 88 bp bands, and a heteroduplex formation (lanes 1, 2, 3, 5, and 8). The homozygous affected individuals were shown as an 85 bp band (lanes 4, 6, and 7).

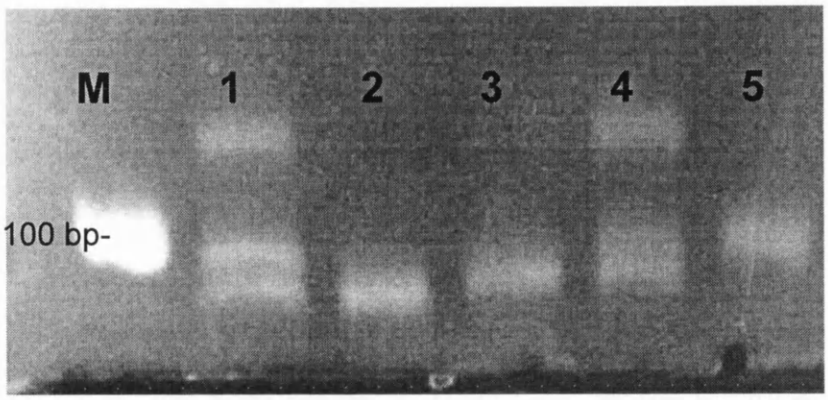


Figure 3.24: Mutation detection of ΔF508 by a simplified polyacrylamide gel. The normal pattern was shown in lane 5. The heterozygous pattern was shown in lanes 1 and 4. The affected pattern was shown in lanes 2 and 3. M: 100 bp DNA ladder.

3.10 Cystic fibrosis detection for stained cells

Another protocol using nested PCR for detecting the ΔF508 mutation was subsequently tested on DOP-PCR-derived DNA from stained cells. The first round PCR aimed to amplify fragments of 380 bp of CFTR exon 10. For normal cases, the second round PCR would produce 160 bp from N tube containing normal primers. For affected cases, the second round PCR would produce 157 bp from M tube containing mutant primers. Heterozygous cases would produce 160 bp from N tube and 157 bp from M tube, respectively (Figures 3.25-3.26).

Of 18 samples analysed, 10 cases (55.5%) revealed a normal pattern; 3 cases displayed a heterozygous pattern (16.7%) while 5 cases (27.8%) were without bands (Figure 3.27). Consequently for the DOP-PCR-amplified DNA derived from stained cells, the coverage rate for CFTR detection was 72%.

First round PCR: amplification of 380 bp within exon 10

Second round PCR:

N tube: contains common primer and normal primer

M tube: contains common primer and mutant primer

Figure 3.25: Strategy for ΔF508 mutation detection by nested PCR.

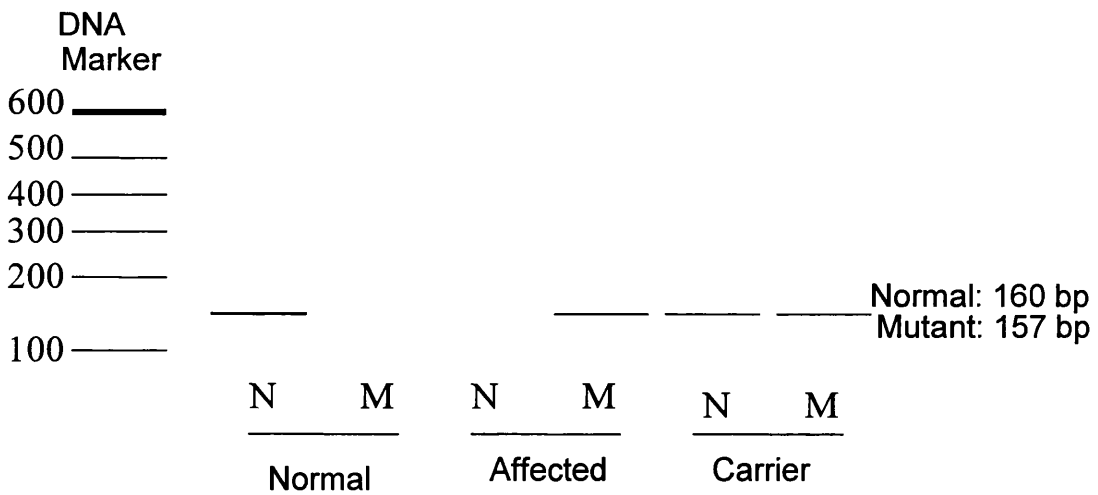


Figure 3.26: Band patterns for ΔF508 mutation detection using nested PCR. Normal individual will display one 160 bp band in N (Normal) tube. Affected case will have one 157 bp band in M (Mutant) tube. Heterozygous carrier will have one 160 bp band in N tube and one 157 band in M tube.

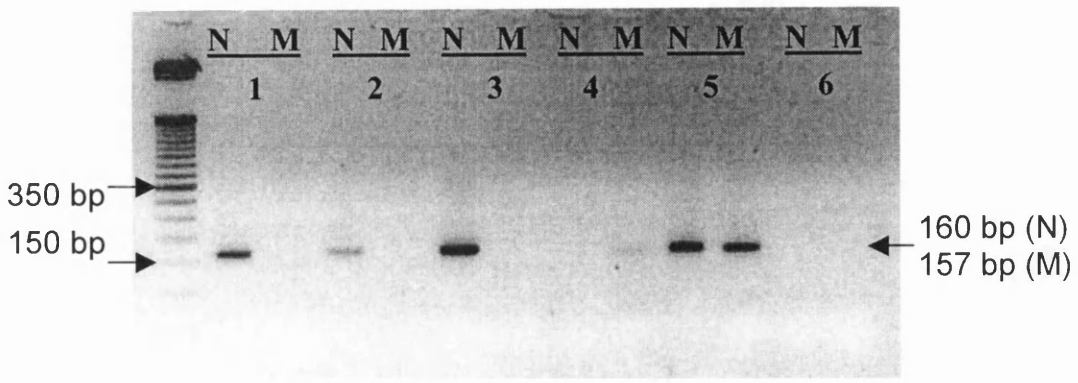


Figure 3.27: Nested PCR for ΔF508 mutation detection. Cases 1-2: DOP-PCR-amplified DNA from stained cells. Both displayed normal pattern. Case 3: normal control. Case 4: affected control. Case 5: Heterozygous carrier control. Case 6: Negative control. M: 50 bp DNA ladder.

3.11 CGH technique

3.11.1 Preparation of genomic DNA for CGH

Reference DNA was prepared from peripheral blood of both a normal male and a normal female. High molecular weight total genomic DNA (>4 kb) was required for successful CGH. The purity of the DNA was not critical, but degraded DNA was avoided because it would yield probes that were too small on nick translation, thereby resulting in poor CGH quality.

3.11.2 Labelling of genomic DNA by nick translation

Painting probes were directly labelled with fluorochrome-conjugated nucleotides. Test DNA was labelled with Fluorescein-12-dUTP and reference DNA was labelled with Rhodamine-5-dUTP. CGH experiments were initially carried out according to the previous report (Kallioniemi et al 1994). However, the protocol required further modification in order to generate consistent and reliable CGH images. Instead of using 200 ng for both test and reference DNA, the amount of test DNA ranged from 400 ng

to 1 µg, while reference DNA ranged from 200-600 ng. Instead of using 1 µl, the amount of fluorochrome-conjugated nucleotides was increased to 2 µl (1 mM). Heterochromatic regions were relatively less stained with green/red by adding 10 µg of human Cot-1 DNA. This represented effective in situ suppression of repetitive sequence in these areas. The probe size was adjusted by varying both the concentration of DNase and the reaction time. To obtain optimal probe size, it usually took 2-3 hours for the nick translation reaction. An increased concentration of DNase and reaction time would decrease the size of probes.

3.11.3 Labelling of DOP-PCR-derived DNA

Labelling of DOP-PCR-derived DNA was carried out by either nick translation or the PCR method. When 1 µg of DOP-PCR-derived DNA was used in the nick translation reaction for one hour, the resulting probes did not show uniform hybridisation on metaphases. The results demonstrated that the current nick translation protocol used for labelling total genomic DNA was not suitable for labelling DOP-PCR-derived DNA because optimal probe size distribution was difficult to obtain. The size distribution was usually too small under such conditions.

In comparison, probes prepared by the PCR method were efficient and the process could be automated. The PCR labelling method generated reliable and consistent painting probes for CGH experiments from 5 µl of primary DOP-PCR products. Since only one fifth of the probe was used for each of the CGH experiments, the remaining probe could be used for additional experiments if there was any uncertainty from the primary CGH result.

3.11.4 Preparation of metaphase slides for CGH

A batch of 50-100 metaphase slides was prepared at a time, and the morphology of metaphase chromosomes was evaluated using a phase contrast microscope. Slides, which did not reach the acceptance criteria, were abandoned. Each batch preparation required a further test to

determine the proper temperature and time requirement for denaturation. Optimal metaphase denaturation was usually achieved at 73-75°C for 2.5-5 minutes. The temperature of the water bath and denaturation solution was carefully monitored. The ideal denaturation time was the one where morphology was maintained for chromosome identification, whist sufficient denaturation had occurred to allow proper hybridisation.

The quality of the slides that passed the morphological check was also evaluated by a rapid denaturation test. The batch of the slides would be discarded if the appearance of C-banding patterns were revealed. Despite the efforts to screen appropriate batches of slides, the only reliable criteria to judge the quality of metaphase spreads was through a real CGH experiment.

Metaphase slides were also commercially available, although these slides required the same scrutinizing criteria to select proper area for hybridisation. Commercial metaphase slides might avoid laborious metaphase preparation and selection procedures; however, it did not guarantee the successful performance of CGH experiments. Despite the recommended temperature of 75°C for 5 minutes according to the manufacturer's protocol, the denaturation time occasionally required adjustment in order to yield optimal CGH images. Compared to homemade slides, commercial slides resulted in more successful CGH results during the initial stage of CGH optimisation. Therefore, commercial slides were employed to test the reliability of single cell CGH.

3.12 Assessment of CGH image quality

Before quantitative analysis, all the images were checked in order to ascertain whether their quality was adequate for ratio profile analysis. Only high-quality images were utilised as accurate interpretation could not be made if hybridisation was not uniform along the majority of the chromosomes (Figure 3.28). During image acquisition and analysis, a number of quality indicators could be estimated in order to accept or reject

for further analysis. The criteria for assessing the quality of hybridisation included (Kallioniemi et al 1996):

(1) Relatively low intensity of fluorescence for both the test and reference DNA probes in the pericentromeric and heterochromatic regions of chromosomes 1, 9, and 16 indicated adequate suppression of the repeated sequences.

(2) All chromosomes showed uniform and smooth high-intensity hybridisation. Metaphase spreads with granular patterns and fluorescent spots were excluded.

(3) Relatively straight chromosomes should be selected to reduce the artefacts that result from bending. Crossover and crowded chromosomes were avoided.

(4) Green and red hybridisation patterns are uniform between the two chromatids of a chromosome, the two homologous chromosomes in each metaphase, and in different metaphases.

(5) Chromosomes showed intense DAPI staining with visible bands.

(6) Background noise should be minimal.

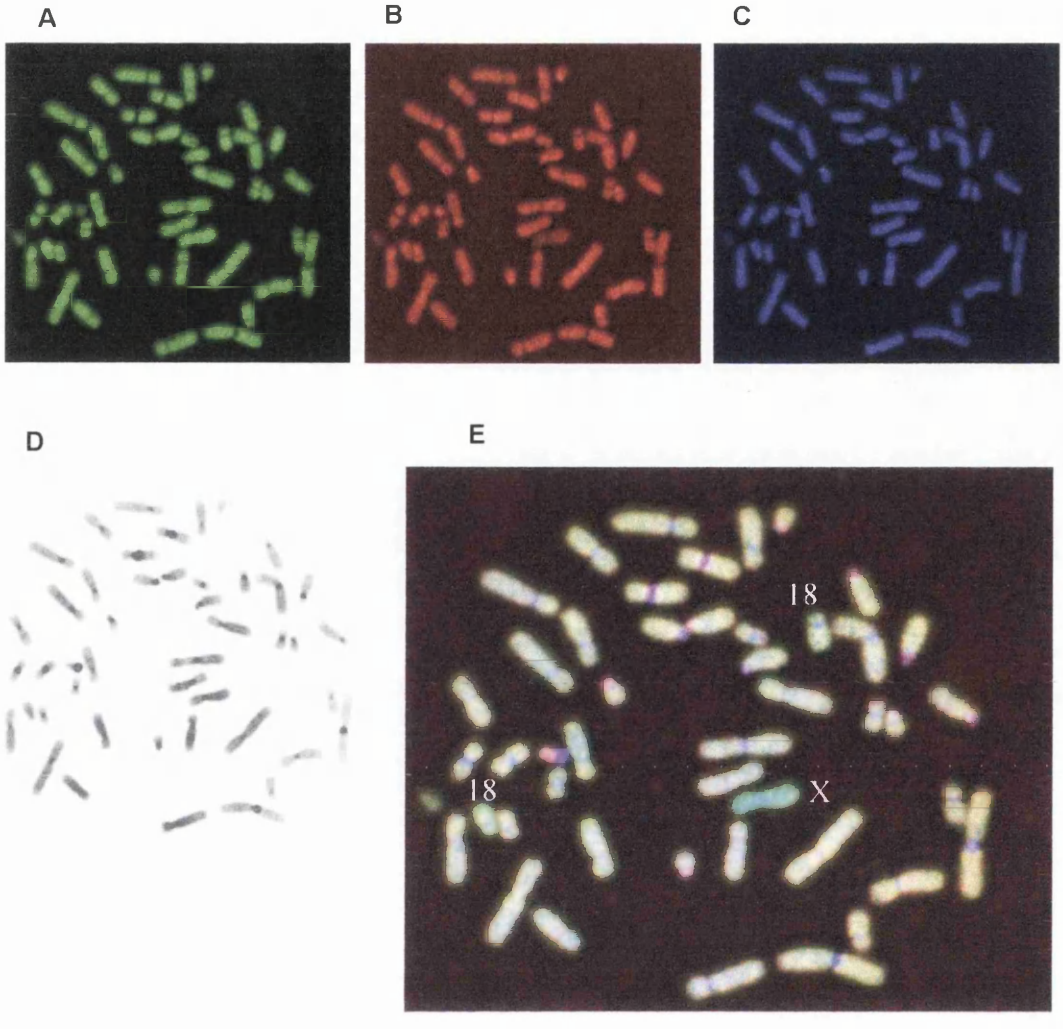


Figure 3.28: Qualitative assessment for CGH experiments. (A) Test DNA labelled with FITC. Using an appropriate single band-pass filter, a CCD image of only FITC staining was obtained. This grey-scale image was then digitally pseudocoloured in green. (B) Reference DNA labelled with rhodamine. The image was psuedocoloured with red. (C) CCD image of a normal metaphase spread counterstained with DAPI. (D) Digital inversion of image shown in C. This procedure provides a banding pattern resembling G-banding. (E) Mixed-colour image obtained by digital overlaying of the images shown in A, B, C. This CGH image was from a female trisomy 18 amniocyte co-hybridised with normal male DNA.

3.13 Chromosome identification with DAPI staining

Chromosomes stained by DAPI alone displayed Q-band-like fluorescence bands along their lengths with prominent staining at the pericentromeric regions of chromosome 1, 9, 16 and the distal long arm of the Y chromosome. Acrocentric chromosomes and pericentromeric regions of chromosome 4, 7, 10, 19 might show fluorescence of variable intensity. The Q-band-like pattern of DAPI staining in CGH experiments, following denaturation and washes, was less obvious than the chromosomes stained directly with DAPI alone.

For quantitative analysis, CGH software would automatically classify the chromosomes by size or by banding-specific classifier. However, errors might occur and part of the chromosomes could not be identified due to similar size and/or poor banding. Further correction could be reached by manually classifying these chromosomes. For successful CGH experiments, DAPI staining usually displayed adequate and clean signals over both interphase cells and chromosomes. Over or poor DAPI staining usually accompanied poor CGH results. However, optimal CGH experiments did not always result in optimal Q-banding pattern of DAPI staining.

3.14 Troubleshooting hybridisation with CGH kits

Unsatisfactory CGH results could be due to poor hybridisation (dim or no signals), granular hybridisation, poor metaphase morphology, and/or background noise. The underlying causes were varied and were relatively difficult and tedious to resolve. To produce ideal CGH images, all parameters including test/reference DNA, metaphase slides, labelling procedures, denaturation, and DAPI staining must be optimal. Since various factors were involved, it was not uncommon to have difficulty in identifying the causes of failed CGH experiments.

Commercial products offer a control to assess and troubleshoot the performance of CGH experiments. These kits contain differently-labelled normal DNA from both sexes, and a positive control from SpectrumGreen

MPE 600, which was extracted from an immortal female breast cancer cell line containing well characterized genetic abnormalities. The confirmed selected chromosome abnormalities included: (1) small deletion near 1pter; gain of 1q (2) loss of 9p (3) distal deletion on 11q (4) loss of 16q.
In troubleshooting CGH experiments, two hybridisation areas were simultaneously tested on the same metaphase slide. Under the same experimental conditions, metaphase quality could be determined. The factor of metaphase quality could be ruled out if the area of controlled CGH experiment demonstrated good hybridisation, whilst single cell CGH displayed poor results. The underlying causes of failed CGH might, therefore, point to the preparation of the test DNA. If both areas showed poor hybridisation, then CGH experiments were repeated with a new batch of slides. However, other factors such as denaturation should be taken into consideration. A reliable reference DNA could help to identify the underlying cause of problems with single cell CGH (Table 3.4).

Test DNA	**Reference DNA**	**Possible causes and solutions**
Poor hybridisation	Good hybridisation	1. Failed single cell PCR (e.g. incomplete lysis, contamination) 2. Labelling errors.
Poor hybridisation	Poor hybridisation	1.Poor metaphase quality. Change with another batch of slides. 2. Improper denaturation.
Good hybridisation	Poor hybridisation	1.Unmatched probe size. Verify the probe size. 2. Out-of-date reference DNA.

Table 3.4: Troubleshooting single cell CGH based on a reliable reference DNA

Using commercial reagents for positive and negative controls, the quantitative CGH analysis also displayed fluctuation. Several extra abnormalities of MPE 600 were noted when cut-off value of 1.2 and 0.8 was selected to represent chromosome gains and losses, respectively (Figures 3.29, 3.30).

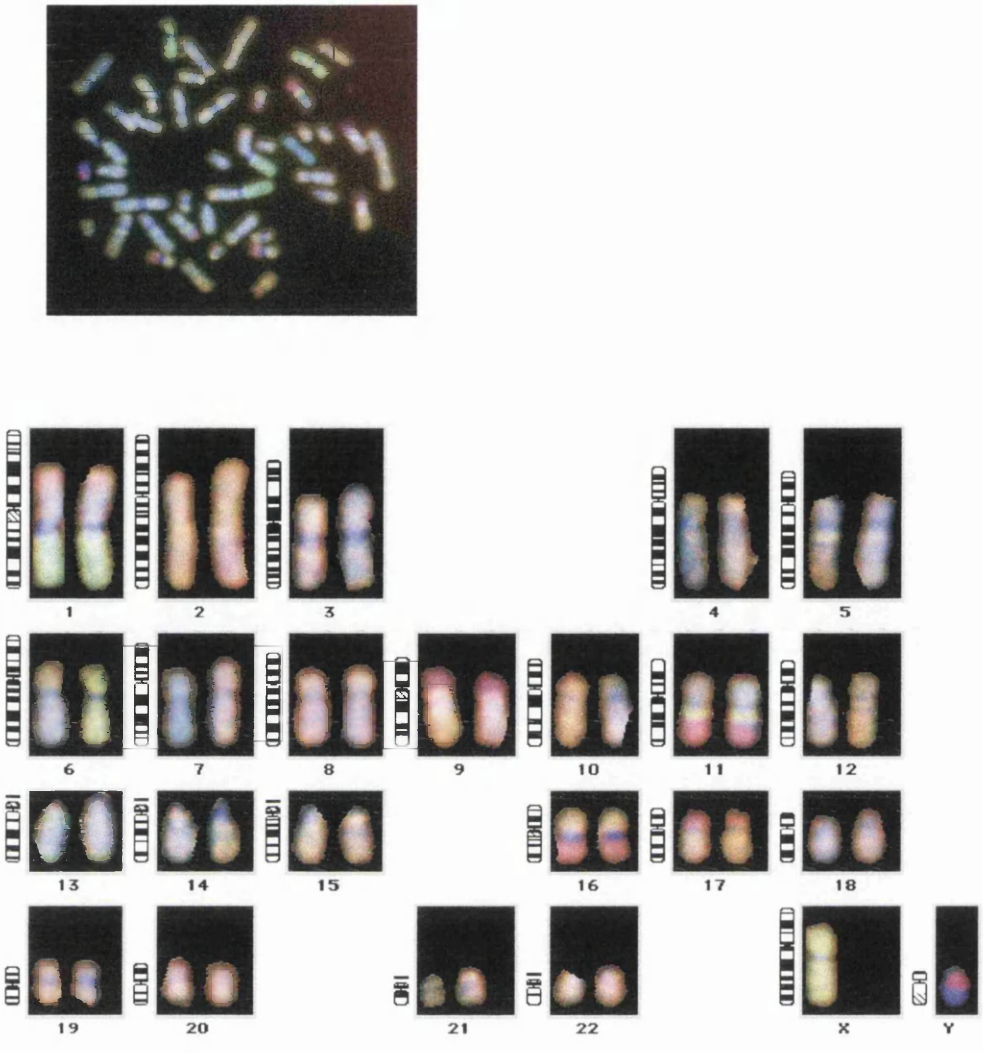


Figure 3.29: CGH karyotyping from SpectrumGreen MPE 600. Test DNA was from immortal female breast cancer cell line and co-hybridised with normal male DNA. CGH image demonstrated greenish colour over chromosome 1q, and X and reddish colour over chromosome 9p, 11q, 16q, 17p and Y.

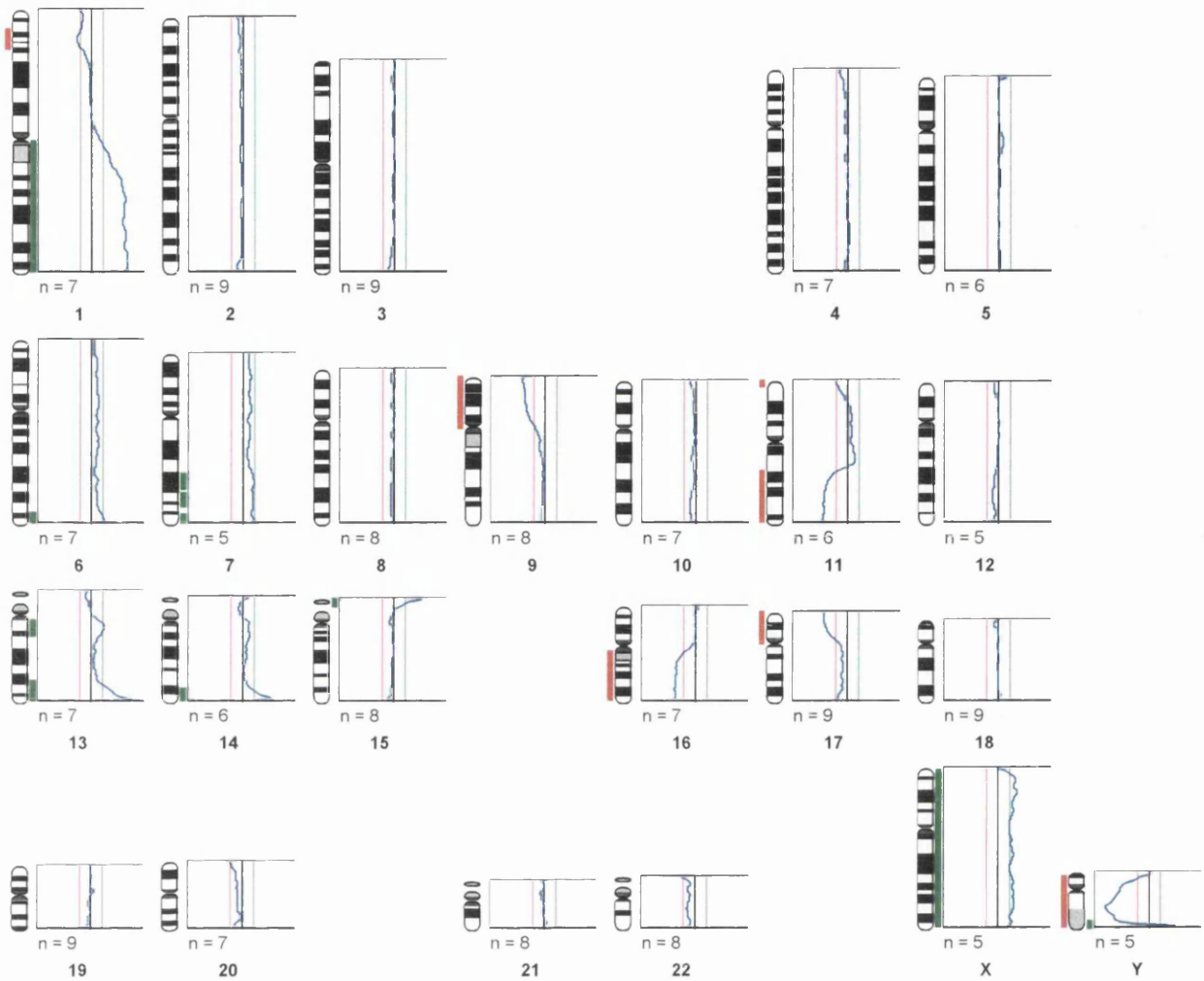


Figure 3.30: Fluorescence ratio profile from breast cancer cell line co-hybridised with normal male DNA. Chromosome gains were noted over chromosome 1q and X, and chromosome losses were noted over chromosome 9p, 11q, 16q, 17p and Y when the threshold value was set at 1.2/0.8.

3.15 Optimisation of single cell CGH

During the optimisation of single cell CGH, the normal reference DNA used was from either amplified DNA (e.g. matched number of single cells or 1-10 ng diluted genomic DNA) or non-amplified DNA. Theoretically, reference DNA for single cell CGH should be from a matched number of single cells. However, this approach usually produced inconsistent results and a high failure rate perhaps because of difficulties involved in single cell manipulation and single cell PCR.

During the initial stage, it was common to have results showing good hybridisation of test DNA, but not the reference DNA, or vice versa. It was also not uncommon to have results showing poor signals from both test and reference DNA, which might result from failure of single cell PCR, poor-quality metaphase slides, or inappropriate denaturation conditions. Success rates of single cell CGH increased only when as many variables were kept relatively constant.

DOP-PCR-derived DNA from a single cell was further labelled either by nick translation or PCR. PCR labelling was more convenient and efficient than nick translation, because the former method could be performed using an automated PCR machine, while the latter one required adjustment of reaction temperature, time, and DNase concentration in order to result in optimal probe size. PCR labelling with 20-25 cycles yielded 10-20 μg of test DNA in a 50 μl reaction volume. A series of test DNA ranging from 5 μl to 40 μl was tested in order to yield optimal CGH images. The results demonstrated that 10 μl (2 μg) was the smallest amount required to produce consistent and reliable hybridisation in CGH experiments.

To validate the effectiveness of labelling from a single cell, a one-colour hybridisation could be applied as a pre-CGH test before the real two-colour CGH experiments. The procedure was similar to CGH, while only one labelled DNA was used on any metaphase slide. If amplification and labelling were successful from a single cell, the hybridisation signals would appear homogeneous and intense. Failed hybridisation usually presented

with background noise or coarse granularity over the metaphase chromosomes. One-colour hybridisation was used to evaluate the efficiency of the test probes in order to prevent lengthy, uninformative CGH experiments. No further CGH experiment would be carried out if one-colour hybridisation appeared dim or produced no signal. A poor one-colour hybridisation test might indicate single cell PCR failure, which might result from cell loss, contamination or inefficient labelling.

