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Molecular and Cytogenetic Approaches to the Analysis of Chromosomes in Human Preimplantation Embryos.

by

Danny Diamantis Daphnis

A thesis submitted for the degree of
Doctor of Philosophy
at the
University of London.

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To my mother, brother, mum and father for believing in me.

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Abstract

The original focus of the research for this thesis was concentrated on establishing strategies to detect chromosome imbalance as well as exploring the phenomenon of mosaicism and its underlying mechanisms in human preimplantation embryos. High levels of chromosomal mosaicism have been detected in human preimplantation embryos mostly by fluorescent *in situ* hybridisation (FISH) but also by comparative genomic hybridisation (CGH) and karyotyping. Mosaicism could arise through several mechanisms including abnormal cell divisions (mitotic non-disjunction or anaphase lag), failure of cytokinesis or endoreduplication. The FISH procedure has been criticised, as it is prone to failure. Two separate studies were developed and carried out in order to detect the level of mosaicism in embryos.

In the first study a FISH protocol for the use of two different probes per chromosome was developed. The aim was to gain information on mechanisms leading to aneuploidy mosaicism and its true incidence. Three colour FISH was performed in three sequential rounds. In the first and second round different probes were used for chromosomes 1, 11, 18. In the third round probes were used for chromosomes X, Y and 18. Each FISH procedure included a control slide to assess FISH efficiency in all rounds of FISH. Two groups of embryos were spread on day 5 of development; embryos grown in cleavage media throughout and embryos transferred to blastocyst media after day 3. A total of 21 embryos were analysed in each Group. The FISH results revealed one uniformly diploid and 20 mosaic embryos for Group I and 2 uniformly diploid and 19 mosaic embryos for Group II. Use of 2 different probes per chromosome was able to detect FISH artefacts and failure of hybridisation. Post-zygotic chromosome loss was the predominant mechanism leading to aneuploidy mosaicism for both groups, followed by chromosome gain, with only a few examples of mitotic non-disjunction. The relatively high percentage of tetraploidy in the blastocyst medium group was considered to reflect normal embryonic development.

The use of CGH was investigated as an alternative strategy to detect the true level of mosaicism in the whole genome. The second part of the research for this thesis involved assessing the efficiency of CGH, improving the protocol for optimised use on single cells, and its application to human embryonic material. Results suggested

that CGH is a laborious and technically demanding technique however, can provide extra information when used as a research tool. CGH was combined with FISH in order to assess chromosomal abnormalities in day 3 and day 5 embryos respectively. CGH was employed in 1-2 biopsied cells from a day 3 embryo, which was grown up to day 5 and further analysed by multi-colour FISH. The aim of this study was to observe the full chromosomal status of 1-2 blastomeres biopsied at the cleavage stage (day 3) of development followed by FISH analysis of the rest of the embryo on day 5. This would allow the assessment of abnormalities in day 3 embryos by a full karyotype and then confirm whether the abnormality persists until day 5 using FISH for the chromosome(s) involved. In summary 30 embryos were fully analysed and only 3 (10%) were uniformly normal, while the rest were mosaic or chaotic. CGH was able to provide results in 83.3% of the embryos subjected to analysis. FISH and CGH showed either agreeing or complimentary results for all embryos analysed. The predominant mechanism of aneuploidy mosaicism was whole chromosome loss. Furthermore, partial aneuploidy was also detected, with partial chromosome loss being the principal mechanism.

In the final part of the thesis the development of PGD protocols for a single gene disorder, namely DM, were devised using polymerase chain reaction (PCR) techniques. Two PGD protocols were devised and employed clinically in two patients undergoing PGD for DM using fluorescent PCR. Due to the extensive workup needed to develop the specific PCR protocols for each patient, a universal-like protocol was researched. Such a protocol would involve production of a sufficient amount of DNA through whole genome amplification techniques i.e. DOP-PCR from a single cell to carry out subsequent analysis with F-PCR markers as well whole chromosome analysis using CGH. DOP-PCR amplified DNA was subjected to amplification of five markers that would have been used during a PGD workup for DM and also subjected to CGH analysis. Initially genomic DNA was tested which produced high fidelity of amplification. Single cell DNA was then utilised in order to assess the amplification rate, allele dropout (ADO) and contamination levels. It was shown that there was relatively low amplification and ADO rates of the five markers at the single cell DNA level compared to the results obtained when the markers were amplified directly from single cells during the development of the two F-PCR PGD protocols. However, CGH

analysis was successfully performed indicating that novel WGA methods might overcome the problem of low fidelity of the F-PCR markers.

In conclusion, several molecular and cytogenetic techniques were employed to analyse human embryos either to provide answers to phenomena such as aneuploidy mosaicism and chaos or to select normal embryos in a clinical setting for PGD for patient carrying single gene defects.

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List of Abbreviations

μg	microgram
μl	microlitre
μM	micromolar
6-FAM	6-carboxyfluorescein
AMA	advanced maternal age
ARMS	Amplification Refractory Mutation System
ART	assisted reproduction techniques
bp	base pair
CCD	coupled charged device
CGH	comparative genomic hybridisation
cm^3	cubic centimetre
CPM	confined placental mosaicism
CVS	chorionic villus sampling
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DM	Myotonic dystrophy
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylene-di-amine-tetra-acetic acid
FBS	fetal blood sampling
FISH	Fluorescent *in situ* hybridisation
FSH	Follicle-stimulating hormone
g	gram
h	hour
HA	heteroduplex analysis
HEX	4,7,2',4',5',7'- hexachloro-6-carboxyfluorescein
HFEA	the Human Fertilisation and Embryology Authority
GnRH	Gonadotrophin-releasing hormone
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IU	international unit
IVF	*in vitro* fertilisation

Kb	kilobase
LH	Luteinizing hormone
MCC	maternal cell contamination
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MNB	multinucleated blastomeres
N	normal
NP-40	nonidet-40
NT	nuchal translucency
PB	polar body
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnosis
pmol	picomole
pM	picomolar
PZD	partial zona dissection
QF-PCR	quantitative fluorescent polymerase chain reaction
RFLP	restriction fragment length polymorphism
RIF	recurrent implantation failure
RM	recurrent miscarriage
RNA	ribonucleic acid
rpm	rounds per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
sec	second
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SSCP	Single Strand Conformational Polymorphism
SSC	standard saline citrate
STR	short tandem repeat

TAMRA	N, N, N', N' – tetramehtyl-6-carboxyrhodamine
Taq	*Thermus Aquaticus*
TE	trophectoderm
TET	4, 7, 2', 7' – tetrachloro-6- carboxyfluorescein
UPD	uniparental disomy
WGA	Whole genome amplification
YAC	yeast artificial chromosome
ZP	zona pellucida

CHAPTER 1

Introduction

1.1 Human Development

Humans, compared with other species, display low fecundity since the chance of conception in any ovulatory cycle is low, being 25% for women under 35 who are trying to conceive and are of proven fertility (Wilcox *et al,* 1988). Despite the advent of *in vitro* fertilisation (IVF) in 1978 (Steptoe and Edwards, 1978) and the major advances in reproductive medicine, there still remains a relatively high failure rate after embryo transfer (Wells and Delhanty, 2000).

1.1.1 Gametogenesis and Meiosis

Gametogenesis is the process of meiosis and cytodifferentiation that converts germ cells into mature male and female gametes (Larsen, 1997). During the fourth week of embryonic development primordial germ cells differentiate within the yolk sac and actively migrate to the posterior body of the embryo. These cells populate the developing gonads and differentiate into the gamete precursor cells which are known as spermatogonia in the male and oogonia in the female. When these cells produce gametes, the process is known as gametogenesis (spermatogenesis in the male and oogenesis in the female).

Meiosis involves a single round of DNA replication followed by two successive chromosome segregations; meiosis I and meiosis II. Meiosis I involves chromosome pairing and recombination between non-sister chromatids, and yields two haploid daughter cells. In meiosis II the duplicated chromosomes divide, yielding four haploid daughter cells. The chromosome number is restored to its diploid state with the fusion of the male and female gametes at fertilisation. The recombination of homologous chromosomes during meiosis leads to exchange of material between maternally and paternally derived chromosomes. This process called crossing over is the source of new combinations of genes in the next generation.

1.1.1.1 Spermatogenesis

Spermatogenesis is a unique process of continuing differentiation since the DNA content of the product is half that of the progenitor cells (Cooke *et al*, 1998). Furthermore, spermatogenesis is a non-stop process involving many mitotic divisions,

possibly around 20-25 per annum, and is initiated at puberty. Spermatogenesis is a relatively rapid process with an average duration of 60-65 days and most steps are closely controlled by a hormone called testosterone. In the initial stages, spermatogonia undergo mitotic divisions, giving rise to primary and secondary spermatocytes, the cell type in which the first and second meiotic divisions occur. The haploid products of meiosis are round spermatids, which elongate during spermiogenesis and with the aid of the Sertoli cells they compact their chromatin into the sperm head and produce further sperm components. Spermiogenesis, a process of sperm cell differentiation, requires about 24 days (Metz and Monroy, 1985; Eddy and O'Brien, 1993). A spermatozoon consists of a head, a midpiece and a very long tail. The head contains the condensed nucleus and is capped by an apical vesicle filled with hydrolytic enzymes. This vesicle, the acrosome, plays a significant role during fertilisation. The midpiece contains mitochondria and is responsible for generating power to the spermatozoon to swim. The tail contains microtubules, which form part of the propulsion system of the spermatozoon. The final step of sperm maturation is known as capacitation and it involves changes mainly in the acrosome that prepare it to release the enzymes required to penetrate the zona pellucida (ZP). Capacitation is thought to take place within the female genital tract.

The process of spermatogenesis is defective for around 2% of the population resulting in abnormally low sperm counts ($<20 \times 10^6$ /ml, oligozoospermia) or total absence of sperm (azoospermia). The discovery that some of these men had a deletion for a region of Yq termed AZF (azoospermia factor) (Tiepolo and Zuffardi, 1976) lead to characterisation of gene families involved in spermatogenesis, with those mapped including Deleted AZoospermia (DAZ) (Reijo *et al,* 1995) and RNA-binding motif (RBM) (Ma *et al,* 1993). Moreover, a study by Mahadevaiah *et al* (1998) suggested that although in mice RBM deficiencies cause sperm abnormalities, in men deletion of the functional copies of RBM is associated with meiotic arrest rather than sperm anomalies.

1.1.1.2 Oogenesis

Oogenesis is a discontinuous process and begins during fetal life. In the female, all primary oocytes that the individual will ever possess are produced during fetal life.

An estimate of 200,000 germ cells are available for the reproductive life span at puberty when recruitment of some of these primordial follicles begins. However, over 99% of follicles undergo atresia rather than ovulation, a degenerative process leading to cell death. Mature ova develop from oogonia by a complex series of intermediate steps. Oogonia originate from primordial germ cells by a process involving 20-30 mitotic divisions early in embryonic life (Siracusa *et al,* 1985). Between the third and fifth months of intra-uterine life the oogonia mature into primary oocytes which start to undergo meiosis. Shortly after beginning meiosis, however, these cells enter a state of dormancy and meiotic arrest that will persist until after puberty (Baker, 1963). The primary oocytes remain suspended in the meiotic stage at dictyotene until puberty. After puberty, a few oocytes and their enclosing follicles resume development each month in response to the monthly production of pituitary gonadotrophic hormones. These hormones include gonadotrophin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The hormones above play an indispensable part in controlling folliculogenesis, ovulation and the condition of the uterus. Only one follicle matures fully (others undergo atresia) and undergoes the process of ovulation, which is the expulsion of the secondary oocyte from the follicle. The final maturation stage of the oocyte, where the oocyte will complete meiosis, can be concluded only if it is fertilised by a spermatozoon.

1.1.2 *In Vivo* Fertilisation

Fertilisation, the process by which the male and female gametes fuse, occurs in the fallopian tube. This multi-step process begins with the specific recognition of complementary receptors on the surfaces of the two gametes. When a spermatozoon encounters an ovulated oocyte it forces its way through the cumulus mass. The zona matrix is made up of at least three major glycoproteins, ZP1, ZP2 and ZP3 where the sperm are able to bind (Longo, 1997; Acevedo and Smith, 2005). If the spermatozoon reaches the ZP, the sperm binds in a human-specific interaction with the glycoprotein sperm receptor molecule (ZP3) and then the sperm acrosome is induced to release degenerative enzymes that allow the sperm to penetrate the ZP. As soon as the spermatozoon penetrates the ZP and reaches the oocyte, the cell membranes of the two cells fuse. This causes a cascade of events including release of specific substances that interact with the ZP in such a way as to alter the sperm receptor molecules,

rendering the ZP impenetrable to other spermatozoa. Moreover, the fusion of the spermatozoon cell membrane with the oocyte membrane causes the oocyte to resume meiosis where the oocyte will consequently release the second polar body.

The chromosomes of the oocyte and sperm are enclosed within female and male pronuclei respectively, which merge to produce the single, diploid, 2N nucleus of the fertilised zygote. The moment of zygote formation is taken as the beginning of embryonic development. The position of the chromosomes in the sperm nucleus has been found to be significant since location could influence paternal gene expression (Foster *et al,* 2005).

1.1.3 *In Vivo* Preimplantation Embryo Development

Twenty-four hours after merging of the male and female pronuclei, the zygote starts dividing through mitotic cell division known as cleavage. The zygote is subdivided into smaller daughter cells called blastomeres. The first cleavage division divides the zygote in line with the polar bodies, whereas subsequent divisions are asynchronous. The embryo should have divided into 4 cells by 48 hours and 6-8 cells by 72 hours (day 3) post ovulation. By day 4 embryos should reach the morula stage and by day 5-6 embryos form a hollow ball of about 100 cells. At this point the preimplantation embryo, now called a blastocyst, enters the uterine cavity and begins to implant into the endometrial lining of the uterine wall (Figure 1.1).

During these initial stages of embryo development (0-6 days), the zygote travels down the oviduct and undergoes cleavage that subdivides the zygote without increasing its size. In fact, with each division the resulting blastomeres are half the size of the parent cells, and they become increasingly tightly connected as compaction occurs to form the 16-32 cell embryo with an appearance of a small mulberry therefore called a morula (90-120h post-fertilisation) which then becomes a blastocyst (Trounson *et al,* 1982; Gardner and Lane, 2005). The cells of the blastocyst are differentiated into two types: the inner cell mass (embryoblast), which will give rise to embryo proper and its attached membranes and the outer cell mass (trophoblast), which will be the main source of the placenta and related structures. This is a time characterised by intense DNA synthesis and replication.

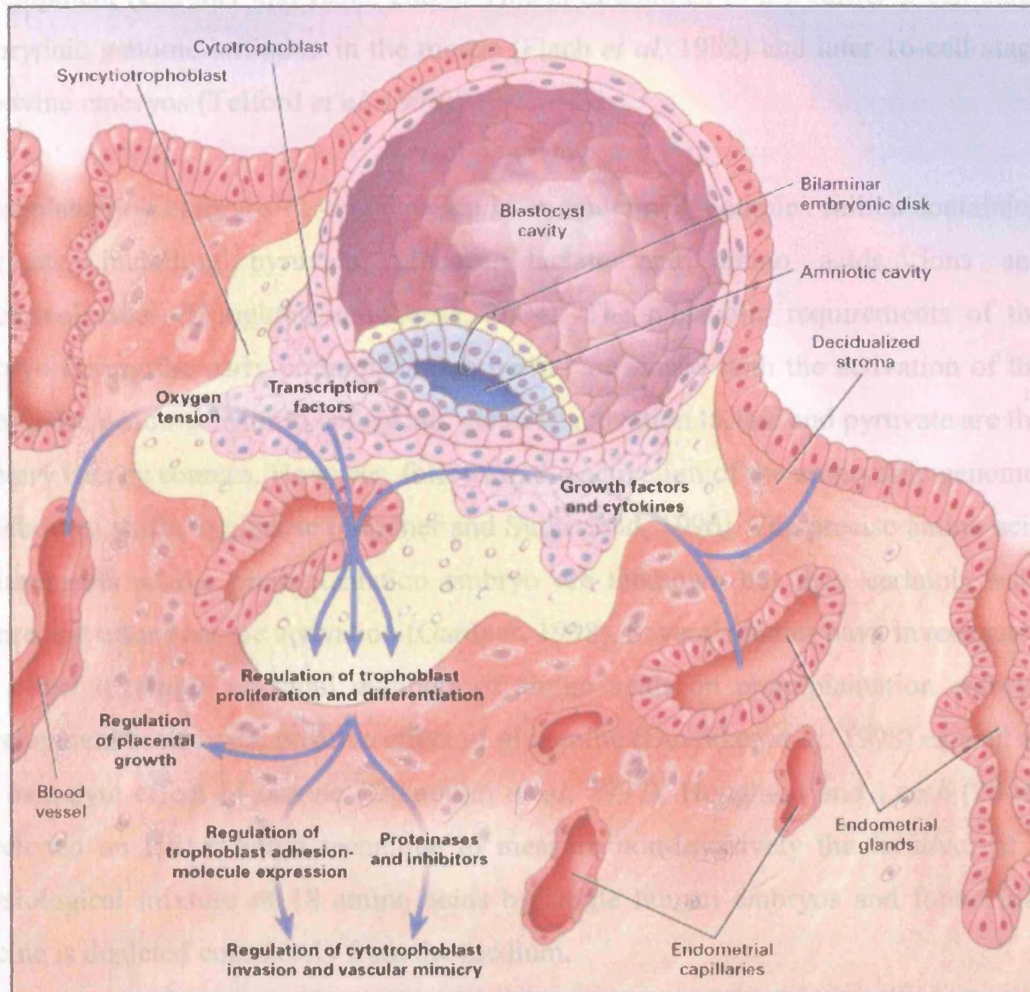


Figure 1.1. Demonstration of blastocyst implantation (picture taken from Larsen, 1997)

During oogenesis, a supply of maternal proteins and mRNA is produced which, following fertilisation, supports the initial development of the mammalian zygote (Daniels and Monk, 1997; Houghton and Leese, 2004). As development proceeds, these maternally derived proteins and mRNAs are degraded and replaced by those produced by the zygote itself. Several studies have tried to determine the timing at which the 'switch' from maternal to embryonic control of development occurs (Tesarik *et al*, 1986; Braude *et al*, 1988; Leese, 1998). Studies involving the incubation of human preimplantation embryos from the 1-cell stage in a medium containing α-amanatin, which inhibits transcription, have shown that development of human embryos may proceed to the 4- to 8-cell stage, presumably supported only by maternal transcripts (Tesarik *et al*, 1986). However, Braude and colleagues (1988)

postulated that beyond this stage embryonic transcripts are required for further development (Gardner and Lane, 2005). This is in contrast to the earlier 2-cell stage embryonic genome initiation in the mouse (Flach *et al,* 1982) and later 16-cell stage in bovine embryos (Telford *et al,* 1990).

Preimplantation embryos in vivo develop in an undefined, complex milieu containing nutrients, including pyruvate, glucose, lactate and amino acids, ions and macromolecules (Houghton and Leese, 2004). The metabolic requirements of the embryo during the early preimplantation period are linked with the activation of the embryonic genome. Prior to embryonic genome activation lactate and pyruvate are the primary energy sources. However, following the activation of the embryonic genome, metabolism shifts to glucose (Gardner and Sutherland, 1996). The precise amino acid requirements of the preimplantation embryo are unknown but they certainly vary before and after genome activation (Gardner, 1998). Several studies have investigated the effect of single or small numbers of amino acids on preimplantation embryo development such as the positive effect of glutamine (Devreker *et al,* 1998) as well as the osmolyte effect of taurine (Dumoulin *et al*, 1997). Houghton and Leese (2004) developed an HPLC-based technique to measure non-invasively the turnover of a physiological mixture of 18 amino acids by single human embryos and found that leucine is depleted completely from the medium.

Recently, the contribution of mitochondrial activity to embryo competence has been investigated in the human (Jansen, 2000; Cummins, 2002; Van Blerkom, 2004). The mitochondrial complement size, mitochondrial DNA copy numbers and defects and stage-specific spatial distribution, have been found to influence the developmental normality and viability of preimplantation embryos (Van Blerkom, 2004).

During embryo growth and more significantly embryo differentiation two types of intracellular junctions have been described as communicative devices between blastomeres, appearing from the 8-cell stage onwards (Fleming *et al,* 2000). Firstly there are the structural tight junctions and desmosomes forming in the outer most cells, anchoring the cells together and forming a permeability seal isolating the interior of the embryo from the external environment. Tight junction construction initiates at compaction and is dependant upon uvomorulin adhesion (Fleming *et al,*

1993). Certain integral and peripheral proteins such as occluding and cingulin (ZO-1) compose the tight junctions (Fleming *et al,* 1993). Desmosomal proteins are points of intercellular contact that 'bolt' the cells together. Secondly, there are the low resistance junctions known as gap junctions allowing intracellular connection between the cytoplasm of two cells so that small proteins and ions can be exchanged (Levin and Mercola, 1998; Cronier *et al,* 2001). Direct transfer through the gap junctions includes metabolites and second messangers (cAMP) (Bennet *et al,* 1997). In human embryos gap junctions are not apparently well developed until the early blastocyst stage when intracellular communication is clearly seen between inner cell mass (ICM) cells (Dale *et al,* 1991). Gap jucntions have also been linked with the early generation of left–right assymetry in Xenopus embryos (Levin and Mercola, 1998) as well as differentiation of the human placenta (Cronier *et al*, 2001)

1.1.4 *In Vitro* Fertilisation

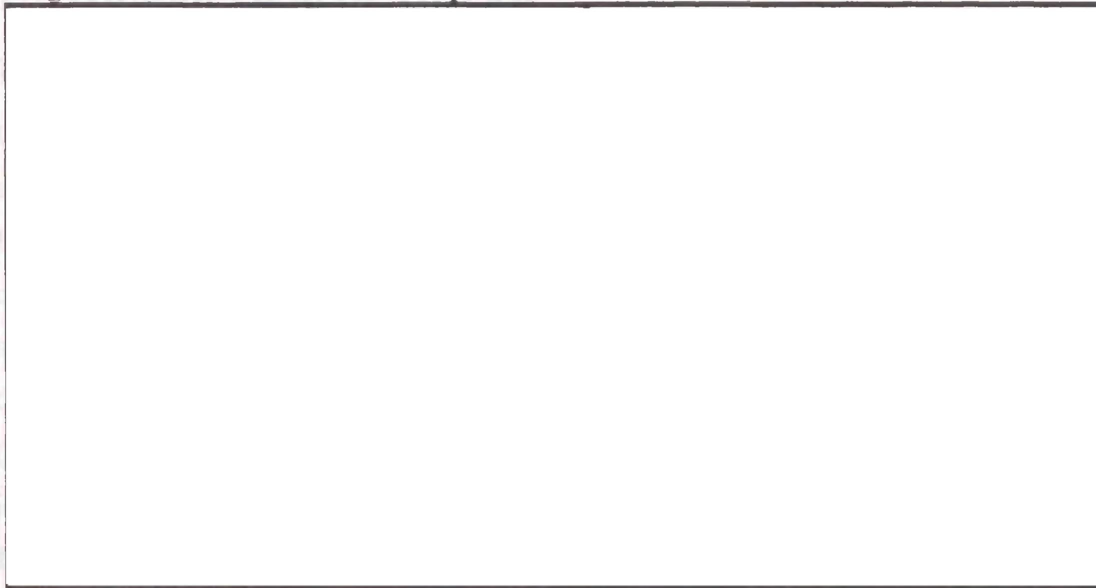
The first successful birth following *in vitro* fertilisation (IVF) treatment was reported in 1978 by Steptoe and Edwards (Steptoe and Edwards, 1978). This medical breakthrough heralded the beginning of real hope for thousands of infertile couples. For almost three decades all aspects of IVF treatment have constantly been fine-tuned in order to improve pregnancy rates and help couples conceive their dream: a baby. Superovulation regimens have been changed to yield a high number of viable and normal oocytes; sperm preparation methods have been improved; culture techniques and media are continuously evolving in association with our understanding of the needs of the early embryo; moreover, laboratory settings have been re-designed to accommodate the gametes and embryos 'normal' growth. The pregnancy rate varies between centres according to the patient group treated but overall the livebirth per cycle or 'take home baby' rate is about 25%, which declines rapidly as maternal age increases over 36 years (HFEA Annual Report, 2004). Of all pregnancies following IVF, 29% are multiple with a significant proportion of triplets (5%). Obstetric and perinatal complications associated with IVF are largely attributable to the high incidence of multiple pregnancies at risk of pre-term delivery and low birthweight. There is no evidence that fetal abnormalities are more common in children conceived after assisted conception (about 2-3%), compared to their peers conceived normally. In longitudinal studies, child development appears normal (SART, 1992; FIVNAT,

1995). Human births from cryopreserved/thawed embryos do not differ significantly from those from fresh IVF embryos or natural conceptions (Wennerholm *et al,* 1997).

1.1.4.1 Intracytoplasmic Sperm Injection

Micro-assisted fertilisation techniques (MAF) based on IVF technology have been developed since the late 1980s specifically to address the problem of male factor infertility. These include partial zona dissection (PZD) where a breach is made in the zona by mechanical dissection to facilitate sperm entry (Malter and Cohen, 1989), and sub-zonal insemination (SUZI) involving placement of two to five single sperm into the perivitelline space (Laws-King *et al,* 1987). Both techniques mentioned above have been replaced with intracytoplasmic sperm injection (ICSI). ICSI involves direct microinjection of a single sperm into the cytoplasm of a mature metaphase II oocyte that has been denuded of all surrounding cumulus and corona cells (Palermo *et al,* 1992; Figure 1.2). This procedure enables men who were previously thought to be irreversibly infertile, the chance to have their own child. However, the clinical application of ICSI has evoked an intense debate on possible associated risks (Cummins and Jequier, 1994; Griffin *et al,* 2003), because bypassing natural mechanisms of sperm selection has been suspected to support the propagation of gene mutations and chromosomal abnormalities. These concerns are based on the assumption that the ZP stops the penetration of genetically abnormal sperm. Furthermore, ICSI has been widely used as the only method of fertilisation in PGD cases for chromosomal abnormalities and single gene defects (Harper *et al,* 2000). The underlying reason is to reduce the chance of maternal or paternal contamination during the biopsy procedure since only one sperm is injected into an oocyte where the surrounding cells i.e. cumulus and corona cells have been removed using hyaluronidase

Figure 1.2. Illustration of an ICSI procedure.