3.16 CGH from a single amniocyte

To prove the feasibility of single cell CGH and its performance in predicting chromosome aberrations, a single cell with a known numerical chromosomal abnormality was retrieved from cultured amniocytes. For visual assessment of colour change over designated chromosomes, trisomy 18 was a preferred candidate over chromosome 21, because the size of the chromosome 18 was relatively easier to identify than chromosome 21. Test DNA was firstly co-hybridised with normal genomic DNA from opposite sex DNA. Subsequent experiments used amplified reference DNA with same sex.

3.16.1 Trisomy 18

Test DNA probe was prepared from a single trisomy 18 female cell, while normal reference DNA was from SpectrumRed male total human genomic DNA. The CGH displayed uniform hybridisation for both test and reference DNA. The fluorescent intensity of sex chromosomes could be viewed as a qualitative internal control for optimal CGH results. In this case, the X chromosomes displayed more green while Y chromosomes displayed more red over upper part of the Y chromosomes (heterochomatic region was suppressed with Cot-1 DNA and stained with DAPI). Chromosome 18 homologues also displayed more green than other chromosomes (Figure 3.31). Similar findings were noted over the majority of CGH images, and these were then utilised for quantitative analysis.

Global analysis demonstrated green colour over chromosome 18 and X chromosome, while the Y chromosome was painted with red (Figure 3.32). Ratio profiles analysis using 0.8 and 1.2 to represent chromosomal losses and gains demonstrated chromosome gains over X and chromosome 18, while Y chromosome displayed losses (Figure 3.33). The quantitative ratio profiles findings were concordant with the preliminary visual assessment of the CGH images. Since reference DNA was from a normal male, the presence of chromosomal gains over chromosome X, 18 and chromosomal losses over Y chromosome indicated that test DNA was from female DNA with extra copy of chromosome 18. In this single cell CGH experiment, test DNA was prepared from amplified DNA, whereas reference DNA was prepared from total genomic DNA. The results showed that heterochromatic regions of chromosome 1, 9 and acrocentric chromosomes displayed more red than green. The euchromatic region of Y chromosome displayed red while the heterochromatic region was painted with blue.

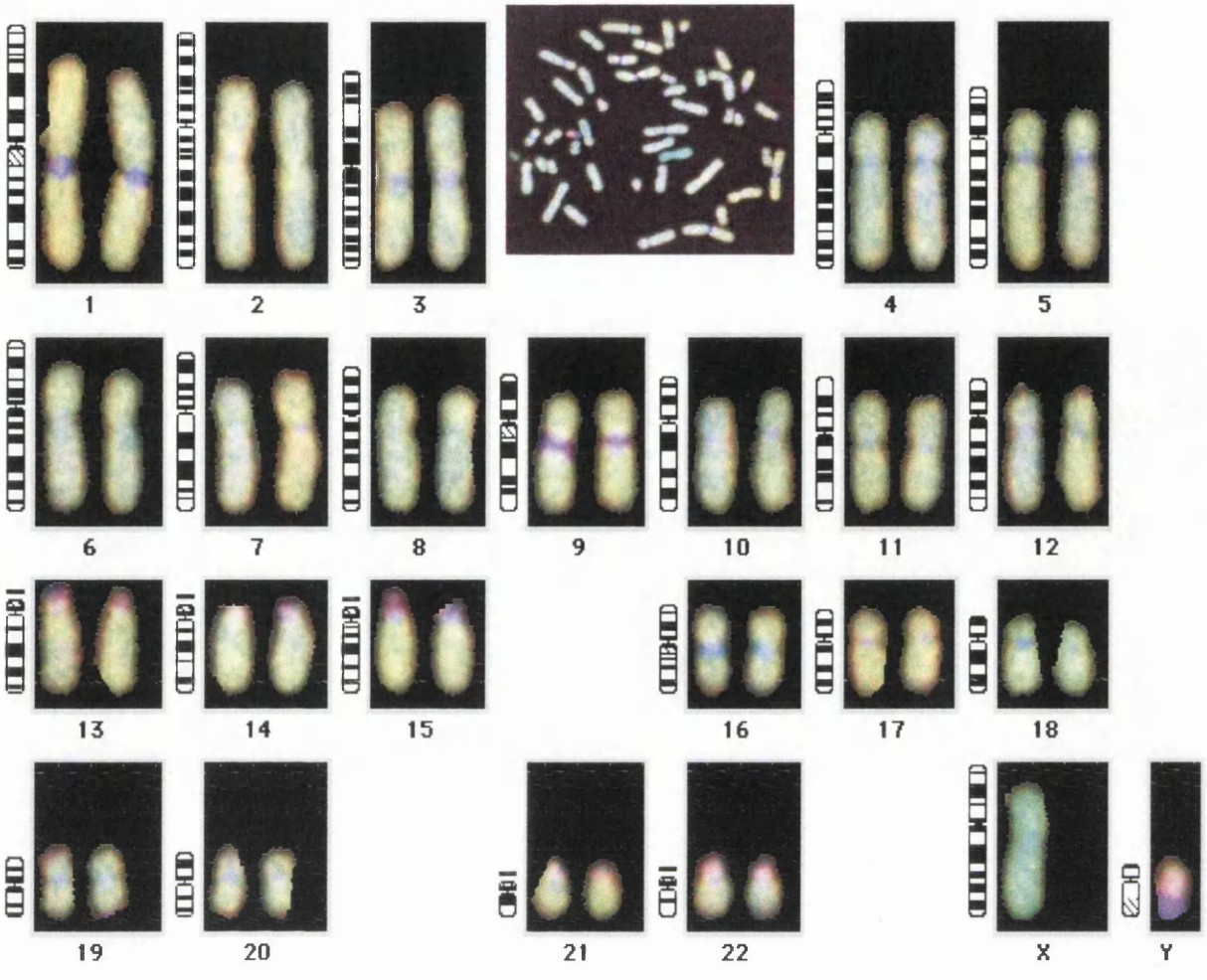


Figure 3.31: CGH karyotyping from a female trisomy 18 amniocyte. By computer software, karyotyping was carried out automatically with manual correction. Five to ten metaphases were required for objective quantitative fluorescent ratio profile analysis. Since normal male DNA was used for co-hybridisation, the X chromosome showed a greenish colour (2X/1X) while the Y chromosome showed a reddish colour (0Y/1Y). Chromosome 18 also showed a greenish colour (3/2).

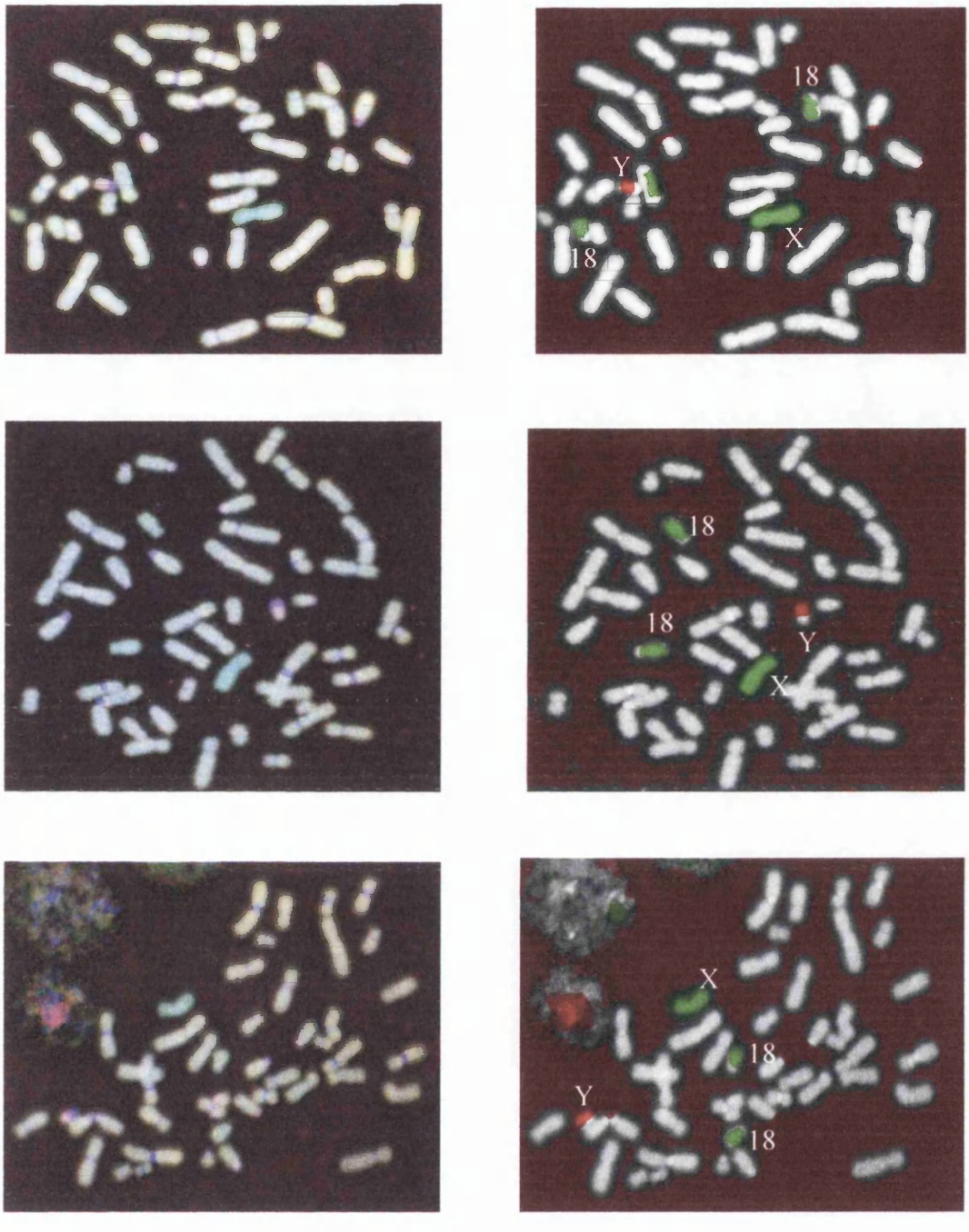


Figure 3.32: Single cell CGH of female trisomy 18 co-hybridised with normal male DNA. Left column: CGH images. Right column: global analysis derived from respective CGH images on the left. In global analysis, a three-colour lookup table in ratio image indicates chromosomal gains and losses. Gains are indicated in green, losses are indicated in red, and normal chromosomal complements are indicated in white.

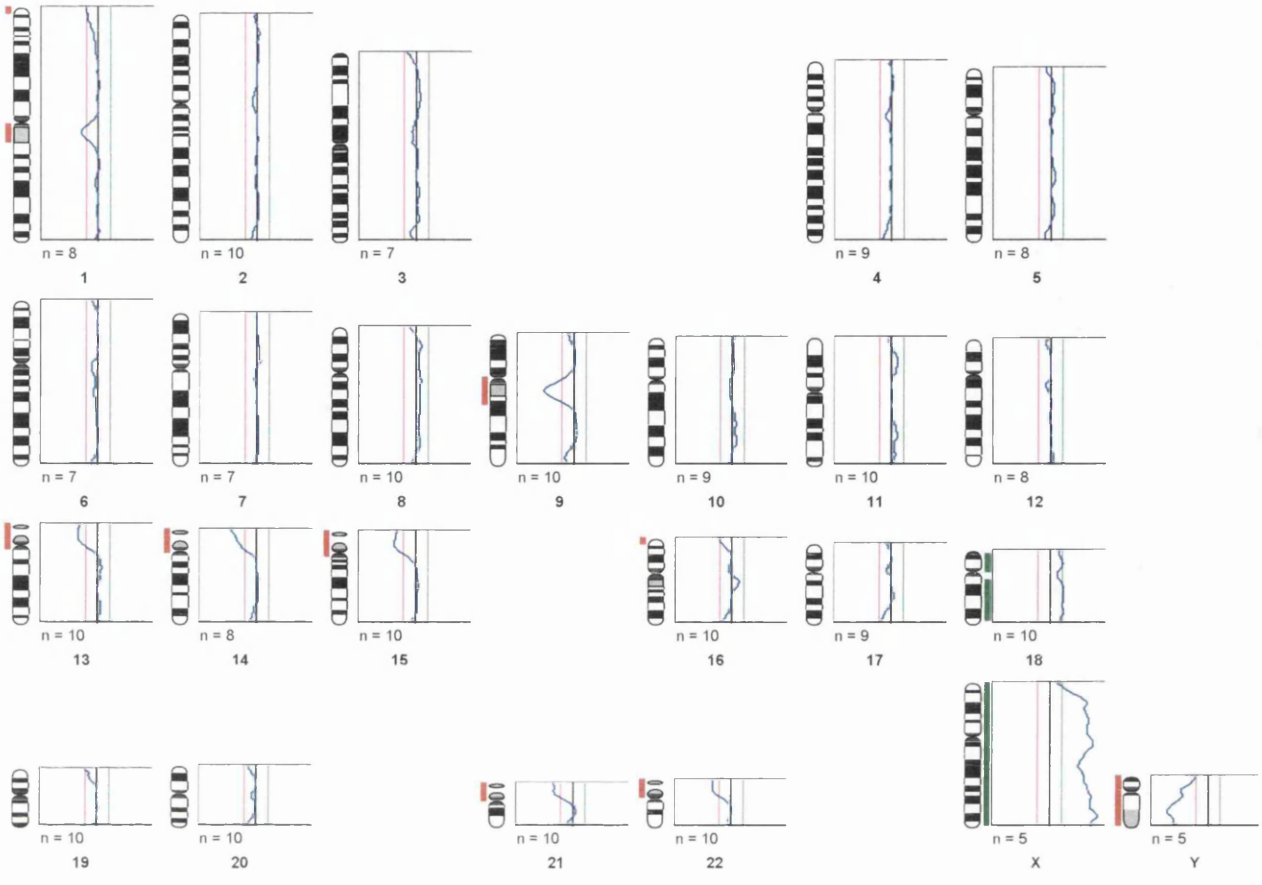


Figure 3.33: Quantitative fluorescence ratio profile from a female trisomy 18 amniocyte co-hybridised with normal male DNA. Test DNA was from amplified DNA while reference DNA was from total genomic DNA. Quantitative CGH analysis used a fluorescence ratio of 1.2 (green line) to represent chromosome gains and 0.8 (red line) to represent chromosome losses. Chromosome gains were noted over chromosomes 18 and X and were represented as green bars to the right of idiogram. The red bar to the left of Y idiogram represented reference DNA was from a male. Heterochromatic and telomeric regions were excluded from analysis.

3.16.2 Trisomy 21

Test DNA probe was prepared from a single, trisomy 21, female cell, while normal reference DNA was from SpectrumRed male total human genomic DNA. Qualitative CGH images displayed more green over chromosome X and 21, while chromosome Y showed a red colour (Figure 3.34). Global analysis showed chromosome gains over the majority of the chromosome 21 homologue. False positives were noted over other chromosomal regions; however, these false chromosome gains did not involve chromosome homologues in a single CGH image. Unlike universal gains over chromosome 21, false positives involving other chromosomes were usually episodic and not consistent. For ratio profile assessment, the number of false positives was 17 when cut-off value was set at 0.8 and 1.2 to represent chromosomal losses and gains, respectively (Figure 3.35). However, false positives were reduced to minimum if a more stringent cut-off value was employed (Figure 3.36). The experiment was repeated, and the results still showed 8 false positives when a low stringency cut-off value was applied.

Another experiment involving female trisomy 21 was carried out by using reference DNA from amplified DNA (e.g. 10 ng female diluted DNA). Despite some false positives, the majority of chromosome 21 homologues demonstrated more green when global analysis was applied (Figure 3.37). Since both test and reference DNA were from a female, the Y chromosome did not display obvious green or red as was seen in experiments employing mismatched sex DNA. The X chromosome showed no gain or loss and was depicted in white colour in global analysis. Centromeric and heterchromatic regions were mainly stained with DAPI (Figure 3.38). The ratio profile showed 6 false positives when low stringent cut-off value was applied (Figure 3.39). However, the number of false positives was reduced to one chromosome region when more stringent value was applied (Figure 3.40).

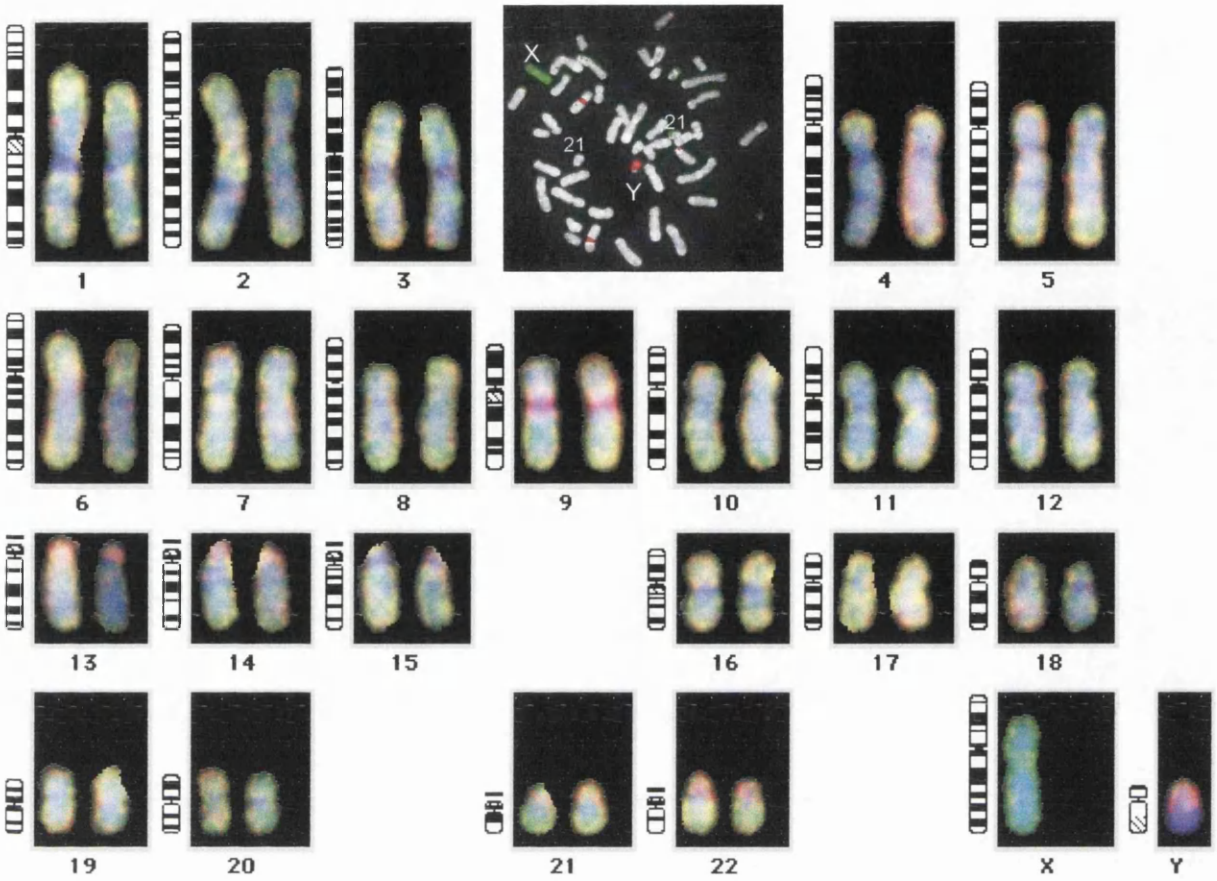


Figure 3.34: CGH karyotyping from a female trisomy 21 amniocyte. Test DNA was from amplified DNA and co-hybridised with total genomic DNA from a normal male. The intersected picture of global analysis demonstrated chromosomal gains over chromosome X and 21, while the Y chromosome was painted with a red colour.

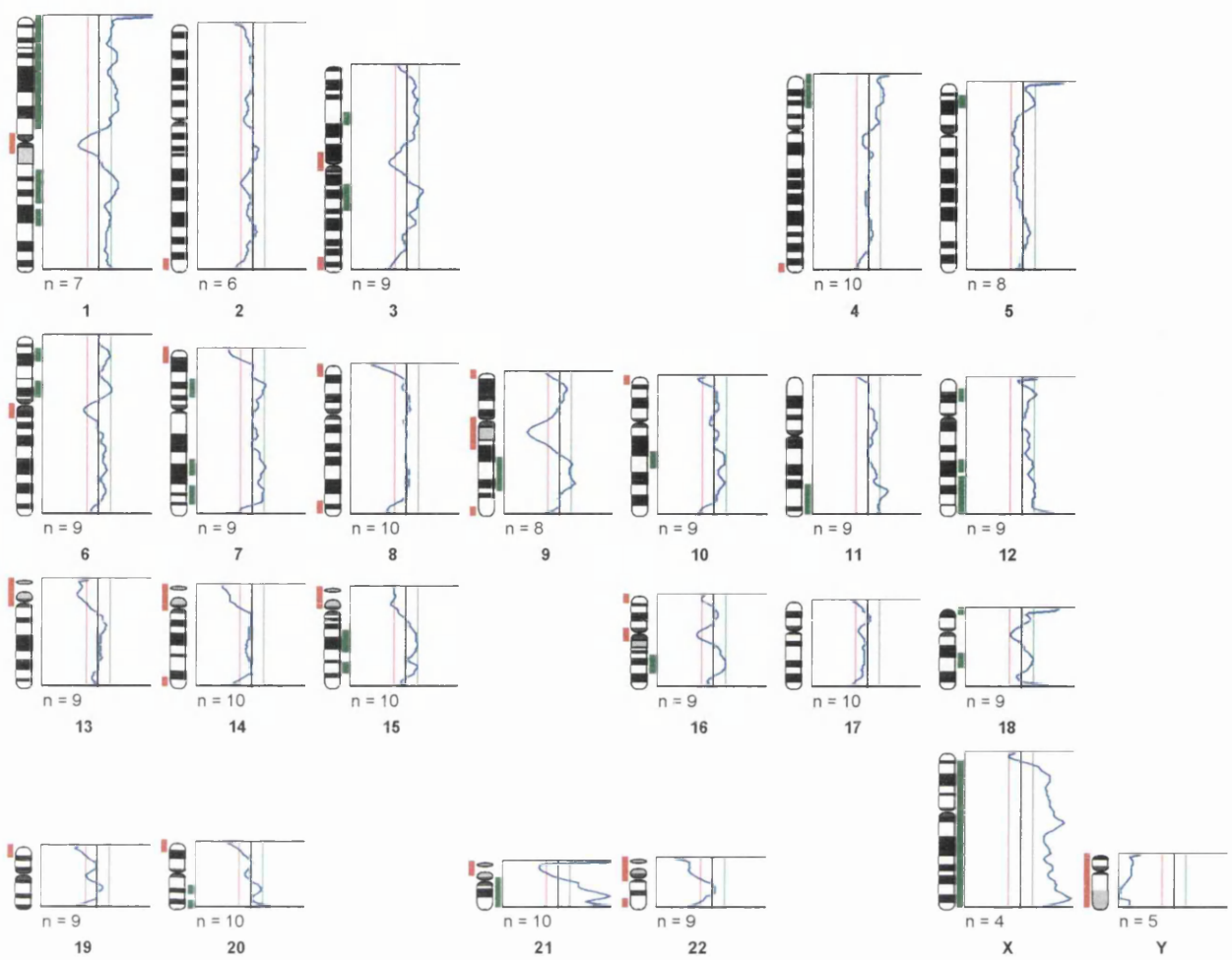


Figure 3.35: Fluorescence ratio profile from a female trisomy 21 amniocyte co-hybridised with normal male DNA. Numerous false positives were noted when the threshold value was set at 1.2 and 0.8. Chromosome gains over the X chromosome and losses over the Y chromosome show that test DNA was from a female. (2X/1X, 0Y/1Y)

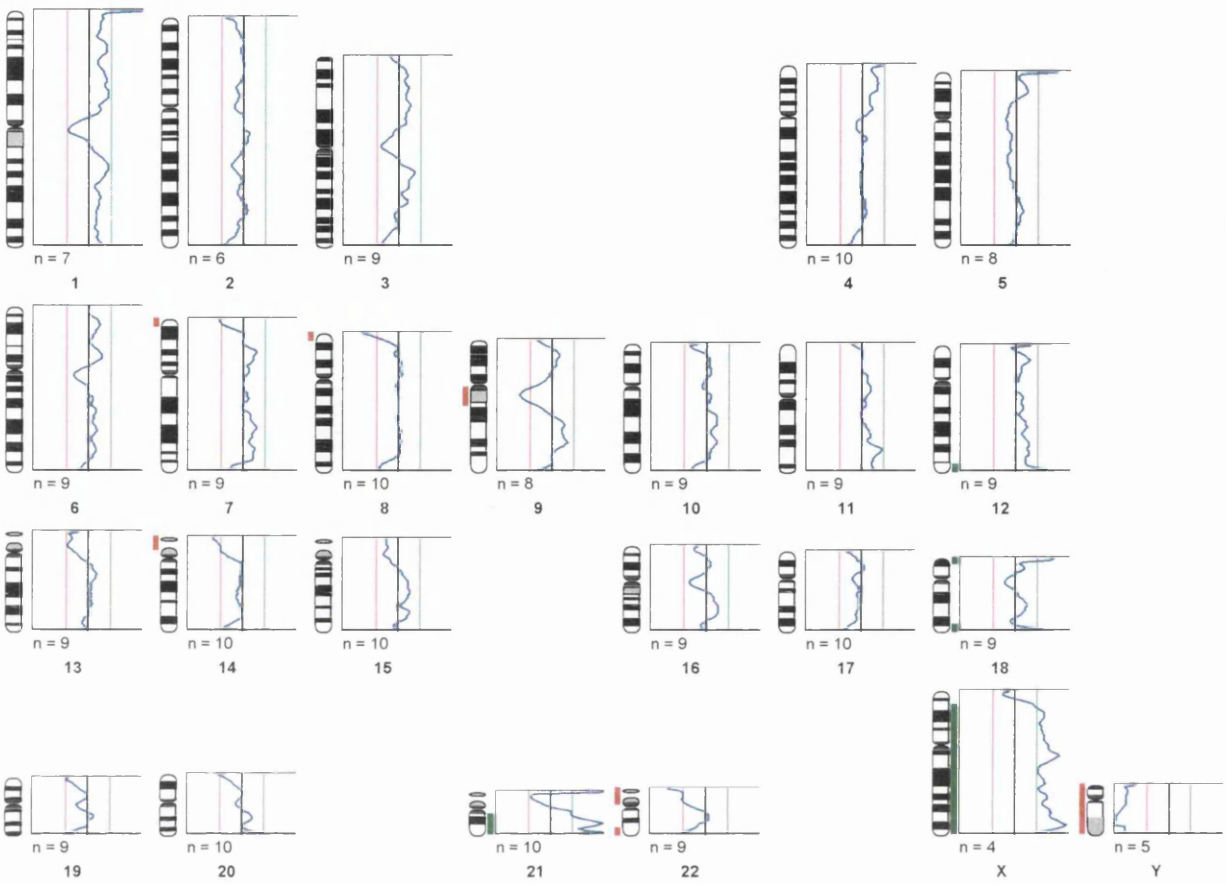


Figure 3.36: False positives were reduced by using a more stringent threshold value. Detection of chromosome 21 was achieved and internal control of chromosome X (from mismatched sex) was still maintained. Compared to figure 3.38, false positives were eliminated when the cut-off values were set at 1.4 and 0.6 to represent chromosomal gains and losses, respectively.

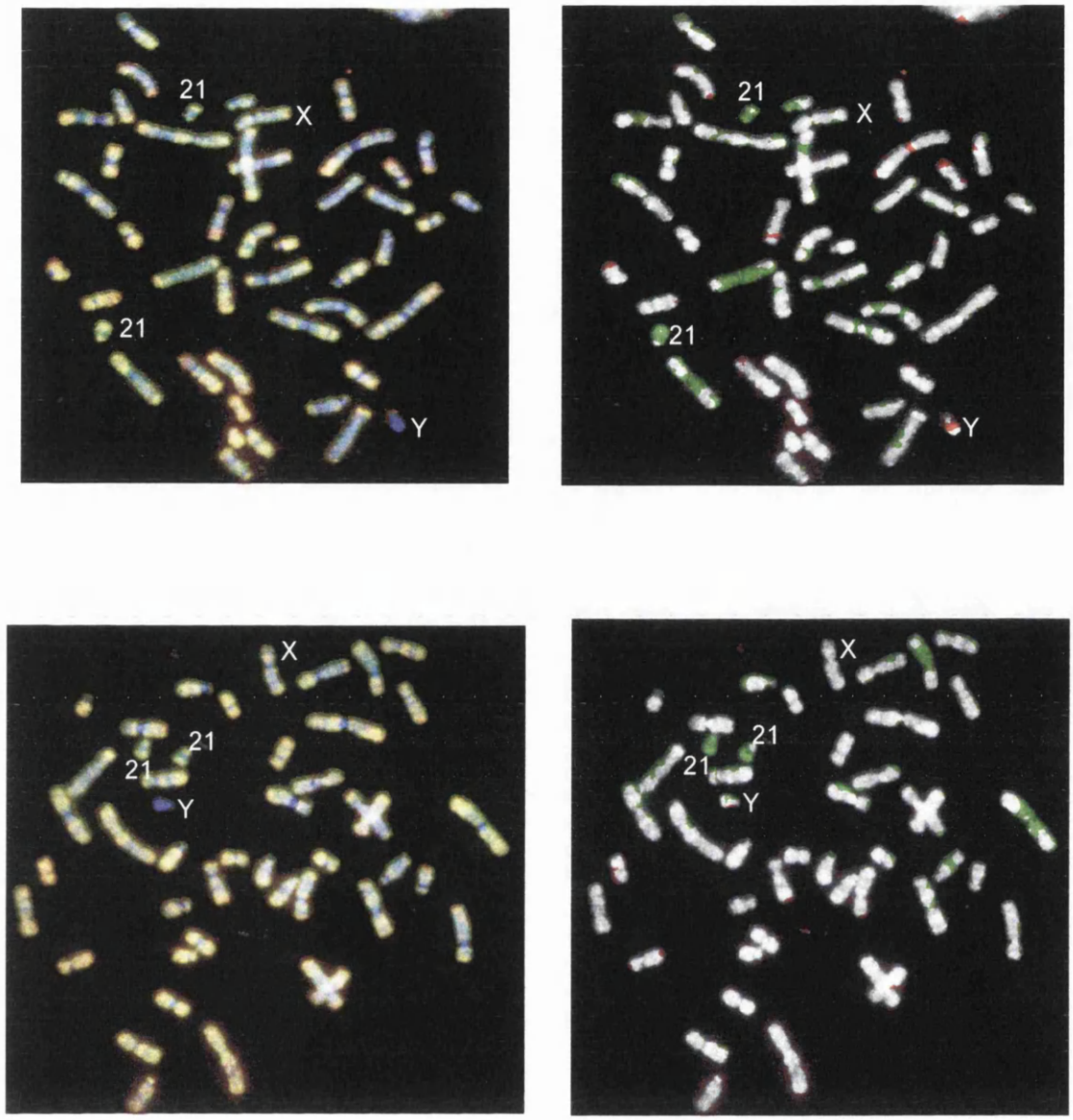


Figure 3.37: CGH images of amplified test DNA vs. amplified reference DNA. Test DNA was from a female trisomy 21 amniocyte while reference DNA was from 1ng female DNA. The Y chromosome was painted with DAPI (blue) in qualitative CGH images (left column). Global analysis (right column) showed a greenish colour over chromosome 21 homologue (3/2). The X chromosome was balanced (white, 2X/2X). Over-representation of other chromosomal regions was noted. However, they did not manifest in both chromosomal homologues.

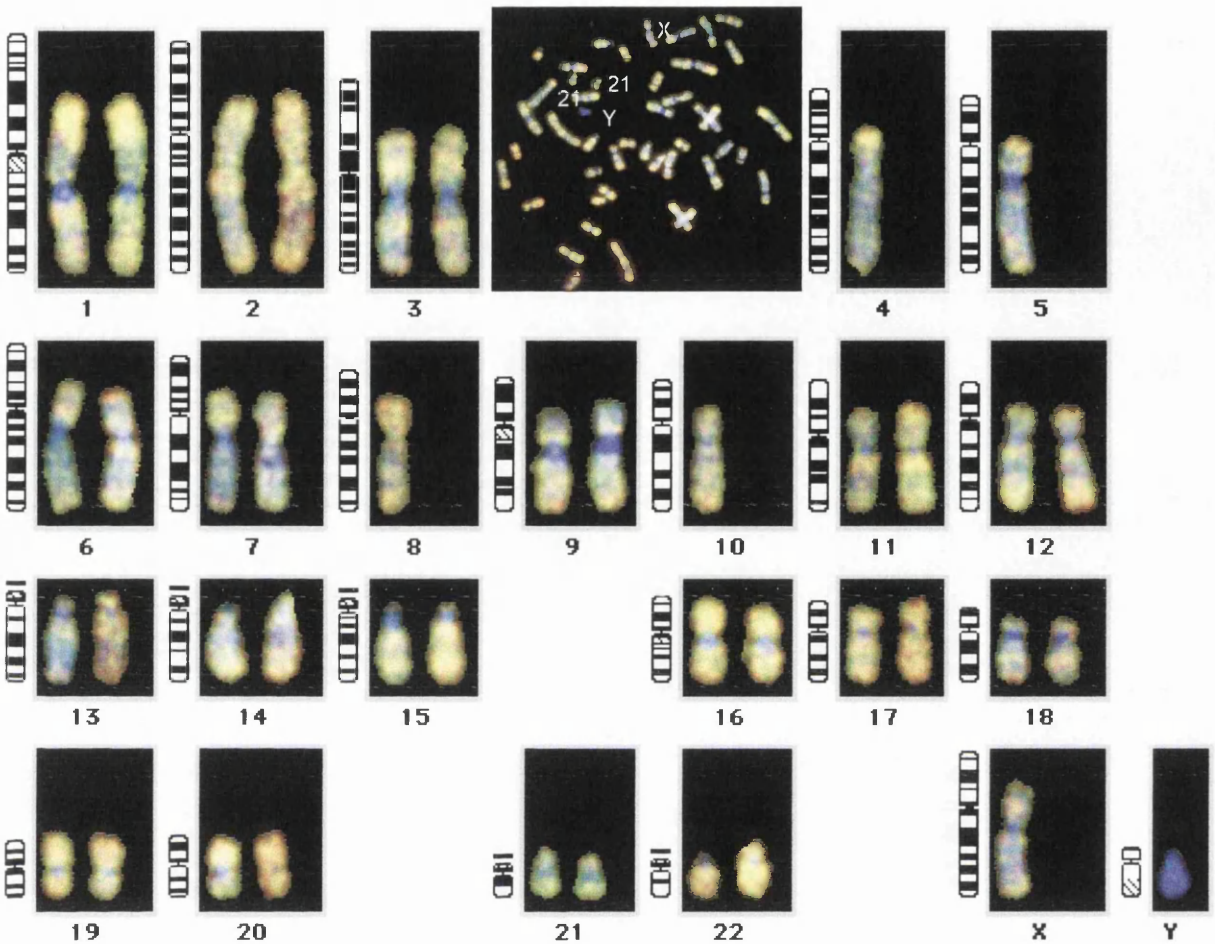


Figure 3.38: CGH karyotyping from a female trisomy 21 amniocyte. Test DNA was from amplified DNA and was co-hybridised with amplified DNA prepared from 1 ng female DNA. The intersected CGH image demonstrated that both chromosome 21 homologues are greenish. The X chromosome was balanced and the Y chromosome was painted with blue.

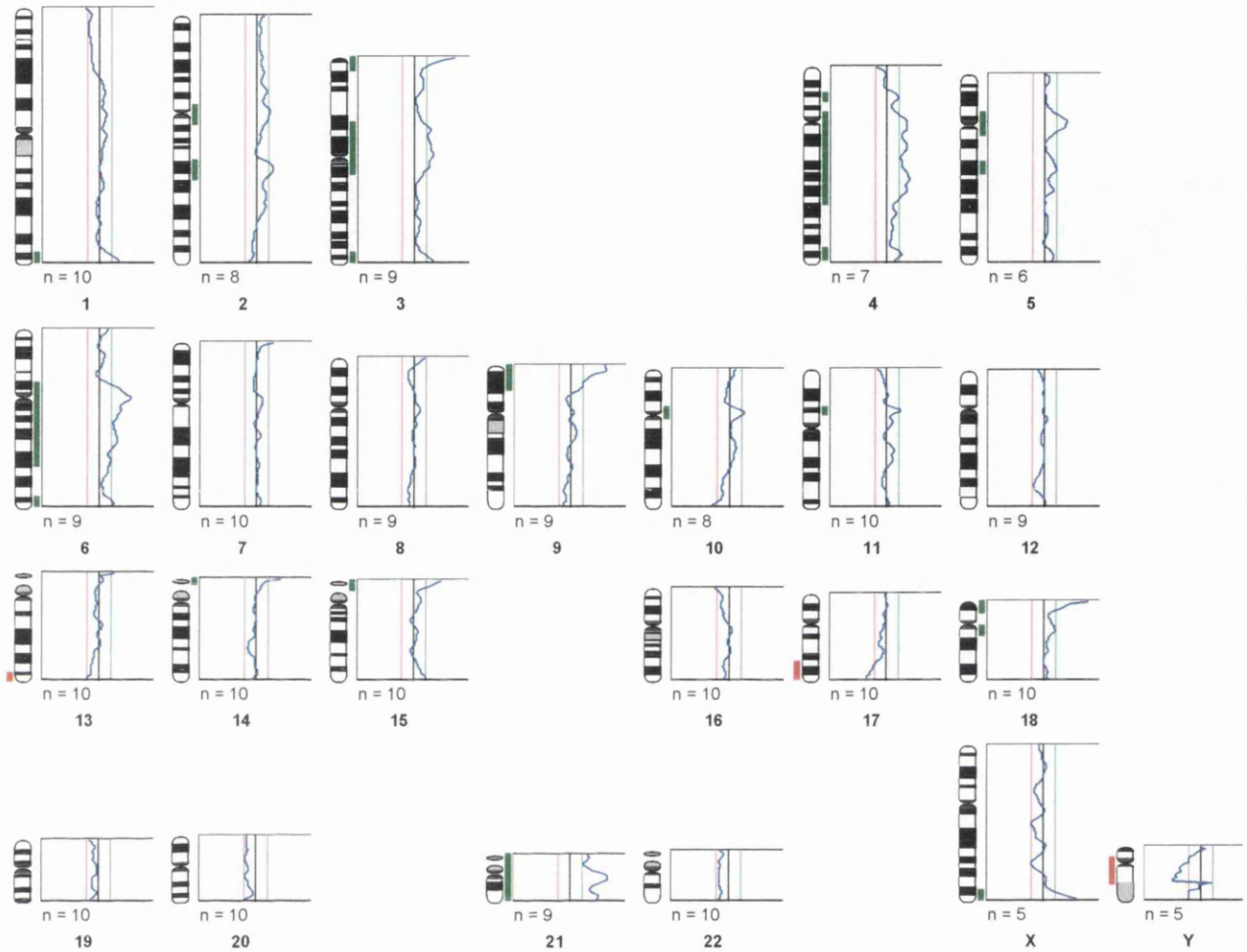


Figure 3.39: Fluorescence ratio profile from a female trisomy 21 amniocyte co-hybridised with amplified normal female DNA. False positives were noted over chromosomes 2, 3, 4, 5, 6, and 11 when the threshold values were set at 1.2 and 0.8. No chromosome gain or loss was seen over the X chromosome (2X/2X).

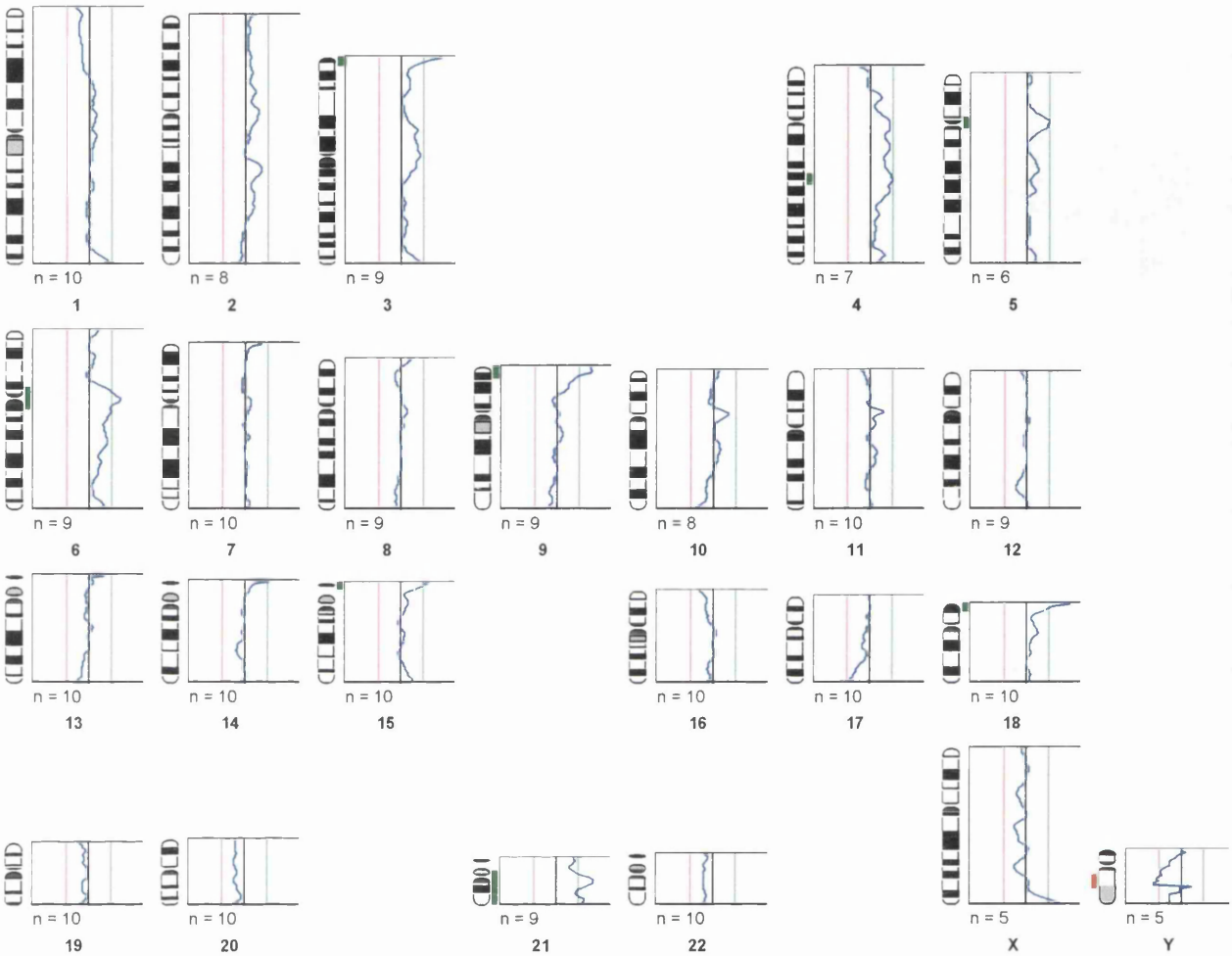


Figure 3.40: Fluorescence ratio profile using stringent cut-off values. The number of false positives was reduced to one chromosomal region on chromosome 4q when the cut-off values were set at 1.4 and 0.6. Heterochromatic and telomeric regions were excluded from ratio profile analysis.

3.17 CGH for microdissected stained cells

To test the sensitivity and reliability of CGH from minute quantities of DNA, archived slides afforded a range of specimens for CGH experiments. The size distribution of PCR products from archived fixative-treated stained cells was usually smaller than those prepared from fresh cells. DOP-PCR-CGH procedure was similar except for the lysis conditions. Compared to a one-hour lysis for single amniocytes, stained cells required lengthy incubation in order to achieve cell lysis. DOP-PCR-CGH experiments failed when a one-hour lysis protocol was applied, whereas it improved substantially when a longer lysis time was adopted (e.g. 1-3 days).

3.17.1 Trisomy 18

Stained cells from a male trisomy 18 were co-hybridised with SpectrumRed female total genomic DNA. The CGH results displayed that the X chromosomes were stained with red while euchromatic parts of the Y chromosomes were stained with green (Figure 3.41). Heterochromatic regions of chromosomes 1, 9, 16, and acrocenric chromosomes showed more red than green. The majority of chromosome 18 homologues also displayed more green than other chromosomes. Global analysis revealed chromosome gains over chromosome 18 and Y while chromosome loss was noted over the X chromosome. Although false positives were noted over other chromosomal regions, these were sporadic and did not show universal pattern as chromosomes X, 18 and Y (Figure 3.42). Ratio profile analysis displayed 6 false positives over other chromosomal regions when cut-off values were set at 1.2 / 0.8. However, a correct diagnosis was made if more stringent values of 1.4 / 0.6 were set (Figure 3.43).

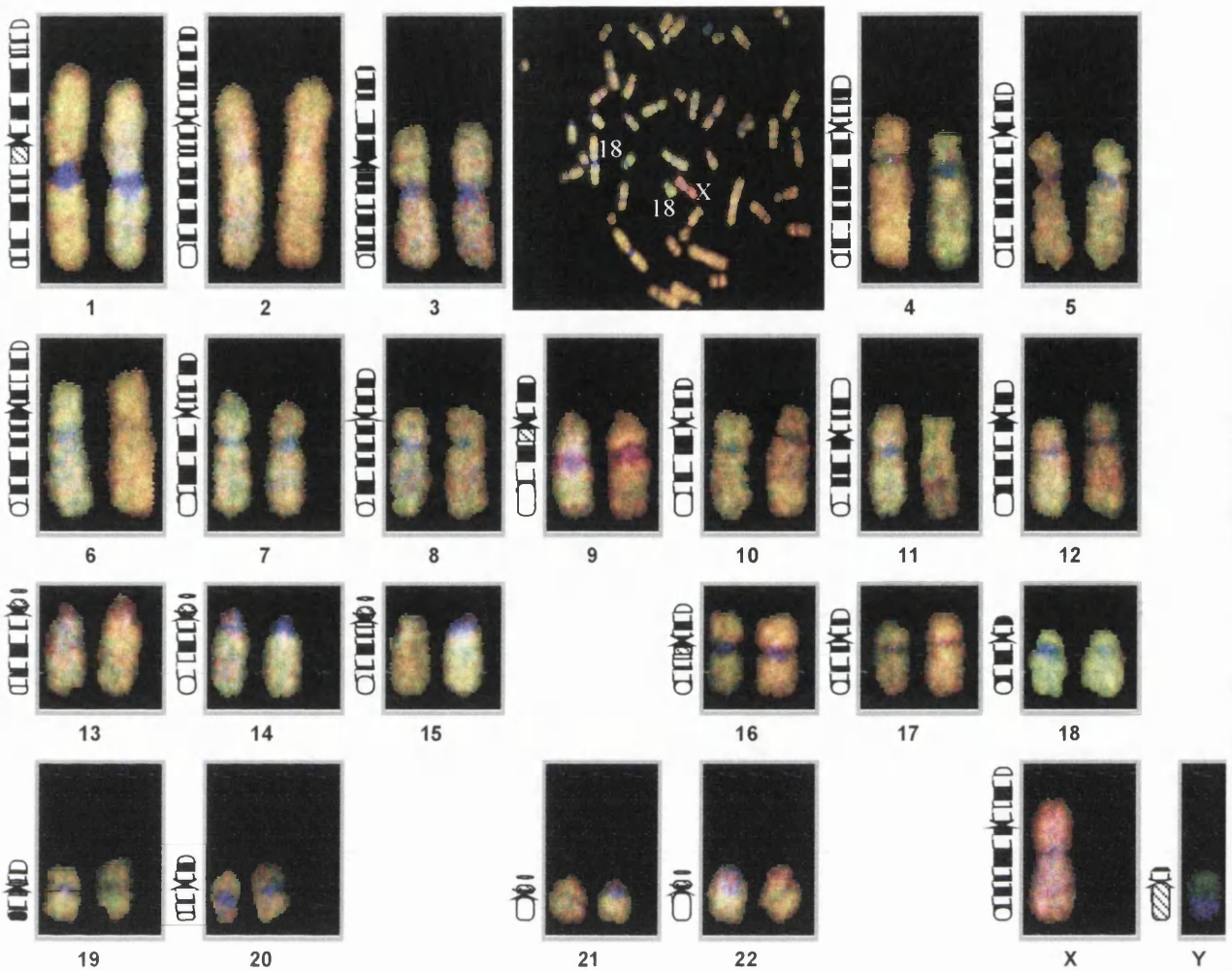


Fig 3.41: CGH karyotyping from male trisomy 18 stained cells. Test DNA was from amplified DNA and co-hybridised with normal female total genomic DNA. The intersected CGH image demonstrated that both chromosome 18 homologue and euchromatic parts of the Y chromosome are painted with a greenish colour, while the X chromosome was reddish.

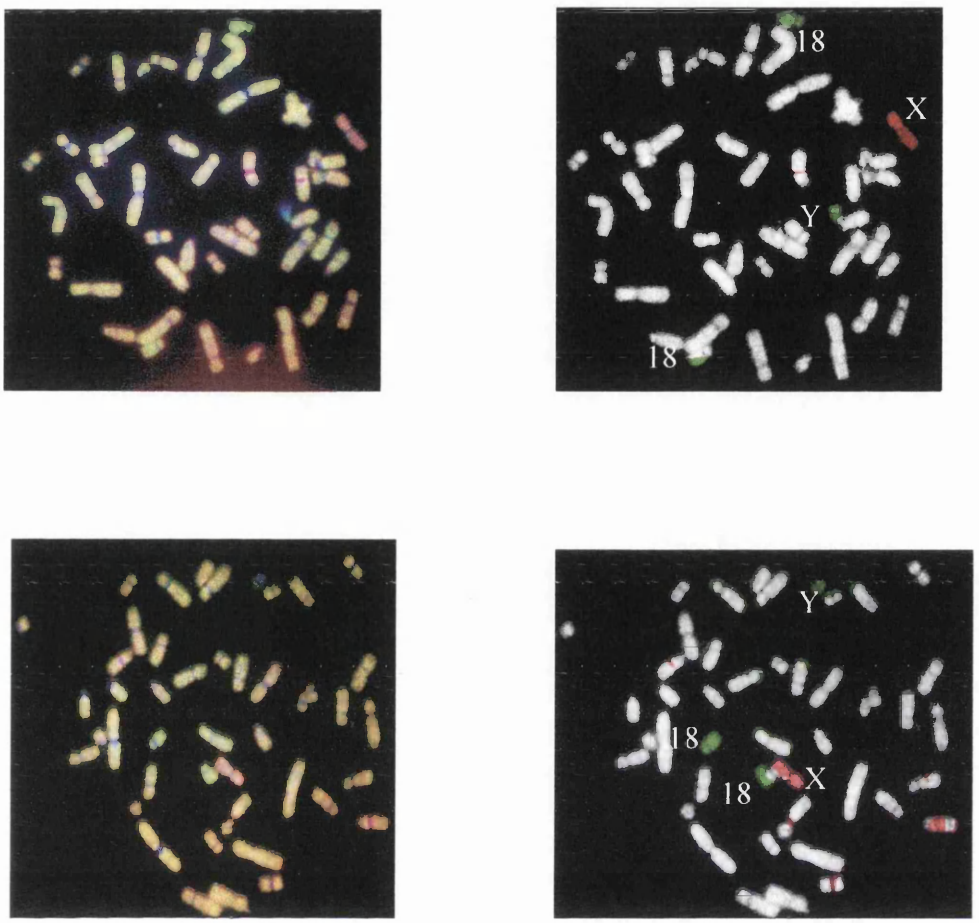


Figure 3.42: Global analysis for trisomy 18 from stained cells. Over-representation of chromosome regions was painted with green, while a red colour represented chromosome losses. A high consistency of chromosome gains was noted on both chromosome 18 homologue, whereas false positive regions did not display a similar phenomenon. The X chromosome was painted with red while the euchromatic part of the Y chromosome was painted with green.

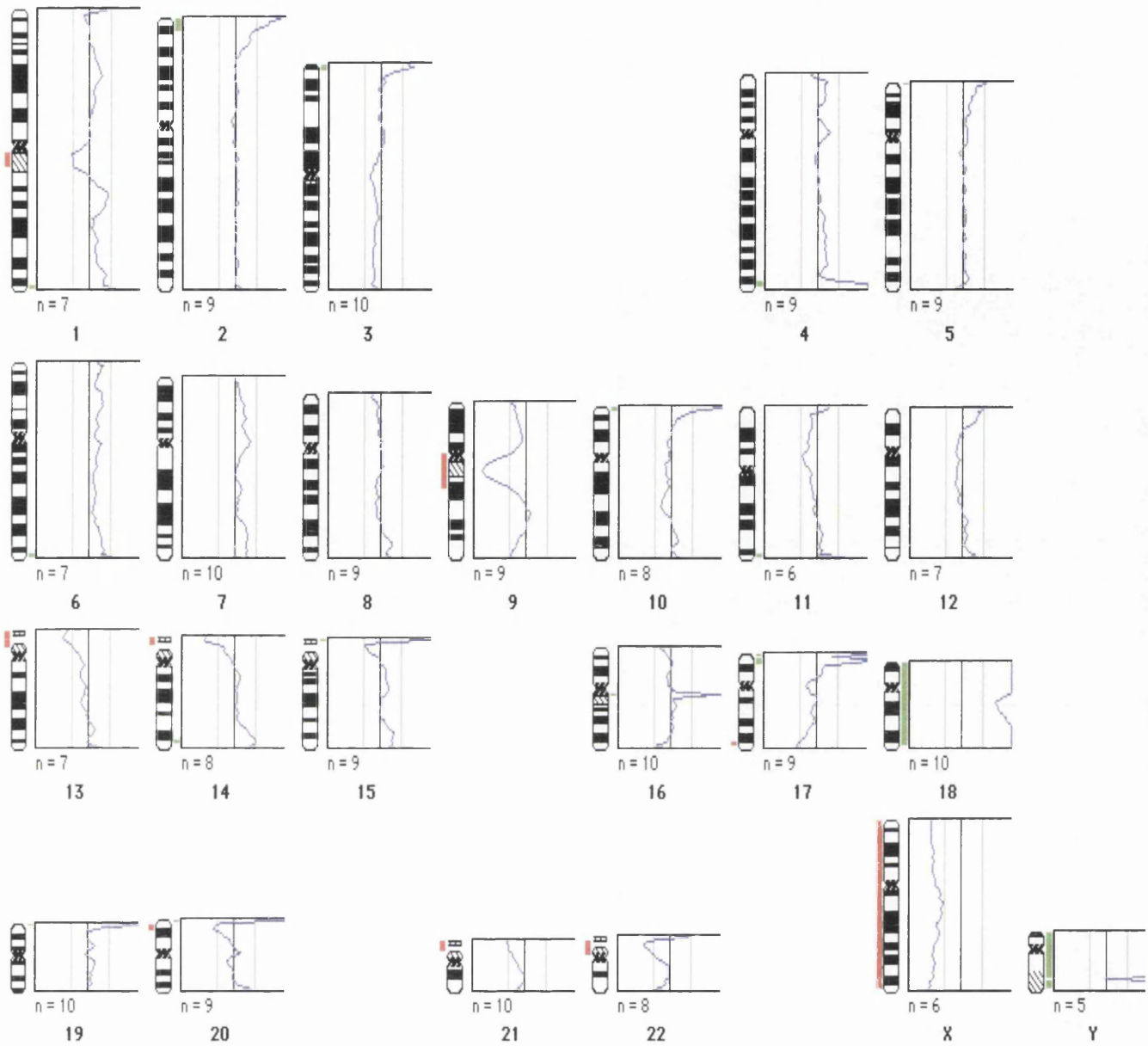


Figure 3.43: Fluorescence ratio profile from male trisomy 18 stained cells co-hybridised with normal female DNA. Chromosome gains were noted over chromosome 18 (3/2) and the Y chromosome (1/0), while the X chromosome (1X/2X) displayed chromosome losses when the threshold values were set at 1.4 and 0.6.