A: 1) Suction applied by a glass holding pipette keeps the oocyte from moving during the injection. A single sperm is picked up in a tiny micro-needle. 2) The needle has a sharp tip and can be gently pushed through the shell of the oocyte and into the cytoplasm and 3) and (B) The sperm is deposited deep inside the oocyte and the empty needle is withdrawn (www.google.com/images)

These concerns appeared to be somewhat confirmed with reports of an increase in sex chromosome aberrations in ICSI babies (Bonduelle *et al.*, 1995; 1998; In't Veld *et al,* 1995; Tournaye *et al,* 1995; Scholtes *et al*, 1998) and the inheritance of paternal chromosome rearrangements (Testart *et al,* 1996; In't Veld *et al,* 1997; Meschede *et al,* 1997) and Y deletions (Kent-First *et al,* 1996). However, other studies have concluded that the chromosomal abnormality rate is not higher in ICSI compared to IVF children (Palermo *et al*, 1996; Engel *et al*, 1996). These discrepancies between studies may be due to differences among laboratories in scoring criteria as well as intrinsic (e.g. age) and extrinsic (e.g. environment) factors may play a role (Griffin *et al,* 2003). Recently, Griffin *et al* (2003) prepared a questionnaire for IVF staff assessing their views whether or not to screen patients for sperm aneuploidy before undergoing ICSI treatment. It was found that staff was not against screening for sperm chromosomal abnormalities and that there would benefits in doing so, however, others argued that most would undergo the ICSI procedure regardless of the screening results (Grffin *et al*, 2003).

1.2 Human Genetic Disease

1.2.1 Chromosome Abnormalities

In 1956 the human chromosome number was identified by Tijo and Levan being 46, and three years later Lejeune *et al,* (1959) connected genetic disease with deviations from the right number of chromosomes. Chromosomal abnormalities comprise cytogenetically detectable alterations in the normal karyotype, and may be either structural involving physical rearrangements of chromosomes, or numerical involving the loss or gain of individual chromosomes (aneuploidy) or whole chromosome sets (polyploidy). Furthermore, in cases where more than one genetically distinct cell line is present, if they arise from one zygote it is called mosaicism whereas if it results form more than one zygote it is referred as chimerism.

Chromosome abnormalities are a major category of genetic disease. They account for a large proportion of reproductive wastage, congenital malformations and mental retardation. The incidence of abnormalities of the autosomes and sex chromosomes are approximately the same (Jackson, 2002). Approximately 60% of spontaneous abortions in the first trimester and 5% of stillbirths are caused by chromosomal abnormalities (Hassold *et al,* 1986; Eiben *et al,* 1990). There has been a variety of factors linked to the high early pregnancy failures, with chromosome abnormalities, present or induced, being the most important (Munne *et al*, 1999). Embryonic aneuploidy, numerical chromosomal abnormality involving the gain or loss of one or more chromosomes, is one of the major causes of reproductive failure in women above 35, at least following IVF (Warburton *et al*, 1986; Munne *et al*, 1995a; Munne, 2002). The chance of a fetus being affected with a chromosomal disorder increases with the age of mother from approximately 1:250 at age of 35 to 1:65 at 40 and 1:20 at 46 years of age (Jackson, 2002). It has been postulated that the high level of early embryonic death must contribute significantly to the observed low fecundity (Wells and Delhanty, 2000). The importance of chromosome abnormalities was demonstrated by karyotyping aborted foetuses, which showed that >60% of spontaneous abortions at <12 weeks carried a chromosomal abnormality (Boue *et al*, 1985).

The major type of chromosomal abnormality found at all gestational ages is aneuploidy, either trisomy or monosomy, for the X chromosome. Trisomies for all the chromosomes have been found in abortuses, including chromosome 1 (Dunn *et al*, 2001) but monosomies with the exception of monosomy X are extremely rare (Boue *et al*, 1985). The incidence of the most common live-born aneuploidies has been shown to increase with maternal age (Nicolaidis and Petersen, 1998). Trisomy 21 is the most common aneuploidy found at birth and the majority of cases are the result of non-disjunction occurring at maternal meiosis (Nicolaidis and Petersen, 1998), whereas only 5% are attributable to mitotic error (Yoon *et al*, 1996). Furthermore, it has been suggested that aneuploidy of gonosomes and disomy 18 by non-disjunction can be due to increasing paternal age (Griffin *et al,* 1995)

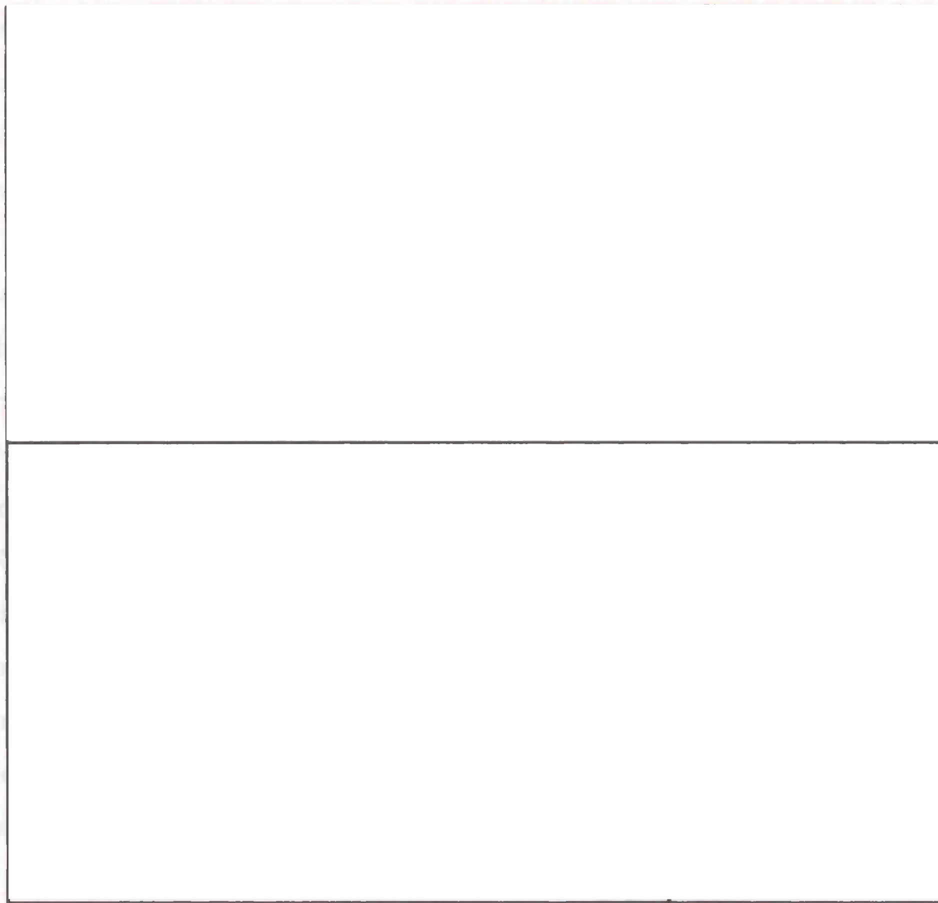
1.2.1.1 Numerical Aberrations

Aneuploidy involves the loss or gain of an extra chromosome causing monosomies, trisomies and tetrasomies (Griffin, 1996). The incidence of aneuploidy in humans is an order of magnitude greater than for other animals and most commonly leads to a high rate of mental retardation and pregnancy wastage between 6 and 20 weeks of gestation (Mahmood *et al,* 2000). Trisomy is the most common class of chromosome abnormality occurring in at least 0.3% of all newborns (Bond *et al,* 1983) and 25% of spontaneous abortions (Hassold *et al,* 1985). Approximately 30% of pregnancies result in fetal wastage due to aneuploidy (Wilcox *et al,* 1988). The most frequently surviving trisomies include trisomy 21 (Down syndrome), 13 (Patau syndrome), 18 (Edwards' syndrome) and aneuploidies involving the sex chromosomes such as monosomy X (Turner syndrome). Trisomy 21 is the most common aneuploidy among liveborns with an incidence of 1:800 births and increases markedly with maternal age, whereas trisomy 16 is the most frequent cause of pregnancy loss (Hassold *et al,* 1986). The incidence of trisomy 21 at conception is far higher but only 24% survive to term (Hassold *et al,* 1996). Furthermore, translocations with an emphasis on Robertsonian translocations, accounting for 5% of trisomy 21, are another cause of aneuploidy. Edwards' syndrome has an incidence of 1:10,000 and Patau's syndrome has an incident of 1: 20,000 livebirths (Hassold *et al*, 1996). Sex trisomies, such as 47XXY or 47XYY, have an incidence of 1:1100 and approximately 1 in 500 liveborn males have either a 47XXY or a 47XYY chromosome constitution (Hassold *et al*,

1998). The only monosomy reported to show compatibility with life is monosomy XO, termed Turner's syndrome, with an incidence of 1:5,000-10,000 female births. This represents only a fraction of all conceptions with this condition, as 98-99% are spontaneously aborted and only 0.3% are born alive (Hassold *et al,* 1996).

There are essentially three developmental stages when chromosomal defects, and especially aneuploidy, can arise: gametogenesis, fertilisation and embryogenesis (Delhanty *et al*, 1995). Errors in gametogenesis are usually considered to be meiotic in origin (Delhanty *et al*, 1995), whereas fertilisation errors are caused due to dispermy which occurs in 1% of spontaneous miscarriages *in vivo* (Jacobs *et al*, 1978). Mitotic errors in embryogenesis are implicated in 5-20% of cases, but this may be underestimated as errors in pre-mitotic divisions during gametogenesis may also appear as being of meiotic origin (Antonarakis *et al,* 1993). Aneuploidy largely arises as a result of an error of chromosome segregation at cell division called nondisjunction (Griffin, 1996) (Figure 1.3). The classical model for the mechanism of nondisjunction in maternal meiosis I is the failure of homologous chromosomes to segregate properly to opposite poles during meiosis resulting in the production of gametes that have an incorrect chromosome complement (Day *et al*, 1998). Non-disjunction of bivalents leading to loss or gain of dyads during meiosis I results in a disomic and a nullisomic gamete (Nakaoka *et al,* 1998). According to Angell and colleagues, premature division of the chromosome centromere during anaphase I leads to loss or gain of monads (Angell *et al*, 1994). Studies in oocytes have revealed that during anaphase I univalents can migrate either as a whole chromosome towards the meiotic spindle, or divide prematurely and move as separate chromatids, with subsequent random segregation (Angell *et al*, 1994). Both types of nondisjunction have been confirmed in subsequent studies (Cozzi *et al*, 1999).

Figure 1.3. Segregation at meiosis of a single pair of chromosomes



A: Non-disjunction occurring during meiosis I at anaphase I will lead to four unbalanced gametes. B: Non-disjunction occurring during meiosis II at anaphase II will lead to two disomic gametes and two unbalanced gametes. (www.google.com/images)

Lamb *et al,* (1996) proposed a two hit system to explain the predominance of maternal MI errors in human trisomy. The first event establishes a susceptible pairing configuration in fetal meiosis whilst the second event is an age-related impairment of the meiotic process, such as defective spindle apparatus (Battaglia *et al,* 1996), which gives an increased risk of nondisjunction. This general interpretation is broad enough to encompass other factors that may contribute to spindle disturbances linked to aneuploidy such as hormonal imbalance and reduced intrafollicular vascularity (Gaulden, 1992; Van Blerkom, 1998).

There is a long list of factors known to affect the process of non-disjunction such as: parental age, recombination of chromosomes, chromosome mover components, differential chromosome susceptibility and certain chemicals. The most significant

factor affecting nondisjunction is advanced maternal age and subsequent aberrant recombination (Griffin, 1996). Errors in maternal meiosis I account for 75% of trisomy 21 cases (Antonarakis *et al*, 1998) and all (100%) of trisomy 16 (Hassold *et al*, 1996). In trisomy 18 cases most arise due to errors occurring during maternal meiosis II (Fisher *et al*, 1995).

Polyploidy (multiple copies of all chromosomes) is the second most common group of chromosomal abnormalities resulting in a spontaneous abortion in the first or second trimester as progression to term is rare (Book and Santesson, 1960; Cassidy *et al*, 1977). Errors in cell division can result in polyploidy. However, it is mainly due to polyspermic fertilisation (Hassold *et al*, 1980; Angell *et al*, 1986, Zaragoza *et al* 2000), with dispermy being the most common cause of triploidy. Complete non-disjunction at MI or MII can also lead to triploidy. In digynic cases this meiotic failure can be caused by non-extrusion of a polar body which then becomes incorporated into the embryo (Penrose and Delhanty, 1961; Zaragoza *et al*, 2000). Following DNA replication any meiotic or post-zygotic mitotic failure in cytokinesis can cause tetraploidy or higher orders of ploidy

1.2.1.2 Structural Aberrations

Structural chromosomal abnormalities arise from chromosome breakage with subsequent reunion in a different configuration. They can be balanced, where the chromosome complement is complete with no loss or gain of genetic material or unbalanced, where the chromosome complement contains an incorrect amount of genetic material. Structural abnormalities include translocations (reciprocal or Robertsonian), deletions, inversions, insertions, ring chromosomes and isochromosomes (Figure 1.4). Structural chromosomal aberrations may be familial or de novo in nature with an estimated mutation rate of $1/1x10^{-3}$ (Jacobs, 1981) and are seen in 5% of spontaneous abortions (Hassold *et al*, 1986; Eiben *et al*, 1990) and between 0.2-0.6% of livebirths, the majority balanced (Hook and Hamerton, 1977; Nielsen and Wohlert, 1991).

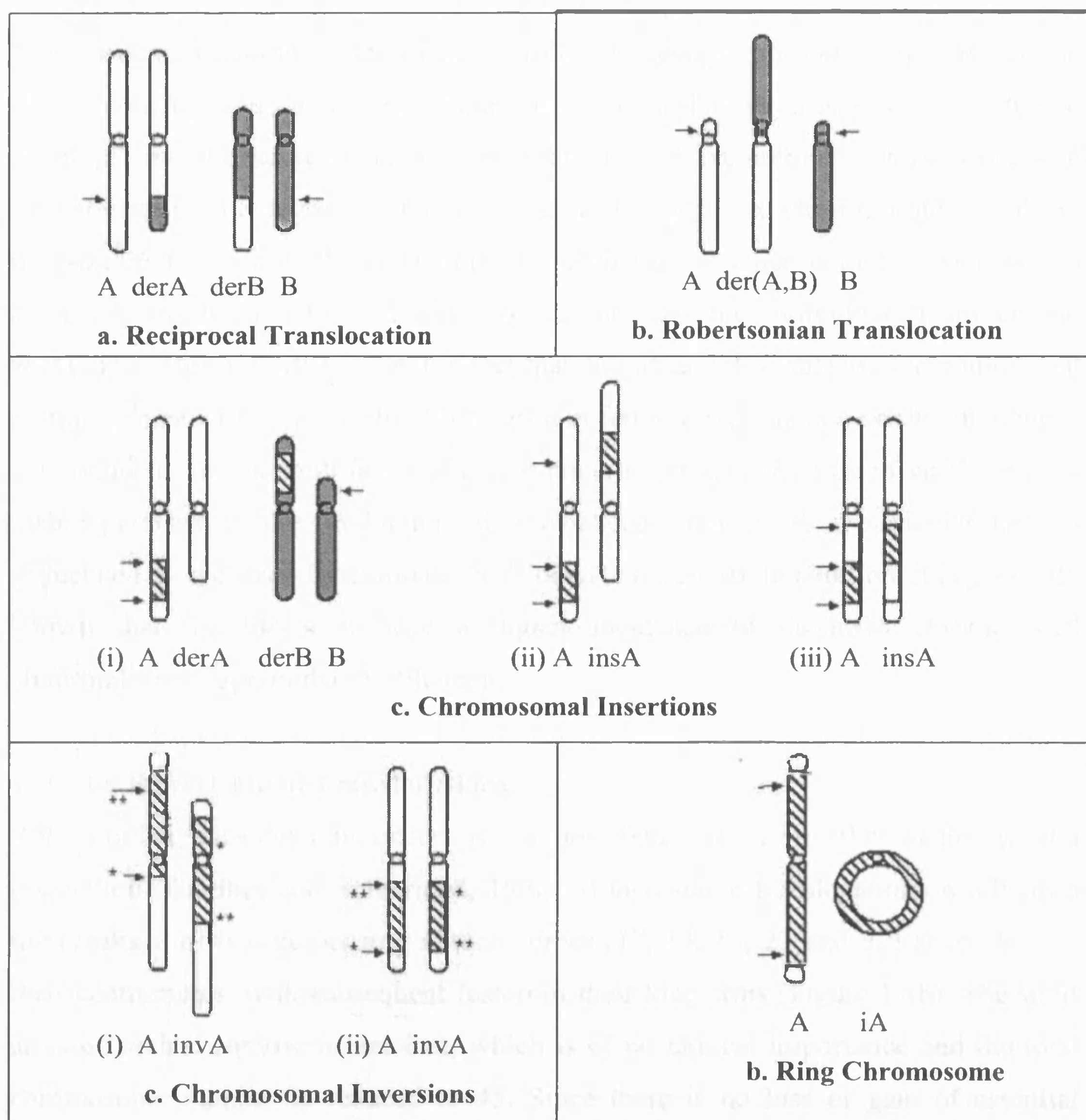


Figure 1.4: Eight types of balanced rearrangements are illustrated, Chromosomal translocations a) Reciprocal, b) Robertsonian, c) Insertions (i) interchromosomal, (ii) intrachromosomal between-arm and (iii) intrachromosomal within-arm. d) Chromosomal inversions (i) pericentric and (ii) paracentric. e) Ring chromosome.
A, B – Normal homologue. der, ins, inv, r – Rearranged chromosome. (Therman *et al.*, 1989)

Translocations are the most common structural abnormality in man (approximately 1.6 in 1000 newborns) and carriers of balanced translocations show an increased risk of having chromosomally abnormal offspring in addition to infertility and spontaneous abortions due to the formation of unbalanced gametes during meiosis (Iwarsson *et al*, 2000). Impaired spermatogenesis has also been frequently seen in male balanced translocation carriers. Most of the male translocation carriers that have reduced fertility are considered to be phenotypically normal (Sutton, 1980).

Most structural anomalies are only identified if a couple presents with a history of chromosomally abnormal conceptions. These reproductive problems arise due to abnormal meiotic segregation and/or production of recombinant chromosomes at gametogenesis, the degree of which varies according to the chromosomes involved, the size and location of the rearranged segments, whether complete synapsis is achieved and the position of crossover events and the individual (Gardner and Sutherland, 1996). Furthermore, the fact that certain couples carrying a chromosomal rearrangement suffer only a slight reduction in fertility and others a detrimental one is a consequence of the multifactorial nature governing the production of viable or non-viable gametes as the production of unbalanced gametes of a particular type is influenced by the size of the imbalance (Cohen *et al,* 2000). In addition, it is generally known that infertile men have a higher incidence of de novo chromosomal abnormalities in sperm than fertile men.

1.2.1.2.1 Robertsonian Translocations

Robertsonian translocations occur with a prevalence of ~1 in 1000 in the general population (Gardner and Sutherland, 1996). Robertsonian translocations result from the breakage of two acrocentric chromosomes (13, 14, 15, 21 and 22) at or close to their centromeres, with subsequent fusion of their long arms (Figure 1.4b). The short arms of each chromosome are lost, which is of no clinical importance and the total chromosome number is reduced to 45. Since there is no loss or gain of essential genetic material this is a functionally balanced rearrangement (Conn *et al*, 1999). The most common forms are non-homologous, involving two different acrocentric chromosomes such as two chromosomes from the D or G group (13, 14 and 15 or 21 and 22 respectively) or a D group and G group chromosome. There are three ways for the formation of a Robertsonian translocation: fusion at the centromere (centric fusion), giving a monocentric chromosome, a whole arm reciprocal translocation, with breakage in one short arm and one long arm giving a monocentric chromosome, and finally, union following breakages in both short arms giving a dicentric chromosome (or after the suppression of one centromere, a monocentric). The most common Robertsonian translocation is between chromosomes 13 and 14 and makes up 75% of all Robertsonian translocations (Gardner and Sutherland, 1996).

1.2.1.2.2 Reciprocal Translocations

Reciprocal translocations are characterised by the exchange of chromosomal genetic material between two non-homologous chromosomes (Figure 1.4a). They are very common in the general population, occurring in 1 in 500 livebirths (Hook *et al,* 1977). Carriers of these translocations are nearly always phenotypically normal, as no loss or gain of genetic material is involved (abnormalities can arise if breakpoints disrupt important genes) (Scriven *et al*, 1998). Translocations are detected when the patient presents with recurrent pregnancy loss or phenotypically abnormal offspring due to the production of unbalanced gametes (Scriven *et al,* 1998; Iwarsson *et al*, 2000). The risk of miscarriages, stillbirths or chromosomally unbalanced live births with multiple congenital abnormalities depends on the probability of different types of unbalanced gametes being produced and, after fertilisation of such gametes, on the probability of different types of unbalanced embryos being able to survive (Van Assche *et al*, 1999). Impaired spermatogenesis is also frequently seen in male balanced translocation carriers.

1.2.1.2.3 Deletions, Inversions, Insertions, Ring Chromosomes and Isochromosomes

Deletions involve loss of a chromosomal segment, resulting in chromosome imbalance. A carrier of a chromosomal deletion (with one normal homologue and one deleted homologue) is monosomic for the genetic information on the corresponding segment of the normal homologue. The clinical consequences generally reflect haploinsufficiency (Jackson, 2002) and appear to depend on the size of the deleted segment and the number and function of the genes that it contains (Gardner and Sutherland, 1996). Deletions have an incidence of approximately 1 in 7000 births and can be terminal or interstitial. Deletions may originate by chromosome breakage and loss of the centric fragment or due to unequal crossing over between misaligned homologous chromosomes or sister chromatids. Furthermore, it has been suggested that deletions are also derived due to abnormal segregation from a balanced translocation or inversion (Iwarsson *et al*, 2000).

Insertions or insertional translocations involve three breaks; the first two breaks release an interstitial segment, which is then inserted into the gap created by the third break (Figure 1.4c). If a single chromosome is involved, this can be described as a

shift. In the interchromosomal insertions a segment of one chromosome is inserted interstitially into another chromosome. The insertion could be direct when the inserted segment is inserted with the same orientation with respect to the centromere, or it could be inverted when the orientation towards the centromere is reversed (Harnden *et al.,* 1985). Interchromosomal insertions are rare chromosomal rearrangements with an incidence of 1:80000, of which nearly 80% are referred because of congenital abnormalities and mental retardation (Van Hemel and Eussen, 2000). In the case of intrachromosomal insertion either within or between an arm crossing over can occur in one or the other insertion loop following complete synapsis. The risk of having a child with an unbalanced karyotype when carrying an intrachromosomal insertion has been estimated to be 15% by Madan and Menko (1992). Insertions are amongst rearrangements implying the highest reproductive risk. This risk is greater for the small segment insertion than the large segment insertion however in the case of the meiotic recombination product carrying a duplication there appears to be no clear relationship between the phenotype and the length of the duplicated segment (Wilson *et al,* 1985).

Inversions involve a two-break rearrangement in a chromosome and the segment formed by the breaks is reversed. If the inverted segment includes the centromere then the inversion is pericentric [Figure 1.4d(i)], if however, the centromere is not involved the rearrangement is known as paracentric [Figure 1.4d(ii)]. Excluding the common inversions of heterochromatin 1qh, 9qh, and 16qh generally considered normal variants, inversions occur in about 1 in 2-5000 births (Gardner and Sutherland, 1996). In pericentric inversions the smaller the inverted fragment the greater the chromosomal imbalance and the likelihood of miscarriage of the conception. Conversely, with a large inverted segment, the unbalanced regions will be small and offspring carrying the recombinant chromosomes may be viable (Daniel, 1981). In the case of paracentric inversions the crossing-over during synapsis at meiosis will lead to the formation of recombinant chromosomes that are either acentric, and incompatible with survival or dicentric and unstable during cell division and therefore are not compatible with life either (Worsham, 1989). Therefore, the risk of the birth of an abnormal child resulting from a carrier parent of a balanced paracentric inversion is almost non-existent.

Ring chromosomes are formed by breakage in both arms of a chromosome, with fusion of the points of the fracture and loss of the distal fragments (Figure 1.4e). Thus, this would result in a partial monosomy for the distal short arm and the distal long arm. Ring chromosomes are often unable to complete mitotic division so that it is not unusual to find the ring chromosome in only a proportion of cells. In carriers of this abnormality where reproduction is an option, meiotic and mitotic disturbances presumably lead to high levels of gametic chromosomal imbalance and impaired fertility, although reports of stable familial rings show that this is not inevitable (McGinniss *et al,* 1992).

Isochromosome is a chromosome where one arm is missing and the other is duplicated in a mirror-image fashion, thus a person carrying an isochromosome has partial monosomy of one arm and partial trisomy of the other. The basis for isochromosome formation is not precisely known. However, the most probable explanation is that the centromere has divided transversely rather than longitudinally. The isochromosome for the long arm of the X chromosome, i(Xq), is the most common isochromosome and is found in some individuals with Turner's syndrome.

1.2.1.3 Mosaicism

Mosaicism is defined as the presence in an individual or in a tissue of two or more cell lines, which differ in their genetic constitution but are derived from the same zygote. The phenomenon of mosaicism will be analysed in section 1.6

1.2.1.4 Chromosomal Abnormalities in Human Gamete Studies

1.2.1.4.1 Karyotyping Studies

Karyotypic analysis of 710 oocytes (with or without polar bodies) revealed that cytogenetic abnormalities are closely associated with abnormal gametes, parthenogenesis, cytoplasm immaturity and division asynchrony after fertilisation (Benkhalifa *et al*, 1996). Angel (1997) after analysis of 200 oocytes by karyotyping postulated that there are two major factors regarding oocyte aneuploidy and maternal age: a) Vulnerable recombinants arise by chance distribution of recombination events at the pachytene stage of meiosis in fetal life and b) the physical structure of all chromosomes in the oocyte gradually deteriorates during the extended dictyate stage.

1.2.1.4.2 FISH Studies

Studies on sperm have tried to approach aneuploidy and especially trisomy by employing FISH (Hixon *et al*, 1998). Sperm studies are advantageous in studying nondisjunction over female studies since they have: a) accessibility to the origin of aneuploidy, the gametes, b) limitless supply of the products of meiosis, making possible to detect subtle differences in nondisjunction among different individuals and c) examination of nondisjunction in a setting that is unaffected by the maternal age effect. Recently, Tempest *et al* (2004), was able to find an association between infertile male individuals (of variant infertility) and sperm disomy for the sex chromosomes and chromosome 21. Overall, it was concluded that oligozoospermic male have significantly elevated levels of sex chromosome disomy only, whereas asthenozoospermic males have elevated levels of disomy 21 and not the sex chromosomes.

Studies on metaphase II oocytes have revealed an unexpected class of chromosome abnormalities, which manifest as single chromatids, rather than whole chromosomes comprising a dyad of chromatids. Mahmood *et al* (2000), determined that extra material in 127 oocytes and 57 polar bodies (PB) was seen in chromosomes 13, 16, 18, X and notably 21, but none were observed in chromosomes 1 and 9. Furthermore, the previous group found clues suggesting that an additional mechanism of maternal aneuploidy operating at anaphase II, might be the underlying reason of prematurely separated chromatids in the metaphase II oocyte Dailey *et al* (1996), used FISH on oocytes to investigate the involvement of specific chromosomes in aneuploidy, especially chromosomes 13, 15, 16, 18, 21, 22, X and Y. Sequential multi-probe FISH was employed for chromosomes 1, 9, 12, 13, 16, 18, 21 and X by Cupisti and colleagues (2003) to investigate the chromosomal status of unfertilised polar bodies and associated polar bodies. The same group found that mechanisms leading to aneuploidy include nondisjunction of whole univalents. In a recent study by Martini and co-workers (2000) on oocytes analysed by FISH, it was revealed that almost half (44% overall) of the material examined was aneuploid with chromosome specific patterns. Furthermore, Durban *et al* (1998) described an oocyte spreading procedure which was able of allowing detection of aneuploidy as well as structural chromosomal abnormalities during PGD using FISH.

1.2.1.5 Chromosomal Abnormalities in Embryo Studies

1.2.1.5.1 Karyotyping Studies

Ideally, cytogenetic investigation would involve analysis of metaphase chromosomes from the blastomeres or polar bodies (Ruanvutilert *et al*, 2000b). Karyotyping embryos has been applied using conventional techniques including culture synchronisation, disruption of the mitotic spindle and G-banding (Papadopoulos *et al*, 1989; Clouston *et al*, 1997; Clouston *et al*, 2002), however it is considered to be technically challenging.

Cytogenetic analyses of human preimplantation embryos have revealed extremely high levels of chromosome imbalance at this early stage of development (Papadopoulos *et al*, 1989; Clouston *et al*, 1997). Many embryos are karyotypically normal, however >50% of embryos are reported to carry a variety of chromosomal abnormalities including aneuploidy and chromosome breakage, polyploidy (addition of one or more complete haploid complements) and haploidy (one set of chromosomes instead of two) (Voullaire *et al*, 2000). These chromosome abnormalities arise during gametogenesis and/or fertilisation. Alternatively, they can be present in a proportion of nuclei due to post-zygotic errors during mitosis. This percentage indicates that pregnancy loss is frequently a result of natural selection against chromosomal aberrations

Limited, useful information can be extracted from processing intact day 2 and day 3 embryos (Angell *et al*, 1986; Papadopoulos *et al*, 1989; Jamieson *et al*, 1994) after analysis using banding techniques. Jamieson *et al* (1994) published a study of 816 embryos, however was able to karyotype only 195 (23.9%), finding 19.1% being aneuploid, 3.5% being tetraploid, 2.3% being triploid and only 0.6% being haploid. This was due to reduced quality of metaphases and G-banding and long colcemid exposure times resulting in highly contracted chromosomes. Clouston and colleagues (1997) devised a novel and inexpensive method for obtaining better quality G-banded metaphases from human blastocysts. They were able to provide full ploidy analysis on 64% (55/86) of the embryos and reported high levels of diploid embryos (67%); however, this percentage included uniformly abnormal karyotypes. Several studies reported chaotic findings while trying to detect mosaic and non-mosaic chromosome

abnormalities (Jamieson *et al,* 1994; Clouston *et al*, 2002) and were able to show that mitotic non-disjunction is significant for the production of aberrations in human pre-embryos (Papadopoulos *et al,* 1989). Furthermore, karyotypic studies on preimplantation embryos were among the first to reveal significant levels of tetraploidy, usually mosaic of about 19-23% (Papadopoulos *et al*, 1989; Clouston *et al*, 1997). Papadopoulos *et al* (1989) observed variable levels of structural chromosome damage, which was later confirmed by Clouston and co-workers (1997), including chromosome branching, chromosome breakage, anomalous chromatid pairing and apparent interchanges. However, it has been suggested that damage in early cell divisions may be a consequence of the ovarian stimulation stage of the IVF process rather than a characteristic of early preimplantation embryos per se (Eibling and Colot, 1985).

1.2.1.5.2 FISH Studies

Although karyotyping studies of chromosomal abnormalities on human preimplantation embryos had already provided important results, it was the advent of FISH that enabled the examination of every cell within the embryo that revealed the true extent of chromosomal abnormality in human development. The widespread use of IVF for treatment of infertility has stimulated research into chromosomal abnormalities in human preimplantation embryos. Most of the studies on embryos have been carried out using FISH. Nowadays, several FISH protocols have been devised for complex translocations (Simopoulou *et al*, 2003) as well as aneuploidy detection of up to 13 chromosomes (Abdelhadi *et al*, 2003). FISH studies have been able to demonstrate that the incidence of aneuploidy, haploidy, polyploidy and mosaicism in embryos is much higher than the incidence observed in clinically established pregnancies (Bielanska *et al*, 2002a). FISH has been carried out in arrested (Munne *et al*, 1995a; Bahce *et al*, 1999), frozen-thawed (Munne *et al*, 1997; Iwarsson *et al*, 1999) and fragmented (Munne *et al*, 1994a,b; 1995b) embryos, as well as embryos of good quality (Harper *et al*, 1995; Delhanty *et al*, 1997; Bielanska *et al*, 2000).

An essential part of FISH is its ability to be carried out on the same sample sequentially or as it is know in the circles of FISH laboratories, re-FISHing. The need

for sequential rounds of FISH on the same tissue/cell came about due to the lack of fluorescent colours of DNA probes thus the number of chromosomes that can be examined simultaneously e.g. in a blastomere is limited to a maximum of five (Gianaroli *et al*, 1997b) or six (Munne *et al*, 1998c). Liu and colleagues (1998a) were able to devise an accurate FISH protocol analysing more than six chromosome in 3 consecutive FISH rounds in a clinical setting. It has been shown that by using centromeric or telomeric probes in all three rounds of FISH, an efficiency of 88-94% and 87-96% respectively can be achieved (Liu *et al*, 1998b).

Delhanty and co-workers (1993) showed that surprisingly even apparently normally developing IVF embryos were often chromosomally abnormal, in agreement with the original karyotyping data (see section 3.1.1). Several factors have been proposed from studies on embryos for this level of abnormality. These factors including maternal age, embryo culture conditions (Munne *et al*, 1995), multinucleation and freezing of human embryos (Laverge *et al*, 1998), ovarian stimulation regimes and patient specificity (Ruangvutilert *et al*, 2000a) have been shown to affect chromosomal constitution of embryos. Almost all studies carried out on preimplantation embryos regarding chromosome abnormalities have tried, through their FISH results, to explain the mechanisms behind these aberrations. Staessen *et al* (1998) whilst analysing the genetic constitution of multinuclear blastomeres using M-FISH, revealed that more than half of bi-or multinuclear blastomeres were abnormal, however, 45% of the those blastomeres had a diploid status.

1.2.1.5.3 CGH Studies

More recently high rates of aneuploidy have been reported whilst applying metaphase CGH in human preimplantation embryos (Wells and Delhanty, 2000; Voullaire *et al*, 2000; Wilton *et al*, 2001; Malmgrem *et al*, 2002; Voullaire *et al*, 2002; Wilton *et al*, 2003; Trussler *et al*, 2004) as well as polar bodies and MII oocytes (Wells *et al*, 2002; Gutierez-Mateo *et al*, 2004). CGH on single cells from human preimplantation embryos has been performed and has provided the opportunity to assess the copy number of all chromosomes and thus the genuine abnormality and mosaicism level at this stage (Wells and Delhanty, 2000).

The application of single cell CGH, although being laborious, provides the opportunity to assess all the chromosomes and thus identify the true level of

mosaicism (Wells and Delhanty, 2000, Voullaire *et al*, 2000, Wilton *et al,* 2001; Trussler *et al,* 2004). CGH has allowed the investigation of chromosomal aberrations in human preimplantation embryos generated by IVF. Wells and colleagues (1999) revealed a WGA protocol that was powerful enough to generate sufficient quantities of DNA from a single cell to perform CGH as well as >90 independent amplification reactions. Instantly, two published studies showed the extent of chromosomal abnormalities in preimplantation human embryos. Voullaire *et al* (1999) obtained results for 63 blastomeres from 12 cleavage stage embryos and found that the proportion of chromosomally normal embryos was 25%, lower than that found from FISH studies (Delhanty *et al*, 1997; Munne *et al*, 1998d). Wells and Delhanty (2000), in a similar study found high levels of chromosomal mosaicism, non-mosaic aneuploidy, and chromosome brakeage. Furthermore, it was postulated that there was a low number of uniformly normal embryos (normal chromosome numbers in every cell), which may have superior developmental potential, but their low frequency might explain the relatively low success rates in assisted conception in humans (Wells and Delhanty, 2000). In a recent study which combined CGH and FISH, the high numbers of abnormal embryos were thought to have arisen as a result of culture artefact or inadequate cell cycle surveillance, rather than meiotic error (Trussler *et al,* 2004). CGH has also been performed in the context of PGD for aneuploidy screening (see section 1.5.2.3).

1.2.1.5.4 SKY-FISH and M-FISH

To overcome the limitations of FISH, it has been suggested that metaphase transformation by cell fusion would allow the differentiation between normal, balanced and unbalanced karyotypes (Munne and Cohen, 1998). Methods to obtain metaphase stage chromosomes by interphase conversion have been published by two teams. They are based on the fusion of blastomeres to bovine eggs or mice zygotes (Verlinsky and Evsikov, 1999; Willadsen *et al,* 1999). The Willadsen approach has been used for two PGD clinical cases for translocations resulting in chromosomally normal offspring (Willadsen *et al,* 1999) whereas the Verlinsky and Evsikov approach has been performed in the context of clinical PGD in 19 patients (Verlinsky and Evsikov, 1999). The methodology of Verlinsky and Evsikov (1999) was performed by fusing individual human blastomeres with enucleated or intact mouse zygotes. After blastomere-cytoplasm fusion, heterokaryons were fixed at metaphase of the first

cleavage division or treated with okadaic acid to induce premature chromosome condensation. The effectiveness of the proposed technique with blastomeres from day 3 diploid embryos was reported to be 91%, since 63 metaphases were obtained from 69 blastomeres (Verlinsky and Evsikov, 1999). However, interphase conversion is extremely labour intensive and has to provide an interpretable metaphase spread from a single cell, which in turn will render a number of metaphases unsuitable due to poor chromosome morphology, overlapping chromosomes and even loss of chromosomes (Harper and Wells, 1999). Furthermore, this technique might cause ethical tension in some countries where it would be impossible to fuse human blastomeres or polar bodies with enucleate oocytes from other species (Harper and Wells, 1999).

Multiplex-FISH (M-FISH) (see section 1.3.2.2.2) and spectral karyotyping are whole genome screening techniques that have been successfully applied for cytogenetic diagnostics of constitutional chromosomal abnormalities (Speicher *et al,* 1996; Schröck *et al,* 1996; 1997). Both techniques utilise 24 chromosome-specific paint probes labelled with different combinations of fluorochromes, thus all chromosomes can be analysed at one time. Schröck *et al* (1996) employed a dedicated custom-designed imaging spectrometer system, in a method known as spectral karyotyping or SKY-FISH. This finely samples each pixel for all fluorescence across the spectrum simultaneously producing a set of interferograms, before data processing to form a spectral image. Analysis is based on comparison of the interference pattern for each pixel with stored data on chromosome interference spectra; the pixel is then allocated to a matching chromosome (Schröck *et al,* 1996). SKY-FISH has been employed to successfully identify particular de novo supernumerary marker chromosomes as well as de novo unbalanced structural rearrangements, proving to be of beneficial role for diagnostic and counselling purposes, due to its reliability and speed (Haddad *et al,* 1998). SKY-FISH has already been used to examne chromosomes from oocytes and polar bodies (Marquez *et al.,* 1998). Sandalinas *et al* (2002), whilst carrying out SKY-FISH on fresh non-inseminated oocytes found increased non-disjunction, increased balanced and unbalanced predivision in the group of patientsars of over 35 years of age, Furthermore, all types of metaphase spreads, including metaphases obtained after nuclear conversion, can be analysed with M-FISH or SKY-FISH. Hence, SKY-FISH was the preferred method to analyse blastomeres and polar bodies where the nucleus

had been converted to a metaphase (Evsikov & Verlinsky, 1999; Willadsen *et al.*, 1999).

However, both SKY-FISH and M-FISH require expertise and are unable to detect small deletions, duplications and translocations (Kirchhoff *et al,* 2000). Also, they are relatively time-consuming and fail to yield metaphase chromosomes of suitable quality for routine diagnostic purposes. Ultimately, a method capable of a full chromosome analysis in single cells without the need for metaphases has been devised, based on the technique known as comparative genomic hybridisation (CGH).

1.2.2 Single-Gene Defects

Analysis of syndromes with Mendelian inheritance patterns has identified over 7000 single-gene defects. They are categorised as autosomal dominant (>3000), autosomal recessive (>3000) and X-linked disorders (>1000). A series of these mutations would be expected to affect the function of essential housekeeping genes and may therefore be lethal either in preimplantation development following the initiation of embryonic gene transcription and loss of maternally inherited products or in post-implantation development. Most *de novo* mutations causing inherited disease are paternal in origin (McKusick, 1992). This is thought to be related to differences between spermatogenesis and oogenesis. Since, in the females, the mitotic expansion of primordial germ cells continues until the late gestation when oogonia enters meiosis and arrest at the dictyate stage of meiosis I until menstrual cycles are initiated at puberty (see section 1.1.1.2). However, in males mitotic division of spermatogonia is continuous throughout life (see section 1.1.1.1). Therefore, the number of mitotic divisions preceding gametogenesis is much greater in the male which consequently may increase the risk of replication errors (Delhanty and Handyside, 1995).
An autosomal dominant trait is one which manifests in the heterozygous state, i.e. in a person possessing both the abnormal or mutant allele and the normal allele. Dominantly inherited diseases are generally caused by mutations resulting in gain of function protein alterations (Ranum and Day, 2002). Autosomal dominant traits can involve only one organ or part of the body and can have a multisystemic effect (pleiotropy) which has been seen in myotonic dystrophy (Ranum and Day, 2004) as

well as tuberous sclerosis (Ess *et al*, 2005). During this study a triplet repeat dominant disorder will be discussed, namely myotonic dystrophy 1.

1.2.2.1 Myotonic Dystrophies

Steinert as well as Batten and Gibb in 1909 identified myotonic dystrophy (DM [MIM 160900 and MIM 602668]) as a multisystemic disorder. Myotonic dystrophy (DM1) also know as dystrophia myotonica is the most common form of inherited muscular dystrophy seen in adults, with an overall incidence of approximately 1 in 8000 (Shaw and Harper, 1989). DM1 shows autosomal dominant inheritance with anticipation and an early onset form with rather different clinical features. The clinical picture of DM1 is well established but exceptionally variable (Harper, 1989). One of the striking features of this disorder is the variability of phenotype both within and between families (Harper, 1989).

Shortly after genetic testing became available, a second multisystemic disorder was identified referred to as either proximal myotonic myopathy (PROMM) or myotonic dystrophy type 2 (DM2) (Ricker *et al,* 1994; Thornton *et al,* 1994). The DM2 mutation was linked to a 3cM region of the 3q21 and it is caused by a transcribed but untranslated CCTG repeat expansion located in intron 1 of the zinc finger protein 9 (*ZINF9*) gene (Ranum *et al*, 1998; Liquori *et al,* 2001).

During this study only DM1 was investigated therefore for simplicity DM1 will be referred to as DM.

1.2.2.1.1 Clinical Pathology of DM

In contrast to most forms of muscular dystrophy, in individuals suffering from DM the clinical features are not limited exclusively to the neuromuscular system. Patients with DM have been found to suffer in other organs in addition to myopathy i.e. bone, skin, eyes, gastrointestinal organs and the endocrine system. Most commonly persons with DM present in their adult life with slowly progressive weakness and myotonia. The latter term refers to tonic muscle spasm with prolonged relaxation, which can manifest as a delay in releasing the grip of shaking hands. Other clinical abnormalities can include cataract, cardiac conduction defects, disturbed gastrointestinal peristalsis,

frontal balding and testicular atrophy. The age of onset can be very variable and the disorder usually runs a benign course. In the congenital form affected babies present at birth with hypotonia, talipes and respiratory distress, which can prove life threatening (Harper, 1975). Children who survive tend to show a lack of facial expression with delayed motor development and mild retardation. The congenital form of DM is mostly maternally transmitted (Brook *et al,* 1992; Zeesman *et al,* 2002). The phenomenon of anticipation in which the disease symptoms become more severe and age at onset earlier in successive generations, is often manifested in a family producing a congenitally affected child (Brook *et al,* 1992).

Early diagnosis is based on clinical symptoms including myotonia, muscular weakness and atrophy, cataract and hypogonadism. In contrast to other dystrophies, DM attacks the distal and then proximal muscles of extremities. Originally the diagnosis of DM patients was based on electromyography, which has now been superseded by mutation analysis (Shaw and Harper, 1989).

1.2.2.1.2 Genetics of DM

Initial biochemical studies failed to identify the defective protein in DM, although some have highlighted a link with abnormalities in calcium transport (Seiler and Kuhn, 1970), membrane fluidity (Butterfield *et al,* 1974) as well as apamin receptor expression (Renaud *et al,* 1986). It was Brook and colleagues (1992) that employed positional cloning strategies in order to identify the CTG triplet repeat that undergoes expansion in DM patients.

The molecular genetic defect of DM involves an unstable expansion of a CTG repeat at the 3' untranslated region (exon 15) of the DM gene on the long arm of chromosome 19 (19q13.3) (Brook *et al*, 1992; Fu *et al*, 1992). The number of repeats relates to the age of onset and the severity of the disease (Harley *et al*, 1993). Normal individuals possesses 5-35 repeat copies, patients with 36-49 copies are said to have the premutation, patients with 50-150 copies are mildly affected, patients with 100-1,000 copies are severely affected and the most severe or neonatal form have 2,000 copies or more [The International Myotonic Dystrophy Consortium (IDMC), 2000]. The mutant gene is almost always transmitted from the mother; however, paternal transmission cases have been reported (Nakagawa *et al,* 1994; de Die-Smulders *et al,*

1997). Variation in the DM triplet repeat has been observed in sperm and somatic cells of the same individual (Jansen *et al,* 1994), between muscle and lymphocyte cells (Anvret *et al,* 1993) as well as in affected identical twins (Dubel *et al,* 1992). The length of the expanded CTG repeat remains fairly homogenous in different tissues during embryonic development (Mankodi and Thornton, 2002). However, during postnatal life a marked variability of repeat length emerges in different cells and tissues of an individual (Thornton *et al,* 1994). This somatic heterogeneity has been found to increase with age (Thornton *et al* 1994). During PGD cases for DM significant increases have been found in the number of repeats in embryos from affected female patients and in their immature and mature oocytes, whereas, in spermatozoa and embryos from affected male patients, smaller increases have been detected (De Temmerman *et al,* 2004).

The estimated risk of any heterozygous woman with DM to have a congenitally affected child is 4-9%, though the risk is significantly increased during conception of a second child to 20-37% (Koch *et al,* 1991). Another feature of DM is the reported tendency for healthy individuals who are heterozygous for DM alleles in the normal size range to preferentially transmit alleles which are greater than 19 CTG repeats in size (Magge and Hughes, 1998). Such phenomenon might help maintain the mutant DM alleles in the population. However, recently Zunz *et al (*2004) found no evidence of statistically significant deviation of the frequency of transmission of the mutated alleles from the 50% expected in autosomal dominant disorders and suggested that previous studies showed ascertainment bias.

1.2.2.1.3 The Myotonin-Protein Kinase

The myotonin-protein kinase (*DMPK*) gene, which consists of 15 exons occupying over 13,000 bases of genomic DNA, encodes a polypeptide of 624 amino acids that functions as a member of a protein kinase family (Shaw *et al*, 1993). At present it is not known how or indeed if the *DMPK* gene causes muscular weakness or other clinical problems. This is due to the fact that the expanded repeat lies in the 3' untranslated region of the *DMPK* gene, a region that is transcribed into RNA, but not translated into protein. Rather surprisingly it has been demonstrated in mice that the over-expression and under-expression of the *DMPK* gene does not display the typical features of DM (Harris *et al,* 1996). However, it has been suggested that the RNA

produced by the mutant *DMPK* gene may influence the cellular processing of RNA produced by other genes i.e. the gene encoding DM locus-associated homeodomain protein (DMAHP) which is located immediately downstream to the CTG repeat (Harris *et al,* 1996). It has been recently proposed that the RNA produced from the mutant DMPK gene has a toxic effect on muscle cells (Mankodi *et al,* 2000). Cell culture models have been used to demonstrate that mutant DM mRNA takes on a gain-of-function and inhibits myoblast differentiation (Amack *et al,* 1999). Although the molecular mechanism(s) by which this mutant mRNA disrupts myogenesis is not fully understood, recent findings suggest that anomalous RNA-protein interactions have downstream consequences that compromise key myogenic factors (Amack and Mahadevan, 2004). Several studies have attempted to determine the effects of the expansion in nearby genes. Fillipova *et al* (2001) suggested that changes in chromatin structure, and the resulting misregulation of genes in the vicinity could be relevant to the defects of muscle and brain development that occur in congenital DM patients.

1.2.2.1.4 Mechanisms of DM Pathogenesis

Most dominant disorders are caused by the expression of an abnormal protein with an altered function, it has not been clear how the multisystemic clinical features of dominantly inherited DM could be caused by a trinucleotide repeat that did not affect the protein coding portion of a gene (Tapscott, 2000). Four different mechanisms have been proposed for the explanation of the DM pathogenesis (Ranum and Day, 2004):

1. Haploinsuffiency of *DMPK*; Earlier studies suggested that alteration of the *DMPK* expression might cause the multisystemic features of the disease (Fu *et al,* 1992), however, studies in *DMPK* knockout mice showed only a very mild, late-onset myopathy (Jansen *et al,* 1996).

2. Haploinsuffiency of *SIX5* and Neighboring genes: A second mechanism is that the expanded repeat affects the expression of multiple genes in the region. It has been proposed that the mutation interferes with expression of multiple genes in the DM1 region, possibly through regional effects produced by repeat-induced alterations in chromatic structure (Otten and Tapscott, 1995). Studies using *Six5* knockout mice provided some support to this mechanism (Klesert *et al,* 2000) however was not conclusive.

3. RNA pathogenesis: Another proposed mechanism has been that the enlarged CUG-containing transcripts accumulate as intranuclear foci and disrupt cellular function which came from a transgenic mouse model (Mancodi *et al,* 2000).
4. Additive model: This model of DM1 has been suggested where all the above mechanisms contribute to disease pathogenesis (Larkin and Fardaei, 2001).

1.3 Molecular Cytogenetic Techniques

Extensive research in the field of chromosome analysis has lead to constant development of laboratory techniques available to detect abnormalities. Nowadays, scientists demand techniques to be powerful, accurate, reproducible and able to provide results in the shortest amount of time possible. Clinical cytogenetics is the study of chromosome structure and behaviour in relationship to clinical syndromes. Karyotyping was the first technique allowing analysis and identification of all 23 pairs of chromosomes with respect to number and morphological structure. Karyotyping is still considered the most widely applicable technique in clinical and research cytogenetics. Fluorescent *in situ* hybridisation (FISH) is a relatively novel technique, which combines cytogenetics and molecular technology. FISH combines DNA hybridisation techniques with fluorescent microscopy, allowing direct visualisation of a specific DNA sequence onto metaphase chromosomes, interphase nuclei or DNA strands. The combination of FISH and karyotyping has lead to the development of innovative techniques such as spectral karyotyping (SKY-FISH) (Schrock *et al,* 1996) and multi-fluorochrome karyotyping (M-FISH) (Speicher *et al,* 1996) (see section 4.1). The Polymerase Chain Reaction (PCR) using oligonucleotide primers and a thermostable DNA polymerase (Saiki *et al,* 1985), is another key technique designed to enrich a DNA sample for a specific fragment, amplifying it to a level at which it can be visualised and subjected to further genetic analysis. However, the most novel technique which has united traditional cytogenetics and modern molecular techniques is comparative genomic hybridisation (CGH). CGH allows in a single hybridisation the copy number of all 23 pairs of chromosomes to be assessed in situations that do not allow standard methods of chromosomal analysis to be used (Kallionemi *et al,* 1992).

1.3.1 Karyotyping

1.3.1.1 Karyotyping Principles

Tijo and Levan in 1956 discovered that the normal human somatic cell contains 46 chromosomes rather than 48. The methods they used, with certain modifications, are now employed in all cytogenetic laboratories to analyse the chromosome constitution of an individual, known as a karyotype. Any tissue with living nucleated cells which undergoes division can be used to study human chromosomes. These cells are cultured with nutrients for 3 days to stimulate the T lymphocytes (for whole blood), chorionic villi (for CVS) and amniotic fluid cells to divide e.g. phytohaemogglutinin. While in culture, colchicine is added to prevent the formation of the spindle, thus arresting cell division during metaphase. During metaphase the chromosomes are maximally condensed and therefore more easily visible.

There are several different staining methods that can be utilised to identify individual chromosomes: 1) G or Giemsa banding is the most common method used. The chromosomes are treated with trypsin, denaturing their protein content and giving each chromosome a characteristic pattern of light and dark bands. 2) Q or Quinacrine banding gives a similar banding pattern to G-banding and requires examination of the chromosomes under ultraviolet fluorescence. 3) R or Reverse banding shows reverse light and dark banding patterns to G-banding since the chromosomes are heated before staining. 4) C or Centromeric Heterochromatin banding preferentially stains highly repetitive DNA sequences such as centromeres and heterochromatic regions, by treatment of chromosomes with acid followed by alkali prior to G-banding.

1.3.1.2 Karyotyping Applications

Karyotyping has been widely used in prenatal diagnosis for genetic analysis of amniocentesis and chorionic villus sampling (CVS) in high risk women. Karyotyping is widely in the analysis of different types of cancers e.g. sarcoma (Ravi and Wong, 2006), leukaemia (Scandura, 2005) and myelomas (Schilling *et al,* 2005) Furthermore, it has been used for research purposes on oocytes, polar-bodies, spermatozoa and embryos (Martin *et al*, 1986; Plachot *et al,* 1988; Zenzes *et al*, 1991; Jamieson *et al*, 1994; Clouston *et al,* 1997; 2002). Karyotyping was amongst the first

technique to be used for embryo analysis. Karyotyping has been used extensively for investigating the mechanisms of nondisjunction in human oocytes and polar bodies.

1.3.1.3 Technical Limitations of Karyotyping

Karyotyping provides valuable information at the research and diagnostic level. Karyotyping has been considered the 'mother of all techniques'. However, technical difficulties have created the need to develop newer and superior techniques. Problematic fixation methods resulting in loss of chromosomes and over dispersed or poorly spread cells which are not in a single focal lane restricting the potential of analysis are the main disadvantages (Martin *et al,* 1996). Moreover, long colcemid exposure times are able to increase the mitotic index but this produces highly contracted chromosomes that exhibit chromatid separation and G-band poorly compromising the information obtained (Jackson *et al*, 2002). Furthermore, karyotyping for prenatal diagnosis requires the isolation of metaphase chromosomes and takes 7-10 days for a final result (Reid *et al,* 1992), which can increase the emotional burden on the patient (Goel *et al,* 1998). The most important limitation during G-banding is that only a few metaphases can be obtained hence only a small proportion of the cells can be analysed. In the study of preimplantation embryos basic technical difficulties of handling individual embryos compromises the potential quality of preparations and hence limits the amount of information that can be gained (Harper *et al,* 1995).