3.17.2 Trisomy 21

Test DNA was from female trisomy 21 stained cells, and reference DNA was from female total genomic DNA. CGH results showed more green over chromosome 21 homologues, while the Y chromosome was painted with a blue colour (Figure 3.44). Since there were no chromosome gains or losses (2X/2X), the X chromosome appeared balanced between green and red colour. This status was depicted with a whitish colour in global analysis (Figure 3.45). The ratio profile showed 3 false positives over chromosome 9 and 11 when the cut-off values were set at 1.2/0.8 (Figure 3.46). False positives were eliminated when a stringent value of 1.25 was set to represent chromosomal gains (Figure 3.47).

3.18 CGH performance from coded samples of stained cells

3.18.1 Sample 753

This female test DNA was co-hybridised with male reference DNA. Qualitative CGH images displayed disparity of green colour over the X chromosome (Figure 3.48). The long arm of chromosome X appeared unanimously greener than the short arm. To confirm the diagnosis of a potential sex chromosome anomaly, test DNA was further co-hybridised with female reference DNA. Qualitative CGH images displayed apparent red over Xp, while Xq remained a green colour (Figures 3.48; 3.49). The euchromatic part of the Y chromosome appeared bright red when test DNA was co-hybridised with male DNA, whereas it stained with DAPI when both test and reference DNA were from the same sex (i.e. female). Combining the results of two CGH experiments, the chromosomal gain over Xq and loss over Xp was interpreted, and a diagnosis of isochromosome Xq was made. Quantitative ratio profiles were compatible with the preliminary qualitative CGH impression (Figures 3.50; 3.51). The karyotype result of sample 753 was 46,X, i (X)(q10) (Figure3.52).

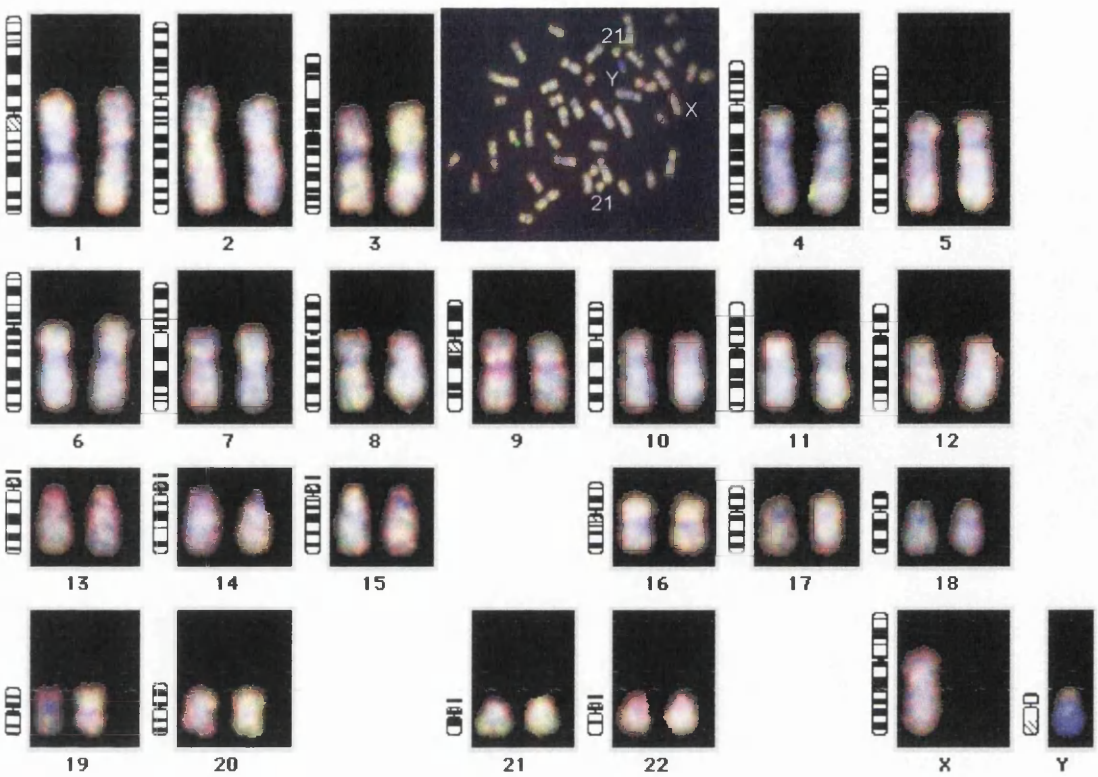


Figure 3.44: CGH karyotyping from female trisomy 21 stained cells. Test DNA was from amplified DNA and co-hybridised with normal female total genomic DNA. The intersected CGH image demonstrated that both chromosome 21 homologues are greenish (3/2). The X chromosome was balanced (2X/2X) and the Y chromosome was painted with blue (0Y/0Y).

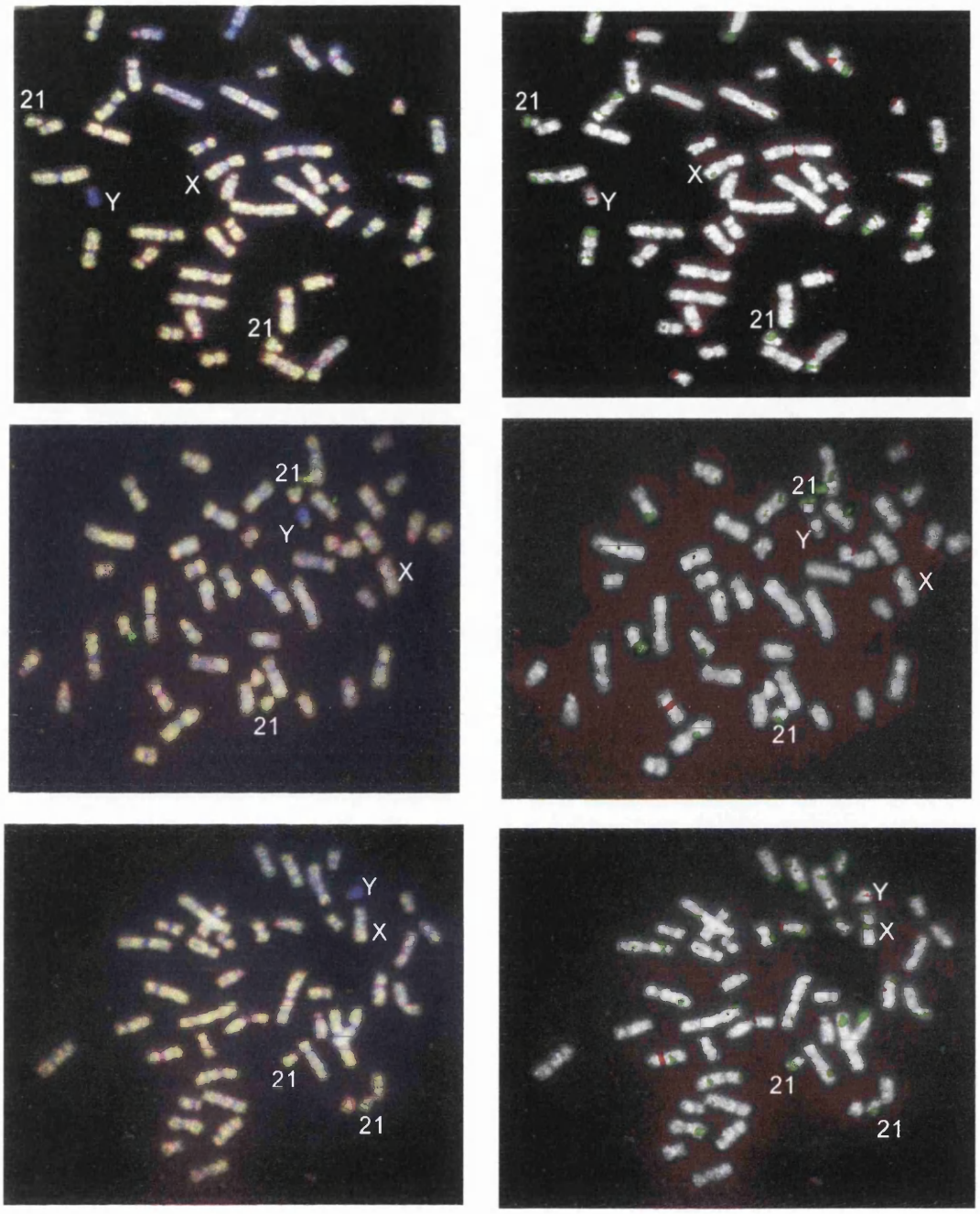


Figure 3.45: Global analysis for trisomy 21 from stained cells. Over-representation of chromosome regions was noted on both chromosome 21 homologs (3/2), while other false positive regions did not display a similar phenomenon. The X chromosome was painted with white (2X/2X) while the Y chromosome was painted with blue.

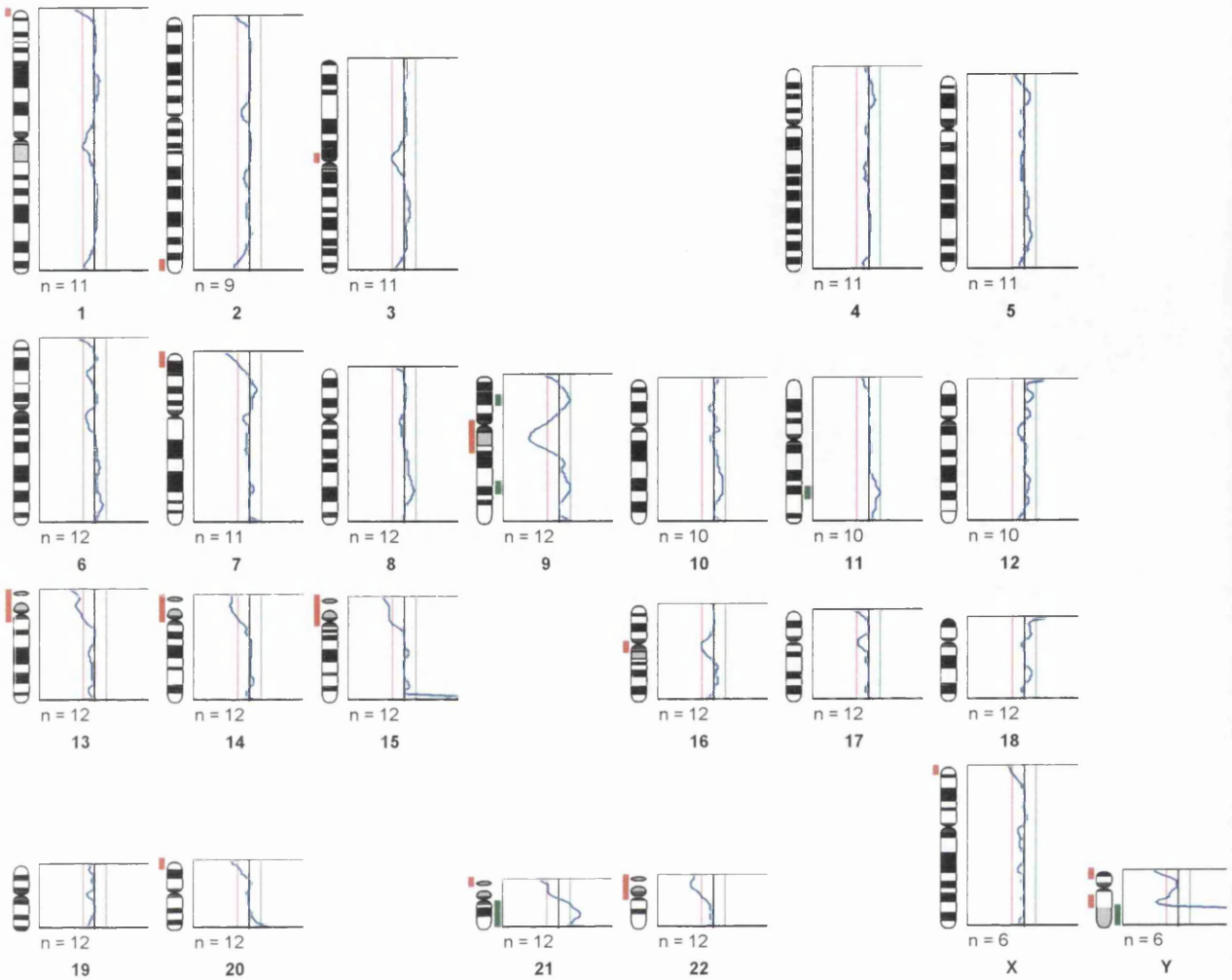


Figure 3.46: Fluorescence ratio profile from female trisomy 21 stained cells co-hybridised with normal female DNA. Chromosome gains were noted over chromosomes 9, 11 and 21 when threshold values were set at 1.2 and 0.8 to represent chromosome gains and losses, respectively. No chromosome gain or loss was seen on the X chromosome (2X/2X).

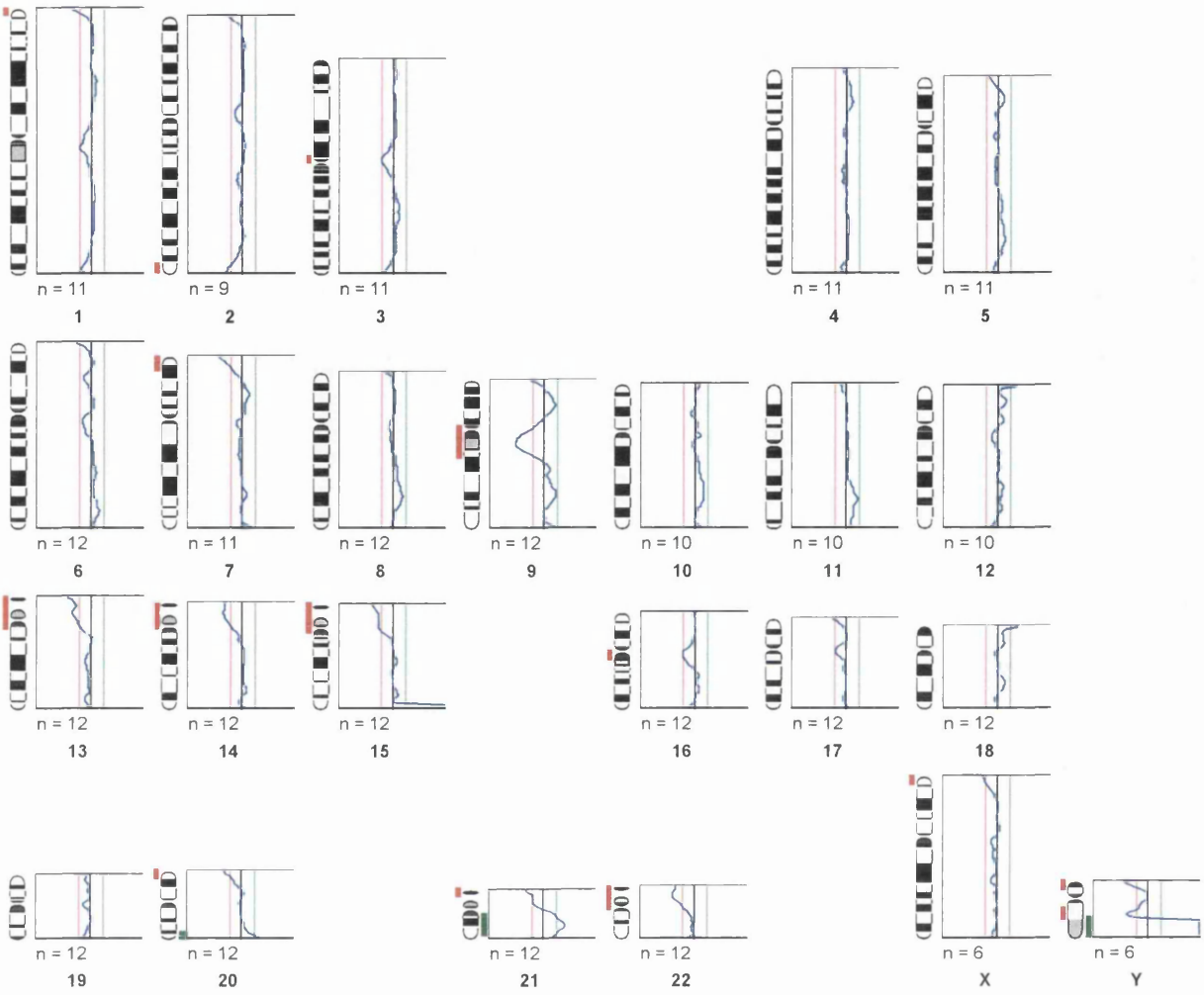
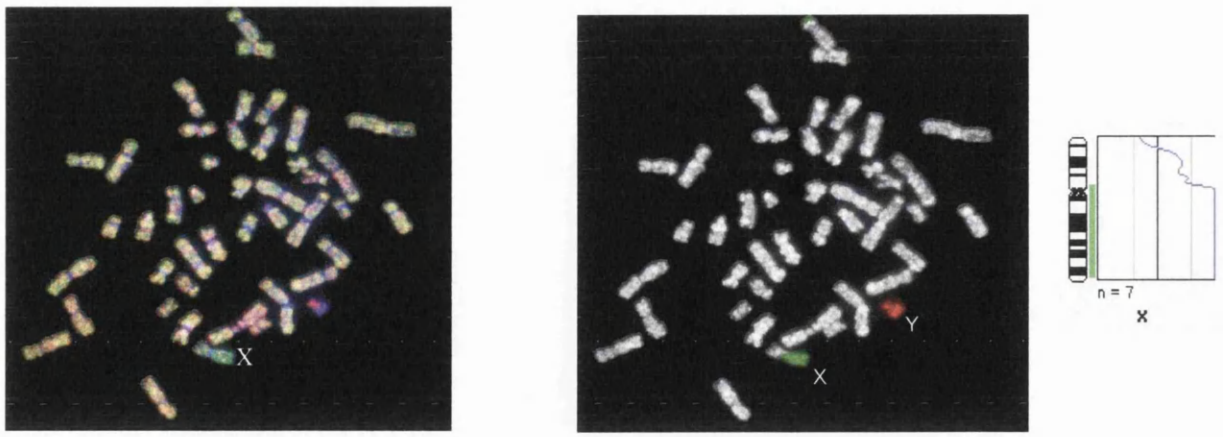
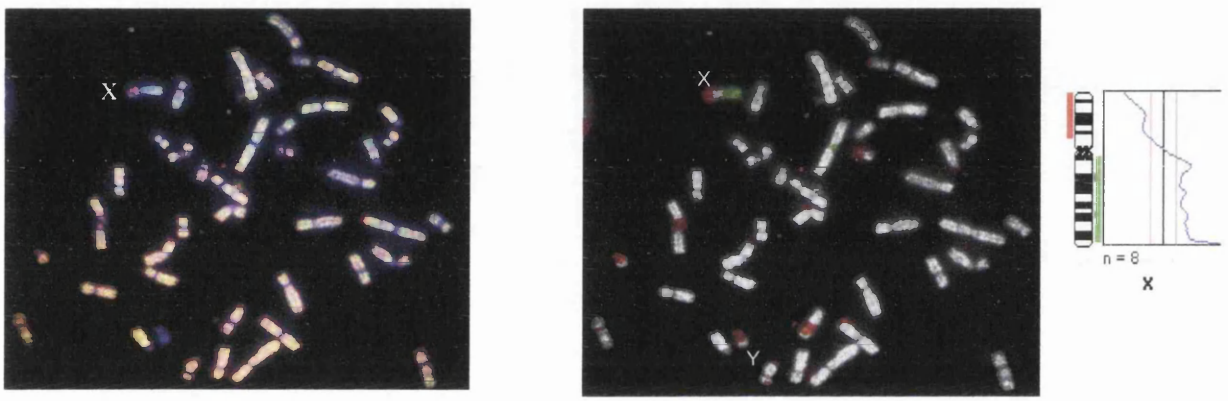


Figure 3.47: Fluorescence ratio profile using stringent cut-off values. False positives were eliminated when stringent cut-off values were set at 1.25/0.75 to represent chromosome gains and losses, respectively.



iXq vs. normal male



iXq vs. normal female

Figure 3.48: CGH and global analysis for an isochromosome Xq. The upper images showed test DNA co-hybridised with normal male DNA. Chromosome gain was noted over Xq (3Xq/1Xq) and was shown painted with green, while Xp was in balanced status (1Xp/1Xp). The idiogram of the X chromosome showed chromosome over-representation (green bar) over Xq arm. The lower images showed test DNA co-hybridised with normal female DNA. Chromosome gain was noted over Xq (3Xq/2Xq) and was painted a greenish colour, while Xp was under-represented (1Xp/2Xp) and was painted a reddish colour. The idiogram of the X chromosome showed chromosome over-representation (green bar) over the Xq arm and under-representation over the Xp arm (red bar).

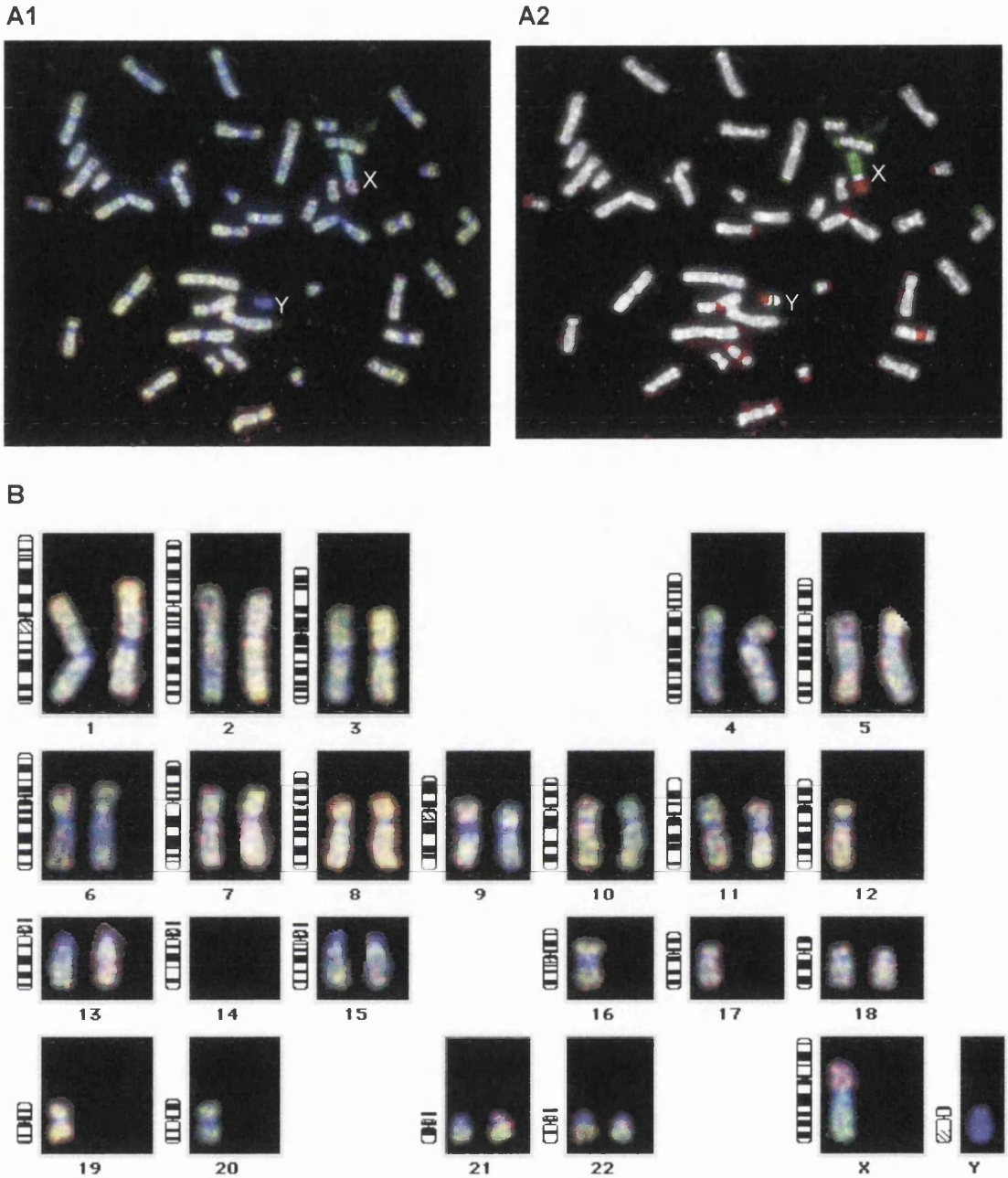


Figure 3.49: CGH karyotyping for an isochromosome Xq. A sex chromosome anomaly was confirmed when iXq stained cells were co-hybridised with normal female total genomic DNA. A1: CGH image displayed a greenish colour over Xq while Xp displayed a reddish colour. A2: Global analysis from A1 also displayed chromosome gains over Xq and chromosome loss over Xp. B: CGH karyotyping from A1. The Y chromosome was painted with blue (0Y/0Y).

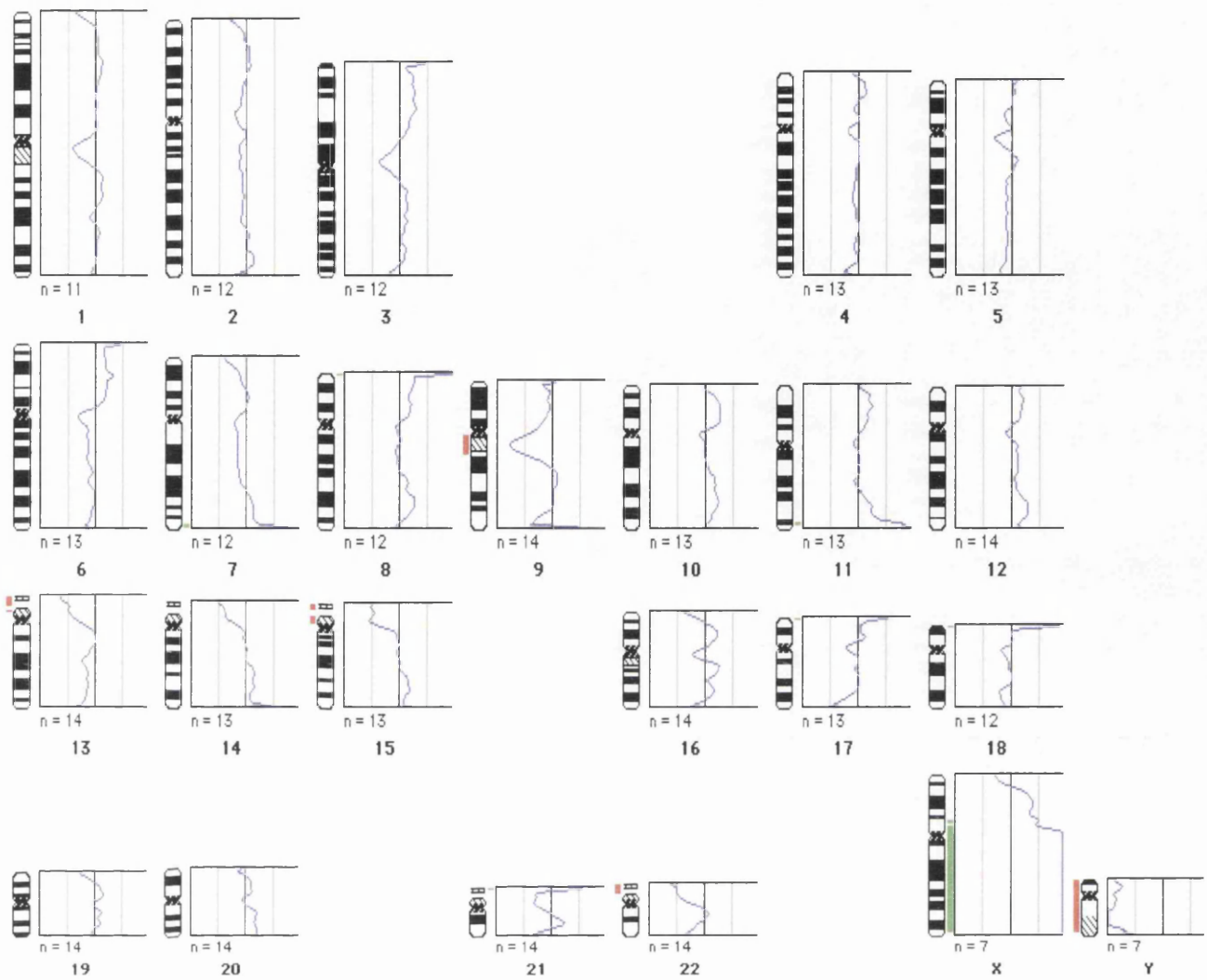


Figure 3.50: Fluorescence ratio profile from iXq stained cells co-hybridised with normal male DNA. Chromosome gains were noted over chromosome Xq (3Xq/1Xq) when threshold values were set at 1.2 and 0.8 to represent chromosome gains and losses, respectively. The Y chromosome showed chromosome loss (0Y/1Y) and was depicted as a red bar to the left of Y idiogram.