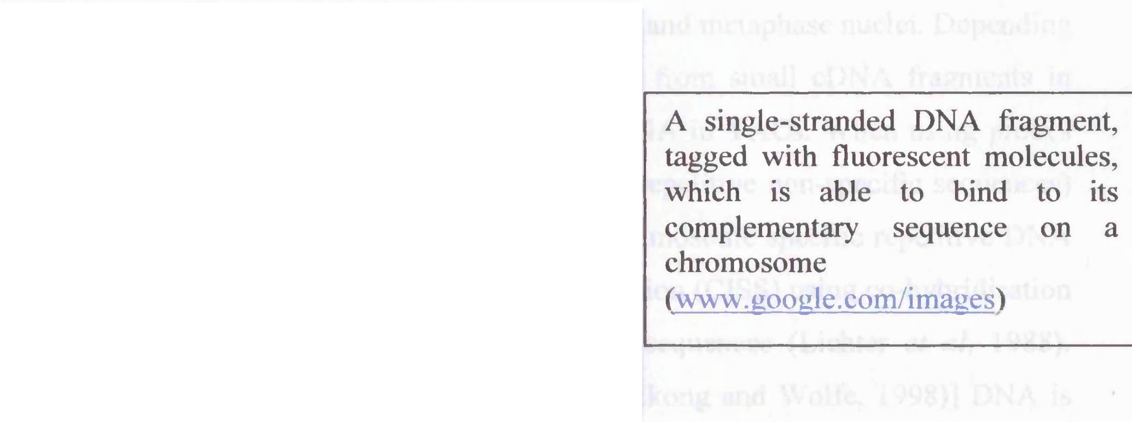
1.3.2 FISH

1.3.2.1. FISH Principles

The combination of chromosome banding techniques with the revolutionary technique of *in situ* hybridisation has aided in the research and diagnosis of structural abnormalities not previously possible. *In situ* hybridisation was first introduced in 1969 using radiolabelling detection (Pardue and Gall, 1969; John *et al,* 1969). In 1986, Pinkel *et al* and Cremer *et al* reported FISH using non-radioactively labelled probes. Fluorescent *in situ* hybridisation (FISH) is a molecular cytogenetic technique for enumerating chromosomes combining DNA hybridisation techniques with fluorescent microscopy. FISH utilises fluorescently labelled DNA probes, which are essentially single stranded DNA fragments capable of binding to their complementary

sequences in an interphase nucleus or a metaphase spread that are fixed and located on a microscope slide (Pinkel *et al,* 1986; Tonnies, 2002) (Figure 1.5).

Figure 1.5. Illustration of a FISH probe



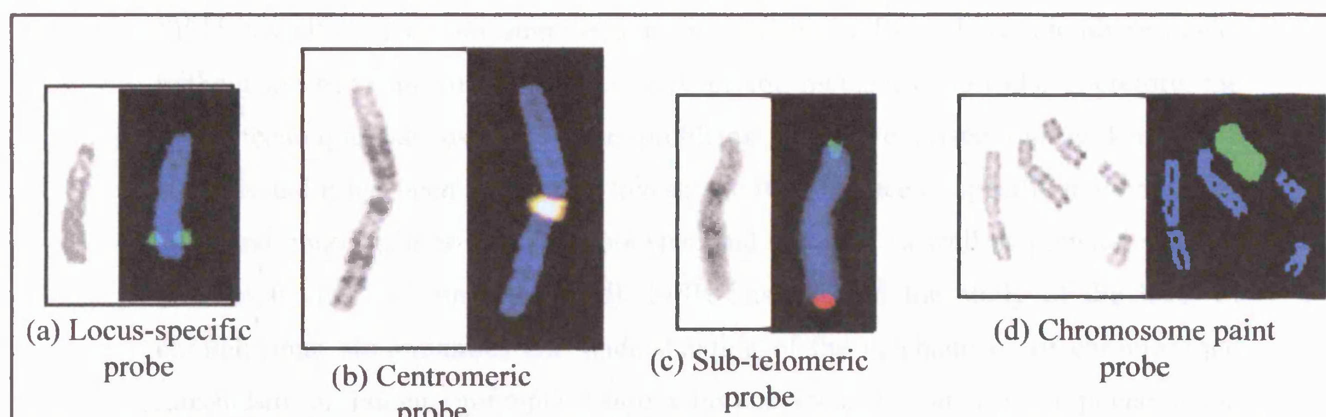
A single-stranded DNA fragment, tagged with fluorescent molecules, which is able to bind to its complementary sequence on a chromosome (www.google.com/images)

FISH probes are quite sensitive DNA molecules being able to detect regions as small as 0.5 kb on metaphase chromosomes (McNeil and Ried, 2000). Cloned DNA fragments are usually the constitution of FISH chromosomal probes, which are characterised by the ability to anneal only to their complementary DNA sequences (Pinkel *et al,* 1986). Probes employed for FISH can be directly or indirectly labelled. The indirectly-labelled probes are labelled with a hapten such as biotin and digoxygenin, which are detected using affinity reagents labelled with fluorophore-linked immunoglobin reagents. This immunocytochemical detection of 'indirectly-labelled' probes allows the flexibility to amplify a weak FISH signal, necessary by sequential applications and is especially useful for visualising smaller probes. However, indirect labelling is a time-consuming process with non-specific background fluorescence potentially making interpretation difficult. The development of directly labelled probes, where the fluorochrome is attached to the probe itself, reduced the time-frame of the FISH experiments and further improved the specificity of the fluorescent detection (Wiegant *et al,* 1993). The most common fluorophores include those based on fluorescein (green) or rhodamine (red), although this selection has widened considerably in the last few years with the introduction of a new generation of commercial fluorophores such as the Cyanine, Blue, and Gold dyes (Yurov *et al,* 1996).

There are three types of probes that are used in FISH with respect to their size, location and how they bind onto the chromosome.

a. **Locus specific probes** (Figure 1.6a), are probes able to bind to a unique sequence of a particular chromosome. Often called band-specific, they require more than six hours to hybridise and can be used in interphase and metaphase nuclei. Depending on the type of clone, these probes may range from small cDNA fragments in plasmids, to much larger blocks of genomic DNA in YACs. When using probes containing unique DNA sequences (as well as repetitive non-specific sequences) suppression of the hybridisation of the non-chromosome specific repetitive DNA is accomplished by chromosome in situ suppression (CISS) using co-hybridisation with labelled probe and unlabelled repetitive sequences (Lichter *et al*, 1988). Currently, CoT-1 [coefficient of temperature (Ekong and Wolfe, 1998)] DNA is added to the probe DNA, binding and blocking the repeat sequences and consequently allowing the probe to bind to the unique sequence that it targets.
b. **Repetitive probes** (Figure 1.6b&c), bind to repetitive sequences located in the centromeric, heterochromatic and telomeric regions of a chromosome. These include the widely used α-satellite/centromeric probes (b), which hybridise specifically to the centromeres of individual chromosomes (Willard, 1985). Heterochromatic probes include those binding to the heterochromatin usually situated below the centromere, seen in chromosomes 1, 9 and Y. Telomeric probes specifically recognize the repetitive sequence TTAGGG, and can be used for the simultaneous identification of all telomeres. They can be used in interphase and metaphase chromosomes and require only one hour to hybridise. The signals obtained are very bright and the whole FISH procedure can be performed within two hours (Harper *et al*, 1994c)
c. **Chromosome paints** (Figure 1.6d), paint the whole chromosome and can only be used in metaphase preparations. They contain a cocktail of DNA sequences hybridising to an entire chromosome or chromosome arm and are derived either from a pool of clones picked from a chromosome specific library (Fuscoe *et al,* 1989) or from flow-sorted chromosomes amplified by PCR (Vooijs *et al,* 1993).
d. **Whole genome probes.** Label the whole chromosome whilst the cells are in metaphase in one fluorescent colour either red or green. These types of probes are widely used whilst carrying out CGH on metaphase chromosomes.

Figure 1.6. Different types of FISH probes.



(a) Illustration of a locus-specific probe labelled in Spectrum Green; (b) Illustration of a centromeric probe labelled in Spectrum Orange. Illustration of two sub-telomeric probes; one for the short arm and one for the long arm, labelled in Spectrum Green and Spectrum Orange respectively and (c) Illustration of chromosome paint. (Harper, *et al*, 2000)

FISH protocols vary between laboratories; however the same basic principles apply. The tissue/cells are obtained and placed on a microscope slide by some sort of fixation, either by methanol/acetic acid fixation or by applying adhesive agents on the actual slide prior to fixation. The slide with the tissue/cells is pre-treated with proteolytic enzymes and /or RNase digestion which render the nucleus of the sample accessible to the DNA probe. Denaturation of the slide and probe follows, causing the double DNA strands to separate. The sample is then left to re-anneal under optimal conditions, the length of time depending on probe type. The stringency with which the probe binds is controlled during post-hybridisation treatment by varying the temperature, ionic strength and concentration of formamide washes to destabilise and remove imperfectly base-paired probe/target duplexes. In this way only probe bound stably to its complementary target remains. After hybridization the slide is observed under ultraviolet light of the proper wavelength, and any region where the labeled DNA fragment has bound fluoresces. The scoring criteria that are most frequently applied are adopted from Hopman *et al* (1991) which state that signals must be a minimum of a signal's width apart in order to be scored as 2 individual signals.

1.3.2.2. FISH Applications

1.3.2.2.1 Interphase FISH applications

FISH has allowed chromosome enumeration to be performed on interphase nuclei without the need for culturing cells or preparing metaphase spreads. Therefore, the FISH technique has overcome the problems that have arisen during karyotypic analysis and it has been possible to investigate the presence of specific numerical and structural abnormalities in sperm, oocytes, and embryos as well as prenatal samples (chorionic villi and amniotic fluid). FISH has allowed the study of the level of chromosomal abnormalities and understanding of the mechanisms of chromosomal mosaicism in human preimplantation embryos (see section 1.6), a phenomenon whereby two or more cell lines are present in a tissue, which differ in their genetic constitution but are derived from the same zygote.

The growing availability of commercially produced probes and probe-cocktails has enabled FISH to become an outstanding routine diagnostic tool in the cytogenetic laboratory. The wide selection of existing centromeric probes allows determination of chromosome-specific ploidy on solid tumours, fresh or archived biopsy specimens, and on cultured and uncultured preimplantation, prenatal, postnatal and hematologic samples. In malignant solid tumors, genomic imbalances resulting from either gains or losses of whole chromosomes or segments of chromosomes are frequently observed using FISH. FISH has enabled the observation and further understanding of how the presence of the large genomic abnormalities are believed to contribute to the disease phenotype by alteration of normal patterns of gene expression. Examples of these genomic aberrations include the aneuploidy seen using FISH in breast, bladder and prostate tumors (Hopman *et al,* 1991; Cornelisse *et al,* 1992) as well as chromosomal rearrangements seen in hematopoietic cancers (Hilgenfeld *et al,* 1999).

In gynaecology and fetal medicine, multicolour FISH has assisted in investigating fertility for example in women with endometriosis (Shin *et al*, 1997) and men suffering from Klinefelter's syndrome (Guttenbach *et al*, 1997) where both conditions cause infertility. The chromosomal constitutions of these patients were analysed by FISH in an attempt to attain causes of the infertile status of these patients. Furthermore, the effects of clinical treatment such as chemotherapy or radiotherapy

on chromosomes has been examined by FISH, whereby a sperm sample was taken before and after treatment and its effects of studied (Monteil *et al*, 1997).

The FISH technique has been found to be highly effective for rapidly determining the number of specified chromosomes in interphase cells. FISH thus seemed to be especially appealing for the prenatal detection of chromosomal aberrations. The first developed probes were derived from DNA of flow-sorted whole chromosomes and used for prenatal diagnosis of trisomy 13, 18 or 21 by Kuo *et al* (1991) on uncultured amniotic fluid cell nuclei. Interphase FISH with a specific probe set for familial rearrangements also allows rapid exclusion of an unbalanced translocation in the fetus of a balanced translocation carrier (Kilby *et al*, 2001).

Aneuploidy and chromosomal rearrangements diagnosed by FISH can be performed on preparations made from around 1.0-1.5ml of amniotic fluid or chorionic villus samples (sections 1.4.1 and 1.4.2). Many prospective studies have been carried out examining the efficacy and accuracy of the FISH probes and subsequently the FISH technique and have revealed more than 90% accuracy (Hulten *et al*, 2003). The most significant point of doing this is a rapid diagnosis without having to wait for culture and karyotype. It has also been shown the risk of over- or under-diagnosis of aneuploidy for the target chromosomes is limited by interphase FISH compared to the 'gold standard' of karyotyping. FISH on uncultured interphase amniocytes and chorionic villus cells, might aid diagnosis of constitutional mosaicism (Feldman *et al*, 2000). Quilter *et al* (2001) stipulated that interphase FISH may be of special advantage with respect to the problem of confined placental mosaicism in chorionic villus sample.

1.3.2.2.2 Metaphase FISH applications

Speicher *et al*, (1996) attempted combinatorial labelling of twenty-four chromosome paints using five fluorophores, reporting the first study of Multiplex-FISH (M-FISH). Following hybridisation, a monochrome CCD camera with multiple optical filters was used to capture each channel sequentially before merger to form a final image. The basis for M-FISH analysis depends on the presence or absence of probe signal for each fluorophore at each pixel that is then compared to the labelling strategy to identify the chromosome (Speicher *et al*, 1996). However, many groups have tried to

maximise the number of probes that can be used employing combinatorial or Boolean labelling i.e. combining labelled probes in different proportions much earlier that 1996. Nederlof and colleagues (1990) attempted FISH utilising three fluorophores to detect four chromosomes and Wiegant *et al* (1993) developed combinatorial labelling, to the detection of six loci on a single chromosome. Combinatorial labelling remains a strategy that although in theory many combinations are possible, in practice the number of combinations is dependent on the sensitivity of the imaging system.

Chromosomal microdeletions are associated with a number of syndromes including Di-George and Williams syndromes. Microdeletion probes consist of a probe specific for the locus or region of deletion associated with the microdeletion syndrome, as well as a control probe for accurate identification of the chromosome of interest. Their use has proved very useful for the detection of a range of microdeletion syndromes, providing rapid same-day results (Ligon *et al,* 1997). Idiopathic mental retardation accounts for approximately 36% of the moderately to severe handicapped population and are associated with subtle subtelomeric chromosomal rearrangements (Raynham *et al,* 1996). Although these abnormalities remain undetected by traditional cytogenetics, they can be readily detected using a panel of FISH probes specific for the subtelomeric regions of all non-acrocentric chromosomal arms (Knight *et al,* 1999)

1.3.2.3. Technical Limitations of FISH

FISH has been hailed as one of the most robust and efficient techniques however, it has certain limitations. There are conflicting reports as to the true sensitivity and specificity of FISH diagnoses with ranges of diagnostic accuracy reported between 70-98% (Christensen *et al,* 1993; Ward *et al,* 1993; Pergament *et al,* 2000). The availability of FISH probes has increased in recent years but it is still not possible to obtain probes that cover all different parts of chromosomes rendering some specific chromosomal rearrangements impossible to diagnose. FISH is unable to distinguish between samples of balanced and normal chromosomal complements in structural aberrations in interphase nuclei (Warburton, 1991; Conn *et al,* 1998). The lack of fluorescent dyes also limits the scope of diagnosis since not many different chromosomes can be analysed simultaneously.

1.3.3 Polymerase Chain Reaction

1.3.3.1 PCR Principles

In 1983, Dr Kary Mulis conceived a novel concept of performing a test-tube process of repetitive DNA synthesis, termed polymerase chain reaction (Mulis *et al,* 1986; Mulis, 1990). The polymerase chain reaction (PCR) is used to amplify a sequence of DNA using a pair of oligonucleotide primers each complementary to one end of the DNA target sequence. These are primers extended towards each other by a thermostable DNA polymerase in a reaction cycle of three steps: denaturation, primer annealing and polymerisation or primer extension. PCR is a powerful molecular technique for quickly amplifying a particular DNA fragment to a stage that can be further analysed by other methods (Saiki *et al,* 1985). The success of PCR in achieving this objective has enabled it to become one of the most important methods in genetic testing having numerous applications in basic research and medicine.

The first step, denaturation, is able to separate the two strands. The temperature is then reduced depending on the primer length and sequence to allow the specific primers to anneal. After annealing, the temperature is increased for optimal polymerisation which uses a mix of deoxynucleoside 5'-triphospates (dNTP's) and a polymerase, which are substrates for DNA synthesis and Mg^{2+}. In the first polymerisation step, the target is copied from the primer site for various distances on each target molecule until the beginning of cycle 2, when the reaction is heated up again in order to denature the newly synthesized molecules. In the second annealing step, the other primer can bind to the newly synthesized strand and during polymerisation can only copy till it reaches the end of the first primer. Therefore, at the end of cycle 2, some newly synthesized molecules of the correct length exist. The successive cycles of DNA synthesis result in an exponential amplification of the target DNA sequence leading to a 10^5-10^6-fold increase in the amount of target DNA. If the PCR technique is 100% efficient, one target molecule would become 2^n after n cycles. In practice, 20-30 cycles are carried out for a PCR experiment, however, during single-cell PCR up to 50 cycles have been reported (Piyamongkol *et al,* 2001a).

PCR primers usually need to be about 18-30 base pair long (bp) and to have similar G+C contents in order to anneal to their complementary sequences at similar temperatures. The primers are designed to anneal on opposite strands of the target sequence so that they will extend towards each other by addition of nucleotides to their 3'-ends. Short target sequences amplify more easily, so often this distance is less than 500bp, however, with optimisation, PCR can amplify fragments over 10kb in length (Reiss and Cooper, 1990).

Thermostable DNA polymerases have been mostly isolated and cloned from a number of thermophilic bacteria. The most common is *Taq* polymerase, which is isolated from *Thermus Aquaticus*. It survives the high temperature denaturation step, usually 95°C, having a half-life of more than two hours at this temperature. However, Taq polymerase is known to introduce errors when it copies DNA since it does not have associated 3' to 5' proofreading exonuclease activity. Therefore, other thermostable DNA polymerases have been introduced with greater accuracy including AmpliTaq™.

1.3.3.1.1 Multiplex PCR

The technique of amplifying multiple loci concurrently is termed multiplex PCR (Findlay *et al*, 1995a, b; Pertl *et al*, 1996; Sherlock *et al*, 1998). This can facilitate the diagnosis of a specific genetic disease or of multiple diseases since it provides information for multiple loci at the same time. One example of multiplex PCR in a non-single-cell PCR is the amplification of nine regions of the DMD gene in a single reaction (Chamberlain *et al*, 1988). Each multiplex PCR needs to be optimised for the combination of primers involved with regard to primer concentrations, annealing temperature, the reaction buffer utilised and number of cycles carried out. Multiplex PCR is usually achieved using F-PCR, as several primers can be multiplexed together employing different fluorescent dyes even if the product ranges overlap each other (Kimpton *et al*, 1993). Strategies for multiplex PCR reactions have been widely reported in prenatal (see section 1.4.5) and PGD (see section 1.5.3) setups.

1.3.3.1.2 Whole Genome Amplification

An alternative method to multiplex PCR that can be used for amplification of low copy numbers of the entire genome is whole genome amplification (WGA) (Zhang *et*

al, 1992; Snabes *et al*, 1994). With WGA a single genome can be amplified numerous times, thus providing sufficient DNA templates for many independent PCR amplifications (Wells and Sherlock, 1998). There are three types of WGA described:

Primer Extension Preamplification (PEP) is a WGA technique whereby at least 70% and 90% of the genome is amplified more than 30 times according to Zhang *et al*, (1992), and Wells *et al* (1998) respectively. PEP can be viewed as essentially a pre-diagnostic PCR treatment, since the PEP product can be subsequently used in a further PCR to diagnose a specific disorder. It has been suggested that PEP is unsuitable for clinical PGD (Findlay, 2000) (see section 1.6.3.3).

Degenerate Oligonucleotide Primed PCR (DOP-PCR) has also been considered as a valuable WGA technique (Telenius *et al*, 1992). DOP-PCR amplifies a similar proportion of the genome to PEP, but to a much more significant level. DOP-PCR followed by comparative genomic hybridization (CGH) analysis of tumour DNA has been described by several investigators (Speicher *et al*, 1993; James and Varley, 1996). A significant drawback of DOP-PCR and of other WGA techniques is that amplification of repetitive DNA sequences, such as short tandem repeats is error-prone when performed on WGA products (Wells *et al*, 1998). In some studies over 50% of fragments amplified are found to differ from their expected size, presumably due to slippage of the DNA chain during product generation (Wells and Sherlock, 1998). The low annealing temperatures that characterize all WGA protocols may underlie this problem (Wells *et al*, 1998).

Multiple Displacement Amplification (MDA) is a rolling-circle amplification (Lizardi *et al*, 1998) method that was developed for amplifying large circular DNA templates such as plasmid and bacteriophage DNA (Dean *et al*, 2002). Using *φ29* DNA polymerase and random exonluclease-resistant primers, DNA is amplified in a 30°C reaction not requiring thermal cycling. This is made possible in part by the great processivity of *φ29* DNA polymerase, which synthesises DNA strands 70kb in length (Blanco *et al*, 1989). Dean and colleagues (2002) showed that MDA-generated DNA product is >10kb, and its performance can be demonstrated for a variety of applications including single nucleotide polymorphism (SNP) analysis, restriction fragment length polymorphism (RFLP), Southern blotting, DNA sequencing as well

as comparative genomic hybridisation (CGH). However, in the same study the capability of MDA was not assessed on single cells. More recently Spits *et al* (2006), was able to carry out 22 locus-specific PCR's from minute quantities of DNA.

1.3.3.2 Methods of Detection

PCR is designed to enrich a DNA sample for one specific fragment, amplifying it to a level where it can be visualised and subjected to further genetic analysis (Wells and Sherlock, 1998). The methods of mutation analyses most commonly used are referred to as the 'scanning' methods, which can be used for diagnostic purposes and applied for searching of uncharacterised mutations. Scanning methodologies are optimal for diagnosis of inherited disorders caused by a heterogeneous spectrum of mutations.

1.3.3.2.1 Fluorescent PCR

The traditional methods for visualizing PCR products following electrophoresis include ethidium bromide or silver staining. Both techniques benefit from nested PCR to ensure sufficient amplified fragments for visualization. The utilisation of radioactively labelled primers or nucleotides can be employed for visualization for greater sensitivity however it has been characterised as a time consuming technique as well as harmful. Furthermore, all of the above techniques are limited by their qualitative nature, with quantities of DNA as well as products of a similar size being indistinguishable (Wells and Sherlock, 1998). The advent of fluorescent PCR technology (Hattori *et al,* 1992) has enabled more far reaching diagnostic applications to be considered especially at the single cell level. Fluorescent-PCR (F-PCR) is a modification of PCR technology using fluorescent primers and an automated DNA sequencer and has improved both PCR accuracy and sensitivity (Ziegle *et al,* 1992). The application of oligonucleotide primers attached to fluorescent molecules gives rise to amplified products labelled with a fluorescent dye. When these F-PCR products migrate under electrophoresis to the position where the laser bisects the products, the fluorescent molecules are activated by the laser and give a signal of a specific wavelength, subsequently detected by a CCD detector and analyzed by computer software. The fluorescence dye is detected at a much lower threshold level than conventional agarose or acrylamide gel analysis. The size analysis is as precise as a single base pair difference. It is of major importance that fluorescent PCR is

compatible with heteroduplex analysis (Rommens *et al,* 1990; section 1.3.3.2.3), single strand conformational polymorphism (SSCP) (Ellison *et al,* 1993; section 1.3.3.2.4) as well as amplification refractory mutation system (ARMS) (Sherlock *et al,* 1998; section 1.3.3.2.5).

The main advantage of fluorescent PCR is its sensitivity compared to conventional PCR techniques. F-PCR is able to detect minute amounts of the fluorescent product such that for a single cell only 35-40 cycles of PCR are required. This in turn eliminates the need for nested PCR, so with one round of PCR amplification products even from a single cell can be analysed. F-PCR has been used extensively for prenatal (Hulten *et al,* 2003) and preimplantation genetic diagnosis worldwide (Sermon *et al,* 1998a; Piyamongkol *et al,* 2001a and b; Dean *et al,* 2001). It has been postulated that F-PCR assays are powerful adjuncts to conventional cytogenetic techniques and can be applied for the rapid and accurate prenatal diagnosis of the most frequent aneuploidies (Pertl *et al,* 1999). Fluorescent PCR has been used for analysis of amniotic fluid and chorionic villus sampling (see section 1.4.5) as well as multiple single gene defects in the context of PGD (see section 1.5.3)

1.3.3.2.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE), involves mixing a radiolabelled single stranded DNA probe with double stranded DNA being screened, which has been previously heated to make it single stranded. The mixture is electrophoresed on a denaturing gradient gel. The differences in DNA are detected by virtue of altered melting characteristics, which affect the DNA strand as it passes through the polyacrylamide gel with an increasing concentration of a denaturant. Recently, DGGE has been used for the detection of the major histocompatibility complex (Knapp, 2005).

1.3.3.2.3 Restriction Endonuclease Digestion

Differences in DNA sequence e.g. caused by mutation can be shown using restriction endonucleases to digest the DNA. The restriction endonucleases are bacterial enzymes which recognise specific DNA sequences and cleave the DNA strand near the recognition site. Therefore, if the mutation and DNA sequence are known, a

restriction endonuclease will be chosen and will cleave the normal strand, whereas the mutant will remain undigested. The results are visible after electrophoresis.

1.3.3.2.4 Heteroduplex Analysis

Heteroduplex analysis (HA) is based on the ability of heterozygous DNA to anneal, after denaturation, to its complementary strands which will associate recreating the original homoduplexes. Hybrid molecules will form generated by the association of partially complimentary strands from mutant and normal alleles. The latter heteroduplexes have an area of mismatch, since at the site of mutation the two alleles differ in DNA sequence. The area of mismatch does not anneal and retards heteroduplex migration during electrophoresis. Hence, heterozygocity of a sample can be established by the existence of homoduplexes and heteroduplexes (White *et al*, 1992). Recently, sensitivity has been improved by 97% by combining a high-resolution sieving matrix and nucleosides as additives (Weber *et al,* 2006)

1.3.3.2.5 Single Strand Conformational Polymorphism

Single strand conformational polymorphism (SSCP) is able to detect point mutations as well as DNA polymorphisms in DNA fragments ranging in size from 100-500bp (Orita *et al,* 1989). This is accomplished by denaturing double stranded PCR products into single strands of DNA. These single strands will form stable, sequence-specific conformations and alterations in base sequences (e.g. different alleles) will give distinct conformations, which will subsequently migrate in different rates to non-denaturing electrophoresis gel. Hence, different alleles can be distinguished (see section 1.3.3.1.1). However, SSCP is inefficient at detecting mutations with increasing size of the PCR product tested which limits its ability to analyses PCR products >300bp (Frayling, 2002)

1.3.3.2.6 Amplification Refractory Mutation System

Amplification refractory mutation system (ARMS) is based on the annealing of three different allele-specific oligonucleotides. With ARMS these oligonucleotides serve as primers for PCR and are not directly detectable. For example in a nested PCR reaction one of the primers can be designed to contain the mutation site during the outer reaction. The other two primers can be part of two different inner amplifications, one containing the normal and the other the mutant allele. By amplifying both normal and

mutant alleles a 'safety net' is created in case one of the inner reaction does not amplify (see section 1.3.3.1.1).

1.3.3.3 PCR Applications

PCR has become an essential tool in molecular biology as an aid to cloning and gene analysis. A variety of PCR-modified techniques have been developed for different purposes, including forensic analysis, PND and PGD of single gene disorders. More sophisticated and modern techniques have been devised to better suit the needs of its applications.

The advent of PCR has helped in the genetic linkage of genes and diseases. For example, a tetranucleotide repeat region around the gene for myelin basic protein, after amplification, demonstrated the presence of eight different alleles among 14 multiple sclerosis families and was used to exclude a linkage between this candidate gene and the disease (Rose *et al,* 1993). The usefulness of PCR in the elucidation and understanding of genetic diseases can be measured by the number of diseases that have been identified and diagnosed by utilising the PCR technology including myotonic dystrophy (Caskey *et al,* 1992), cystic fibrosis (Collins, 1992) as well as inherited colorectal cancer (Lunch *et al,* 1994) and breast cancer (Mikki *et al,* 1994). Furthermore, PCR can facilitate the diagnosis of infectious diseases caused by viral, bacterial, fungal, protozoal and other infectious agents. Using PCR on peripheral blood mononuclear cells from HIV affected individuals, HIV-1 sequences were detected in 100% of specimens that contained discernible virus by culture (Ou *et al,* 1988). However, PCR has provided extreme aid in the identification and diagnosis of genetic diseases including β-thalassaemia, Tay-Sach's disease, Duchenne muscular dystrophy in addition to deducing and cloning of cancer-causing genes such as the *Rb* gene associated with retinoblastoma, APC gene associated with adenomatous polyposis coli and mismatch repair genes for nonpolyposis colorectial cancer.

PCR has an advantage over FISH in prenatal diagnosis of aneuploidies, since it can accommodate smaller sized samples (Hulten *et al*, 2003). Quantitative Fluorescent PCR (QF-PCR) has been described as more amenable to automation, and a large number of samples can be handled simultaneously, allowing substantial economy of

scale (Grimshaw *et al*, 2003). It has been postulated that it is less time consuming and labour intensive compared to FISH in a prenatal or preimplantation diagnostic setup (Findlay, 2000).

1.3.3.4 Technical Limitations of PCR

Because of the extremely sensitive nature of the PCR process, contamination from carrying over of previously amplified PCR products in the same laboratory can lead to significant problems. The problem of contamination affects both the diagnostic and quantitative PCR and may be the most formidable obstacle in PCR application. Contamination can occur either by intersample contamination during sample processing and reagent contamination from carryover (section 1.5.3.5.1). Precautionary measures are required such as a separate work bench, pipettes, sterile tips and diligent practice of aliquoting all PCR primers and reagents beforehand to reduce carryover contamination. Furthermore, it has been suggested that use of ultraviolet light (254+300nm) to inactivate as much as 30ng of contaminating double-stranded DNA can alleviate the problem (Sarkar and Sommer, 1993). Nested-PCR has also been found to significantly reduce contamination (Wells and Sherlock, 1998) whereas the utilisation of polymorphic markers can detect contamination (Ma, 1995). The human genome contains many dispersed tandem-repetitive DNA sequences that are polymorphic due to variation in the copy number of tandem repeats. Those with longer motifs are called minisatellites (Jeffreys and Thein, 1985), while those with shorter motifs are called microsatellites (Weber *et al,* 1989). These polymorphic markers can act as contamination markers whilst examining a genetic disease in prenatal and preimplantation diagnosis. An important limitation of PCR and specifically single cell PCR is termed allele dropout (ADO) and poses a serious threat of false positive or false negative results (section 1.5.3.5.3)

1.3.4. Comparative Genomic Hybridisation

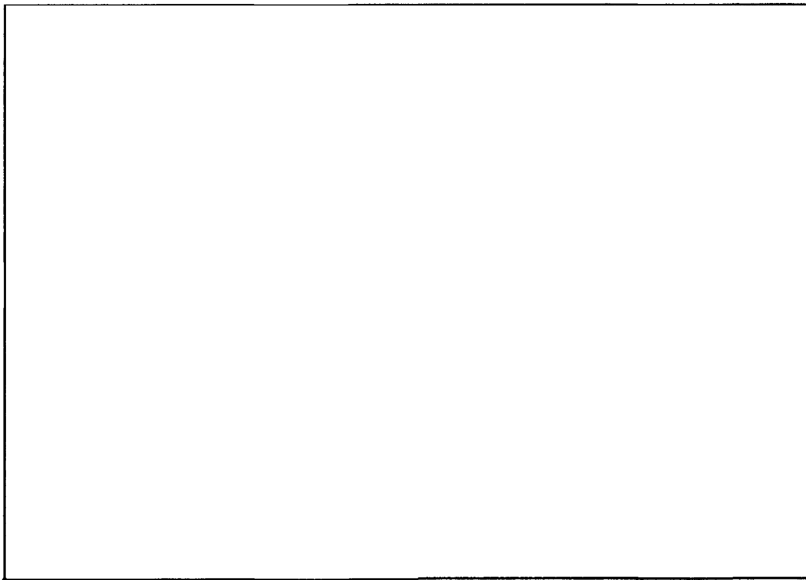
CGH has been described as an alternative method to interphase FISH or G-banding (Kallioniemi *et al,* 1992). It was originally applied for the detection of chromosome copy number changes in tumours and has further identified a variety of unbalanced chromosome complements. As a result quantitative fluorescent analysis using a digital imaging system is able to detect duplications or deletions in the sample DNA mapped

to a target metaphase; ploidy abnormalities and balanced rearrangements however remain undetected.

1.3.4.1 CGH Principles

CGH is a powerful technique that utilises the advantages of the FISH and karyotyping techniques. Even when DNA quantities are minute CGH is able to merge karyotyping, FISH and PCR to view its chromosome complement under a fluorescent microscope. The tissue is tested against a control DNA sample, either a single cell or genomic DNA from a normal individual. The "control" DNA is labelled in spectrum Red and the "test" DNA (tissue under investigation) is labelled in spectrum Green. The test and control samples are mixed and co-hybridised on a target metaphase slide of a normal male individual. The slide is viewed under a fluorescent microscope. Several metaphases are captured, which are analysed using specific software able to give a ratio of the green and red spectra (test and control samples respectively) of the chromosomes therefore, allowing detection and examination for all the chromosome complement. Equal hybridisation of red and green DNA produces a yellow colour. However, if the test sample contains an excess of chromosomal material, trisomy 21 for example, then green DNA fragments for this chromosome will be more abundant than their red equivalents and will out compete the red DNA fragments for hybridisation sites. This effect is only seen on the over-represented chromosome and results in a greenish coloration. Conversely if the test sample has a deficiency of chromosomal material, such as a monosomy, then a predominantly red coloration is seen on the chromosome in question (Figure 1.7).

Figure 1.7: Comparative genomic hybridisation on tumours.



Sample DNA (labelled green) and 46,XY reference DNA (labelled red) are simultaneously hybridised to normal chromosome spreads. The ratio of red:green fluorescence along the axis of each chromosome is calculated by computer. An excess of red fluorescence indicates a deficiency in the sample for the chromosome under analysis, while an excess of green reveals regions of the genome that are over-represented. (www.bu.us; University of Boston)

In Figure 1.7, it can be observed that the ratio of red:green fluorescence along the axis of each chromosome is calculated by a computer. Equal quantities of any loci (disomy) are assigned a profile ratio of 1.0. Changes in the copy number will deviate from this by a factor of 0.5 X n (where n is the number of copies of any locus). Therefore, chromosome gain (trisomy) will produce a ratio of 1.5 and a loss (monosomy), a ratio of 0.5 (Chang and Mark, 1997). The standard resolution of this technique is in the range of 10-40Mb but varies according to the regions analysed and degree of amplification (Kallioniemi *et al,* 1992; 1994; Voullaire *et al,* 1999). However, recent studies have reported sensitivity of their CGH protocols detecting deletions of <10Mb (Kirchhoff *et al,* 1999; Tonnies *et al,* 2001) by averaging CGH profiles. Since the smallest autosome is in excess of 50Mb, CGH analysis provides a very powerful and sensitive method for detecting duplications and deletions of significant size (Wells and Levy, 2003). However, certain regions of the karyotype including centromeres and heterochromatic segments show variation in the profile and these regions are normally excluded from CGH (Kallionemi *et al,* 1994). Certain chromosomes (1p, 17, 19 and 22) are prone to show frequent enhancement of the test signal and are also excluded from the analysis (Moore *et al,* 1997).

1.3.4.2 Single Cell CGH

Most genomic DNA CGH protocols utilise between 100 ng and 1 μg of test DNA, equivalent to more than 10,000 cells. CGH can be applicable when only a single cell, or a few cells are available for analysis such as in the case of PGD when a single blastomere is analysed (Griffin *et al,* 1993) as well as for non-invasive prenatal diagnosis using fetal cells obtained from the maternal circulation (Simpson and Elias, 1995). In these cases universal amplification, using specific PCR, of the minute amount of starting template DNA is required to produce a sufficiently large DNA sample for CGH. Amplification of the whole genome from single cells or a few cells has been reported by performing PEP, DOP, Alu PCR and MDA (Snabes *et al,* 1994; Wells *et al,* 1999; Dean *et al,* 2002; Lasken and Egholm, 2003; Handyside *et al,* 2004; see section 1.6.3.3).

It has been shown that between PEP, DOP, and Alu PCR the most reliable WGA technique is DOP since it provides the most complete coverage of the genome (Wells *et al,* 1999). Wells *et al* (1999) reported successful application of DOP-CGH for diagnosing chromosome imbalance in single cells, which was the first reliable application of CGH in a research context on single cells from fibroblasts, buccal cells, amniocytes, and blastomeres from human preimplantation embryos. Similar results from single fibroblasts and amniocytes were described by Voullaire *et al* (1999) where it was demonstrated that diagnosis of chromosomal aneuploidy in single cells is feasible using CGH with DOP-PCR amplified DNA (see section 1.6.2.3). Single cell CGH has also been successfully employed to assess clonal evolution of genetic variants in complex populations, by subjecting single micrometastatic cells isolated from bone marrow of cancer patients to CGH (Klein *et al,* 1999). Since the first report of successful application of single cell CGH, continuous investigations aim to improve the technique's fidelity. Huang *et al* (2000) conducted a comparative study aiming to define the optimal protocol for single cell CGH. The study employed differently labelled probes and hybridisation combinations and concluded that DOP-PCR-CGH homo-hybridisation (amplified test DNA vs. amplified reference DNA), especially when combined with labelling by nick translation is reliable and reproducible. However, the most important product of the first successful single cell CGH application was the potential use of CGH as a diagnostic tool for detecting

chromosomal abnormalities in human preimplantation embryos (see section 1.6.4). DOP-PCR has been successfully employed as a means to amplify single human blastomere DNA allowing comparative genomic hybridisation to be undertaken by groups worldwide (Wells and Delhanty, 2000; Voullaire *et al,* 2000; Malmgren *et al,* 2002).

1.3.4.3 CGH Applications

CGH was originally developed as a cancer research tool (Figure 1.5), the initial application of CGH involved direct analysis of genomic DNA from tumour specimens. Rising above the usual problems encountered with the use of conventional cytogenetics, CGH did not require time-consuming and laborious techniques of cell culture, or obtaining poor quality metaphase spreads consisting of short chromosomes of low mitotic index unsuitable for G- banding analysis. Therefore, the major advantage of CGH to highlight chromosomal regions of amplification or deletion is that it can be applied to any sample from which DNA can be extracted in a rapid single step, without reliance on the cytogenetic preparations needed for standard FISH or karyotyping..

1.3.4.3.1 Cancer Studies

The use of CGH for the analysis of tumours revealed a number of new recurring chromosomal gains, amplifications, losses and deletion sites. There have been over 1400 articles published that have employed CGH to delineate cytogenetic changes in cancer specimens (Wells and Levy, 2003). A number of groups have employed CGH to show previously unrecorded areas of presumed tumour suppresser gene deletion and oncogene amplification in cell lines and solid tumours (Kallioniemi *et al.,* 1994; Kokkola *et al.,* 1997; Van Roy *et al.,* 1997). Some of the tumours studied included; Uveal melanomas (Becher *et al,* 1997), small-cell lung carcinomas (Ried *et al,* 1994; Levin *et al.,* 1995) and breast cancer (Kallionemi *et al,* 1994). CGH has also shown its prognostic value by detecting chromosomal alterations in neoplasms including node-negative breast cancer (Isola *et al,* 1995), renal cell carcinomas (Moch *et al,* 1996), Uveal and cutaneous melanomas (Prescher *et al,* 1996; Wiltshire *et al,* 1995) and bladder cancer (Kallionemi, *et al,* 1995). CGH has also been applied to neoplastic samples relevant to reproductive medicine, such as: prostate cancer (Joos *et al,* 1995;

Visakorpi *et al,* 1995), testicular germ cell tumours (Korn *et al,* 1996) and ovarian tumours (Kiechle *et al,* 2001).

1.3.4.3.2 Prenatal Studies

Similarly in clinical cytogenetics CGH has been applied to prenatal and paediatric samples as well as mitotically inactive cells derived from products of conception (Bryndorf *et al,* 1995; Levy *et al,* 1997; Wells and Levy, 2003) to characterise marker chromosomes, cryptic deletions and complex rearrangements, and the origin of intrachromosomal duplications (Benzaken *et al,* 1998; Daniely *et al.,* 1999; Kirchhoff *et al,* 2001). Lapierre and co-workers (2000) carried out the first prospective study of CGH on uncultured amniocytes and concluded that CGH is a valuable alternative to interphase FISH for the rapid detection of unbalanced chromosomal aberrations. CGH has also been applied to material from spontaneous abortions (Daniely *et al,* 1998), where it was able to detect 48% of the abnormalities usually detected by conventional karyotyping including trisomies, monosomies and partial gains and losses. Tabet *et al* (2001) used CGH to analyse trophoblast cells from spontaneous abortions, intrauterine fetal death, and malformed fetuses, circumventing the need for culture. The investigations suggested that the contribution of chromosome aberrations to first trimester pregnancy loss is nearly 70%.

1.3.4.3.3 Embryo Studies

Recently PGS has been carried out on using CGH (Wilton *et al,* 2001; Wells *et al,* 2002). The application of CGH for PGS is not straightforward as after embryo biopsy there is only a narrow window of time for the diagnosis to be made. As most protocols for CGH require 72 hours for hybridisation alone, two strategies have been proposed. Embryos can be frozen following biopsy and thawed after the CGH analysis has been completed (Wilton *et al,* 2001) or alternatively Wells *et al* (2002) tried an accelerated protocol following polar body biopsy on the day of fertilisation. Both studies suggested that the major limitation of CGH for PGS is the investigation takes time due to the laborious analysis of template chromosomes. These difficulties will be overcome when the template chromosomes are replaced by microarrays where hundreds or thousands of chromosome specific probes are spotted onto a glass slide (Wilton, 2002). In the study carried out by Wilton *et al* (2001), the need for cryopreservation reduces embryo viability and implantation potential by 30% (Edgar

et al, 2000), whereas in the study by Wells *et al* (2002), post-zygotic abnormalities, which account for more than half of abnormalities, as well as paternally derived aneuploidies are not detected

1.3.4.3 Technical Limitations of CGH

One of the few limitations of CGH is that it only detects relative alterations of chromosome copy number and cannot detect changes that involve the entire set of chromosomes, therefore it is unable to detect abnormalities of ploidy (Wells and Delhanty, 2000). Although providing information on imbalances of all chromosomes, unlike conventional karyotyping, CGH fails to provide information on chromosomal architecture. Furthermore, CGH analysis is suboptimal for both telomeric and pericentromeric regions, because of low fluorescence intensities and highly polymorphic regions that are blocked by COT-1 DNA (Daniely *et al*, 1998). The most significant limitation in utilizing CGH in a clinical setting and especially in IVF, relates to the technical complexities of the technique, as the method has been described as time consuming, labour intensive, and requiring expertise with several cytogenetic and molecular genetic techniques (Wells and Levy, 2003).

1.4 Prenatal Diagnosis

Prenatal diagnosis with a view to identifying fetal genetic disorders started in the early 1970's. Prenatal tests can be divided into two groups: a) screening techniques such as those involving measurements of chemicals in maternal blood and imaging the fetus and b) diagnostic techniques including invasive tests to remove tissues of fetal origin. Women who are at known risk e.g. carrying a translocation or a single gene disorder and women who are found to be at risk due to abnormal screening results undergo invasive tests, which will allow karyotyping or identification of the single gene defect. The appropriate technique is selected based on the evaluation of gestational age, the urgency to obtain a result, the risk of the procedures and the *a priori* risk for a disorder in the fetus. Fetal cells for chromosome diagnosis are obtained invasively either by chorionic villus sampling (CVS) at about 9-11 weeks gestation or amniocentesis at about 15-20 weeks of gestation. Both procedures are invasive and carry an associated risk for induced abortion of 0.5-2% of women tested (Hulten *et al*, 2003).

1.4.1 Chorionic Villus Sampling

CVS can be carried out from 9-11 weeks of gestation. The timing of diagnosis can be crucial in minimising the trauma of an elective abortion caused to the patient when unbalanced chromosomal abnormalities as well as single gene defects are diagnosed. The procedure is done either transcervically or transabdominally. For transcervical sampling different catheters are used including curved biopsy forceps as well as the catheter originally designed by Ward and colleagues (1983). For the transcervical approach, a bendable polyethylene catheter with a metal obturator is introduced through the cervix and guided to the chorionic frondosum under ultrasound surveillance. With an attached syringe (partly filled with medium) a vacuum is applied and approximately 10-50mg are aspirated and rinsed into a Petri dish (Rodeck and Whittle, 1999). The transabdominal technique was initially developed in Denmark by Smid-Jensen and Hahnemann (1984). It can be carried out either as a free-hand ultrasound-guided fine-needle aspiration or with a needle guide ultrasound transducer. In CVS, some fetal cells are spontaneously dividing and cells at metaphase can be obtained. However, the resolution, which is of great importance for the detection of structural abnormalities, may be low due to the condensed nature of the dividing cells, hence cells are cultured.

Two major problems are encountered in fetal karyotyping using cultured cells from chorionic villi: the relatively slow growth of these cells in culture, which subsequently delays the diagnosis and the occurrence of maternal cell contamination (MCC) (Goumy *et al,* 2004). However, chorionic villi are considered an excellent source of DNA supplying sufficient amounts for most molecular genetic techniques without prior culture. Thus, CVS is the method of first choice in pregnancies at risk for monogenic diseases (Stranc *et al,* 1997). Biochemical testing after CVS is possible for most metabolic disorders and is advantageous over amniotic fluid cells due to the high recurrence risks and the usually feasible use of fresh uncultured villi (Holzgreve *et al,* 1999). Confined placental mosaicism (Section 1.4.7.1) has been detected in 2% of viable pregnancies (Kalousek and Vekemans, 1996; Grati *et al,* 2006) and can give false positive results which need to be confirmed by amniocentesis (Lacroute *et al,* 2004)

1.4.2 Amniocentesis

Amniocentesis can be carried out at gestational ages between about 14 weeks and term. Traditionally this test is offered between 15-17 weeks of gestational age when the total amount of amniotic fluid is approximately 200ml and the uterus can be reached transabdominally without major risks of transversing the bladder or bowel. Nowadays, the correct line of insertion of the needle is chosen by ultrasound and subsequently the puncture and aspiration are performed under continuous ultrasound surveillance (Holzgreve *et al*, 1999). In most cases, culture of amniotic fluid cells is required for prenatal diagnosis and this takes a further 1-4 weeks, depending on the number of cells required and individual variations in the speed of cell growth. This is due to the fact that amniotic fluid samples do not contain any fetal cells in division and have to be grown *in vitro* to obtain cells at the metaphase stage

Three problems can occur during analysis of amniocentesis samples including complete failure of culture, maternal cell contamination leading to false-negative results and the incidence of mosaicism. Several studies have been carried out trying to determine the risk of spontaneous abortion after amniocentesis and it is found to be 0.5-1.5% (Tabor *et al*, 1986; Kapel *et al*, 1987).

1.4.3 Fetal Blood Sampling

Fetal blood sampling is usually obtained antenatally by fetoscopy, however, this procedure has been almost abandoned in favour of cordocentesis. Cordocentesis is a sampling technique whereby fetal blood is obtained by direct puncture of the umbilical vein.

1.4.4 Maternal Cells and DNA in the Fetal Circulation

During pregnancy, the fetal and maternal circulations are separated by the placental membranes. However, a variety of evidence has pointed towards the incompleteness of this barrier of cellular trafficking. Fetal nucleated cells were first demonstrated in the maternal circulation by Walknowska *et al* (1969) and have now been widely pursued as potential substrates for non-invasive prenatal diagnosis (Bianchi, 1999). The isolation and analysis of fetal cells in maternal blood for genetic diagnosis has been a matter of great interest in the world of prenatal diagnosis. Attention is being

directed on choice of the best fetal cell type, selection of optimal cell enrichment, consistency and reproducibility of cell recovery and analysis.

1.4.5 Cytogenetics and Prenatal Diagnosis

Prenatal diagnosis of chromosomal abnormalities is routinely accomplished by standard cytogenetic techniques. The major disadvantage of these procedures is the fetal cells must be cultured for up to two weeks (for amniocentesis) before analysis and that cultivation must be prolonged in advanced stages of pregnancy (Pertl *et al*, 1999). This time interval between sampling and diagnosis places a considerable emotional burden on the prospective parents (Tercyak *et al,* 2001). A rapid diagnosis is essential when an ultrasound examination suggests an abnormal fetus. The two most common types of rapid molecular method for prenatal diagnosis of chromosome disorders are fluorescent *in situ* hybridisation (FISH) and quantitative fluorescent polymerase chain reaction (QF-PCR). Both methods are used, but, QF-PCR is applied routinely for rapid and simple diagnosis of aneuploidy (numerical chromosome abnormalities) including trisomies 21, 13 and 18 which give rise to Down, Patau and Edward syndromes respectively and sex chromosome abnormalities.

In studies where either FISH or QF-PCR have been compared to karyotyping it has been postulated that both rapid techniques are more economical, rapid and easier to perform, however, they cannot be carried out independently (Eiben *et al,* 1998; Billi *et al*, 2002). Eiben *et al* (1998) concluded that all FISH analysis should be followed by karyotyping in order to cover the 35% of aberrations which cannot be detected by FISH. In a recent study on uncultured chorionic villus samples using FISH for chromosomes 13, 18, 21, X and Y showed that FISH is able to minimize maternal cell contamination and provide rapid diagnosis which reduces parent anxiety (Goumy *et al*, 2004). Feldman *et al* (2000) found 100% sensitivity, specificity and predictive values for their routine FISH analysis as a method to detect aneuploidies of chromosomes 13, 18, 21, X and Y in amniocytes. The same group, however, quoted that even with 100% accuracy of their test, routine FISH analysis will miss about 25-30% of the abnormalities detected by standard cytogenetic techniques, therefore, the latter must always act as a backup to FISH. Several studies have shown a variety of detection percentages whilst using FISH in normal uncultured amniotic fluid and

uncultured CVS, such as 70-100% (van Opstal *et al*, 1995; Bryndorf *et al*, 1997) and 36-100% (Bryndorf *et al*, 1996) respectively. This substantial range of FISH efficiency is probably due to: maternal cell contamination; true low level mosaicism; the types of probes used; error in scoring' and the efficiency of hybridisation, especially in nuclei interphase (Ruangvutilert *et al*, 2000b).

Pertl and co-workers (1999) after carrying out QF-PCR analyses on 247 chorionic villus samples concluded that the QF-PCR assay provides a fast and economical method for the prenatal diagnosis of major chromosome defects, but it is not a substitute for conventional cytogenetic analysis. Billi *et al* (2002), found no discordance between the results of QF-PCR and karyotyping after CVS where only 4/1100 cases were missed by QF-PCR, indicating the reliability of QF-PCR as a supporting prenatal diagnostic method. Furthermore, it has been shown that QF-PCR is a valuable tool for prenatal diagnosis of multiple pregnancies since it allows the detection of all aneuploid fetuses in just a few hours after sampling, as well as the determination of fetal zygocity in all cases (independently of chorionicity and fetal sex) (Cirigliano *et al*, 2003).

1.4.6 Prenatal Diagnosis of DM

The development of a reliable molecular diagnostic test for DM has meant that pre-symptomatic testing, prenatal testing as well as PGD for DM can be offered to those families for whom it is appropriate and acceptable. The severe congenital form of DM has a prevalence of 2.5-5.5 per 100,000 live births (Geifman-Holtzman and Fay, 1998).

In congenital DM affected pregnancies polyhydramnios, decreased fetal movement and prematurirty often complicate the pregnancies. Since the triplet repeat is expanded to hundreds or even thousands of copies, standard PCR protocols are unable to amplify such long products (Monckton *et al*, 1995). Therefore, the CTG expansion is detected by Southern blot performed from extracted DNA of CVS and amniotic fluid samples digested with the appropriate enzyme using a radioactive probe (Geifman-Holtzman and Fay, 1998). More recently several groups have reported the development of non-radioactive methods to determine the CTG repeat expansion in

DM patients involving the use of fluorescein-labelled probes (Brugnoni *et al*, 1998; Zuhlke *et al*, 2000). The combination of PCR and hybridisation reduces the time required for prenatal diagnosis from 10 days to 2-3 days (Zuhlke *et al*, 2000). Furthermore, smaller amounts of DNA are required compared to conventional Southern blot analysis (Brugnoni *et al*, 1998). Moreover, Amincucci and co-workers (2000) attempted to isolate fetal cells from maternal plasma for PND of DM of an unaffected woman whose husband was affected by DM and concluded that this non-invasive method allowing first–trimester PND, can become an alternative procedure in selected cases.

1.4.7 Prenatal Diagnosis and Mosaicism

Mosaicism, defined as the presence of two or more cell lines with different chromosome constitution in an individual, presents a prognostic dilemma since it influences the postnatal phenotype. Mosaicism has been found present in amniotic cells as well as chorionic villus samples (Eisenberg and Wapner, 2002). In order to identify samples with mosaicism the minimal demand is:

a) Two or more identical aberrant cells in villi analysed by the direct (short term culture) method (CVS)

b) At least one identical aberrant cell in each of the two or more independent culture dishes (CVS/amniotic cells)

c) At least one identical cell found both by the direct and the culture methods (CVS+amniotice cells) (Phillip and Bryndoff, 1998).