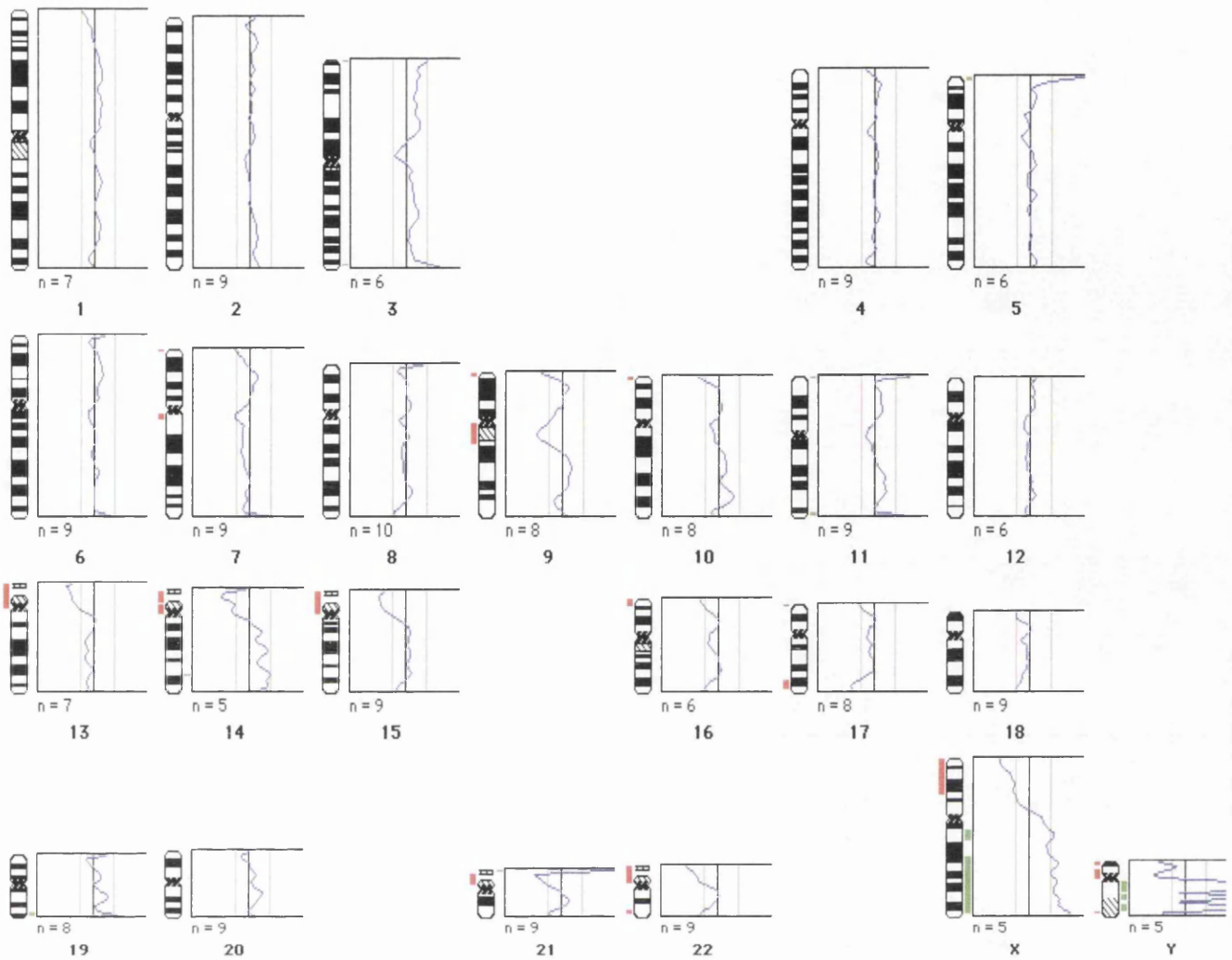


Figure 3.51: Fluorescence ratio profile from iXq stained cells co-hybridised with normal female DNA. Chromosome gains were noted over chromosome Xq (3Xq/2Xq) when threshold values were set at 1.2/0.8, and was depicted as a green bar to the right of chromosome X idiogram. Chromosome loss was noted on Xp (1Xp/2Xp) and was depicted as a red bar to the left of chromosome X idiogram.

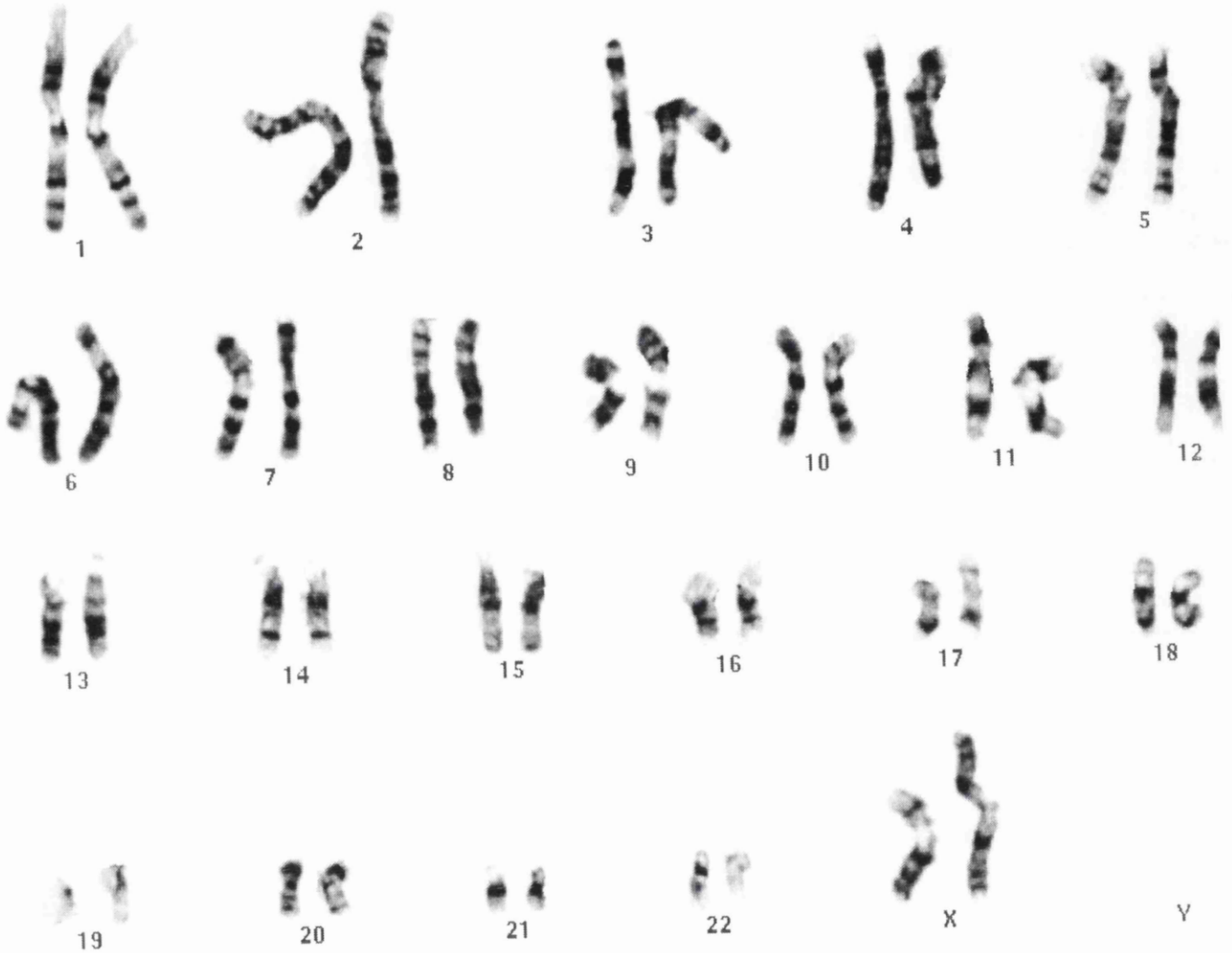


Fig 3.52: A G-banded karyotype of sample 775 shows 46,X, i (X)(q10).

3.18.2 Sample 1495

This female DNA was co-hybridised with male reference DNA. Visual assessment of digital images did not have a definite conclusion initially. Quantitative ratio profiles, using 1.2 and 0.8 as cut-off values, displayed chromosomal gains over the X chromosome together with other tiny regions over chromosome 12q, 12p, 16p, and 22q (Figure 3.53). When more stringent values were adopted (i.e. 1.5), only 12p displayed gains, while internal control of the X chromosome was still maintained (i.e. 2G/1R, 2 copies of X chromosome in test DNA versus 1 copy of X chromosome in reference DNA) (Figure 3.54). Global analysis also demonstrated that chromosome gains occurred over most of the chromosome 12p homologues (Figure 3.55). CGH interpretation was partial trisomy 12. The karyotying of the sample 1459 was 46,XX, der (10) t (10; 12)(q26.13, p11.23)- a karyotype resulting in trisomy for 12p with an effective monosomy of the terminal region of 10q (Figure 3.56).

An additional CGH experiment was carried out using reference DNA from normal male amplified DNA. This amplified DNA was prepared from a matched number of normal stained cells. The results showed multiple false positives even when stringent threshold was set, whereas the internal control of X chromosome was still maintained (Figure 3.57). More red was noted over chromosome 1p, 16, 17, 19, 22. Heterchromatic regions of chromosomes 1, 9 and acrocentric chromosomes were stained with DAPI (Figure 3.58).

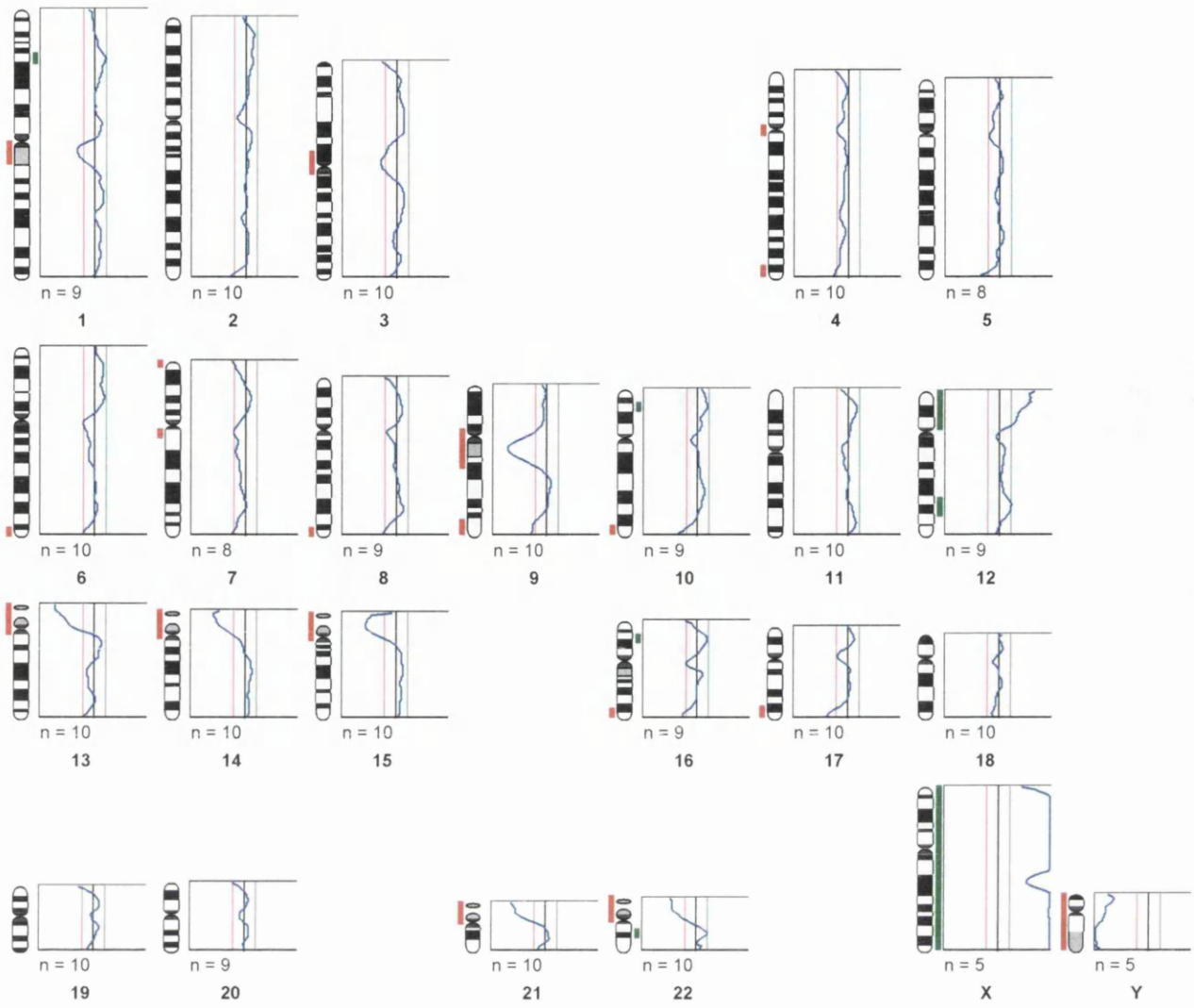


Figure 3.53: Fluorescence ratio profile from female trisomy 12p stained cells. The test DNA was co-hybridised with normal male DNA. Chromosome gains were noted over chromosomes 1, 12, 16, 22 and X when threshold values were set at 1.2/0.8 to represent chromosome gains and losses. Chromosome loss was noted on the Y chromosome.

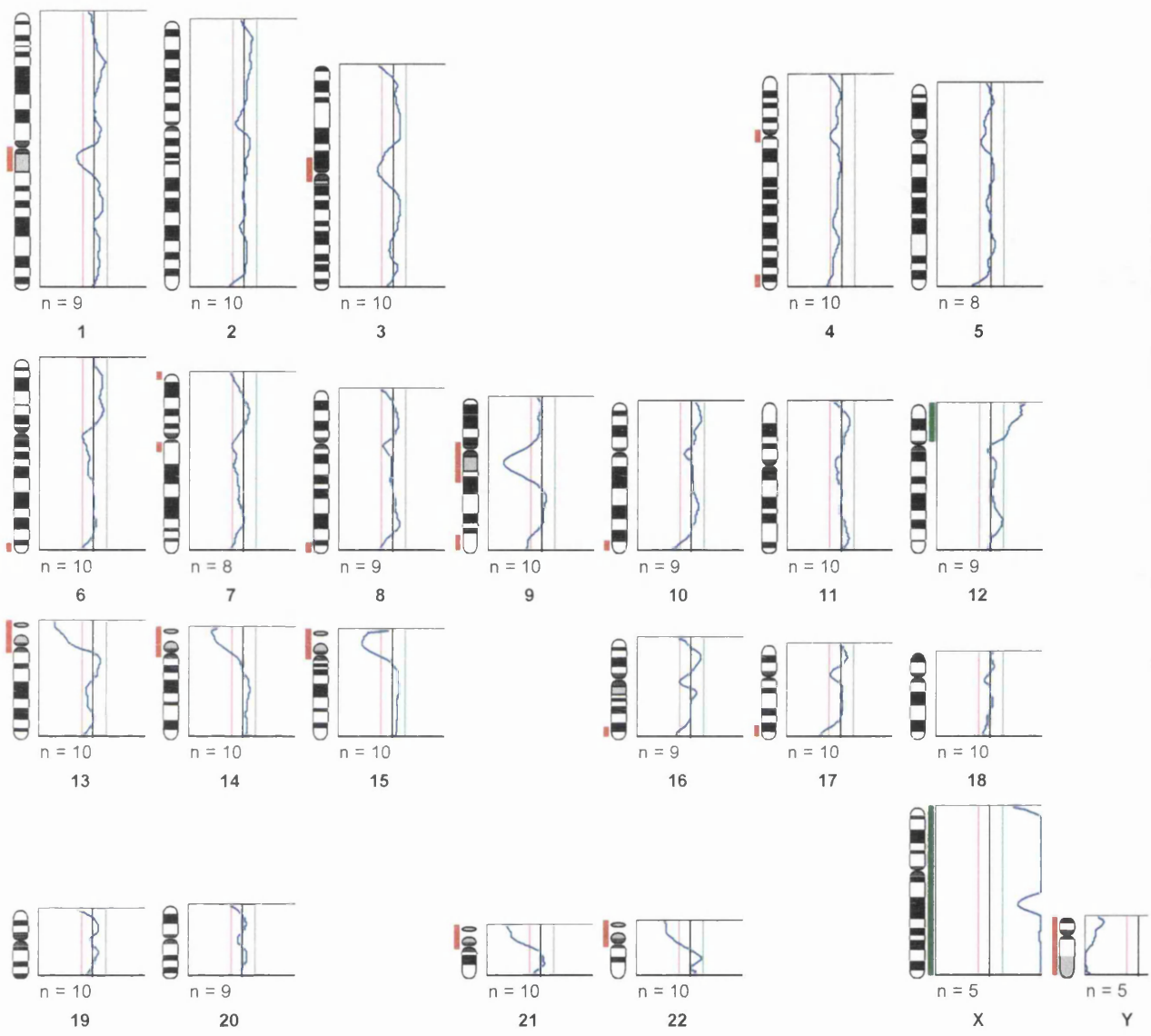


Figure 3.54: Quantitative analysis using stringent cut-off values. Over-representation of chromosome 12p was maintained when stringent cut-off values were set at 1.25 and 0.75 to represent chromosome gains and losses, respectively, while false positives were reduced to a minimum.

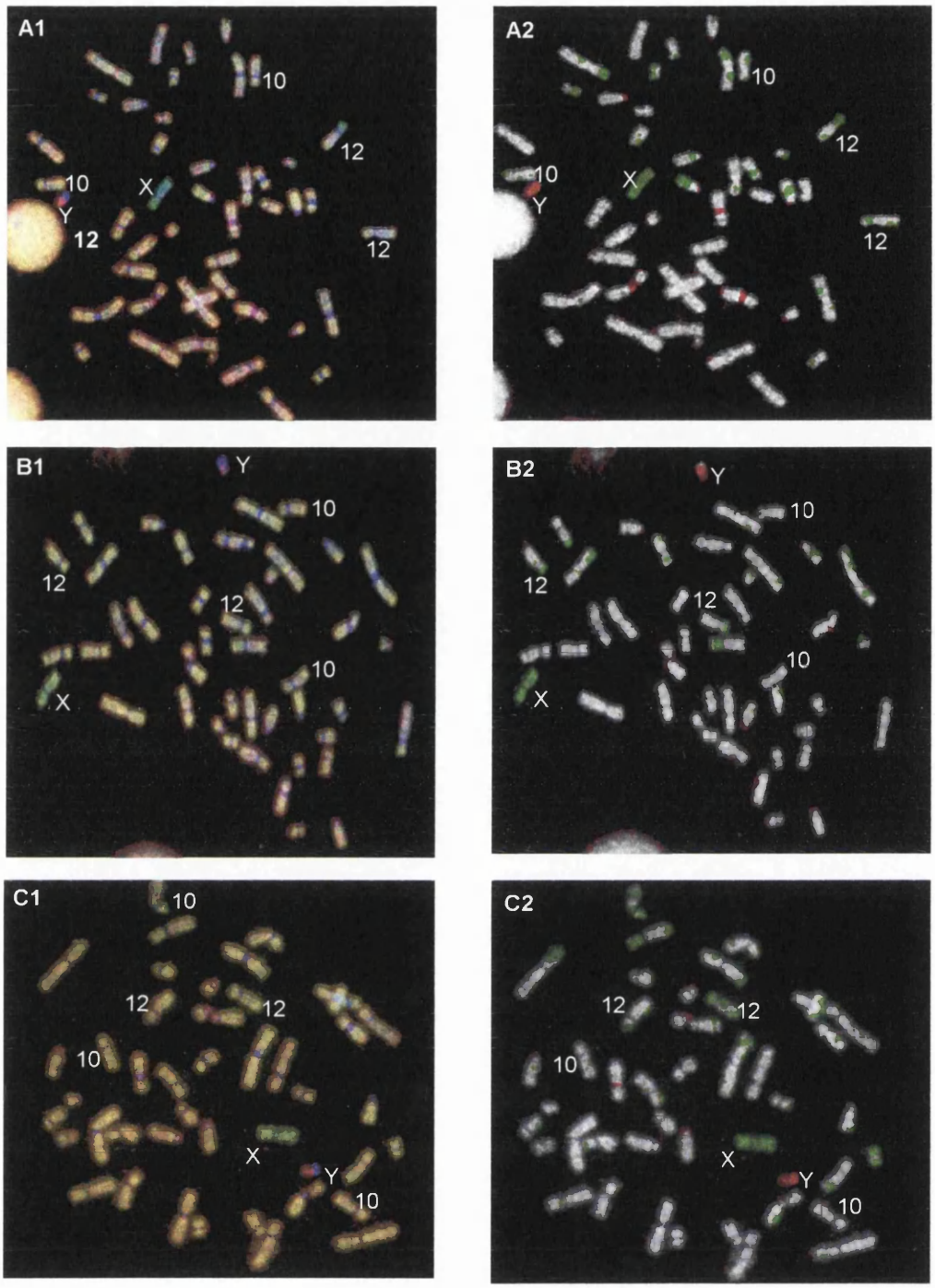


Figure 3.55: Global analysis of CGH from a female trisomy 12p co-hybridised with normal male DNA. Left column (A1-C1): CGH images. Right column (A2-C2): Global analysis of CGH images from the left side. Despite several false positives, only chromosome 12p homologues displayed universal chromosomal gains.

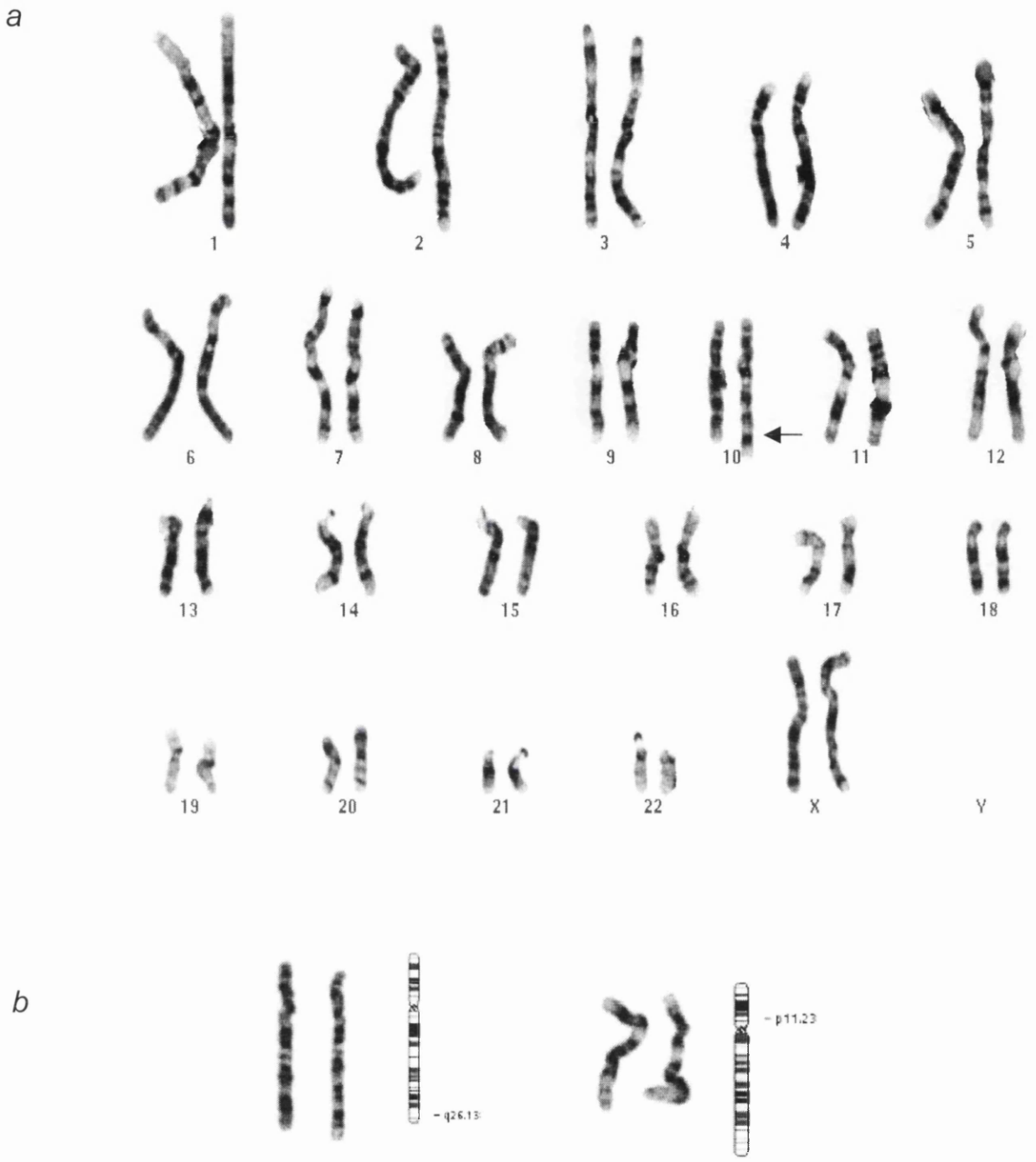


Fig 3.56: *a*: A G-banded karyotype of sample 1495 shows 46,XX,der(10)t(10;12)(q26.13;p11.23). *b*: shows partial G-banded karyotype of parent of 1495 carrying balanced translocation t(10;12)(q26.13;p11.23).

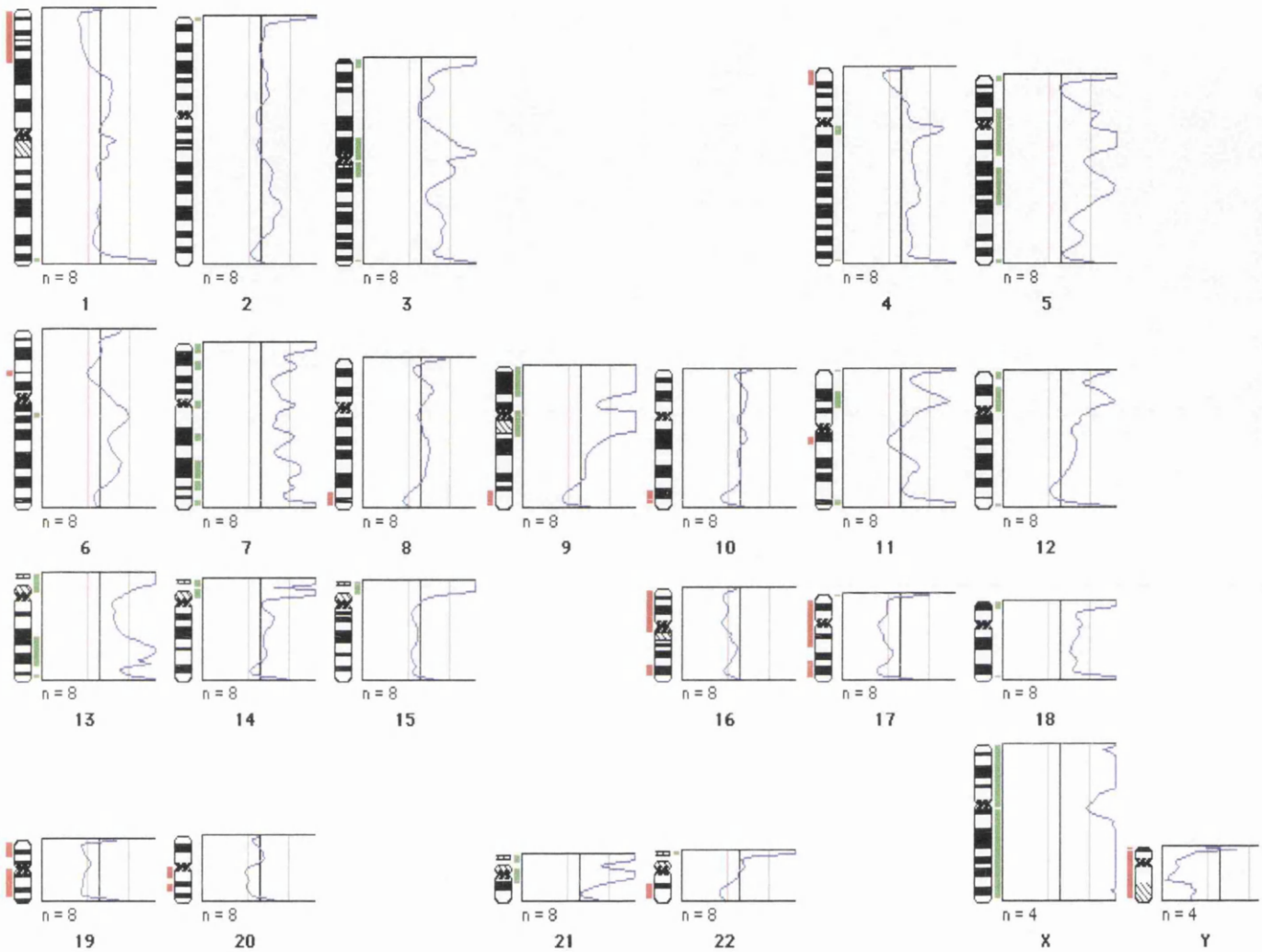


Figure 3.57: Fluorescence ratio profile from female trisomy 12p stained cells. The test DNA was co-hybridised with amplified normal male DNA. Chromosome gains were noted over chromosomes 5, 7, 9, 11, 12, 13 and X. Chromosome losses were noted over chromosomes 1, 16, 17, 19, 20 and Y when threshold values were set at 1.2/0.8.