The confirmation or exclusion of mosaicism detected prenatally by CVS requires repeat sampling of fetal cells (amniotic fluid or fetal blood) (Pergament, 2000). The phenomenon of mosaicism in prenatal diagnosis has also been linked to intrauterine growth retardation (IUGR), uniparental disomy as well as confined placental mosaicism.

1.4.7.1 Confined Placental Mosaicism

In most pregnancies the chromosomal complement detected in the fetus is also present in the placenta. The detection of an identical chromosomal complement in both the fetus and its placenta has always been expected as both develop from the same zygote. However, in approximately 2% of viable pregnancies studied by chorionic

villus sampling (CVS) at 9 to 11 weeks of gestation, the cytogenetic abnormality, most often trisomy, is confined to the placenta (Kalousek and Vekemans, 1996). This phenomenon is known as confined placental mosaicism (CPM). It was first described by Kalousek *et al* (1991) in term placentas of infants born with unexplained intrauterine growth restriction (IUGR). Contrary to generalised mosaicism, which is characterised by the presence of two or more karyotypically different cell lines within both the fetus and its placenta, CPM may be due to a postzygotic nondisjunction event generating a trisomic cell line in an initially normal conceptus (mitotic origin) or the postzygotic loss of one chromosome in an initially trisomic conceptus (meiotic origin and trisomy rescue) (Grati *et al,* 2006).

Cytogenetically, CPM can assume three different forms. In type I the trophoblast is aneuploid, in type II aneuploidy is present in chorionic stroma and in type III both trophoblast and chorion are aneuploid. In trisomic zygote rescue, either reverse type I CPM, when trophoblast is diploid and the rest of the placenta and the fetus are nonmosaic trisomic, or type III are seen (Kalousek, 2000). It has been shown that the effects of CPM on development depend on the origin of the extra chromosome in the placenta as well as the specific chromosome involved. Meiotic origin is highly correlated with type III CPM and increased risk of pregnancy complications, whereas mitotic origin, more frequently found in types I and II, shows a lower risk of pregnancy complications (Robinson *et al,* 1997).

Specific chromosomal trisomies have been observed in CPM more frequently than others, with trisomy of chromosomes 7, 16, and 18 being the most prevalent (Wolstenholm, 1996). Previous studies have shown that the majority of the CPM for autosomes 9, 16, and 22 are meiotic in origin, whereas CPM for autosomes 2, 7, 8, 10, and 12 are predominantly somatic in origin (Robinson *et al,* 1997)

Uniparental disomy (UPD), the inheritance of the two copies of a chromosome from the same parent, may sometimes be associated with CPM. UPD can result from gamete complementation, chromosome loss in trisomy, or duplication in monosomy (with or without residual mosaicism) and somatic recombination (Engel, 1993). In isodisomy, the uniparental pair is a duplicate of a same chromosome DNA template and causes an increased risk of recessive disorder by reduction to homozygosity. In

heterodisomy, the pair remains heterozygous, made up of 2 non-recombinant homologous segments. But both iso- and heterodisomy may also cause disruption of the genomic imprints needed for differential expression of some maternal and paternal genes crucial to growth and development (Engel, 1997).

Conventional cytogenetic methods for detection of CPM depend on the availability of dividing cells and analysis of a large number of metaphase cells (Lestou *et al,* 1999). FISH has been established as a molecular cytogenetic technique for the detection of chromosomal aneuploidy and mosaicism in placental tissues (Lomax *et al*, 1994). More recently, it has been shown that CGH can be reliably performed on DNA obtained form placental (either trophoblast or stroma cells) and fetal tissues in order to detect aneuploidy (Lestou *et al*, 1999; Lestou *et al,* 2000; Barrett *et al,* 2001).

1.4.7.2 Uniparental Disomy

Uniparental disomy (UPD) is the occurrence of both homologous chromosomes from one parent. If an individual inherits two copies from one parent, through an error in meiosis II this is named uniparental isodisomy. If, however, the individual inherits two different homologues from one parent through an error in meiosis I, this is termed uniparental heterodisomy. Recent insights have revealed that the molecular basis for the clinical features of UPD are specific human genes that are only monoallelically active, depending on whether they are located on the paternal or maternal chromosome. UPD will lead to an imbalanced expression of these imprinted genes and cause abnormal development (Eggermann *et al,* 2002). Specific syndromes have been found to be associated with UPD, these include Prader-Willi syndrome (maternal UPD15/mUPD15), Angelman syndrome (paternal UPD15/pUPD15), (transient) neonatal diabetes mellitus (pUPD6), Silver-Russell syndrome (mUPD7), Beckwith-Wiedemann syndrome (pUPD11) and the mUPD14 syndrome.

The Prader-Willi (PWS) and Angelman (AS) syndromes are two clinically distinct syndromes which result from lack of expression of imprinted genes within chromosome 15q11-q13. These two syndromes result from 15q11-q13 deletions, chromosome 15 uniparental disomy (UPD), imprinting centre mutations and, for AS, probable mutations in a single gene. The differential phenotype results from a paternal

genetic deficiency in PWS patients and a maternal genetic deficiency in AS patients. Within 15q11-q13, four genes (SNRPN, IPW, ZNF127, FNZ127) and two expressed sequence tags (PAR1 and PAR5) have been found to be expressed only from the paternally inherited chromosome, and therefore all must be considered candidate genes involved in the pathogenesis of PWS (Glenn et al, 1997; Liehr *et al,* 2005)The mechanisms of imprinted gene expression are not yet understood, but it is clear that DNA methylation is involved in both somatic cell expression and inheritance of the imprint

Maternal UPD(16) is the most often reported UPD other than UPD(15); almost all cases are associated with confined placental mosaicism (CPM). Most of maternal UPD(16) cases are characterised by intrauterine growth retardation (IUGR) and different congenital malformations. Maternal UPD(16) has therefore been suspected to have clinical effects: however, the lack of uniqueness and specificity of the birth defects observed suggests that the phenotype may be related in parts to placental insufficiency (Eggermann *et al*, 2004)

1.5 Preimplantation Genetic Diagnosis

It has been 15 years since the first PGD baby was born (Handyside *et al,* 1989). PGD has been offered for a variety of single gene defects (Verlinksy *et al,* 1994; Sermon *et al,* 1997; Abou-Sleiman *et al,* 1999; Sermon *et al,* 2004), chromosomal abnormalities (Munne *et al,* 1998e; Conn *et al,* 1999; Scriven *et al,* 2001; Braude *et al,* 2002) and sexing (Griffin *et al,* 1991; 1992; 1994; Staessen *et al,* 1994; Handyside and Delhanty, 1997) and offers an alternative to traditional methods of prenatal genetic testing (CVS and amniocentesis), and allows genetic analysis to be performed on early embryos prior to implantation and pregnancy. This provides couples at a risk for certain genetic diseases the opportunity to know that any pregnancy they achieve should be unaffected (Handyside, 1998). Patients requesting PGD undergo in vitro fertilisation (IVF) treatment because in this way multiple embryos can be generated *in vitro* giving an increased probability that a disease free embryo will be identified (Wells and Delhanty, 2001).

FISH and PCR, following extensive groundwork for their suitability in clinical application, are the methods of choice and now form the basis of most PGD strategies used today for a spectrum of genetic defects ranging from gross chromosomal aberrations to single base-pair mutations. Although classical cytogenetic techniques can also be successfully applied to single blastomeres with a view to PGD, the efficiency with which analysable metaphase preparations can be produced per biopsied cell is notoriously low (Kola and Wilton, 1991; Wells and Levy, 2003).

The scope of PGD has been widened and apart from the diagnosis of genetic diseases (Handyside, 1998) and the detection of chromosomal abnormalities, it has been used to try and improve pregnancy rates for certain groups of IVF patients (Munne *et al*, 2000; 2005). Preimplantation genetic screening (PGS) is offered to couples with recurrent IVF failure, recurrent abortions and screening for aneuploidy in older aged women (Egozcue *et al*, 2000; Abelhadi *et al*, 2003).

PGD is a clinical diagnostic procedure that has evolved from the substantial advances both in assisted reproduction technology and molecular genetic analysis. Patients undergo routine IVF procedures which will produce multiple embryos. Polar bodies (first and second) and day 3 or day 5 embryos are biopsied (section 1.5.1) and are analysed either by FISH (section 1.5.2), or PCR (section 1.5.3) or CGH depending on the nature of the aberration. Embryos free of the specific inherited disorder are replaced in the uterus, which will give the parents the chance of starting a 'normal' pregnancy. As the embryos subjected to PGD must be IVF generated, certain difficulties are encountered. Significant limitations are presented involving the number and the subsequent quality of the embryos provided, that have a direct effect on the success rate of PGD. Thus, the success rate of PGD is relative to the highest possible success rate of IVF (Egozcue *et al*, 2000).

1.5.1 Sampling Strategies for PGD

Genetic analysis of preimplantation developmental embryos prior to replacement into the uterus inevitably involves removal of some embryonic cellular material from one of these stages.

1.5.1.1 Polar Body Biopsy

Biopsy of the first polar body has been carried out when the carrier of the mutation or the chromosomal aberration is the female partner (Verlinsky *et al,* 1990; 1997; Munne *et al,* 2000; Strom *et al,* 1997). The first polar body (PB) is biopsied within six hours of oocyte retrieval to preserve optimum chromosome morphology (Verlinsky *et al,* 1990). PB biopsy involves a breech in the zona which can be performed by mechanical (Verlinsky *et al,* 1997) or laser (Montag *et al,* 1998) drilling followed by polar body aspiration. Direct penetration through the ZP is mainly performed using a bevelled pipette (Roudebush *et al*, 1990; Verlinksy and Cieslak, 1993). The bevelled pipette allows mechanical perforation of the ZP, and once inside the perivitilline space, the first and second PB can be aspirated (Gianarolli, 2000).

First PB removal does not seem to interfere with normal fertilisation and the percentage of the embryos entering cleavage (Verlinsky *et al*, 1992). No effect on the viability of the resulting embryos was observed in a mouse model when both polar bodies were removed (Kaplan *et al,* 1995). Moreover, Strom *et al* (2000a and b) did not observe any deleterious effect in a follow-up study of 109 children born after first and second PB biopsy. The previous studies were reassuring for the clinical application and genetic evaluation of human oocytes, which has resulted in clinical pregnancies and livebirths (Verlinsky and Kuliev, 1996b). Munne *et al* used PB biopsy for translocations of female origin, significantly reducing spontaneous abortion rate (Munne *et al.,* 1998a; 1998b; 2000).

However, the main disadvantage of PB sampling is that the paternal contribution cannot be evaluated. Furthermore, the presence of post-zygotic mitotic errors cannot not be detected. Furthermore, this technique is extremely laborious (Wells and Delhanty, 2001), there is a risk of misdiagnosis in case of crossing over during meiosis I when only the first polar body is analysed and there is no information about the paternally derived genetic make-up of the embryo (Ruangvutilert *et al,* 2000b). First and second maternal meiotic errors can be excluded only if information on both polar bodies is obtained (Angell, 1994b). In cases where the paternal contribution needs to be evaluated, biopsy at post fertilisation stages will be required.

1.5.1.2 Cleavage-Stage Biopsy

At present, most of PGD analyses are performed by employing cleavage-stage biopsy, where 1-2 cells are extracted from each embryo (ESHRE PGD Consortium, 1999; 2000; 2001; 2002). The biopsy procedure involves making an opening in the ZP through which a cell can be removed. This can be accomplished either mechanically involving partial zona dissection (PZD) by direct piercing or cutting with a micropipette (Grifo *et al,* 1990; Cieslak *et al,* 1999), chemically by the localised application of acid Tyrode's solution (pH 2.3) (Hardy *et al,* 1990; Inzunza *et al,* 1998) or by employing an extremely precise laser system to puncture a hole into the zona (Palanker *et al*, 1991; Veiga *et al,* 1997; Montag *et al,* 1998; Joris *et al,* 2003). The latter two techniques, using Acid Tyrode's solution and laser, are the most commonly used methods for cleavage or blastocyst stage biopsy (Figure 1.8). Chemical zona drilling followed by blastomere aspiration is normally performed with separate pipettes (one drilling pipette with inner diameter ±5-7 μm, and one aspiration or biopsy pipette with inner diameter ±40 μm) using a double-holder setup (de Vos and Steirteghem, 2001). The resultant local acidification can cause subtle damage to the embryos and may interfere with further embryo development. Hence, Cohen *et al* (1992) suggested immediate washing of micromanipulated embryos. The use of a laser for zona drilling in cases of PGD is an easier procedure and results in more intact blastomeres (Veiga *et al,* 1997). Zona opening by laser drilling is performed by exposing the ZP to laser light. The hole size can be chosen precisely by varying the irradiation time, however extra care should be taken not to harm the embryonic cells with the laser shot. Since similar pregnancy rates are obtained from studies comparing laser and the acid Tyrodes technique (De Vos and Van Steirteghem, 2001). Recently, it was shown that there was no diference in blastomere viability, level of chromosome abnormalities and cytoskeleton damage when comparing embryos biopsied either by acid tyrodes or laser (Chatzimeletiou *et al, 2*005).

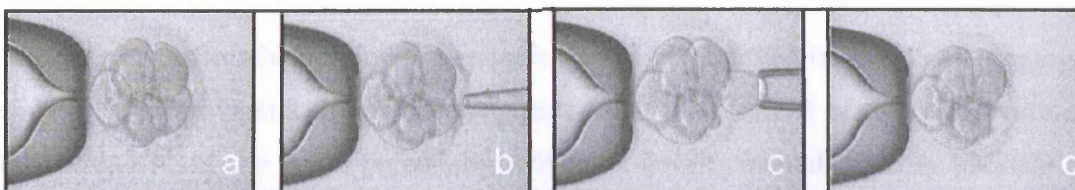


Figure 1.8. Illustrating cleavage stage embryo biopsy. (a): Embryo attached to holding pipette and positioned. (b): Acid Tyrodes is used to create a hole in the zona

pellucida. (c): A single blastomere is carefully removed. (d): The embryo following the biopsy. (Harper and Doshi, 2000)

Prior to cleavage-stage biopsy, embryos are placed into Ca^{2+}/Mg^{2+}-free medium to reduce the tight junctions that occur between human blastomeres at compaction (Dale *et al*, 1991). Blastomeres at the 6-8 cell stage show a strong tendency to adhere to each other, which in turn might cause a high rate of cell lysis whilst performing the biopsy. Hence, Santalo and colleagues (1996) used Ca^{2+}/Mg^{2+}-free medium to loosen the tight junctions of preimplantation mouse embryos, which allowed easier removal of the blastomeres and reduced the biopsy time. The use of Ca^{2+}/Mg^{2+}-free medium thus allows for an easier biopsy procedure during pre-implantation genetic diagnosis, while it does not result in a loss of developmental potential of the embryo to the blastocyst stage (Dumoulin *et al*, 1998).

Early studies focused on measuring the effect of biopsy on human embryonic development revealed that two cells could be removed from 8-cell embryos on day three post-insemination without reducing the number blastulating or disturbing cleavage rates (Hardy *et al*, 1990). De Vos and Van Steirteghem (2001) suggested that at day 3 of embryo development the blastomeres are totipotent and embryo biopsy could be carried out even if the embryo was compacting. Unfortunately, not all embryos reach the 7- or 8-cell stage by the morning of day 3. Six cell embryos might also be included for one or two cell removal. However, biopsies performed at the 4-cell stage may alter the ratio of inner cell mass to trophectoderm cells, if more than one cell is removed, which may be detrimental to embryo development (Tarin *et al*, 1992). Consequently two-cell biopsy procedures should only be carried out on day three post-insemination at the 6-8 cell stage, when up to a quarter of the blastomeres can be removed without disturbing subsequent development (Handyside, 1991; Hardy *et al*, 1990), as the biopsied cells are still undifferentiated (Harper *et al*, 1996).

The decision as to whether one or two cells should be removed from a cleavage stage embryo is controversial. It has been suggested that the removal of two cells reduces the cellular mass and could potentially reduce its developmental capacity (Braude *et al*, 2002). However, diagnosis of inherited diseases can suffer from technical limitations. Single cell FISH and PCR are not 100% accurate and can give false

positives and negatives. Furthermore, the phenomenon of mosaicism, poses a serious threat of misdiagnosis for PGD of chromosome abnormalities (see section 3.1.1). Hence it has been postulated that the accuracy of the diagnosis can be enhanced if embryos are replaced when results from two cells are concordant (Van de Velde *et al,* 2000). For this reason many groups prefer to base their diagnosis on the result of two biopsied cells, particularly for chromosomal analysis and dominant disorders (Delhanty *et al,* 1994; Delhanty and Handyside, 1995; Kuo *et al,* 1998; Van de Velde *et al,* 2000; Simopoulou *et al,* 2003).

1.5.1.3 Blastocyst Biopsy

The limited amount of tissue available in PB and cleavage-stage sampling could be overcome with the use of blastocyst biopsy. Trophectoderm biopsy from blastocyst stage embryos has been attempted in animal models (mouse, Gardner, 1971; cattle, Betteridge *et al*, 1981; monkey, Summers *et al*, 1988), as well as humans (Dokras *et al,* 1990) showing no adverse effect on further development. The technique of zona slitting (similar to PZD) is used to open the ZP with a microneedle (Dokras *et al,* 1990), however, laser zona opening has also been performed (Veiga *et al,* 1997). This will allow a generous amount of embryonic material in order to provide a reliable genetic analysis, overcoming the lack of material made available when carrying out cleavage stage biopsy. As these cells are from the trophectoderm there is no decrease in the ICM and therefore in the embryo proper. De Boer *et al* (2004) showed that blastocyst biopsy permits up to five or six cells to be genetically tested, leaving the inner cell mass intact and enabling embryos to be electively transferred one at a time without diminishing the chance of pregnancy compared with cleavage-stage biopsy and testing. The main drawback of this approach is the limited number of embryos available for biopsy and diagnosis, since only half or fewer of the IVF generated embryos are able to reach the blastocyst stage (Jones *et al,* 1998). Moreover, the time for diagnosis will be severely limited if the biopsy is postponed to this later preimplantation stage, posing serious restrictions in the time available for genetic analysis (De Vos and Van Steirteghem, 2001).

1.5.2 Chromosomal Abnormalities

Chromosomal analysis of embryos is one of the most important research fields in the area of preimplantation development. It has been suggested that fewer than 50% of naturally conceived embryos reach full term with most lost before or shortly after implantation (Edwards an Gardner, 1967). With the advent of IVF and more recently PGD studies to confirm chromosomal abnormalities by analysing embryos at early stages have revealed similar rates of embryonic mortality, where a significant portion of embryos arrest in development during the first days after fertilisation. Chromosomal abnormalities especially in the form of aneuploidy, usually have an adverse effect on the developing embryo by altering the dosage of hundreds of expresses genes (Wells and Levy, 2003). Chromosome analysis of human embryos has shown higher rates of aneuploidy than those reported for prenatal testing (Jamieson *et al,* 1994), suggesting that considerable numbers of chromosomally abnormal embryos are eliminated early in development (Wells and Delhanty, 2000; Sandalinas *et al*, 2001).

1.5.2.1 X-Linked Disorders

Sexing the embryo to avoid X-linked disease was the first application of FISH in this context (Griffin *et al,* 1991; 1992; 1994) and is one of the major indications for PGD (ESHRE PGD Consortium, 1999, 2000 and 2002, 2004). X-linked recessive diseases account for 6-7% of single gene defects and include conditions such as Duchenne muscular dystrophy (DMD), haemophilia, and various metal retardation syndromes. The first application of PGD was to avoid X-linked disease carried by the mother by the selection of female embryos for transfer following diagnosis of embryo sex by employing PCR (Handyside *et al*, 1990). Primers specific for a sequence derived from the long arm of the Y chromosome were designed and diagnosis was performed by negative selection i.e. the embryos for which no amplification was present were diagnosed as female and recommended for transfer. In the second series, of seven fetuses tested following sexing by PCR amplification, one singleton was male and the pregnancy was terminated (Handyside and Delhanty, 1993). This error presumably arose from amplification failure of a XY blastomere, subsequently shown to occur in 15% of cells tested, although biopsy of an anucleate or haploid blastomere would give the same result (Kontogianni *et al,* 1996). Following this, protocols were developed

for the simultaneous detection of both X and Y chromosomes, using either combinations of specific primers (Kontogianni *et al,* 1991; Grifo *et al,* 1992) or more reliably common primers for homologous sex chromosome sequence such as amelogenin (Nakahori *et al,* 1991) steroid sulphatase (Liu *et al,* 1994) or ZFX/ZFY (Chong *et al,* 1993).

FISH with biotinylated probes was introduced Griffin *et al* (1991). Moreover, the same group simultaneously hybridised a biotinylated X probe and two digoxigenin labelled Y probes using FISH to establish dual-colour FISH as the preferred method of for embryo sexing (Griffin *et al,* 1992; 1994). Indirectly labelled probes originally employed were soon replaced by directly labelled probes reducing the time of the FISH procedure from 7 to 2 hours (Harper *et al,* 1994a; Harper and Delhanty, 1996). Nowadays, to misdiagnose a normal male embryo as a normal female embryo two errors must occur; the signal for chromosome Y must be lost and an extra signal for chromosome X must be generated. A further possibility for misdiagnosis can arise in the case when the embryo analysed is chaotic or grossly mosaic and the cells biopsied are not representative of the whole embryo (Kuo *et al,* 1998). However, only one FISH misdiagnosis occurred among 78 cycles of social sexing as reported to the ESHRE PGD consortium (2002).

1.5.2.2 Structural Aberrations

Structurally abnormal chromosomes are formed from the rearrangement, deletion or duplication of chromosomal segments leaving the karyotype either genetically balanced or unbalanced. Structural abnormalities include translocations (reciprocal or Robertsonian), deletions, inversions, insertions, ring chromosomes and isochromosomes (see Figure 1.6; section 1.2.1.2). Balanced translocations occur in 0.2% of the neonatal population, however are at a higher rate among infertile couples and patients with recurrent abortions (Munne, 2002). In a study, it was reported that balanced translocations were found in 0.6% of infertile couples, 3.2% of couples that had failed over 10 IVF cycles and 9.2% among fertile couples experiencing three or more consecutive first trimester abortions (Stern *et al*, 1999).

With the advent of PGD, carriers of balanced translocations can circumvent repeated miscarriages or prenatal diagnosis and termination of pregnancy (TOP) of unbalanced fetuses and opt for PGD. The aim of PGD for translocations is to reduce the rate of spontaneous abortions and to minimise the risk of conceiving an unbalanced offspring. PGD for structural aberrations has been attempted using a variety of approaches with the aid of FISH. A number of reports concerning PGD have been published involving Robertsonian and reciprocal translocations, inversions, insertions, microdeletion syndromes and gonadal mosaicism (Conn *et al,* 1998, 1999; Iwarsson *et al,* 1998; Reubinoff *et al,* 1998; Scriven *et al,* 1998, 2001; Van Assche *et al,* 1999; Simopoulou *et al,* 2003).

Different approaches have been tried to identify structural chromosomal abnormalities including: a) probes spanning the breakpoints of each translocation or inversion (Cassel *et al*, 1997; Munne *et al*, 1998a and b) use of probes distal to the breakpoints or telomeric probes in combination with proximal or centromeric probes (Conn *et al,* 1998; 1999; Munne *et al*, 1998g). Munne *et al* (1998a) employed FISH in polar bodies in order to identify the translocations using chromosome painting probes for the chromosomes involved in the translocation. This technique was later modified by the same group using telomeric probes to further enhance the regions not covered by the chromosome-paint probes (Munne *et al*, 1998b). Furthermore, spectral imaging has been applied in polar bodies to identify all 23 chromosomes, though it was found to be laborious and needed well-spread chromosomes in order to distinguish each one (Marquez *et al,* 1998). The drawback of polar body analysis for translocations is the occurrence of crossing over and predivision of chromatids (Munne *et al*, 1998a, b and f; Marquez *et al,* 1998). The result of the second meiotic division in both cases is unclear and either the second polar body or the blastomeres should be analysed. Moreover, Munne *et al* (1998f) detected interstitial crossover with subsequent segregation of balanced and unbalanced sets of chromosomes during the second meiotic division, which might cause problems during diagnosis.

1.5.2.2.1 Robertsonian Translocations

PGD for Robertsonian translocations has been carried out on biopsied polar bodies as well as blastomeres (Conn *et al,* 1998; Munne *et al*, 1998g; Scriven *et al*, 2001; Ogilvie and Scriven, 2004; Sermon *et al,* 2004). To detect Robertsonian translocations

chromosome enumerator probes are used to count the chromosomes in the interphase nuclei in the cases of cleavage blastomere biopsy (Conn *et al,* 1998; Munne *et al,* 1998g). Probes can be chosen which bind to any point on the long arm of each chromosome involved in the translocation. Studying infertile couples carrying Robertsonian translocations using FISH revealed two factors leading to infertility in some of these cases. Firstly the aneuploid segregation of Robertsonian translocations carried by the parent and secondly a factor acting at the post-zygotic level provoking an uncontrolled chromosome distribution in early cleavage stages giving rise to chaotic embryos (Conn *et al,* 1998). However, this latter factor was not supported by a recent study by Scriven *et al* (2001), which concluded that Robertsonian translocations do not predispose to embryos with abnormal cleavage division. Nevertheless all studies on Robertsonian translocation carriers have reported a high incidence of mosaicism on their resulting blastomeres of >60%. It has been shown that the most common mode of segregation is the alternate (85%) (Iwarsson *et al,* 2000). This finding is in line with studies of meiotic segregation in sperm of male carriers of a Robertsonian translocation which displayed an incidence of 91% (Pellestor *et al,* 1987). However, recent data has shown that the pregnancy rates for maternal or paternal reciprocal or Robertsonian translocations are similar (Sermon *et al,* 2004)

1.5.2.2.2 Reciprocal Translocations

PGD is appropriate for those at high reproductive risk. Due to the complex nature of reciprocal translocations, each case is usually unique, hence difficult to treat by PGD (Harper and Bui, 2002). For reciprocal translocations the prevalence of unbalanced gametes is estimated to be between 50-70% (Gardner & Sutherland, 1996).

As mentioned above (see section 1.5.2.2) two types of FISH protocols have been devised for the investigation of reciprocal translocations; the use of spanning (Munne *et al,* 1998g) or flanking probes (Conn *et al,* 1998; Munne *et al,* 1998e). Breakpoint spanning probes used in interphase nuclei can detect normal, balanced or unbalanced karyotypes resulting from a reciprocal translocation (can also be used for inversions, deletions and duplications). The disadvantage with spanning probes and their seldom use in PGD is due to laborious, time-consuming and expensive probe development required for each breakpoint for each translocation. Methods used to produce these

probes have been described by Fung *et al* (1998). For the flanking-probe approach, two probes would be used flanking the breakpoint of one chromosome and the third probe would be specific for the other chromosome. Conn and co-workers (1998) employed this strategy for studying a reciprocal translocation involving chromosomes 6 and 21, being able to detect all segregation patterns apart from free trisomy and monosomy of chromosome 6, which is not viable. The disadvantage of using flanking probes is that balanced and normal embryos cannot be distinguished. This approach however, is the simplest owing to the commercialisation of sub-telomeric probes for most *p* and *q* arms. Scriven *et al* (1998), suggested a generalized strategy involving chromosome specific sub-telomeric probes specific for the subtelomeric regions of the translocated segments, combined with proximal probes in order to provide a fast and reliable approach to PGD for cases of reciprocal, Robertsonian translocations, inversions and other complex chromosomal rearrangements.