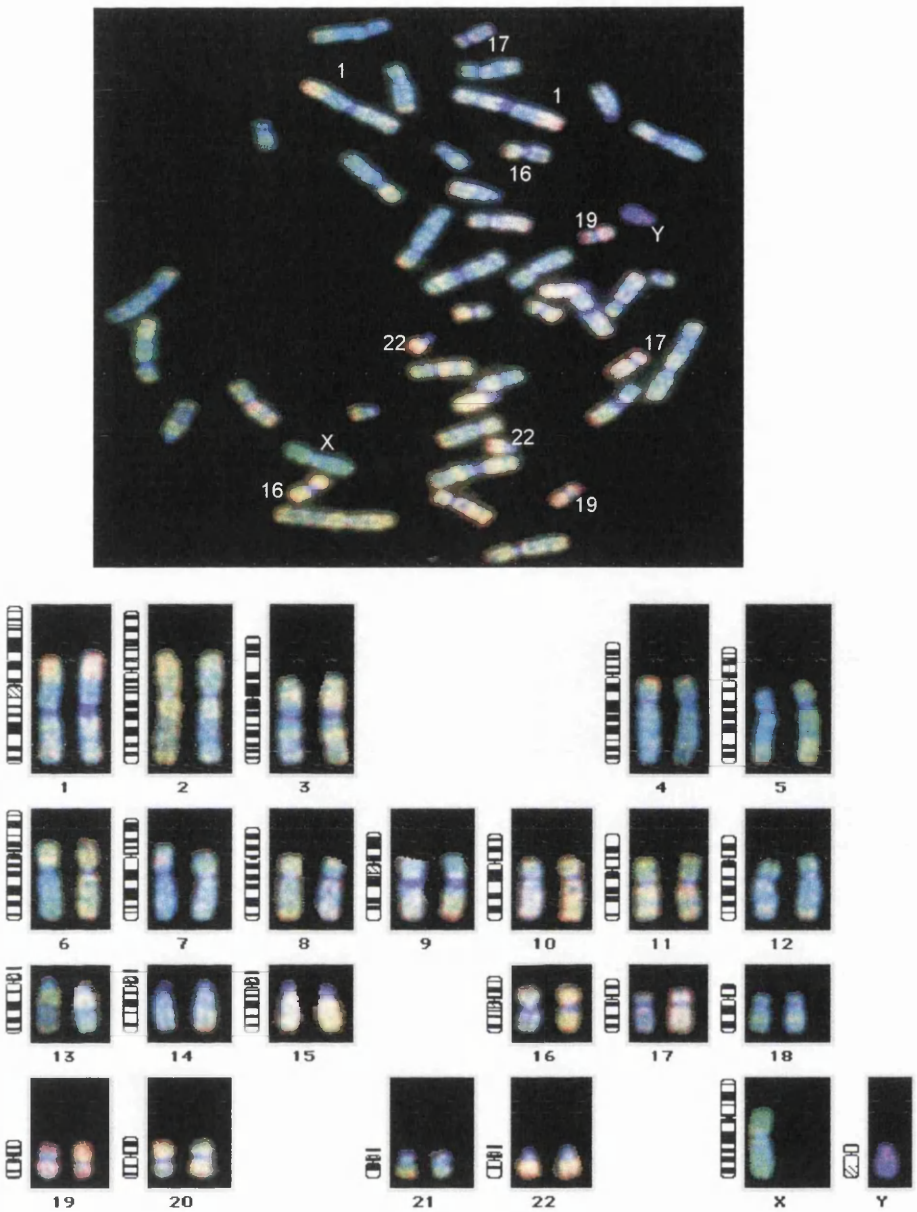


Figure 3.58: CGH karyotyping from female trisomy 12p stained cells. Test DNA was from amplified DNA and co-hybridised with normal male amplified DNA. CGH images showed a greenish colour over the X chromosome and a reddish colour over euchromatic part of the Y chromosome. Under-representation was noted over chromosomes 1p, 16, 17, 19, 20 and 22.

3.18.3 Sample 445

This female DNA was co-hybridised with male reference DNA. No apparent diagnosis was made from CGH digital images except for the sex (Figure 3.59). When cut-off values were set at 1.2 and 0.8, quantitative ratio profiles displayed 5 regions of chromosomal gains and 2 regions of chromosomal losses over two different hybridisation areas (Figure 3.60). Ratio profiles revealed a normal female when stringent values of 1.3 and 0.7 were set, while internal control of the X chromosome was still maintained. The karyotype of this sample was 46,XX, del (2)(p12-p13) del (11)(q25-qter) t (2,11) (Fig 3.61). Chromosome analysis reveals a translocation between the short arm of one chromosome 2 and the long arm of one chromosome 11. This translocation appears to be unbalanced with the region (2)p12->p13 missing from the derivative chromosome 2 and the region (11)q25->qter missing from the derivative chromosome 11. This is an unbalanced karyotype resulting in monosomy for these regions of chromosomes 2 and 11. The CGH results showed that small deletions, at band level, might not be detected at a single cell level.

3.19 CGH performance from manufactured mosaics

The results demonstrated that CGH was unable to detect trisomy 18 at 70% mosaic level using the current protocol (Figure 3.62). Qualitative digital images did not display more green over targeted chromosome 18 and X. The resulting diagnosis would be a normal female under such circumstances (Figure 3.63).

3.20 CGH for blastomeres

DNA prepared from single blastomeres was tested with SpectrumRed reference DNA from both sexes. CGH images showed uniform hybridisation for the majority of the hybridisation area (Figure 3.64). Qualitative ratio analysis demonstrated multiple gains/losses when threshold values were set at 1.2/0.8 (Figure 3.65).

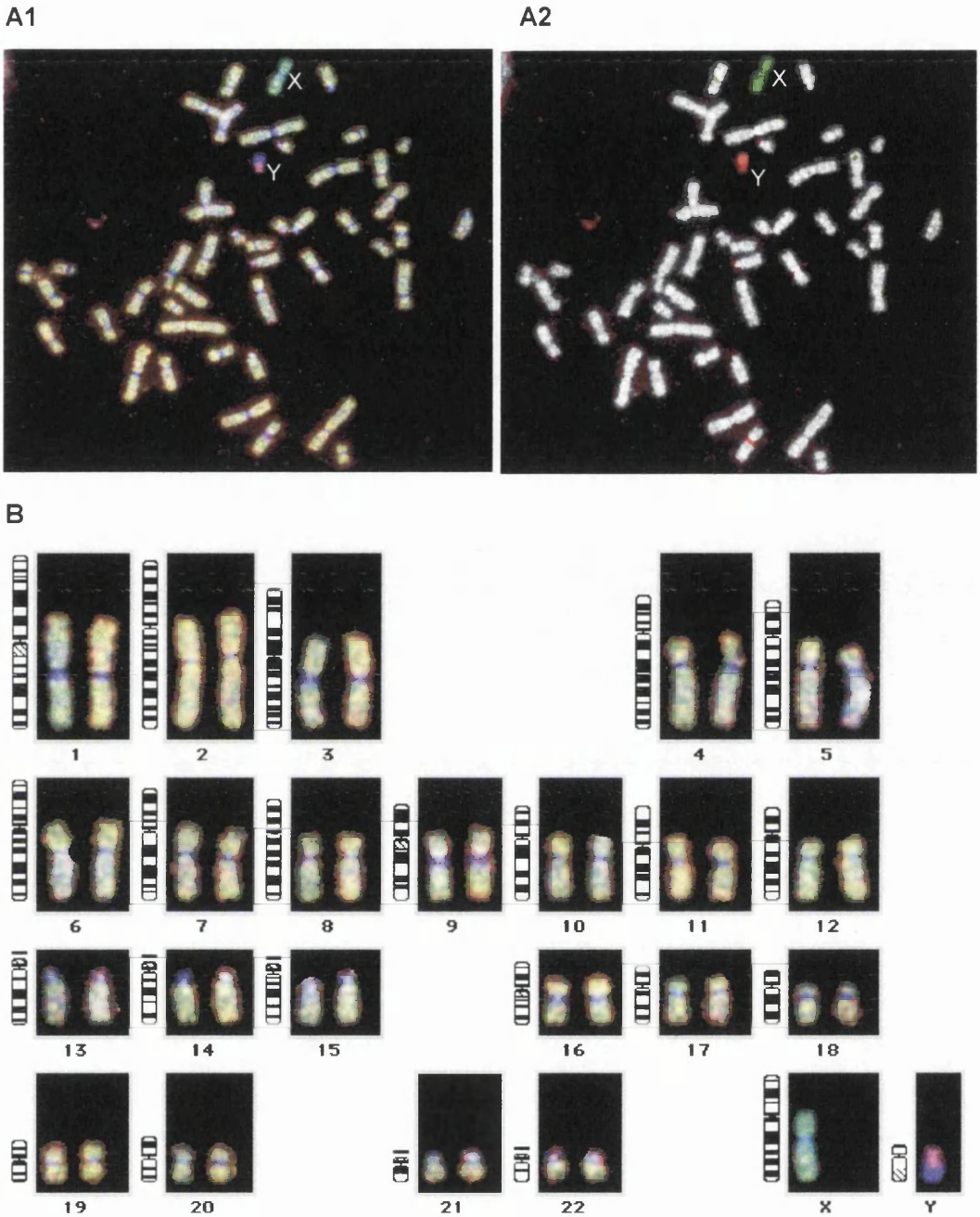


Figure 3.59: CGH images obtained from stained cells with tiny chromosomal deletions. A1: Qualitative CGH image displayed uniform hybridisation for both test and reference DNA. A2: Global analysis showed that the X chromosome was painted with a greenish colour (2X/1X) while the Y chromosome was painted with a reddish colour (0Y/1Y). B: CGH karyotyping revealed a greenish colour over the X chromosome.

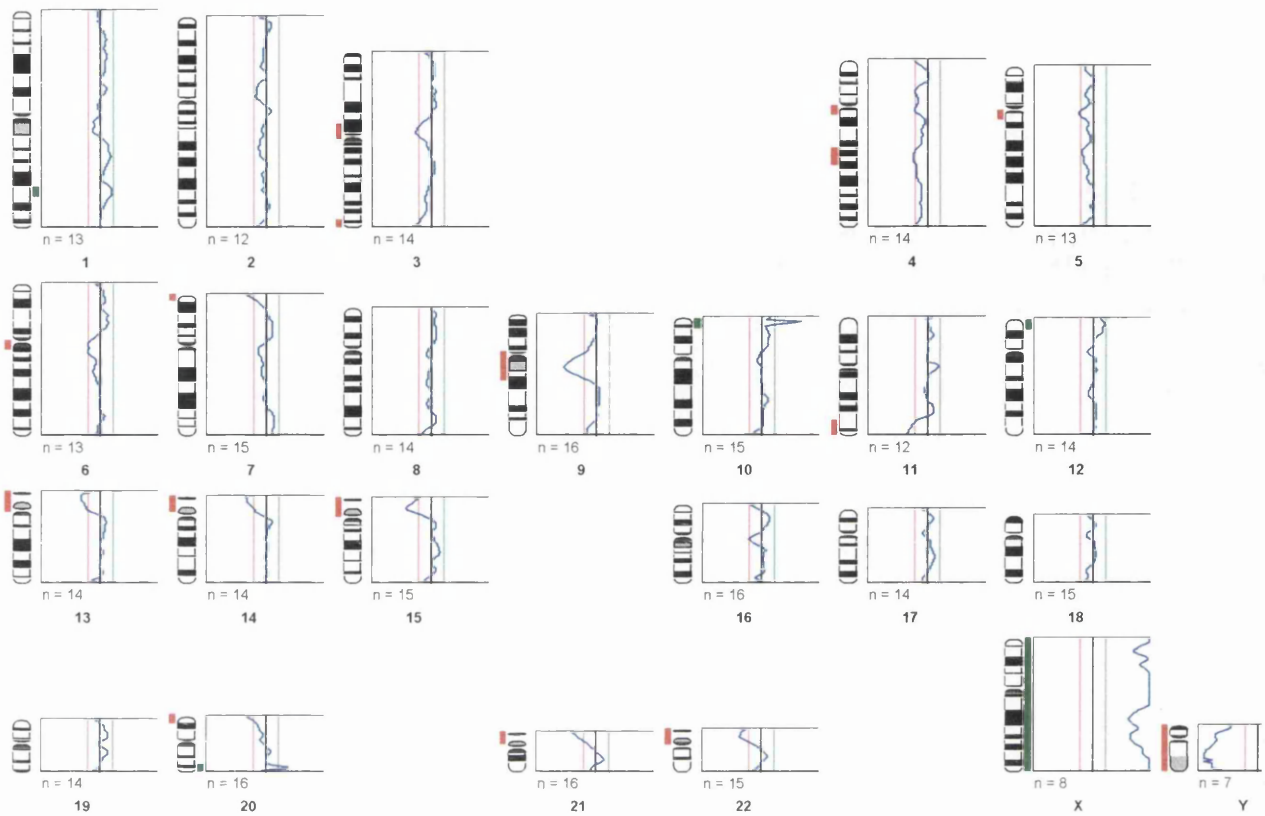


Figure 3.60: Fluorescence ratio profile of small chromosome deletions. The test DNA was co-hybridised with normal male DNA. Chromosome gains were noted over chromosomes 1 and X. Chromosome losses were noted over chromosomes 4, and Y when threshold values were set at 1.2/0.8 to represent chromosome gains and losses, respectively. For single cell CGH, accurate prediction might not be available for small chromosome deletions at a single band level.

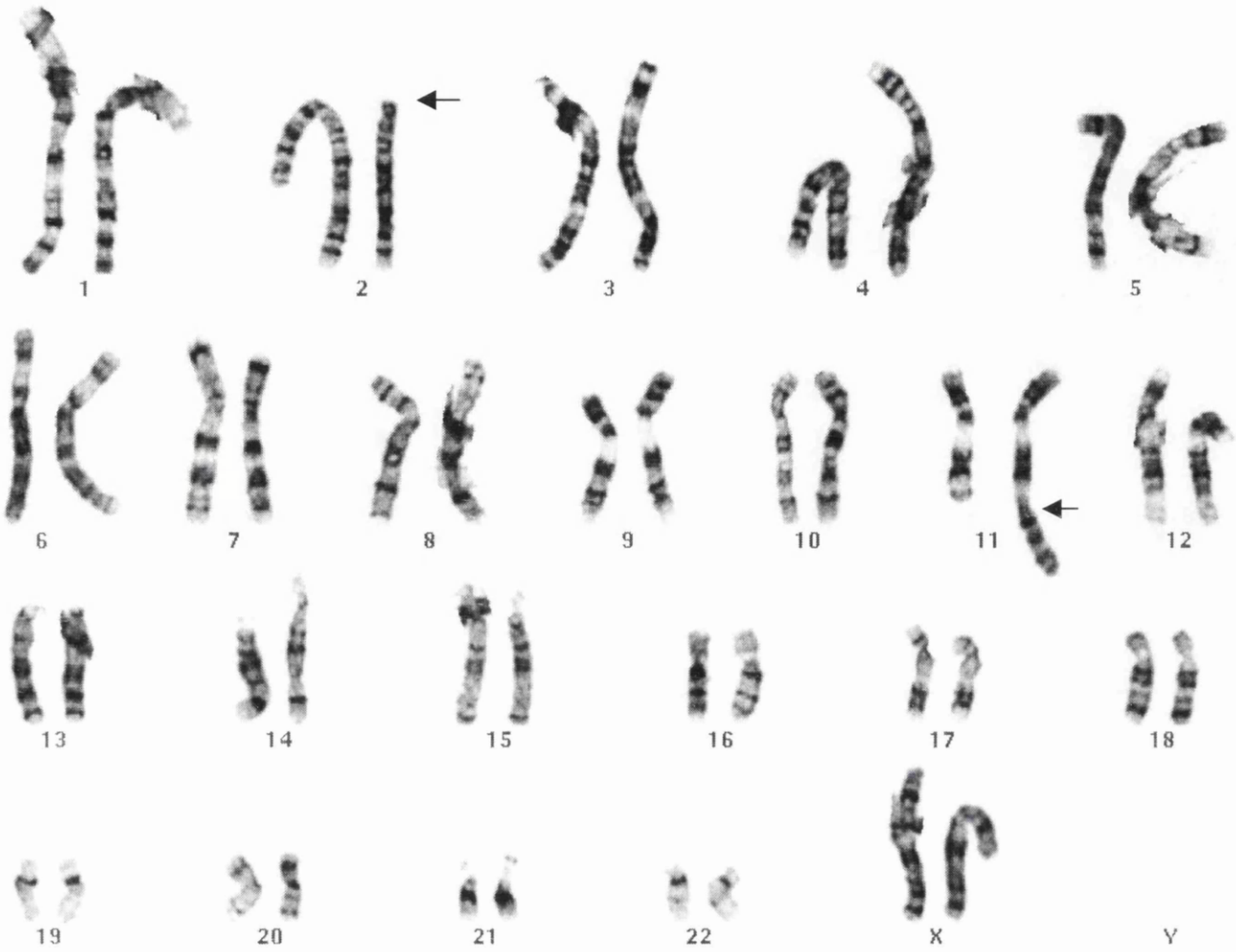


Fig 3.61: A G-banded karyotype of sample 445 shows 46,XX,der(2)del(2)(p12-p13),der(11)del(11)(q25),t(2;11)(p13;q25).

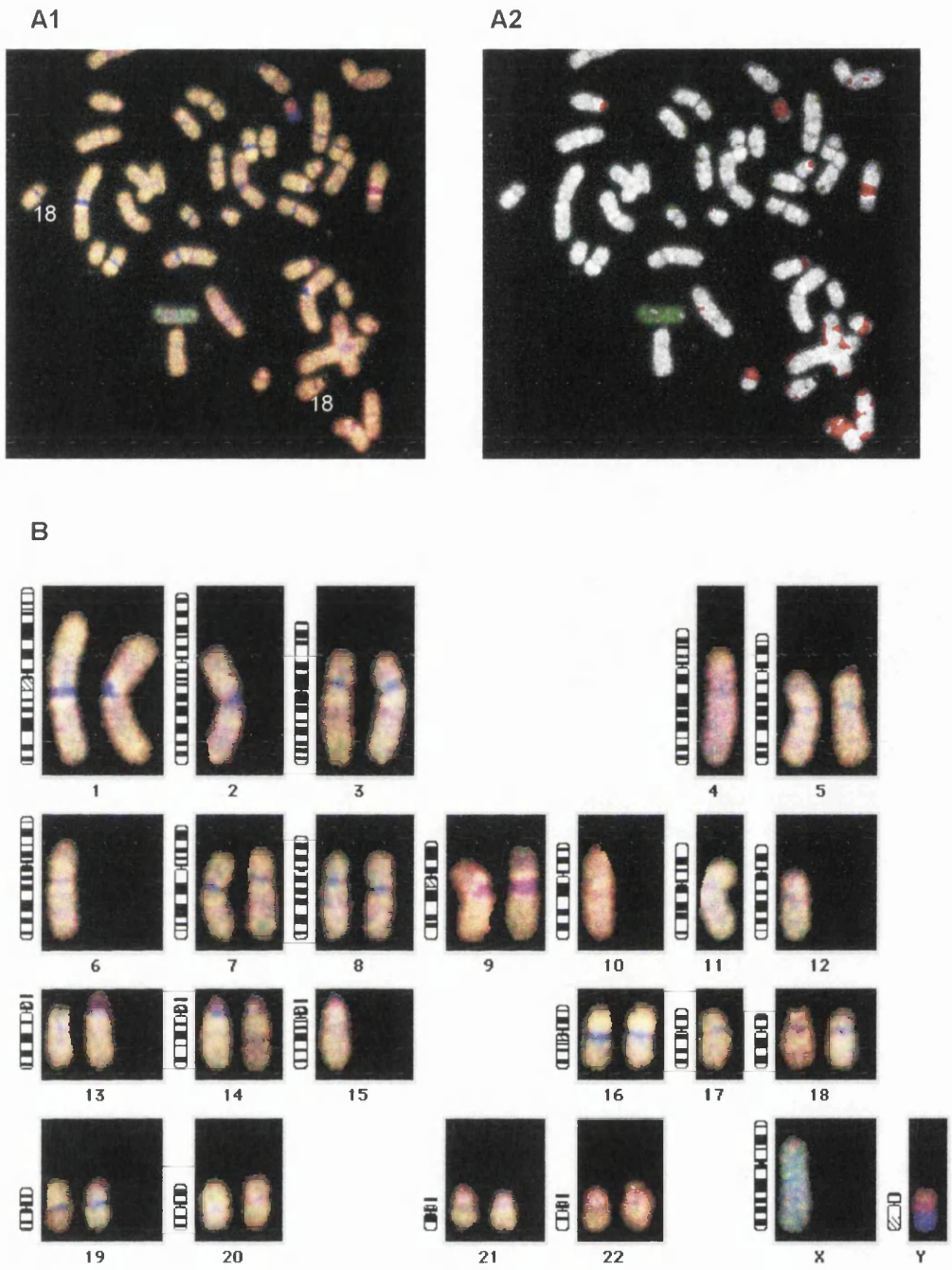


Figure 3.62: CGH images from 70% manufactured mosaics of male trisomy 18. Test DNA was from amplified DNA while reference DNA was from normal male DNA. CGH karyotyping showed a greenish colour over the X chromosome, but chromosome 18 did not show a greenish colour change.

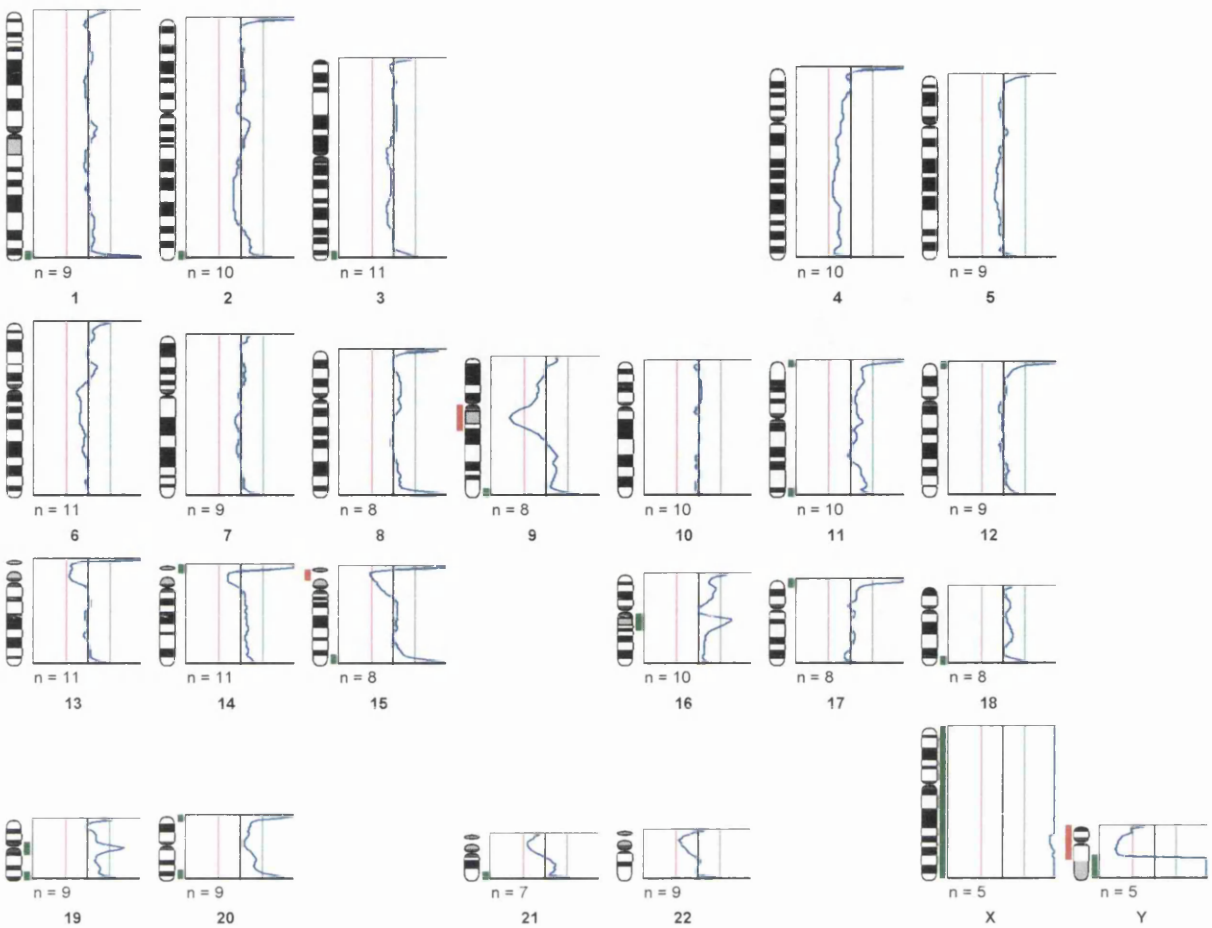


Figure 3.63: Fluorescence ratio profile for 70% manufactured mosaics of male trisomy 18. Chromosome gains were noted over the X chromosome and chromosome losses were noted over the euchromatic part of the Y chromosome when thresholds were set at 1.2/0.8 to represent chromosome gains and losses, respectively.