Malmgrem *et al* (2002) performed CGH on embryos diagnosed as abnormal after PGD for two Robertsonian and four reciprocal translocations. The group was able to confirm the results of CGH supported by the PGD results in 11 out of 15 embryos. It was revealed that all the embryos (100%) were mosaic and it was further observed that some couples were more prone to generate chaotic embryos than others as shown previously by Delhanty *et al* (1997). However, imbalances involving a translocation with a very distal breakpoint could be missed as CGH resolution was limited and unreliable ratio changes appeared at the telomeric regions.

Several groups have revealed high rate of mosaicism and chaos during PGD for reciprocal translocations. Van Assche *et al* (1999) after analyzing 35 embryos from carriers of the most common reciprocal translocation, involving chromosomes 11 and 22 (see section 1.3.1.2.2), revealed that use of four-colour FISH analysis allowed the identification of all the possible segregation modes using commercially available probes. The most common segregation pattern in reciprocal translocations is alternate, although considerable variations in the proportions of segregants in reports of male carriers exist (Iwarsson *et al,* 2000). Estop *et al* (1995) reported an average of 47% of alternate segregation mode and a very low rate of adjacent-2 segregation.

It has been postulated that two mechanisms exist giving rise to the production of unbalanced gametes in patients with reciprocal translocations: a) one from meiotic crossing over involving the critical region between the centromere and the breakpoint and b) the other from abnormal meiotic segregation (Brandriff *et al*, 1986; Scriven *et al*, 1998). By contrast, Robertsonian translocations result in unbalanced gametes only as a consequence of abnormal meiotic segregation because there is no critical region (Munne *et al*, 2000).

1.5.2.2.3 Inversions and Insertions.

The majority of patients requesting PGD for inversions carry a pericentric inversion with a very large inverted segment. The FISH protocol devised for inversions utilises breakpoint spanning probes and were originally presented by Cassel and co-workers (1997) whilst performing PGD for inversions. The probe strategy devised for such cases involves a probe for one of the distal segments of the chromosome involved, which can then detect chromosome imbalance from either of the two possible recombinant chromosomes. Additional probes, with different fluorophores, on the same or different chromosomes can be added as a control as reported by Iwarsson *et al* (1998b).

PGD for insertions can be rather problematic since depending on whether synapsis takes place or not, several types of meiotic behaviour are possible that have to be taken into consideration while devising a strategy. The complexity of such cases is increased as the orientation of the inverted segment is often impossible to detect. Therefore, the use of a subtelomeric probe along with a locus specific for the inserted segment is essential.

1.5.2.3 Numerical Aberrations

The major causes of the decline in implantation observed with increased maternal age and embryo incompetence is aneuploidy. Navot *et al* (1994), transferred embryos from younger women to women >40years of age and observed a high implantation rate suggesting that the latter group's ability to become pregnant is largely unaffected, whereas their oocyte quality is compromised. Altered oocyte metabolism such as ATP production (Van Blerkom *et al,* 1995) and excessive deposition of ZP glycoproteins

(Garside *et al*, 1997) have also been linked with advanced maternal age. The increase in aneuploidy and maternal age seen in spontaneous abortions and live offspring after genetic analysis (Antonarakis *et al,* 1991; Fisher *et al,* 1991) has also been observed in unfertilised oocytes (Dailey *et al,* 1996) as well as human cleavage-stage embryos (Munne *et al*, 1995a and b). However, in the case of preimplantation embryos and oocytes, the rate of chromosomal abnormalities was significantly higher, suggesting that a proportion of chromosomally abnormal embryos are eliminated before any prenatal diagnosis (Munne *et al*, 1999). Close correlation between aneuploidy and declining implantation rates, prompted the hypothesis by Munne and co-workers (1993b) that selection of chromosomally normal embryos could reverse this trend. However, while some research groups have shown a positive attitude towards this kind of selection (Verlinsky and Kuliev, 1996b; Gianaroli *et al,* 1997a; 1999) other have doubted its value and feasibility (Egozcue, 1996; Reubinoff and Sushan, 1996). As well as being labour intensive PGD reduces the number of embryos available for transfer and the error rate is estimated to be 9-15% (Munne *et al,* 1998c). Clear benefits of this technique in terms of live birth rate per initiated cycle have yet to be shown in any large-scale prospective controlled study that would properly evaluate this technology and its effects (Braude *et al,* 2002). An international clinical trial is needed, with a suitably matched control group to determine if this procedure benefits older-aged IVF patients. Such trial would involve clinical settings with similar (identical if possible) stimulation protocols, IVF media, diagnostic procedures in different laboratories. The trial would consist of three groups of patients: women <35 years of afe, women >35 years of age and the last "blind" group would have a mixture of ages. All patients should be assessed for the type of infertility and should be "healthy" individuals.

Couples opting for PGD of aneuploidy are mainly infertile and undergoing IVF/ICSI to overcome their infertility. There are three main indications for aneuploidy screening: 1) advanced maternal age (AMA), 2) recurrent miscarriage (RM) and 3) recurrent implantation failure (RIF). These patients are thought to be predisposed to producing aneuploid embryos which would die at or before the time of implantation (Wilton, 2002).

1) Patients >39 years of age show the highest increase of aneuploidy (Marquez *et al*, 2000), however more recent data has suggested that PGD for aneuploidy can display an increase in implantation even for women between 35-39 years of age (though, not shown to be statistically significant) (Munne *et al*, 2003). Staessen *et al* (2004) in clinical randomised study showed that PGD-AS does not improve clinical outcome per initiated cycle in patients with AMA when there are no restrictions in the number of embryos to be transferred.

2) RM in patients with a normal karyotype is defined as three or more consecutive spontaneous abortions of less than 20-28 weeks' gestation (Stephenson, 1996). PGD–AS has been performed in this category of patients (Pellicer *et al*, 1999; Rubio *et al*, 2003), however, no differences in pregnancy or implantation have been observed.

3) RIF is defined as three or more failed IVF attempts or implantation failure after the replacement of more than 10 embryos (Munne *et al*, 2002). A study by the ESHRE PGD Consortium Steering Committee (2004) which covers cases from 25 centres, reported a pregnancy rate for RIF of only 7% per retrieval, compared to 28% for PGD of aneuploidy cases with the indication of AMA or RM. Gianaroli *et al* (2001b) reported results on 66 PGD cycles of RIF patients, which did not find any statistical significance between the implantation rate of the PGD group (17%) and the control group (10%). From those studies it can be postulated that there is no clear indication that RIF patients have benefited from PGD for aneuploidy.

FISH allows enumeration of chromosomes on preimplantation embryos of common aneuploidies (13, 14, 15, 16, 18, 21, 22, X and Y) testing either blastomeres from cleavage-stage embryos or oocyte polar bodies (Munne *et al*, 1993b; 1995a,b; 1998c,e; Verlinsky *et al*, 1996b; Gianaroli *et al*, 1997a). Currently, probes for chromosomes 13, 14, 15, 16, 18, 21, 22, X and Y are being used simultaneously (with re-probing)(Bahçe *et al*, 2000), with the potential of detecting 70% of the aneuploidies detected in spontaneous abortions. Inclusion of probes for chromosomes 16 and 22 is of particular importance as trisomies of these two chromosomes are the two most common autosomal aneuploidies observed in spontaneous abortions (Wilton, 2002). Thus far, more than 2000 cases of PGS have been performed, resulting in >400 chromosomally normal babies (Sermon *et al*, 2004).

Misdiagnoses have been reported after PGD for aneuploidy (Munne *et al,* 1998c; Gianaroli *et al*, 2001; PGD Consortium 2005). In all of those cases, reanalysis of the misdiagnosed cells with probes binding to a different locus confirmed prior results, indicating that the errors were probably caused by mosaicism (Munne, 2003; see section 3.1.1). Other sources where misdiagnosis can occur include false monosomies produced by signal overlaps due to the failure of the FISH technique to display the remaining signals (Ruangvutilert *et al,* 2000a and b). Causes of reduced FISH hybridisation efficiency can be attributed to loss of DNA during denaturation or fixation, poor probe penetration, insufficient binding of detection reagents or overlap of chromosome-specific signals when multiple probes are used (Ruangvutilert *et al,* 2000b; Munne *et al,* 2002)

Munne and colleagues (1999) revealed that couples undergoing PGD for common aneuploidies showed a significant decrease in cases of spontaneous abortions (from 23% to 9%), whereas the ongoing pregnancies and deliveries in the PGD group of patients increased from 10.5% to 16.1%. Furthermore, several studies have displayed that after PGS, the incidence of multiple embryos being transferred has been significantly reduced (Munne *et al,* 2003; Werlin *et al,* 2003). However, this might be due to the fact that there are fewer chromosomally normal embryos to select after PGD. After 10 years of PGS, only recently has it been shown that PGS can increase implantation rates, when nine chromosomes were screened using FISH (Munne *et al,* 2003a). In this study the PGD group showed a 20% implantation rate compared to a 10% rate in the control group in women with an average age of 40 years (Munne *et al,* 2003).

Recently PGS has been carried out on using CGH (Wilton *et al,* 2001; Wells *et al,* 2002; section 1.4.1.2).

1.5.2.4 Single Cell FISH Limitations

FISH has been hailed as one of the most robust and efficient techniques however, it has certain limitations. When using FISH to analyse chromosome constitutions, several obstacles emerge, including failure of hybridisation, probe inefficiency, signal overlapping yielding false negative results and split/diffused/patchy signals (Munne *et*

al, 1998a; Ruangvutilert *et al*, 2000a). Most importantly FISH is limited by the number of probes that can be simultaneously applied due to an increasing chance of FISH artefacts and FISH failure and lack of colours, since it is not possible to look at all chromosomes in one interphase (Ruangvutilert *et al*, 2000b). However, as many as six different chromosomes have been simultaneously analysed by Munne *et al* (1998c) and Gianaroli and colleagues (1999), achieving a total of nine chromosomes studied per cell with a second sequential hybridisation.

1.5.3 Single Gene Disorders

Almost any source that contains one or more intact target DNA molecules can, in theory, be amplified by PCR, providing appropriate primers can be designed. This has been of great interest to researchers as well as clinical geneticists, forensic scientists and even scientists involved in archaeological biological findings. Single cell PCR has enabled geneticists to carry out PGD. By employing PCR, the 5-10pg of DNA in a single blastomere is amplified in order to make the DNA amenable to conventional forms of mutation analysis (Wells and Delhanty, 2001). Over 8,000 disorders caused by single gene defects have been described. The first autosomal single gene disorder to be analysed by PGD was cystic fibrosis more than ten years ago (Handyside *et al*, 1992). Since then, methods and procedures have improved, allowing more and more PGD protocols to be devised for monogenic diseases. To date many single gene defect diseases can be treated with PGD. Fluorescent dyes, multiplex reactions as well whole genome amplification techniques have been employed during PGD with PCR (see sections 1.5.3.1-3). Several inherent difficulties that are associated with single cell DNA amplification have become evident. These include potential sample contamination, total PCR failure, allelic dropout and preferential amplification, all of which should be minimised for any PGD PCR protocol before clinical application (see section 1.5.3.4). In addition, the chosen method must reliably and accurately characterise the genotype of the embryo relative to the disorder under investigation.

Initial work to develop PGD for single gene diseases began with the common disorders for which mutation profiles and prenatal diagnostic tests were already well established such as CF and the haemoglobinopathies (Monk *et al,* 1988; Monk and Holding, 1990; Liu *et al,* 1992). The first single gene defect to be diagnosed was

cystic fibrosis which was accomplished using simple heteroduplex analysis to identify unaffected homozygous normal and heterozygous embryos for transfer (Handyside *et al*, 1992). Since then increasingly sophisticated PCR-based protocols have been developed and applied for many single gene defects on a list which is growing steadily along with patient demand and technological advances (Wells and Delhanty, 2001). These include Tay-Sachs disease (Gibbons *et al*, 1995), Duchenne muscular dystrophy (Lui *et al*, 1995), Marfan syndrome, (Harton *et al*., 1996), spinal muscular atrophy (Dreesen *et al*., 1998), Lesch-Nyhan syndrome (Ray *et al*., 1999), sickle cell anaemia (Xu *et al*., 1999) as well as the triplet repeat expansion disorders Huntingtons disease (Sermon *et al*, 1999), myotonic dystrophy (Sermon *et al*., 1998a,b; Piyamonkol *et al*., 2001), Fragile X (Sermon *et al*, 1999; Apessos *et al*., 2001) and inherited cancer syndromes, familial adenomatous polyposis coli (FAPC) (Ao *et al*., 1998), Li-Fraumeni syndrome (Verlinsky *et al*., 2001) and neurofibromatosis type-1 and type-2 (NF-1 and NF-2) (Abou Sleiman *et al*., 2002).

PGD on the 1st polar body (PB) was first introduced by Verlinsky *et al* (1992). However, this analysis was not sufficient since the genotype of embryos resulting from diagnosed heterozygous oocytes was not predictable and testing of the 2nd PB was required. Therefore, Verlinsky and co-workers (1997) carried out 1st and 2nd PB analysis for PGD of sickle cell disease, haemophilia B and cystic fibrosis. Furthermore, the same technique was later performed on patients at risk of producing offspring affected with Neurofibromatosis type-I (NF-1) (Verlinsky *et al*, 2002). The same group have continued using the 'two-step PB analysis of oocytes' as their preferred method for PGD of single gene disorders, because they have reported that ADO is at least half as frequent in PB's than in blastomeres (Rechitsky *et al*, 1998), which has not been by corroborated by any other study. Dean and co-workers (2003) introduced the prospect of PGD for heritable mitochondrial diseases on polar bodies using PCR. The group studied the levels of heteroplasmy on polar bodies, oocytes and embryos from a heteroplasmic mouse model to assess the relative proportions of each of the two mitochondrial DNA genotypes in gametes and early cleavage stage embryos. They concluded that PGD for mitochondrial DNA diseases is feasible, although it should be approached with caution (Dean *et al*, 2003). Currently, most

PGD cases involving single gene defects are performed on blastomeres of cleavage-stage human embryos, thus making PB analysis at the single gene level scarce.

1.5.3.1 Microsatellite DNA (STR's)

Microsatellite DNA is a subclass of tandemly repeat DNA found throughout the human genome mostly in non-encoding loci and is also referred to as short tandem repeats (STR's) (Ellegren, 2004). The most common type of microsatellite DNA are dinucleotide repeats, comprising 0.5% of the genome, whilst the total microsatellite DNA comprises approximately 2-3% of the genome (IHGSC, 2001). Other types of microsatellite DNA include mono-, tri- and tetra-nucleotide repeats. A significant feature of microsatellite DNA is that its polymorphic nature i.e. the variability in repetitive DNA is in the number of repeat units, which confers variability in length rather than sequence. STR's have been used extensively for PGD of single gene disorders to overcome the problems of ADO and contamination (Sermon *et al,* 2001; Piyamongkol *et al,* 2001a and b). This has been achieved by carrying out multiplex reactions incorporating one to two STR's in addition to the mutation marker (Piyamongkol *et al,* 2001a)

During STR amplification, the incidence of so-called 'shadow bands' have been reported by many groups especially during single cell PCR. It has been suggested that such bands, termed stutter bands, represent mutations in the repeat sequence and are thought to have arisen due to replication slippage (Hauge and Litt, 1993). Replication slippage occurs due to mispairing between two complementary DNA strands during DNA replication. One strand dissociates from its complementary strand during replication, only to re-associate to its complementary strand incorrectly, causing an increase or a decrease in one repeat unit length. It has been noticed that stutter bands are more prominent in amplification of mono- and dinucleotide repeats and are usually one repeat unit length shorter than the main allele (Ellegren, 2004). Stutter bands usually cause problems when allele scoring of heterozygote individuals whose alleles are close in size.

Multiplex PCR reactions during single cell work are designed to alleviate the problem of ADO and contamination. The need to design protocols which include amplification

of more than one (singleplex) primer has mostly been the case during PGD cases. Incorporation of STR's linked to the disease gene or unlinked i.e. located in another chromosome require alterations in the PCR protocol. Laborious and time-consuming testing is required especially whilst performing on single cell DNA in order to acquire analysable results from more than 2 primers when multiplexing. Primers must be designed, ideally, to have similar annealing temperatures and must not be competing for reagents such as the dNTP's or the Mg^{+2}. Furthermore, when designing PCR protocols for single gene disorders, a variety of STR's may be informative for different families. Thus, the need to develop several multiplex protocols according to each family and in single cell DNA can be time consuming and not cost-effective.

1.5.3.2 Single Cell F-PCR

The introduction of F-PCR (see section 1.3.3.2.1) has boosted single cell analysis driving scientists to achieve analysis as well as diagnosis of single gene mutations accurately. It has been concluded that F-PCR was an efficient tool for PGD of Steinert's disease (myotonic dystrophy) (Sermon *et al,* 1997; Piyamongkol *et al,* 2001a). Currently, most diagnoses before implantation for inherited monogenic diseases are carried out using multiplex F-PCR (see section 1.5.3.2). In this type of diagnosis it amplifies, short tandem repeat (STR) markers which are highly polymorphic loci of differing numbers of a 2-5 base pair repeated unit. The polymorphic nature of STR markers allows the determination of the origin and purity of DNA amplified e.g. from single cells. Hence, if the STR allele sizes are known, any impurities or unexplained sizes can be distinguished. Furthermore, in extreme cases of preferential amplification, with conventional PCR product detection techniques it may seem as ADO, whereas F-PCR may still demonstrate the presence of both alleles with one greatly amplified relative to the other (Sherlock *et al,* 1998).

1.5.3.3 Single Cell Multiplex PCR

Single-cell PCR has the disadvantage that just one amplification reaction can be undertaken. When several different primers are combined within a single PCR assay, each can multiply independently and sufficiently, allowing simultaneous diagnoses to be performed. Furthermore, multiplex PCR can alleviate problems caused by ADO (Kuliev *et al*, 1998). When a disease-causing mutation marker and an informative

polymorphism for the respective mutation are multiplexed, there are two chances of for the detection of a chromosome carrying a mutant gene. Thus, if one primer does not amplify due to ADO, there is another primer for backup. It has been postulated that ADO is independent for each fragment amplified in a multiplex PCR assay and the probability of ADO affecting both the mutation site and the linked polymorphism are very low (Ao *et al*, 1998). Lewis et al (2001), developed a a mathematical model to explore accuracy of PGD using PCR by including both extrinsic technical errors and intrinsic errors related to nuclear and chromosomal abnormalities. It was suggested in that study that a linked marker or a second biopsied cell reduces the probability of replacing an affected embryo (Lewis *et al,* 2001)

1.5.3.4 Single Cell WGA

WGA can be used for amplification of low copy numbers of the entire genome (Zhang *et al*, 1992) and provide sufficient DNA templates for many independent PCR amplifications (Wells and Sherlock, 1998). Hence several studies have been performed assessing the use of WGA in the context of PGD.

Ao *et al* (1998) was able to perform PGD for familial adenomatous polyposis coli (FAPC), after amplifying two biopsied blastomeres from each embryo with PEP and subsequently carrying out nested PCR to amplify two APC fragments. Following PEP the APC mutation and the linked polymorphism achieved 87.5% and 75% amplification respectively.

Applying DOP-PCR to single fibroblasts, buccal cells, amniocytes and human blastomeres, Wells *et al* (1999) showed that it could provide DNA sufficient for performing 100 subsequent PCR amplifications as well as CGH analysis. Voullaire and colleagues (1999) showed that DOP-PCR was able to successfully amplify single cells in such quantities able to provide diagnosis of aneuploidy using CGH. Despite hopes that WGA strategies might reduce the incidence of allele drop out, Wells and Sherlock (1998) found ADO rates after PEP and DOP-PCR to be comparable to those obtained by direct amplification of single cell loci.

MDA is a method of whole genome amplification that utilises the bacteriophage φ29 DNA polymerase for isothermal displacement amplification (Dean et al, 2001; see section 1.3.3.1.3). Recently, Handyside *et al* (2004) reported that isothermal WGA from single and small numbers of lymphocytes and blastomeres isolated from cleavage stage embryos yielded microgram quantities of amplified DNA. 20 different loci were successfully analysed, but a relatively high ADO of 31% at heterozygous loci was found (Handyside *et al,* 2004).

1.5.3.5 WGA and STR's

Whole genome amplification (WGA) has been reported by Wells and colleagues (1999), as an efficient means to generate sufficient quantities of DNA for as many as 90 independent amplification reactions from a single cell. Thus, numerous specific loci and subsequently copy number of every chromosome (using comparative genomic hybridisation, CGH) can be assessed in a single cell.

WGA may be defined as the non-specific amplification of all sequences in the genome (Wells and Sherlock, 1998). There are different types of WGA, with three techniques being the most notable in context of embryo research and PGD, namely PEP, DOP-PCR and MDA (sections 1.3.3.1.3 and 1.6.3.3). PEP has been used clinically in the context of PGD of familial adenomatous polyposis coli (Ao *et al,* 1998). DOP-PCR has been found to be the choice of method, especially for CGH since it results in larger amounts of DNA being produced (Wells *et al,* 1999; Chapter 4). It has been proposed that WGA would be beneficial for patients undergoing PGD for single gene disorders who are also of advanced maternal age since detection of the single gene disorder as well as chromosome complement of the embryos can be performed (Wells, 2004). WGA could reduce the significance of the risks of ADO and achieve enhanced detection of contamination without the need to perform multiplex reactions. However, this is based on the assumption that a single cell can be amplified in full, without bias, such that one could rely on subsequent tests performed on the DOP-PCR product. By performing microsatellite marker genotyping of the DOP-PCR amplified product, this would allow testing for coverage of the genome.

Cheung and Nelson (1996) first attempted to amplify different types of STR's after WGA with DOP-PCR. The study was carried out on genomic DNA and it was concluded that DOP-PCR provides relatively good coverage of the genome, however, is dependant on the amount of starting DNA. Wells and co-workers (1999) studied DOP-PCR on single cells, in addition to other WGA techniques, in order to investigate the most suitable WGA protocol for CGH analysis. The idea of producing enough DNA from a single cell and then perform separate PCR reactions would be ideal for PGD of single gene disorders.

1.5.3.6 Single Cell Mini-Sequencing

The amount of time and resources required for the design and validation of a novel PGD strategy limits the number of protocols that a laboratory can develop. Furthermore, developing a PGD strategy is not cost-effective especially for rare mutations which can only be used for a handful of families; hence, a universal methodology applicable to several mutations would accelerate the rate of which new methods can be developed and reduce the cost (Bermudez *et al*, 2003). Mini-sequencing has recently been proposed as an alternative technique able to tackle disease involving a heterogeneous spectrum of mutations, such as cystic fibrosis, β-thalassaemia or haemophilia A in PGD (Fiorentino *et al,* 2003). The basis of mini-sequencing is similar to that of sequencing apart from the fact that the nucleotides added to the reaction are of the labelled dideoxy type (ddNTP) i.e. only a single nucleotide can be added to the primer thus preventing further extension. The primers designed for mini-sequencing anneal to the template at the 3' end, one nucleotide upstream of a known mutation/polymorphism. The ddNTP added to the primer is able to reveal the identity of the base of this site. Bermudez *et al* (2003) assessed mini-sequencing as a method for single cell analysis and concluded that although expensive compared to established methods, it is rapid and accurate in diagnosing disease-causing mutations in single cells and the near-universal applicability of this method could shorten the time required for devising PGD protocols. Fiorentino and co-workers (2003) were able to interpret results from 96.5% of the 887 blastomeres tested, including 55 PGD cases. This technique may be particularly useful in cases where the mutation involved is difficult to assess by restriction analysis or other commonly used methods.

1.5.3.7 Single Cell PCR Limitations

1.5.3.7.1 Contamination

Single cell PCR assays usually employ a large number of cycles to be carried, especially for sufficient amplification of a single cell. Contamination can be caused by cumulus cells of maternal origin, sperm, culture media and the PCR products present in the laboratory environment (Delhanty, 1998). To improve the PCR technique the protocol has changed over the years to reduce contamination. The introduction of ICSI, where the oocyte is fertilised by the injection of a single sperm into the cytoplasm, thus avoiding excess sperm often left embedded in the zona pellucida following IVF, reduces paternal contamination (Wells and Delhanty, 2001). In addition some PGD protocols attempt to detect contaminants by amplifying a highly polymorphic locus for DNA fingerprinting (Findlay, 2000; Piyamongkol *et al*, 2001a). Single-cell PCR should be set up in a DNA-free environment away from the analysis area, which can reduce the chance of 'carry over' contamination.

Nested PCR was developed to increase sensitivity and specificity (Monk and Holding, 1990) addressing the problem of "carry over" contamination. Nested PCR is widely used in PGD and is based on the use of two sequential amplification reactions in order to enhance the specificity of PCR and reduce the risk of contamination caused by the accidental amplification of DNA fragments (Wells and Sherlock, 1998). The use of nested PCR by aliquoting the first amplified products for use as the target template in a second reaction using another set of primers (markers) situated internally to those used in the first reaction was suggested to prevent carry over contamination (Wells and Sherlock, 1998). Furthermore, Multiplex-PCR may substantially decrease the possibility of misdiagnosis (Lewis *et al,* 2001), by providing the added assurance of a partial "fingerprint" of the embryo, and confirming that the amplified fragment is of embryonic origin (Findlay *et al,* 1995). Piyamongkol *et al* (2000a) showed that the use of DNA fingerprinting can increase the detection of the contaminants. Ideally linked (Rechitsky *et al,* 1998; Dressen et al, 2000; Piyamongkol *et al,* 2001a and b) or unlinked polymorphic markers, which are informative for the family undergoing PGD should be employed (Kuliev *et al,* 1998; Ioulianos *et al,* 2000; Harper *et al*, 2002). For these markers the parents should have four different alleles e.g. A and B (mother), C and D (father); hence, during analysis of the embryo it should have one allele from

each parent e.g. AC, AD, BC or BD. Any other pattern e.g. ABD or ACD would indicate presence of contaminants. Moreover, multiplex F-PCR is considered approximately 1000-fold more sensitive than conventional agarose or acrylamide gel analysis (Findlay, 2002). Despite all efforts, paternal and maternal contamination has caused misdiagnosis (Sermon *et al*, 1998; Harper and Delhanty, 2000).