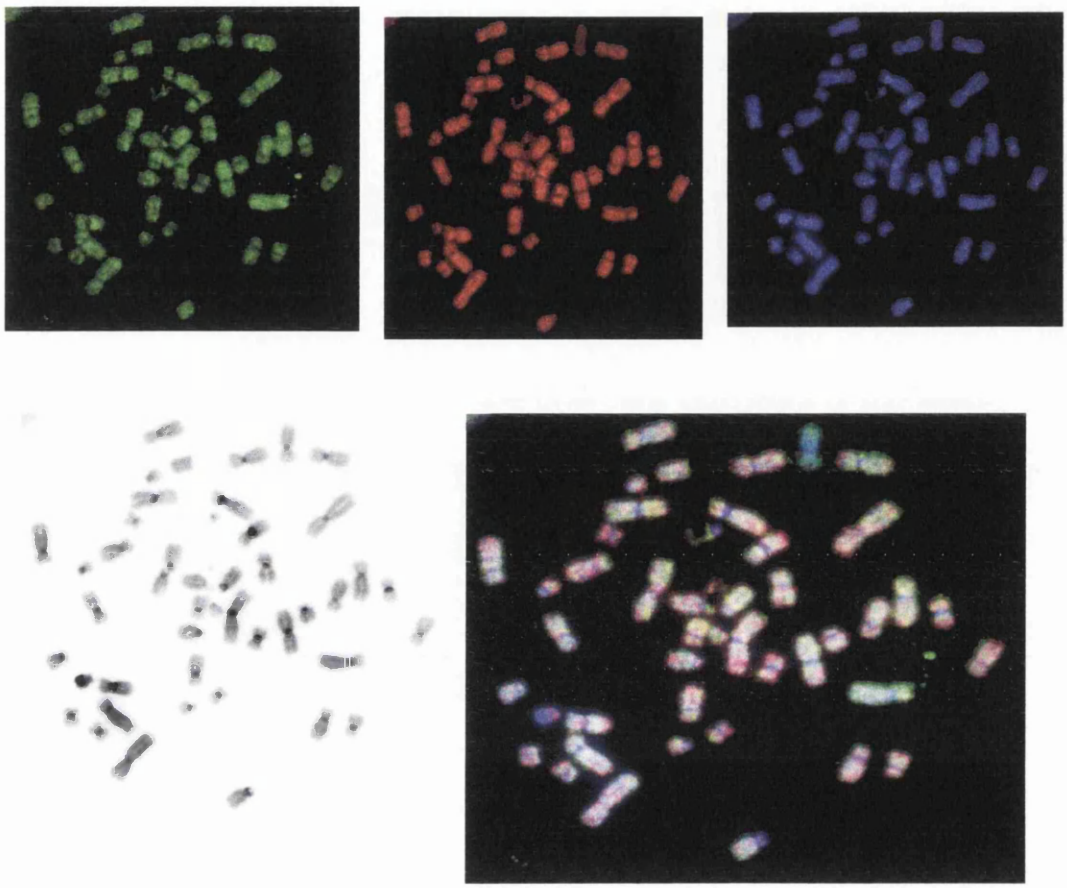


Figure 3.64: CGH images from a single blastomere. The test DNA was co-hybridised with total female genomic DNA. Mixed CGH images were pseudocoloured into green, red and blue colour, respectively.

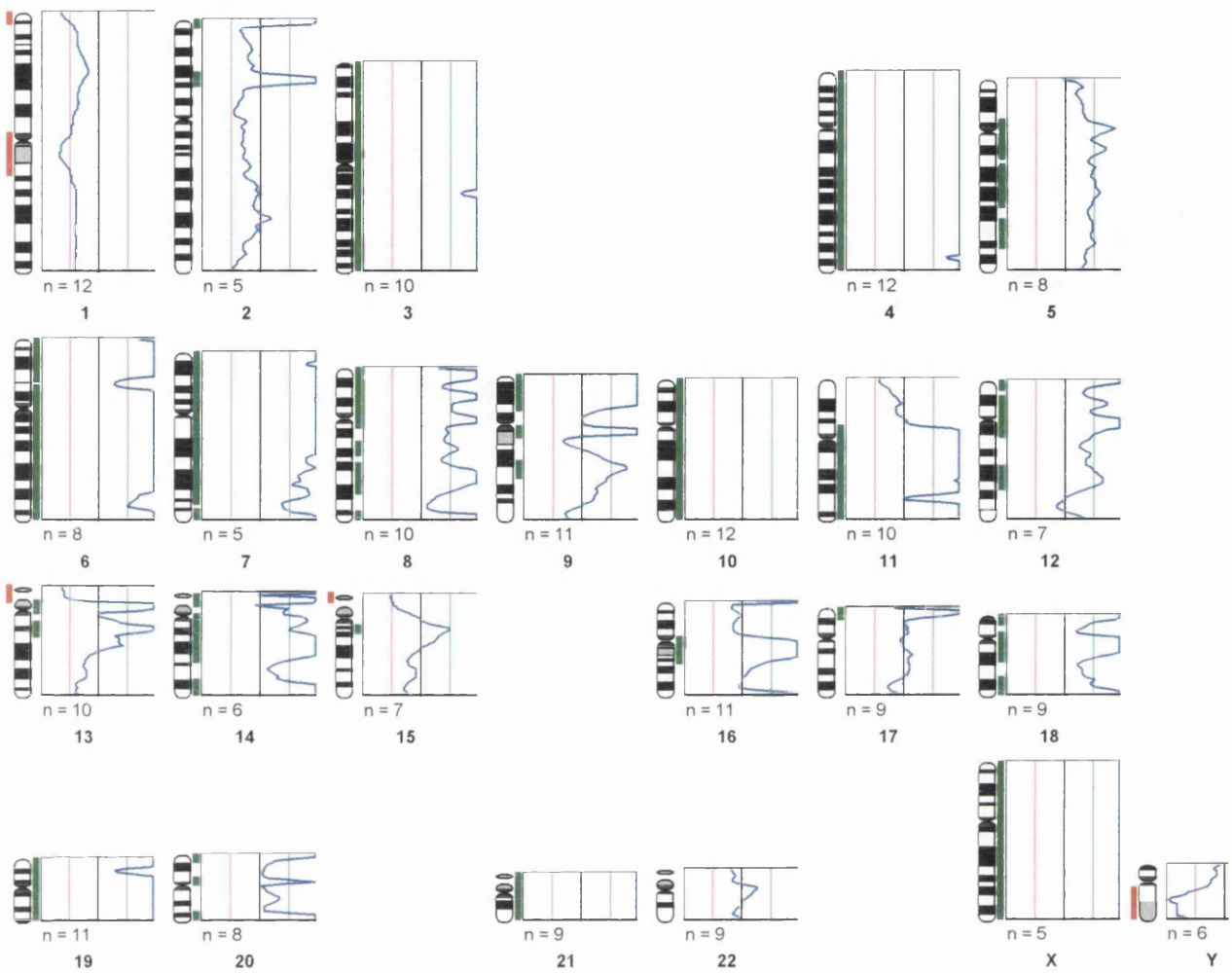


Figure 3.65: Fluorescence ratio profile from a single blastomere. The test DNA was co-hybridised with normal male DNA. Chromosome gains were noted over chromosomes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 18, 19, 21 and X, while chromosome losses were noted over the Y chromosome when thresholds were set at 1.2/0.8 to represent chromosome gains and losses, respectively.

3.21 Time consideration for CGH

The current described procedure, including retrieval of single amniocytes, lysis of cells, and preparation for DOP-PCR, usually took one working day. DOP-PCR, which took around 5 hours, was performed as an overnight procedure. Subsequent PCR labelling took around 3 hours and was carried out on the second day. Preparation for CGH experiments required two hours. After this, it took 48-72 hours for two colour hybridisation, and 2 hours for post-hybridisation washing. Image capture and analysis took around 3-4 hours, depending on image quality and complexity of diagnosis. Therefore, in total it took some 5-6 days to complete the whole process for DOP-PCR-CGH from fresh single amniocytes.

3.21.1 Overnight CGH

To shorten the time requirement, the feasibility of a short protocol involved overnight CGH was tried. CGH results were investigated by testing the current protocol and a protocol using triple DNA concentration. The preliminary results showed that both protocols could produce uniform and intense hybridisation signal within 21-23 hours (Figure 3.66). An ultra-short protocol using a one-hour hybridisation was tried; however, despite most chromosomes being painted under the fluorescent microscope, the hybridisation quality was not suitable for ratio profile analysis. All short protocol results were tested for visual assessment and not analysed quantitatively.

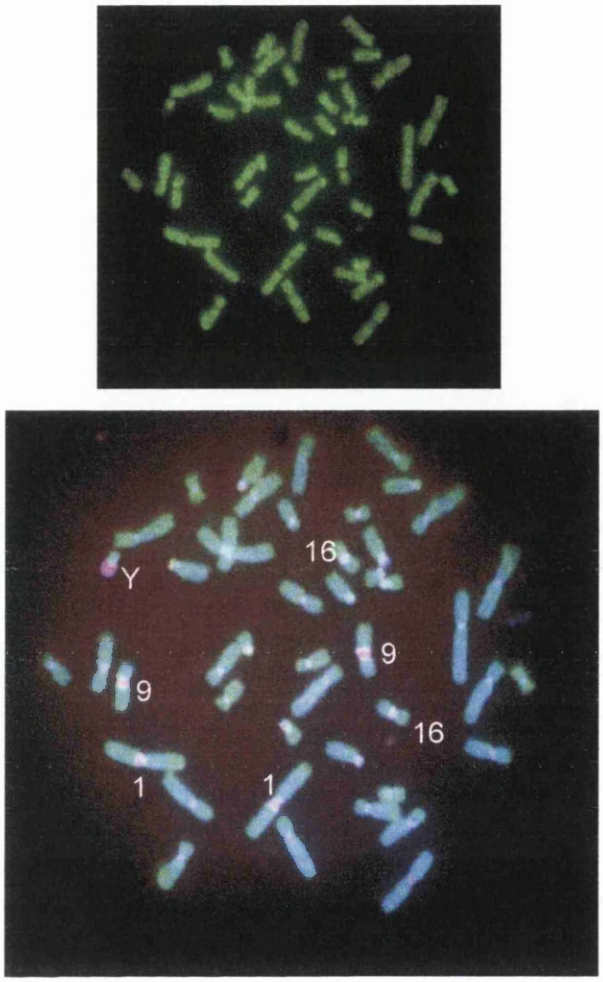


Figure 3.66: CGH images from an overnight protocol. Test DNA was from 30 µl labelling probe, which was prepared from a blastomere. Intense greenish painting was noted after around 21 hours' hybridisation.

3.22 False positives in CGH experiments

False positive findings were a common phenomenon in ratio profile analysis in single cell CGH experiments. Depending on the selection of cut-off values, the number of false positives could vary even when using the same test and reference DNA. Although the theoretical fluorescence ratio cut-off values for trisomy and monosomy in CGH analysis are 1.5 and 0.5 respectively, these values were rarely achieved in real CGH experiments (usually resulting in false negatives at such stringent values). When less stringent cut-off values were set at 1.2 and 0.8 to represent chromosomal gains and losses respectively, smaller copy number changes might be detected at the expense of more false positives. On the contrary, false negatives might occur if a more stringent cut-off value was applied. To minimize false positives, and produce no false negatives, for the targeted chromosome and internal control of X chromosome, adjustment of the cut-off values were retrospectively manipulated to retain the balance between false negative and false positive results (Table 3.5). Ratio profiles using stringent cut-off values would reduce the false positives. When less stringent cut-off values were set at 1.2 and 0.8 to represent chromosomal gains and losses respectively, the average number of false positive regions was 5 (inconclusive cases were excluded).

	Test DNA	Reference DNA	FP	FR*	FP*	CGH results
Single amniocyte						
	47,XX,+21	M	8	0.5-1.5	1	Trisomy 21, female
	47,XX, +21	PCR*-F	6	0.7-1.3	0	Trisomy 21, female
	47,XX, +21	M	17	0.6-1.4	0	Trisomy 21, female
	47,XX, +18	M	0	0.8-1.2	0	Trisomy 18, female
Stained cells						
	47,XY, +18	F	6	0.6-1.4	0	Trisomy 18, male
	47,XX, +21	F	3	0.7-1.25	0	Trisomy 21, female
	47,XX,+21	M	1	0.7-1.3	0	Trisomy 21, female
	46,XY	F	4	0.8-1.3	0	Male
	46,XX	M	0	0.8-1.2	0	Female
	753, female	M	6	0.55-1.45	0	Isochromosome X
	753, female	F	3	0.7-1.3	1	Isochromosome X
	1495, female	PCR*-M	22	0.5-1.5	7	? inconclusive
	1495, female	M	5	0.8-1.25	0	Trisomy 12p, female
	445, female	M	5	0.7-1.3	0	Female
	445, female	M	1	0.7-1.3	0	Female

FP: number of false positives occurring when fluorescence ratio cut-off value was set at 0.8 and 1.2 to represent the chromosomal losses and gains, respectively.

FR*: Retrospective manipulation of stringent cut-off value to result in less false positives and no false negative.

FP*: number of false positives occurring when FR* was set as indicated.

M: SpectrumRed normal male DNA

F: SpectrumRed normal female DNA

PCR*-F: Amplified female DNA.

PCR*-M: Amplified male DNA.

753: 46,X, i (X)(q10)

1495: 46,XY, der (10) t (10; 12)(q26.13, p11.23)

445: 46,XX, del (2)(p12-p13) del (11)(q25-qter) t (2,11)

Table 3.5: CGH results of single amniocytes and stained cells

Chapter 4

Discussion

4.1 Manipulation techniques for single cells

To suit various kinds of research purposes, many studies involving single cell genetic analyses are carried out by testing simplified methods on tissues which are relatively easily available (Beltinger 1997, 1998; Schaaff et al 1996). Prior to real preimplantation diagnosis, a number of studies have also applied their analytical methods on single cells from different sources (Cui et al 1994, Schaaff et al 1996, Wells et al 1999). To retrieve single cells, this study applied heat-pulled micropipettes and simple needles both on cell suspensions and on archival cytogenetic slides. The research aim was to prove the feasibility of single cell genetic analysis using PCR and CGH techniques.

Single cell retrieval is an elaborate and tedious process. To validate that only one cell was retrieved requires stringent negative controls. In comparison, the microdissection used in this study is a relatively simple technique. Using this technique, an approximate number of 10-20 cells can easily be distinguished and isolated. Despite the efforts to completely transfer microdissected cells, small amounts of scraped material may remain on the slides. Incomplete transfer of microdissected materials may not be problematic for allele detection; however, it can lead to false interpretation when global investigation such as CGH technique was applied. For this reason areas of slides containing metaphases was avoided, because the actual chromosome constitution of a sample may not be reflected if only part of a metaphase is included.

In theory, accurate CGH diagnosis is possible only if over 50% of cells transferred remain intact. Correct CGH diagnosis of trisomy 18 and 21 in this study indicates that the current simplified method has successfully transferred at least 5-10 intact cells into the PCR tubes. However, the results may not be accurate if fewer cells are transferred. PCR can be biased particularly when the starting template is from a single cell. Misdiagnosis may occur when part of the genome is damaged by the microdissecting procedure.

Since stained cells are fixed on the slide, microdissection procedures will inevitably damage the cells. So far no reliable method is available to assess whether single cells have been safely transferred into PCR tubes or not. To improve the chance of successful genetic analysis, transfer of more than one cell is preferred. However, a less traumatic design may be required particularly when only one cell is available (Takabayashi et al 1995). The future prospect of applying CGH techniques to a single cell retrieved from a slide is promising if during the transfer the cell genome remains intact.

4.2 Lysis protocols and the effect of cell storage for PCR

Single cell genetic diagnosis may fail due to several causes. One of the hypotheses is a blockade of a particular genomic site due to incomplete lysis. This may explain a proportion of amplification failures and the phenomenon of ADO (Wells et al 1998). The selection of any cell type will theoretically make no difference if the nuclear genome is fully exposed and available for the PCR reaction. However, cell preparation may have an impact for subsequent genetic analysis as the current lysis protocol for fresh cells could not produce similar results from fixative-treated stained cells. Extension of lysis time from 1 hour to one day produced optimal CGH results in the stained cell study. It appears that both the reagents and the time required for cell lysis can affect the PCR outcome.

In this study, samples were selected from fresh amniocytes, frozen amniocytes, buccal cells, fixative-treated lymphocytes, and stained cells. Although one-hour lysis was adequate for fresh single amniocytes, archival stained cells required a longer time. Fresh amniocytes stored in sterile culture medium were the best candidates as they were clean and relatively easy to identify. Any potential lysed DNA in the culture medium could also be eradicated through repeat washes with PBS. Apart from poor PCR performance due to the presence of fixative, cells in fixative suspension were not suitable for cell manipulation for technical reasons. Despite the advantage of easy identification and retrieval, buccal cells had

plentiful debris which might increase the chance of contamination in the DOP-PCR reaction.

It has been suggested that the use of lysis buffer may play a role in decreasing the incidence of ADO. The phenomenon of allele specific amplification failure is at least in part dependent on the lysis buffer used according to one study. El-Hashemite et al (1997) compared two different lysis buffers for PCR performance: KOH/dithiothreitol (DTT) and sodium dodecyl sulphate (SDS)/proteinase K (PK). None of the cells lysed by SDS/PK showed any evidence of allele-specific amplification failure whereas ADO was observed in 4.7% and 17.6% of the HbC and CF cells lysed with KOH/DTT respectively (El-Hashemite et al 1997).

SDS is a strong detergent which disrupts membrane lipids, while proteinase K digests proteins without interfering with the DNA template prior to amplification. In contrast, the contents of alkaline buffer solution may interfere with the DNA template, because KOH will denature DNA and leave it in a single stranded state. Moreover, DTT does not digest protein but breaks the disulphide (S-S) bonds. The denatured DNA and the presence of undigested proteins may lie behind the failure of allele specific amplification, which is not the case with SDS/PK.

For comprehensive chromosome analysis from a single cell, complete removal of DNA-binding protein is necessary as this will help expose the DNA template for whole genome amplification. However, the sub-optimal performance of allele detection may not be entirely associated with lysis buffer. Mechanical damage of the template DNA, the fixative treatment, and the analytical methods may all have influences on PCR performance. Although successful locus detection of single cells is possible by simple boiling (Liu et al 1994), this study prefered inclusion of proteinase K and SDS for removal of nuclear protein prior to WGA. Successful CGH in diagnosing major chromosome abnormalities in this study indicates that inclusion of SDS and proteinase K is an adequate lysis method for current single cell CGH protocol.

It is generally recognised that degraded tissues such as paraffin-embedded samples will contain a large proportion of fragmented DNA which will jeopardise the PCR performance. Further genetic analysis usually fails because of preferential amplification of short products or complete failure of amplification. Nevertheless, it was possible to extract DNA sequences up to 200 bp from paraffin-embedded samples (Gupta et al 1997). The efficiency of single cell PCR from archival pathologic slides revealed 11-25% depending on the size of amplified fragments. The average degree of DNA disintegration in paraffin-embedded, stained tissues gives fragments of around 100 bp (Roehrl et al 1997). For archival cytogenetic slides, Choi et al (1999) reported a 100% detection rate of sex and exon 46 of the dystrophin gene. However, PCR amplification was carried out by scraping all the cells off the slide, and hence this was not performed at the single cell level (Choi et al 1999).

For one single cell, Beltinger successfully combined a simple microdissection procedure and nested PCR on a cell derived from peripheral blood smears (Beltinger et al 1997, 1998). By using ligase-mediated PCR/CGH, Klein et al successfully proved the feasibility of whole genome screening from a metastatic cancer cell in the bone marrow. (Klein et al 1999) This study confirmed that, by using the DOP-PCR/CGH technique, detailed chromosome analysis could be achieved from a starting template of 10-20 stained cells scraped from G-banding slides. Moreover, up to 72% locus detection (i.e. CFTR) was also attained from these samples. As DOP-PCR produced a smear ranging from 200-1500 bp from stained cells, the fragmented sequences encountered from the extracted paraffin-embedded tissues are no longer available for G-banding slides. Compared with the paraffin-embedded specimens, it appears that methanol/acetic acid-treatment causes less damage to the integrity of DNA, and therefore results in a higher success rate for molecular genetic analysis. As the cells used for non-invasive prenatal diagnosis are usually methanol/acetic acid-treated, this will increase the chance of successful

diagnosis. Further work is needed to prove these techniques before they can be incorporated in the field of non-invasive prenatal diagnosis.

4.3 Prevention of contamination

The large number of PCR cycles required for sufficient amplification from a single genome intensifies many of the problems encountered in the routine PCR of relatively large amounts of DNA. With the starting template DNA of one single molecule, the risk of contamination with extraneous DNA sequences is the main problem that must be avoided by implementation of stringent experimental practices.

For PGD, it is recommended to set up single cell reactions in a room designated for this purpose and separated from the laboratory where analyses of PCR products take place. (Wells et al 1998). Some sources of contamination, however, are intrinsic to specific procedures. Extraneous DNA from sperm or maternal cumulus cells is a potential source of contamination. Many sperm may be embedded in the zona pellucida after IVF and may accidentally be sampled along with the blastomeres during embryo biopsy. Based on the poor CGH prediction of manufactured mosaicism in this study, reliable CGH diagnosis is unlikely if contamination occurs at this stage. Although complex mosaicism seems to be a common phenomenon in early embryo development, efforts to screen all the chromosomes using CGH techniques required more studies to confirm its reliability. Currently, the consistency of single cell CGH is still troubled by some technical difficulties such as data interpretations, the problems of false positives, and the tolerance of mosaicism (e.g. contamination). To overcome the hazard of contamination, a combination of DNA fingerprinting prior to single cell CGH may improve the reliability if CGH technique is to be used in PGD. (Kuliev et al 1998; Findley et al 1995)

Precautions to avoid contamination of DOP-PCR protocol have also been mentioned in other reports (Telenius et al 1992a; Speicher et al 1993). In this study, significant contamination is manifested by an intense smear in negative controls, which are indistinguishable from smears derived from

single cells. Throughout the study period involving single cell analysis, two stage DOP-PCR usually resulted in an intense smear which is indistinguishable from the negative control. This long protocol was abandoned in favour of single stage DOP-PCR. Unfortunately, a certain degree of smear was not an uncommon phenomenon in negative controls even in single stage DOP-PCR. Nevertheless, subsequent CGH experiments prepared from the smear of negative controls did not show hybridisation on human metaphases. Based on these experiences, further single cell CGH experiments can be carried on if apparent smear strength was noted between single cell DOP-PCR and negative controls. As DOP-PCR uses degenerate primers to amplify whole genome, certain forms of microorganisms can easily be amplified if containers and solutions are not totally sterilised.

4.4 Whole genome amplification

DOP-PCR is very efficient in producing a great amount of DNA from minute starting quantities. No smear could be detected on an agarose gel after 50 cycles of PEP, whereas an obvious smear was produced by use of DOP-PCR from a single cell. DOP-PCR yielded 10-20 µg of DNA in 50 µl after 28 cycles of DOP-PCR. More than 10 separate PCR experiments could be carried out after primary DOP-PCR amplification and, in theory, limitless experiments could be executed if repeat DOP-PCR using 5 µl of primary DOP-PCR product was performed.

Although variation might occur for individual DOP-PCR reactions, the smear produced usually ranged from 200-2000 bp. The majority of the fragments were less than 1000 bp; however, a wide range of sizes might be occasionally amplified. DOP-PCR amplification from stained cells usually yielded shorter sequences, with the bulk of the smear less than 1000 bp. This variation might be due to degraded DNA or incomplete lysis. All experiments involving two-stage DOP-PCR produced a smear from negative controls. The smear strength could not be differentiated from positive controls. To prevent false amplification from many PCR cycles,

two-stage DOP-PCR involving 60 cycles was not recommended. Preferential amplification of DOP-PCR evidenced by the presence of obvious bands amongst the background smear occurred occasionally. Other researchers had also mentioned the similar findings, which might be caused by extraneous non-human DNA or mitochondrial DNA (Telenius et al 1992; Vollaire et al 2000).

4.5 Genomic coverage and WGA

Several factors may affect the genomic coverage after WGA from a single cell: the degree of cell lysis, the method of WGA, and the strategy of analytical and detection methods. Zhang et al (1992) studied 12 genetic loci and estimated that the probability of amplifying any sequence in the genome to a minimum of 30 copies was no less than 78%.

Based on the number and sizes of DOP-PCR products, there were about one million fragments generated from the entire genome because degenerate primers probably could anneal every 4 kb. It is possible to anticipate that an arbitrary stretch of DNA has a one in six chance of being included in DOP-PCR product, if the average 500 bp sized product is produced in the haploid human genome (i.e. 3×10^9 bp). DOP-PCR usually amplified one-third of the genome, and the reaction is characterised with:

(1) production of non-specific DNA created by non-specific priming
(2) over-representation of highly repetitive DNA
(3) production of overlapping sets of DNA fragments (Cheung et al 1996).

Compared with primers flanking long sequences, primers designed for short fragments usually produce higher detection rates; however, there are significant locus-dependent variations in amplification efficiency for specific WGA protocols (Schaaff et al 1996; Roehrl et al 1997; Wells et al 1998). Therefore, each locus of interest should be tested independently. Wells studied the genomic coverage at various loci (e.g. CFTR, amelogenin, APC, ß-globin, and DNA markers at chromosome 21 and 18) after several

versions of WGA (e.g. PEP, Tagged-PCR, DOP-PCR, Alu-PCR) (Wells D et al 1998). In their report, the PEP and DOP-PCR methods provided the most complete coverage of the genome with 91% and 89% of loci successfully amplified, respectively. Alu-PCR performed worst with only 59% of loci being successfully amplified.

In this study the genomic coverage after DOP-PCR was investigated on two different loci by a strategy of nested PCR. Unlike fluorescent PCR, nested PCR is a relatively easy and cheap method, while its products can be analysed on a simple agarose gel. The results displayed 48% success rate for amelogenin locus and 72% success rate for CFTR locus. Instead of producing both X and Y bands for male cases, the phenomenon of single Y band might indicate that short Y fragments (371 bp) were amplified more efficiently than the long X fragments (555 bp) from DOP-PCR products. The occurrence of single Y band was not reported in the original report in which the sexes were analysed on PEP-amplified-DNA from 102 single amniocytes (Schaaff et al 1996). This demonstrated the altered detection patterns after different WGA methods. The results also indicated that the average size of DOP-PCR products might be too short if longer sequences were to be produced at the first round of nested PCR.

Since DOP-PCR tends to amplify shorter fragments more effectively than longer ones, primers preferably aiming at short fragments may yield higher success rates than those aiming at longer fragments. This may also explain the different performance between the amelogenin locus (555 bp) and the CFTR locus (380 bp).

To improve the length of DOP-PCR fragments, an Expanded High Fidelity PCR (EHF-PCR) system aiming at longer fragments was tried; however, the results did not improve greatly in terms of producing longer fragments. Failure to produce longer products by an EHF-PCR system indicates that the nature of DOP-PCR or incomplete lysis may be the underlying causes which lead to the production of shorter fragments. To improve the success rate of locus detection using nested PCR, further study may focus on developing WGA protocols which will amplify longer fragments (Dietmaier

et al 1999). Other improvements may depend on designing primers aiming at shorter fragments or on using more sensitive detection methods.

4.6 Optimisation of nested PCR for sex determination

A simple and reliable method of sex determination is important for prenatal and preimplantation diagnosis of X-linked recessive diseases. Simultaneous amplification of X- and Y-homologous regions is a convincing tactic because X-chromosomal fragments serve as an internal control for successful amplification. There will be no misdiagnosis due to amplification failure as seen in methods relying on amplification of Y-specific fragments only. A nested PCR strategy using ZFX/ZFY and AMGX/AMGY systems has been successfully applied to the PEP-amplified-DNA from single cells (Kristjansson et al 1994; Schaaff et al 1996).

For sex determination, a nested PCR strategy was applied directly to single cell or DOP-PCR-derived DNA in this study. The concentration of primers and the PCR cycling conditions were performed as the method described by Schaaff et al (1996); however, the results displayed extra bands. The production of unwanted bands indicated that preferential PCR amplification occurred between outer and inner primers during the secondary PCR reaction. Not only did the first PCR products remain but also extra fragments were produced. This phenomenon disappeared when the primer concentration was reduced from 400 nM to 100 nM. Compared with the original methodology, both methods transferred 1 µl of the first round PCR product into the second round PCR reaction mixture. Schaaff's method applied 5 µl (1 tenth volume) PEP products for the first part of nested PCR, whereas 5 µl (1 fifth volume) DOP-PCR products were added to a total volume of 25 µl in this study. Therefore, an excess of outer primer proceeded into the second PCR reaction, and competed with the inner primers.

The ADO phenomenon, which involved the failure of one allele being amplified during the first several cycles of PCR, might occur in male cases

with only a Y band or in male cases being diagnosed as females. Therefore, this might occur in 17.2% (5/29) in this study. ADO generally affected 5-20 % of single cell amplifications (although in some instances the frequency was higher) and could be caused by sub-optimal PCR conditions and rapid degradation of the target DNA during thermocycling (Ray et al 1996; Wells et al 1998).

4.7 Mutation detection on amplified DNA

The advantage of using WGA in single cell genetic analysis is that more than one locus can be analysed from the same cell and repeated aliquots may be taken for independent tests in the event of ambiguous results (Ao et al 1998). This strategy also helps monitor ADO in dominant conditions (Ray et al 1996). For a recessive condition ADO will not lead to serious misdiagnosis because the affected embryo will still be detected as such. However, it may lead to failure to detect the abnormal mutant allele in dominant conditions. By analysing the mutation and polymorphism, the risk of misdiagnosis resulting from ADO at a single locus is significantly reduced.

In this study, the common 3 bp CF Δ508 deletion could be detected by nested PCR after DOP-PCR. Similar results were also achieved by using one-stage PCR. The underlying causes for detectable products from one-stage PCR might lie on the fact that longer PCR cycles were used and the shorter sequences were amplified.

4.8 Optimisation for single cell CGH

4.8.1 Selection of WGA for single cell CGH

Various WGA protocols have been applied in the cloning of microdissected chromosomes, single cell genotyping and preimplantation multi-locus analyses. These include DOP-PCR, prime-extension preamplification (PEP), Alu-PCR, Tagged-PCR, and restriction enzyme digestion followed by ligation-mediated PCR.

To the best of knowledge in 2000, there have been no successful CGH results using PEP protocol as the amplification method from minute DNA, whereas reverse painting generated by DOP-PCR from chromosome microdissection has been reliably established by a number of studies (Speicher et al 1993; Meltzer et al 1992; Viersbach et al 1994). In this study, WGA using the PEP protocol was abandoned because of its inefficiency in producing adequate amount of DNA from single cells. In contrast, DOP-PCR proved to be an efficient technique involving multiple priming, which allows a more general amplification than other methods. Two rounds of DOP-PCR can yield 10 μg probe which allows more than five separate CGH experiments. As the current CGH technique is still limited by several technical factors, this productivity will help to re-confirm the diagnosis if the initial CGH experiments fail or produce equivocal results.

Apart from high yields of DOP-PCR, this study also demonstrated that uniform signals without any preference for centromeric and telomeric regions could be produced from single cell DOP-PCR/CGH protocol. However, the centromeric and telomeric regions are mostly stained with red colour when amplified DNA is co-hybridised with non-amplified DNA. As the experiments use more test DNA than reference DNA, the underlying cause due to the disproportion between amounts of test and reference DNA is unlikely. The best explanation may indicate DOP-PCR does not amplify preferentially over these regions from single cells.

Several other WGA protocols have been investigated in order to compare their reliability in producing uniform CGH images. Alu elements are preferentially found in the G-light bands of human chromosomes. Chromosome paints amplified by Alu-PCR showed a banding pattern (Telenius et al 1992). According to Wells' study, single cell CGH produced from Alu-PCR showed extreme bias towards regions rich with Alu repeats with low CGH sensitivity in the Alu-deficient areas (Wells et al 1998). Tagged-PCR could give accurate single cell CGH results, but displayed a disproportionately high level of hybridisation to centromeric and telomeric

regions and reduced signal intensity elsewhere on the chromosomes (Wells et al 1998).

4.8.2 Target metaphases

Selection of high-quality slides is crucial to the success of CGH experiments. Although proteinase K can remove nuclear protein, cytoplasm and debris, reducing the background and increasing probe penetration, this treatment did not significantly improve CGH results in this study. In addition, it may also diminish the quality of chromosomal morphology and banding patterns. Therefore, it is preferable to select metaphase slides containing little or no cytoplasm or debris rather than treating the slides with proteinase K.

Kahru et al (1997) developed a rapid denaturation test to identify sub-optimal metaphase spreads without having to perform unnecessary, lengthy, and expensive hybridisation procedures. Optimal metaphase slides have to tolerate denaturation to achieve complete uniform hybridisation along all chromosomes. Denaturation may have been excessive if a C-banding pattern emerges during the denaturation. Unfortunately, failed CGH experiments did still occur in this study despite the careful selection of metaphase slides both by morphology and by rapid denaturation tests.

Many of the unsuccessful CGH experiments could have been attributed to poor metaphase quality when slides were homemade in the early stage of this study. It appeared that metaphase preparations ideal for G-banding analysis were not necessarily optimal for CGH experiments. Although the number of successful CGH experiments increased when commercial slides were used, the quality of metaphase slides remained one of major obstacles in performing single cell CGH.

Commercial slides were convenient and usually presented with adequate numbers of metaphase spreads. However, the same morphological criteria and quality assessment should be evaluated prior to CGH experiments because some of these batches were not necessarily of uniform standard

and high quality. Although denaturation at 75°C for 5 minutes was generally adequate for these slides, some batches required modification at lower temperature for less time (e.g. 73°C for 3-4 minutes). One-colour hybridisation might help select appropriate slides prior to real two-colour CGH.

So far the only certain criterion for slide quality is through performing an actual CGH experiment. The preparation and testing of slides for ideal CGH experiments is a labour-intensive process. Commercial slides afford a better choice for beginners or a laboratory where metaphase slides are not regularly produced. It also shortens both preparation time and the learning curve particularly for a difficult procedure such as CGH from minute DNA.

4.8.3 Labelling procedures

DNA was labelled indirectly in the early days of CGH development. Reporter molecules such as biotin or digoxigenin were detected via fluorochrome-coupled reporter binding molecules (Kallioniemi et al 1992). The major advantage of the indirect labelling method over the direct one is the strong intensity of hybridisation signals obtained by indirect labelling through several layers of signal amplification. Compared with indirect labelling, deoxynucleotides that have been directly conjugated with fluorochromes render a smooth but weak hybridisation signal along the chromosomes. This disadvantage can be overcome by the use of a sensitive CCD camera and high power illumination such as 100 W mercury lamps. The direct labelling method has become more popular because the procedure is simpler and requires less time. The accumulated experiences in this laboratory also show that direct labelling is a simple method and can yield intense hybridisation signals, low background and homogeneous coverage of target chromosomes.

One of the main applications of CGH to date has been in the field of malignancy. Most studies use tumour DNA as test DNA and normal total genomic DNA as reference DNA. These non-amplified DNA sources are

further labelled by nick translation. Following the guidelines described by Kallioniemi et al (1994), the CGH results were usually inconsistent. However, the conditions improved when the amount of fluorochrome-conjugated nucleotide was raised from 2 μM to 4 μM, and the DNA amount was increased from 200 ng to 1 μg. Hybridisation artefacts across the genome can be inherent to specific fluorochromes. It may reflect an underlying difference in the way green and red molecules bind to DNA, or it can be related to a differential affinity of the fluorochromes to certain types of DNA families not homogeneously spread throughout the genome (Larramendy et al 1998). To produce a hybridisation with signal intensity equivalent to both SpectrumGreen and SpectrumRed labelled DNA, the commercial guidelines recommend the co-hybridisation of 200 ng SpectrumGreen to 100 ng SpectrumRed in a CGH experiment. Although the precise mechanism underlying hybridisation and labelling is not clear, interchanging the labels between test and reference DNA in CGH has been suggested as a method to confirm the CGH results (Kallioniemi et al 1994; Larramendy et al 1998).

In various conditions involving small amounts of DNA, amplification of the starting DNA to a detectable level is a requisite for probe preparation. For amplified DNA, further labelling can be achieved either by nick translation or using a PCR method. Wells et al (1998) used a PCR method to label amplified-DNA and reported accurate diagnosis of major aneuploidies from several cell types such as amniocytes, fibroblasts and buccal cells. However, Voullaire et al (1998) used nick translation to label amplified-DNA and also reported reliable results in predicting common aneuploidies such as trisomy 13, 18, and 21 from single amniocytes. The single cell CGH results in this study also demonstrated that common aneuploidies (e.g. trisomy 21, 18, partial trisomy and sex chromosome aberration) could be reliably diagnosed by PCR labelling.

So far these limited reports have demonstrated that both labelling methods can produce accurate CGH interpretation from a single cell. However, the advantage of the PCR method over nick translation is the feasibility of

automation. In nick translation, parameters such as reaction time, temperature, and probe sizes require close monitoring, whereas PCR labelling can be applied automatically in a PCR thermal cycler.

Other reports claimed that amplification of genomic DNA with standard Taq DNA polymerase yielded poor amplification (Kuukasjarvi et al 1997). By using T7 DNA polymerase in their study, larger DNA fragments could be amplified from minute DNA in the primary DOP-PCR reaction. Fluorochromes could be added in the subsequent high stringency amplification steps. Alternately, if unlabelled DOP-PCR products were produced in the primary DOP-PCR reaction, the unlabelled DOP-PCR products could be labelled by PCR method or nick translation. However, the result of this study and others confirm that appropriate probes could be produced by using standard Taq polymerase (Vollaire et al 1999; Wells et al 1999). Although an EHF-PCR system aiming at amplifying larger fragments was tested in this study, the results were not superior to those obtained by standard Taq polymerase. Moreover, the yield tended to diminish. Longer DNA fragments might be achieved in PCR labelling using an EHF-PCR system; however, similar results were also obtained by using Taq polymerase with increased magnesium concentration.

Fluorochrome-conjugated nucleotides can be added to the primary DOP-PCR amplification reaction. However, this will interfere with the capability of DOP-PCR amplification from a single cell. Apart from this study, two-stage DOP-PCR is preferred by most other researchers so far (Wells et al 1999; Kuukasjarvi et al 1997). DNA can be labelled with various fluorochrome-conjugated nucleotides by nick translation, random priming or using temperature cycled amplification methods. In all instances, a fraction of input dTTP is substituted with fluorochromes-conjugated-dUTP. Instead of using 2.5 µl fluorochrome-conjugated nucleotides as described in Wells' report, a substitution ratio of 20% (2 µl) fluorochromes-conjugated-dUTP relative to dTTP also worked reliably in this study (Wells et al 1999).

The distribution of probe size has been stressed in many reports. Similar size distribution between test and reference DNA was important because this will allow, in theory, an unbiased competition on target chromosomes between test and reference DNA. Discrepancy of probe size may lead to biased competition between green and red probes and produce false results. However, no reliable method can be applied so far in accurately quantifying the incorporation rate of fluorochromes. Only a tiny percentage of fluorochromes (1-3% of total nucleotide) are incorporated into the probes. In addition, the size distribution of probes cannot be accurately quantified by current methods. Therefore, the optimal size distribution generated by a second DOP-PCR should only be viewed as a requisite to carry on further CGH experiments and not a guarantee for successful results.

4.9 Homo-hybridisation and hetero-hybridisation

During the optimisation of single cell CGH, reference DNA was prepared from either amplified DNA (homo-hybridisation) or non-amplified DNA (hetero-hybridisation). Theoretically, reference DNA for single cell CGH should be prepared from a matched number of single cells. However, such a strategy of homo-hybridisation usually produced high failure rate and inconsistent results in this study. The underlying causes might be due to the difficulties involved in single cell manipulation or single cell PCR errors. Furthermore, incomplete lysis plus preferential amplification through many cycles of random priming could also result in part of the genome being either over- or under-represented. The situation deteriorated when both test and reference DNA were prepared by PCR from single cells. This theory was supported by the observation that CGH resulted in unreliable prediction when test DNA prepared from 20 aneuploidy stained cells was co-hybridised with reference DNA prepared from 20 normal stained cells. However, correct CGH interpretation was made when such test DNA was co-hybridised with non-amplified reference DNA.

Since the development of CGH in 1992, the procedure has been plagued with technical limitations and difficulties. Among these, DNA copy number changes in chromosomes 1p, 16p, 17p, and in the whole chromosomes 19 and 22 are considered difficult to interpret, leading to false-positive observations (Kallioniemi et al 1994). A similar observation was also noted in this study and in other reports when both test and reference DNAs were prepared from amplified DNA (Vollaire et al 1999; Wells et al 2000). However, when reference DNA was prepared from non-amplified DNA, the CGH results did not show this phenomenon. Different DNA preparation and complex hybridisation dynamics could have been the factors responsible for the hybridisation artefacts. It might also reflect an underlying difference in the way FITC and rhodamine molecules bind to DNA molecules, or it could be related to a differential affinity of the fluorochromes to certain types of DNA families not homogeneously distributed in the genome, such as the GC rich areas or small interspersed repeated sequence elements (Larramendy et al 1998).

Huang et al (2000) developed a reproducible DOP-PCR-CGH protocol by evaluating different labelling methods and different hybridisation mixtures (including amplified test DNA vs. amplified reference DNA, termed homo-hybridisation; and amplified test DNA vs. non-amplified reference DNA or vice versa, termed hetero-hybridisation). In their conclusion, a DOP-PCR-CGH homo-hybridisation method, especially when combined with labelling by nick translation, was reliable and reproducible (Huang et al 2000). Although correct single cell CGH interpretation was achieved in a case of trisomy 21 using homo-hybridisation, other homo-hybridisation experiments usually displayed considerable variations with numerous false gains and losses in this study. Compared with homo-hybridisation, hetero-hybridisation produced higher success rate and reliable single cell CGH interpretations in this study. The cause of the different results is not clear, although it may be due to different methodology in terms of DNA preparation and labelling methods.

Single cell CGH using hetero-hybridisation produced accurate prediction for major chromosome abnormalities (e.g. trisomy 21, 18, partial aneuploidy and sex chromosome anomaly) in this study. Apart from increased success rate, a reliable non-amplified reference DNA also help to identify the underlying causes which might lead to failed single cell CGH (e.g. single cell PCR errors, sub-optimal quality of metaphase slides, improper temperature or time for chromosome denaturation).

Preparing reference DNA from single cells was a laborious process and requires tedious assessment to ensure probe quality. In comparison, preparing reference DNA from genomic DNA is relatively straightforward. Similar products are also commercially available. Considering the high failure rate and poor CGH prediction related to homo-hybridisation, preparing reference DNA particularly from a matched number of single cells is not recommended.

4.10 Qualitative and quantitative analysis of single cell CGH

Qualitative assessment by inspecting all CGH images is mandatory prior to quantitative ratio profile analysis. Guidelines for accepted images have been described by a number of studies (Kallioniemi et al 1994; Kallioniemi et al 1996). Using the current protocol of hetero-hybridisation, pericentromeric regions of chromosome 1, 9, 16 are usually painted with red colour. This may suggest these areas tended not to be amplified in the DOP-PCR reaction. Lapierre et al (1998) reported the possibility of detecting numerical trisomy without the need of digital analysis system. In this study obvious colour change of chromosome 18, 21 and sex chromosomes can also be differentiated by the naked eye from most single cell CGH images involving trisomy 18, 21, and mismatched sex, respectively. However, colour intensity cannot be differentiated from separated green or red images in trisomy cases. Changes in copy number of tiny chromosomal regions however require the utilisation of a CGH software programme for detailed analysis. Therefore it is better to rely on

computer software rather than the naked eye in the interpretation of CGH. This would be mandatory in a diagnostic setting.

For quantitative ratio profile analysis, the most widely used thresholds are based on fixed cut-off values. Others use statistical concepts to set the definition of diagnostic thresholds (Du Manoir et al 1995b, Moore et al 1997). The choice between the two approaches may strongly influence the number of gains and losses detected. Barth et al (2000) recommended the use of a fixed-threshold procedure because of its higher reliability of positive test results, even at the cost of not detecting all possible aberrations.

This study manipulated a flexible threshold value to evaluate the risk of false positive results in single cell CGH. In the initial stage, when trying to test the feasibility of single cell CGH, each experiment was accomplished by mismatching sexes between test and reference DNA. Consequently, the fluorescent intensity change of the X chromosome and the designated chromosome in trisomy cases could be viewed as an internal control for optimal CGH results. Although the theoretical fluorescence ratio (FR) cut-off value for trisomy and monosomy in CGH analysis is 1.5 and 0.5 respectively, these values were rarely achieved in real CGH experiments, which usually resulted in false negatives at such stringent values. To minimize false positives, and produce no false negatives, for the targeted chromosome and internal control of X chromosome, adjustment of the cut-off values were manipulated to retain the balance between false negative and false positive. Ratio profiles using stringent cut-off values would reduce the false positives. When less stringent cut-off values were set at 1.2 and 0.8 to represent chromosomal gains and losses respectively, the average number of false positive regions was 5. The problems of false positives were not widely reported in the clinical-associated CGH reports. Further investigation is required in order to clarify the balance between the false positives and the risk of missed detection.

4.11 Applications and limitations of DOP-PCR/CGH

4.11.1 Detailed chromosome analysis for early embryo development

So far two CGH studies involving blastomeres have demonstrated around 75% chromosomal aberrations in early embryo cells (Voullaire et al 2000, Wells et al 2000). The proportion of totally normal embryos seen in Voullaire's study (25%) is slightly lower than other groups using a FISH technique (Delhanty et al 1997, Munne et al 1998b). As CGH allows a much more extensive analysis of the karyotype than is possible with interphase FISH analysis, this result is to be expected (Voullaire et al 2000). In Wells' report, the CGH karyotype of blastomeres also showed a high level of mosaic and chaotic results. Only 3 of 12 embryos studied showed a normal CGH karyotype (Wells et al 2000).

Numerous chromosome gains and losses were also noted in this study involving a single blastomere. Although DOP-PCR/CGH from single blastomeres of human cleavage embryos appears to be a reliable technique for the determination of whole and partial aneuploidy (Voullaire et al 2000), verifying the CGH results of blastomeres with smaller duplications or deletions is extremely difficult. Currently, most studies report the use of inverse CGH, FISH, microsatellite and LOH analyses to validate CGH findings. However, it is impractical to confirm numerous gains and losses in early embryo cells. Besides, the accumulated experiences in CGH studies show that a number of technical difficulties (e.g. the identification of chromosomes, the choice of cut-off threshold, and the problem of false positive or false negative findings) will further complicate the CGH interpretation of blastomeres, which can have such an enormous spectrum of chromosome aberrations. Utilizing ultra sensitive charge-coupled device detection of labelled probes in a miniaturized format (DNA chip technology) may help to determine the wide spectrum of abnormalities currently encountered in the study of early embryos.

PGD and transfer of embryos is usually completed within 24 hours. This limited time window can be extended if embryos are frozen during the

PGD process. Technical advances in the culture of human embryos to the blastocyst stage will allow an additional two days in which to complete CGH. Current single cell DOP-PCR-CGH protocols take around 72 hours to perform, with this length of time hampering its application to PGD. By increasing the amount of test and reference DNA, an overnight CGH protocol produces intense hybridisation signals similar to those from the conventional protocols. The preliminary results in this study illustrated that a total time of less than 1 ½ days for the DOP-PCR/CGH protocol is possible. The implications of an ultra-rapid protocol for whole chromosome painting (WCP) probes may indicate further reduction of time requirement for CGH is possible (Liu et al 1998b). Combining the advance in techniques which allow an extended time window for PGD and a short protocol, DOP-PCR/CGH may well be applied in PGD in the future.

4.11.2 Potential application for non-invasive prenatal diagnosis

It has been estimated that around 10-20 fetal erythroblasts can be identified from mid-trimester maternal blood, and these cells may provide a source for various kinds of genetic analyses (Kuo et al 1999). In order to simulate the potentially retrievable fetal cells in maternal blood and prove the feasibility of using these cells for multi-purpose diagnosis, this study investigated and successfully proved the performance of DOP-PCR/CGH on cell clusters containing 10-20 stained cells scraped from G-banded slides.

Sex chromosome aneuploidy is among the most common chromosome abnormality present at birth. Its incidence at amniocentesis is even greater and is estimated to be 1/250 in woman over 35 years of age (Ferguson-Smith et al 1984). Therefore, the possibility of sex chromosome aneuploidy should always be covered for any modern technique involved for prenatal genetic diagnosis. In this study, successful application of DOP-PCR/CGH in the diagnosis of isochromosome Xq from stained cells demonstrated that these techniques are reliable and could be used as a supplement for current prenatal diagnostic procedures (Lapierre et al

2000) and non-invasive prenatal diagnosis in the future. Compared with Xq, the hybridisation over Xp showed less green when test DNA was co-hybridised with normal female reference DNA. Although this was indicative of deletion on Xp, further hybridisation with normal male reference DNA confirmed the diagnosis. The resultant hybridisation showed distinctive red colour over Xp; therefore, unequivocal diagnosis of isochromosome Xq was made. It is generally recommended that normal female reference DNA is used in the CGH experiment as Y chromosome provides less genetic information than X chromosome (Weiss et al 1999). However, it is worthwhile co-hybridising prenatal test DNA with both sexes as the result will provide more information of sex chromosomes and confirm the diagnosis of sex chromosome aneuploidies.

Similarly the correct diagnosis of trisomy 21 and 18 from a few stained cells in this study also proved that DOP-PCR/CGH is a reliable technique for diagnosis of complete aneuploidy. However, the correct diagnosis of partial trisomy 12p required a stringent cut-off value because false positives occurred if less stringent value was set. In retrospect, a consistent greener colour was noted over chromosome 12p using global analysis; however, this result was not conclusive during the initial CGH evaluation because several other chromosome regions also presented with gains. It would be preferable to re-confirm the diagnosis using another technique (e.g. FISH) if extra cells are available. The occurrence of false positives commonly encountered in the CGH experiments remained an issue to be resolved. Although CGH is a powerful tool for universal screening of whole genome in various research areas, it can only be viewed as supplementary tool for prenatal diagnosis at present.

4.11.3 Limitation of DOP-PCR/CGH

The resolution of CGH from using DOP-PCR-amplified DNA has not been well verified in the literature. Successful detection of trisomy 21 in this study and other reports shows that resolution power is at least 60 Mb for DOP-PCR/CGH techniques involved with single cell genetic analysis

(Voullaire et al 1998, Wells et al 1997). The results of the current study demonstrate that complete or partial aneuploidies can be detected using DOP-PCR/CGH techniques. However, further studies are needed to confirm the resolution limit of using this technique for single cell genetic analysis.

CGH has limitations in detecting genetic abnormalities such as balanced translocations, ploidy change, small intragenetic rearrangements and low level mosacism. It is also unable to resolve the chromosome sequence copy changes smaller than 10 Mb (Bents et al 1998). To increase the sensitivity of CGH, a high resolution modification makes it possible to detect submicroscopic deletions or duplication but the method does not permit precise identification of breakpoints and cannot detect balanced rearrangements (Jens Pedersen-Bjergaard 2001). In the future, an array of large-insert genomic clones placed in the exact genomic order on a glass slide (DNA chips) might replace the metaphase chromosomes as a hybridisation target and substantially increase the resolution of the CGH technique (Solinas-Toldo et al 1997, Pinkel et al 1998).

It has been generally recognised that CGH can detect copy number changes if more than 50% of cells analysed contained chromosomal gains or losses (Kallioniemi et al 1994). Although some researchers had reported the possibilities of detecting lower mosaics, these studies used FISH or conventional cytogenetic counting to decide the mosaic level and CGH experiments were not carried out at single cell level (Lesto et al 1999; Gaffari et al 1998). To simulate the sub-optimal identification of fetal cells from maternal blood (i.e. maternal cell contamination), this study manufactured 50-70% mosaics by mixing male aneuploidy cells with normal female cells. At 70% trisomy 18 mosaic levels, this study was unable to detect the trisomy 18 and sex using the current protocols. Preferential amplification of normal female cells may be the underlying cause; however, incomplete lysis of aneuploidy cells could not be ruled out. The CGH tolerance of maternal cell contamination in detecting common fetal aneuploidy remains unknown. Failure to detect aneuploidy

from manufactured mosaicism illustrates that certain prerequisites should be met if the strategy of pooling erythroblasts for DOP-PCR/CGH diagnosis is to be used in the field of non-invasive prenatal diagnosis. In the future, this may necessitate the improvement in a specific antibody for fetal erythroblasts detection or the inclusion of DNA fingerprinting prior to DOP-PCR /CGH (Wells et al 1998).

4.12 Multi-purpose diagnosis from single cells

There is an increasing demand for fetal karyotyping due to the prevalence of maternal serum screening for common aneuploidies, increasing ultrasound use in characterising chromosomal anomalies (e.g. nuchal translucency thickness) and the tendency for elevated maternal age. It is also increasingly common as molecular pathologies are pinpointed for a family with a history of a single gene disorders to have a need for genetic analysis as well. However, the use of such diverse strategies in the field of preimplantation genetic diagnosis and non-invasive prenatal diagnosis has not been developed since only one or several single cells are available for diagnosis. Current prenatal diagnosis is based mainly on conventional cytogenetic karyotyping, polymerase chain reaction (PCR) or fluorescence in situ hybridisation (FISH). When analysis is for a specific locus by PCR on a single cell, this has the drawback of sacrificing further detection of various chromosomal abnormalities.

To date, FISH using centromeric-specific probes, can detect common aneuploidies such as trisomy 21, 18, or 13, and abnormal sex chromosome constitution simultaneously (Bischoff et al 1998). In addition to the possibility of cross hybridisation occurring between some chromosomes, simultaneous detection of more than 5 colours is costly and technically demanding. To broaden the diagnostic spectrum, various strategies including recycling of single cells, multicolour FISH, and whole genome amplification followed by specific locus detection have been reported (He et al 1999; Marquez et al 1998; Wells et al 1998). This study also investigated the feasibility of concomitant diagnosis at both molecular

and cytogenetic levels using DOP-PCR/CGH and nested PCR (Figure 4.1). Using this strategy, numerous specific loci and the copy number of every chromosome could theoretically be assessed from a single cell.

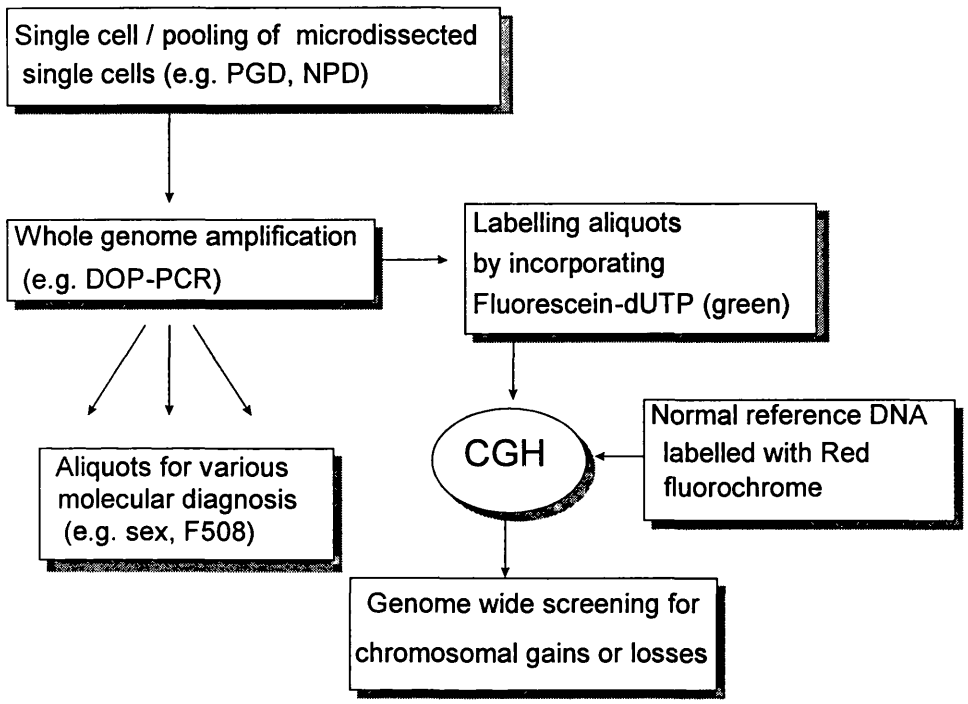


Figure 4.1: Combining WGA and CGH for various loci detection and genome wide screening of chromosomes.

4.13 Conclusions

The methods described in this study suggest a novel strategy for diagnosis encompassing both molecular and cytogenetic analysis. By using a combination of DOP-PCR, nested PCR, and CGH, a wider range of genetic analysis is possible from a single cell. Simultaneous detection of sex, cystic fibrosis mutation status and diagnosis of chromosomal gains or

losses across the whole genome from a single cell is possible. This would increase the diagnostic scope of current clinical practice.

Although single cell CGH is a lengthy and relatively difficult technique, the success rates were optimised when reliable metaphase slides and non-amplified reference DNA were used. The results of current studies involving single cells, the quantitatively accurate CGH data and the fidelity at the specific sequence level, may pave the way for further application of this technique in the field of non-invasive prenatal diagnosis and preimplantation genetic diagnosis.

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