1.5.3.7.2 Amplification Failure

The problem of AF of single cell PCR emerged when a misdiagnosis from the first series of PGD for X-linked disorders was reported (Hardy and Handyside, 1992). Whilst amplifying single cells, even an experienced PCR practitioner might face the obstacle of total failure of amplification. AF can between 10-20% (Kontogianni *et al,* 1996; Wells and Sherlock, 1998; Piyamongkol *et al,* 2003) and the underlying reasons of this relatively high incidence are difficult to determine. The cause of AF cannot be distinguished empirically. However the loss of the isolated single cell during transfer into the PCR tube or the chance that the cell is anucleate or in the process of degeneration have been considered as likely candidates. Furthermore, DNA in the cell nucleus might not have been made accessible to the PCR reagents due to failure of cell lysis (Piyamongkol *et al,* 2003).

1.5.3.7.3 Allele Dropout

ADO is the amplification of only one of the two parental alleles present in the single cell, which is usually caused due to sub-optimal PCR conditions and rapid degradation of the target DNA during thermocycling (Handyside *et al,* 1997). Several factors have been linked with the struggle against ADO including increased denaturation temperature (Ray *et al,* 1996; Lissens and Sermon, 1997), different lysis methods (Sermon *et al*, 1995) and F-PCR (Findlay *et al,* 1995a). When ADO is encountered it gives the perception that the cell is homozygous which can lead to misdiagnosis when applying PCR for PGD analyses in dominant disorders. If the affected allele does not amplify, the embryo will be diagnosed as normal, when actually it is affected. For autosomal recessive diseases in couples carrying the same mutation, the consequences of ADO are minimal; the loss of the normal allele in carrier embryos will result in an apparently affected embryo that will not be replaced. In the case where the affected allele is lost, this will lead to diagnosis of a homozygous normal embryo instead of a carrier (Fasouliotis and Schenker, 1998). In

a recent large study of single blastomeres and single buccal cells all possible aspects of PCR were investigated regarding their effect on allele drop-out. It was revealed that ADO is affected by amplicon size, amount of DNA degradation, freezing and thawing, the PCR programme and the number of cells simultaneously amplified (Piyamongkol *et al,* 2003). In the same study, factors which had little or no affect on ADO were local DNA sequence, denaturing temperature (94°C or 96°C) and type of cell (buccal or blastomere). Fluorescent PCR technology (See section 1.2.3.1.2) is having a wide impact on the PGD of single gene disorders proving to be more sensitive, reliable, accurate, and fewer cycles are required, thereby reducing the time taken to reach diagnosis (Findlay *et al,* 1996; Sermon *et al,* 1998). Furthermore, F-PCR technology has been found to be amenable to automation.

A different approach, which involves the combination of FISH and PCR techniques, has been reported by Thornhill and colleagues (1994) termed cell recycling. Cell recycling is performed by fixing a cell onto a glass slide and initially carrying out PCR and then FISH. These combined analyses can serve independently for sexing for X-linked diseases or for PGD of single gene defects. The combined efficiency of both techniques ranges from 65-85% (Monk and Thornhill, 1996; Rechitsky *et al*, 1996). However, the ADO rates were reported to be considerably higher than in conventional single cell PCR, in both studies above.

1.5.4 PGD for DM

Sermon and colleagues (1997) were the first group to develop a single cell PCR assay able to detect DM affected embryos during clinical application of PGD. It was reported, in this first attempt of PGD for DM, that ADO affected 24% of the biopsied cells in the clinical setting and 21% of the cells in the research setting (Sermon *et al,* 1997). Although, these figures are considered high, there was no use of multiplex or fluorescence PCR techniques. The same group was able to increase their amplification efficiency from 87% to 95% during another study where F-PCR and automated fragment analysis was performed and significantly decrease their ADO rate (4.5%) in a clinical setting (Sermon *et al,* 1998a). However, the same group reported a misdiagnosis case during the latter study (Sermon *et al,* 1999). Piyamongkol *et al* (2001a) was among the first to carry out PGD on DM patients using the combination

of multiplex and fluorescent PCR. By incorporating informative polymorphic markers (single tandem repeats), which are linked to the disease gene and a marker for the mutation, affected embryos can be distinguished thus reducing ADO and AF rates. Furthermore, polymorphic markers, which not linked to the disease gene, can act as contamination markers able to detect contamination of any sort. In an ideal PCR protocol a mutation marker, a linked marker and a contamination marker should be multiplexed (as long as they are informative for the respective patients) to minimise the risks associated with single cell PCR. Dean *et al* (2001) were able to reduce the ADO rates (during clinical PGD) using a hemi-nested multiplex F-PCR protocol to 0% for the DM locus and 18% for the linked polymorphic marker.

1.6 Mosaicism in Human Preimplantation Embryos

The advent of IVF as a treatment for infertility has created the opportunity to study the chromosomal constitution of surplus human preimplantation embryos. An increasing body of evidence suggests that the incidence of chromosomal abnormalities in embryos is extremely high (Wilton, 2002) and even good embryo morphology does not necessarily exclude an abnormal chromosome constitution (Magli *et al*, 2000).

Mosaicism is defined as the presence in an individual or in a tissue of two or more cell lines, which differ in their genetic constitution but are derived from the same zygote. Little is known about the mechanisms of mosaicism. Sometimes mosaicism is described for chromosomal trisomy and diploidy however, the abnormal cell line may be represented by other chromosomal abnormalities such as sex chromosome monosomy, triploidy or structural rearrangement (Kalousek, 2000).

Delhanty *et al* (1993) first noted the presence of mosaicism as a common feature in preimplantation embryos whilst analysing the X and Y chromosomes using interphase FISH. A high degree of mosaicism has been detected in several FISH studies (Delhanty *et al*, 1993, 1997 (Table 1.1); Harper *et al*, 1994, 1995; Munne and Weier, 1996 (Table 1.2); Munne *et al*, 1998c, d, e; Iwarsson *et al*, 1999; Ruangvutilert *et al*, 2000a), karyotyping studies (Jamieson *et al*, 1994; Clouston *et al*, 1997, 2002) and CGH studies (Wells and Delhanty, 2000; Voullaire *et al*, 2000; Malmgrem *et al*,

2002). The frequency of chromosomal abnormality in early cleavage embryos has also made a major impact in IVF and human reproductive biology. It is well known that in routine IVF, at the cleavage stage, each transferred embryo has only about a 1 in 5 chance of implantation (Delhanty and Harper, 2000).

Table 1.1 Classification of human embryonic mosaicism adapted from Delhanty *et al* (1997)

Classification	Explanation
Uniformly Normal	Embryo uniformly normal for the chromosomes tested
Uniformly Abnormal	Embryo uniformly abnormal for the chromosomes tested
Diploid Mosaic	Majority of embryo euploid but one or a few cells differ (i.e. aneuploid, polyploid or haploid).
Aneuploid or Polyploid Mosaic	Majority of embryo uniformly aneuploid or polyploid but one or a few cells differ
Chaotic	Chromosome constitution varies randomly from cell to cell and status of original zygote cannot usually be determined

Table 1.2. Chromosomal patterns in cleavage stage embryos as described by Munne and Weier (1996)

(i) **Normal** – Embryos whose chromosome constitution is >90% either normal or aneuploidy or haploid or polyploidy.

(ii) **Mosaic embryos containing a majority of diploid cells***

   a. Diploid mosaics
      i. Majority of diploid embryos with few being aneuploid, haploid or teraploid
      ii. Embryos which contain a diploid and a polyploid cell line (2n/4n)
   b. Polyploid mosaics – embryos with a polyploid cell line
   c. Haploid mosaics – embryos with a haploid cell line

(iii) **Mosaic**

   a. Extensive – embryos with >38% of abnormal cells
   b. Limited – embryos with <38% of abnormal cells

(iv) **Chaotic** - nuclei showing randomly different chromosome complements

*This category includes mosaic embryos that contain a majority of abnormal cells (e.g. due to presumptive meiotic error and also embryos in which the majority of cells are normal).

It has been suggested that all types of mosaicism have the same impact on embryo development (Sandalinas *et al*, 2000). Delhanty *et al* (1997) classified the chromosome patterns in cleavage stage embryos into four groups: uniformly normal, uniformly abnormal, mosaic and chaotic (Table 1.1). These observations were confirmed by Clouston *et al,* (1997), who karyotyped nuclei from 6- to 8-day human blastocysts and proposed that all four groups were also observed at that stage. Furthermore, the same patterns have been observed in human blastocysts (Ruangvutilert *et al* 2000a).

The delineation of the extent and nature of mosaic and non-mosaic chromosome abnormalities in early human preimplantation embryos is important for understanding the origins and selective processes leading to the anomalies seen later in gestation. Therefore it is important to try to understand the underlying mechanisms responsible for mosaic cell lines. The different types of mosaicism and their underlying mechanisms are better understood by reviewing the different types of analysis that have been used to investigate embryos

1.6.1 Mosaicism and Karyotyping

Karyotyping was amongst the first technique to be used for embryo analysis. It is a robust staining technique using a variety of dyes including DAPI (4, 6-diamino-2-phenylindole) and Giemsa to produce characteristic bands along each chromosome in order to carry out chromosome enumeration and structural analysis (Ronne *et al*, 1990; Glassman, 1997; section 1.3.1).

Angell *et al* (1983) whilst examining 8-cell stage embryos revealed a high incidence of chromosomal abnormalities which she suggested was contributing to early embryonic loss and to the high failure rate after embryo transfer. This was the first report on haploid human embryos with an incidence of 20% suggesting parthenogenic activation of the oocyte, which was later confirmed by Plachot (1985). Angell *et al*

(1986) carried out another karyotyping study trying to assess the contribution of lethal chromosome abnormalities to implantation failure and found non-disjunction giving rise to trisomy, monosomy, nullisomy, as well as structural abnormalities, haploidy and triploidy. In the same study it was revealed that chromosomally abnormal embryos could not be distinguished on morphological criteria from embryos of normal chromosomal constitution based on similar cleavage rates, which was also supported by Jamieson *et al* (1994). However, it was later concluded that only the embryos which were very fragmented and degenerated were shown to display a higher rate of chromosomal abnormalities (78%) when compared with morphologically healthy embryos (Plachot *et al,* 1989). Similar findings were observed by Pellestor *et al* (1995) which showed that the rate of abnormalities were significantly higher in dysmorphic embryos (86.6%) than in good quality embryos (36.6%). Hence, these findings confirm the prognostic value of the grading system as a means of eliminating a large proportion of chromosomally abnormal embryos (Pellestor *et al,* 1995). The most significant findings whilst carrying out cytogenetic analysis on human preimplantation embryos was the correlation of chromosomal aberrations and maternal age. Maternal age was found to be directly proportional to aneuploidy frequency (Angell *et al,* 1986; Plachot *et al,* 1987; 1989), particularly affecting aneuploidies of the small satellited chromosomes of Group G (Zenzes and Casper, 1991).

Chromosomal abnormalities on human preimplantation embryos have also been linked to certain parameters of IVF such as oocyte retrieval and constitution of culture media (Angell *et al,* 1983; Pellestor *et al,* 1995). Ovarian hyperstimulation might be involved in the immaturity or overmaturity of the oocytes retrieved having a serious effect on the fertilization process (Testart *et al,* 1989) as well as delayed IVF might also lead to triploidy, abnormal cleavage, and fragmentation (Plachot *et al,* 1988). Moreover, delay in gamete fusion could also lead to asynchronisation of both formation and migration of the male and female pronuclei, resulting in cleavage disturbance and chromosome set fragmentation (Ron-El *et al,* 1991).

Several karyotyping studies reported chaotic findings while trying to detect mosaic and non-mosaic chromosome abnormalities (Angell *et al,* 1986; Plachot *et al,* 1989; Papadopoulos *et al*, 1989; Jamieson *et al,* 1994; Clouston *et al*, 1997; Clouston *et al*,

2002) (Table 1.3). It has been proposed that hypodiploidy, hyperdiploidy and structural chromosome damage reflect the same basic phenomenon, termed uncontrolled or chaotic division (Clouston *et al*, 1997), predicted by FISH studies (Harper *et al*, 1995). Studies have shown significant levels of tetraploidy, usually mosaic, whilst karyotyping of about 19-23% (Angell *et al*, 1987; Jamieson *et al*, 1994; Clouston *et al*, 1997). From karyotypic analysis of human blastocysts it was suggested that mosaic tetraploidy might result due to failure of cytokinesis (Hardy *et al*, 1993). The production of mosaic tetraploidy as a common event in early embryogenesis was also confirmed by a recent study on blastocysts (Clouston *et al*, 2002). Mosaic monosomy was not detected in a study by Clouston *et al* (2002), due to technical difficulties and haploid cells were completely lacking in the same study

Table 1.3. Frequency of mosaicism in human preimplantation embryos after karyotypic analysis

Study / Observation	**Papadopoulos *et al*, 1989**	**Jamieson *et al*, 1994**	**Clouston *et al*, 1997**	**Clouston *et al*, 2002**
No. of embryos observed	35	178	73 (blastocysts)	182(blastocysts)
Diploid (including abnormal cells)	40%	22.5%	67%	68%
Mosaic polyploid (mainly tetraploidy)	3%	2.2%	18%	28%
Mosaic aneuploid (mainly trisomies)	9%	19%	7%	5%
Chaotic cells	5/35	6/178	1/73	-
Structural chromosome damage	26%	1.1%	26%	%

* Some of the studies do not add up to 100%. This is due to the presence of abnormalities that cannot be classified

Although results observed from karyotypic analysis have shown that a certain level of mosaicism exists, several technical difficulties have limited the conclusion that can be drawn from these studies. These technical difficulties include problematic fixation

methods resulting in loss of chromosomes and over dispersed or poorly spread cells which are not in a single focal lane restricting the potential of analysis (Harper *et al*, 1995). Also, long colcemid exposure times are able to increase the mitotic index but this produces highly contracted chromosomes that exhibit chromatid separation and G-band poorly compromising the information obtained (Jamieson *et al*, 1995). The most important limitation during G-banding is that only a few metaphases can be obtained hence only a small proportion of the cells can be analysed.

Spectral karyotyping (SKY) is a technique which employs 24 chromosome specific probes and has been considered an alternative to conventional cytogenetic analysis (Schrock *et al*, 1996). Each probe is labelled with different proportions of five separate fluorochromes and observed by spectral imaging, providing a different colour for each human chromosome. It has been applied to human oocytes and polar bodies, being able to simultaneously detect specific aneuploidies as well as de novo structural abnormalities, such as acentric fragments, translocations and marker chromosomes (Marquez *et al*, 1998). The analysis on first polar bodies provided useful data for polar body genetic diagnosis (Marquez *et al*, 1998). However, currently the technique is known to be fairly unreliable especially as it requires good quality chromosome spreads (Wells and Levy, 2003). Therefore, it would be more suitably employed in a research rather than in a clinical setting

1.6.2 Mosaicism and FISH

FISH has been widely applied in studies of oocytes, polar bodies, spermatozoa, blastomeres and blastocysts (sections 1.3.2.2). High levels of mosaicism and chaotic embryos have been reported in all embryonic stages (Delhanty *et al*, 1997; Clouston *et al*, 1997). Chaotics or those with a majority of abnormal cells are unlikely to survive beyond implantation (Harper and Delhanty, 2000; Table 1.1). Chromosomal abnormalities and mosaicism have been described in arrested and morphologically abnormal embryos (Munne and Cohen, 1998) as well as in normally developing embryos (Harper *et al*, 1995; Delhanty *et al*, 1997). Different types of mosaicism have been reported through FISH analysis including aneuploid mosaics, olyploidy and haploid mosaics, chaotic mosaics and multinucleation. Thus each type should be explored individually to understand their underlying mechanisms. Each type of

mosaicism observed in human preimplantation embryos using FISH is explored in section 3.1.4, in order to fully understand the different forms of mosaic embryos and explain the mechanisms that various FISH studies have revealed. In Table 1.4, the data obtained from several studies carrying out FISH in human cleavage stage embryos and blastocysts is summarised. The table reveals high levels of mosaicism found in several studies both on cleavage stage embryos and human blastocysts.

Table 1.4. Summary of studies carried out on normally developing human cleavage-stage embryos by applying FISH to investigate the extent of mosaicism

Study/Chromosomes observed		No. of embryos	Normal	**Abnormal**	**Mosaic Diploid**	**Mosaic Abnormal** [§]	**Chaotic**
Delhanty *et al* (1993) / X, Y		4	50%	25%	25%	-	0
Munne *et al* (1994) / 18, X & Y		67	0	2%	52%[#]	40%	6%
Coonen *et al* (1994) / 1, 7, X and Y		37	38%	8%	55%	-	-
Harper *et al* (1995) / 1, 7		35	54%	9%	23%	0	14%
Kligman *et al* (1996) / 13, 18, 21, X and Y		47	23%	21%	51%	4%	
Delhanty *et al* (1997) / 1, 7, X and Y		93	48%	2%	19%	4%	26%
Munne *et al* (1997) / 13, 18, 21, X and Y		138	66%	9%	12%[#]	-	14%
Laverge *et al* (1997) / 1, X and Y		97	40%	25%	23%		12%
Munne *et al* (1998d) / 13, 16, 18, 21, X, Y		381	36%	12%	40%[#]	12%	
Laverge *et al* (1998) / 1, X and Y[¥]		60	20%	45%	15%	-	20%
Evsikov & Verlinsky (1998) / 13, 18, 21 *		86	**	4%	86%	7%	3%
Iwarsson *et al* (1999) / 15, 16, 17, 18, X and Y		40	28%	2%	45%	10%	15%
Staessen *et al* (1999) / 18, X and Y		94	35%	11%	40%	3%	11%
Veiga *et al* (1999) / 13, 18, 21, X, and Y *	Blastocysts	8	12.5%	0	75%	12.5%	0
	Arrested Embryos	8	37.5%	0	25%	0%	37.5%
Ruangvutilert *et al* (2000a) /13, 18, 21, X and Y *	Blastocysts	19	10.5%	-	68.5%	10.5%	10.5%
	Arrested Embryos	20	20%	-	70%	-	10%
Harrison *et al,* (2000) / 1, 4, 7, 8, 9, 13, 16, 18, 21, X, Y		6	0%	17%	50%	17%	17%
Sandalinas *et al,* (2001) / 1, 13, 15, 16, 18, 21, 22, X, Y		216	15%	47%[&]	15%[#]		23%

Gonzalez-Merino *et al*, (2003) / 13, 18, 21, X and Y	50	10%		68%	20%	2%
Baart *et al*, (2004) / 1, 7, 13, 15, 16, 18, 21, 22, X, Y	22	45%	5%	45%	-	5%
Coonen et al, (2004) / 18, X and Y*	295	25%	-	26%	31%	17%[@]

* These studies were carried out on human blastocysts
¥ The embryos were analysed after the freezing and thawing process
+ No diploid/euploid nuclei present with no evidence of chaotic division
§ Major cell line has an abnormal chromosome complement but diploid cells present
** The study did not distinguish between the normal and diploid mosaic
\# Diploid mosaics are embryos with >38% of abnormal cells (Table 3.2)
& Including the 2n/aneuploid cell
@ Including embryos classified as unexplained

When using FISH to analyse chromosome constitutions, several obstacles emerge, including failure of hybridisation, probe inefficiency, signal overlapping yielding false negative results and split/diffused/patchy signals (Munne *et al*, 1998; Ruangvutilert *et al*, 2000a, b). Most importantly FISH is limited by the number of probes that can be simultaneously applied due to an increasing chance of FISH artefacts and FISH failure and lack of colours, since it is not possible to look at all chromosomes in one interphase (Ruangvutilert *et al*, 2000b). As many as six different chromosomes have been simultaneously analysed by Munne *et al* (1998c) achieving a total of nine chromosome studied per cell with a second sequential hybridisation. However, by analysing many chromosomes simultaneously the efficiency of the FISH technique drops (Conn *et al*, 1998; Ruangvutilert *et al*, 2000b) thus producing false negative and positive results. Conn *et al* (1999) proposed that double locus analysis might decrease the number of chromosomes analysed per FISH analysis will however, provide true information about the extent of mosaicism present in human preimplantation embryos. Magli and co-workers (2001) carried out double locus analysis of chromosome 21 for PGD to reduce any false positives and/or negatives and thus the risk of misdiagnosis

1.6.3 Mosaicism and CGH

To date only a small number of embryos have been studied using CGH to analyse every cell (Table 1.5). Two studies were carried out on a series of good quality cleavage stage embryos. Both studies aimed to reveal the true extent of chromosomal abnormalities. Combining the results of the two similar studies conducted by Wells

and Delhanty (2000) and Voullaire *et al* (2000), the most striking finding was that mosaicism was found to be extremely common affecting 67% and 64.6% respectively. However, Trussler *et al* (2004) observed a lower incidence of mosaicism of 50% whilst analysing 40 embryos. It was concluded in the latter study that the difference in normality in the three studies might be due to variations in maternal age, embryo quality, stimulation protocols and culture (Trussler *et al,* 2004).

Table 1.5. Summary of CGH data carried out on human preimplantation embryos revealing the level of mosaicism.

Study	**No. of embryos**	**Normal**	**Abnormal[a]**	**Mosaic Diploid**	**Partial Aneuploidy[b]**	**Chaotic**
Wells & Delhanty (2000)	12	25%	8%	50%	-	17%
Voullaire *et al,* (2000)	12	25%	8.3%	58.3%	-	8.3%
Wilton *et al,* (2001)	5	20%	40%	-	-	40%
Malmgrem *et al,* (2002)	28	0%	-	14%[c]	54%[d]	32%
Voullaire *et al,* (2002)	126	40%	25%[a]	-	6%	29%
Trussler *et al,* (2004)	40	42.5%	7.5%	37.5%	5%	7.5%

[a] Including mosaic aneuploid complements
[b] Partial aneuploidies or structural damage induced by chromosome breakage
[c] The embryos were mosaic diploid or mosaic aneuploid but balanced regarding the chromosomal rearrangement
[d] The embryos were mosaic diploid or mosaic aneuploid but unbalanced regarding the chromosomal rearrangement

However, all three CGH studies have confirmed the FISH and karyotyping studies performed on cleavage embryos. In the study by Wells and Delhanty (2000) only one embryo (8.3%) was completely aneuploid where all six cells were trisomic for chromosome 21 and monosomic for the X chromosome and was thought to have arisen due to meiotic errors. The same results were obtained from Voullaire *et al* (2000), though Trussler and co-workers found slightly lower (7.5%) consistent abnormality resulting from a meiotic error. In both early studies 3/24 (12.5%) embryos revealed evidence of chromosome breakage resulting in imbalance of specific regions, rather than whole chromosomes, were detected, with one embryo showing reciprocal gains and losses of regions of chromosomes 2 and 7 in sibling blastomeres (Wells and Delhanty, 2000). In the recent study of Trussler *et al* (2004) chromosome breakage was again reported (7.5%). Furthermore, such chromosome

breakage has also been recorded in karyotyping studies (Papadopoulos *et al.*, 1989; Zenzes and Casper, 1992; Clouston *et al.*, 1997).

Voullaire *et al* (2002) used CGH for aneuploidy screening and detected chromosome abnormality in 60% of single blastomeres biopsied prior to implantation from 20 women with repeated implantation failure. The abnormalities included aneuploidy for one or two chromosomes (25%) and complex chromosomal abnormality (29%). Mosaicism involving a complex abnormality (i.e. chaotic) is a more frequent occurrence in these patients than in the previously studied cohort of surplus embryos (Voullaire *et al,* 2000), and is therefore likely to be related to the history of recurrent implantation failure. This study supports the observation that some individuals are more prone to chaotic embryos than others as suggested by previous FISH studies (Delhanty *et al,* 1997; Harrison *et al,* 2000). In addition, the complex abnormality seen in morphologically normal and actively dividing embryos supports the idea that mitotic checkpoints may not function in the cleavage embryo (Delhanty and Handyside, 1995; Wells and Delhanty, 2000; Harrison *et al,* 2000), and it suggests that disturbance of the normal early embryonic cell cycle might be a pathology associated with infertility and implantation failure (Wilton *et al,* 2003). Recently, Wells *et al* (2005), after carrying out gene expression studies on human oocytes and embryos found that BUB1, MAD2 and APC genes were expressed in low quantities in 2-4cell stage embryos. These findings prompted the author to suggest that this might be significant for the level of mosaicism since these genes are involved in producing proteins that interact in the spindle assembly checkpoint which ensures accurate chromosome segregation.

A recent study by Malmgren *et al* (2002) analysed 94 blastomeres from 28 embryos generated from 13 couples carrying a balanced chromosomal rearrangement undergoing PGD. The single cell CGH confirmed most of the unbalanced translocations detected by PGD. As the embryos made available for this study were previously diagnosed as unbalanced regarding the chromosomes involved in the translocation or were considered unsuitable for transfer for other reasons a higher degree of mosaicism was expected in comparison to the other CGH studies (Wells and Delhanty, 2000; Voullaire *et al,* 2000). Indeed all of the embryos (100%) were classified as mosaic (containing more than one chromosomally uniform cell line) or

chaotic. In this study, a tendency for some couples to be more prone to generate chaotic embryos than others was also seen, as previously described by Delhanty *et al* (1997). In the study by Malmgrem *et al* (2002) there was a significant reduction in the efficiency of the CGH technique, compared to the previous studies (Wells and Delhanty, 2000; Voullaire *et al,* 2000) from 98% and 97% respectively to 70%. However, the dissimilarity was attributed to the fact that normal IVF generated embryos were used in the earlier studies in contrast to the unbalanced or not suitable for transfer embryos analysed in the Malmgrem study (Malmgrem *et al,* 2002). However, it is more likely the underlying reason was the use of different CGH techniques between the Malmgrem study (Malmgrem *et al,* 2002) and the other two early CGH studies (Wells and Delhanty, 2000; Voullaire *et al,* 2000).

Following the application of CGH on single blastomeres, concerns about the reliability of PGD using FISH to identify chromosomally normal from abnormal embryos were raised. In the case of FISH, probes target a defined region on a chromosome so the status of the rest of the chromosomes is simply assumed to be normal, but lacking conclusive proof. This fact strengthens the argument for the adaptation of CGH for clinical screening of embryos (Wells and Levy, 2003). However, hypothetically more than half of the abnormalities found in the CGH embryo studies could have been excluded using a limited FISH probe set (13, 18, 21, X and Y). Recently, in a study carried out by Trussler *et al* (2004) the combination of CGH and FISH was assessed in cleavage stage embryos. A total of 1-4 cells were biopsied from 40 embryos and analysed with CGH and their sibling blastomeres were examined by FISH. From the forty embryos investigated FISH results were in agreement with the CGH results in all 22 embryos where both tests were informative (Trussler *et al,* 2004).

1.6.4 Types and Mechanisms of Mosaicism

Different types of mosaicism have been reported in preimplantation embryos including aneuploid mosaics, polyploid and haploid mosaics, chaotic mosaics and multinucleation. Thus each type should be explored individually to understand their underlying mechanisms.

1.6.4.1 Aneuploidy Mosaicism

Aneuploid mosaicism is considered to be the most frequent form of mosaicism observed in human embryos (Munne *et al,* 1994b). Aneuploid mosaicism arises as a somatic postzygotic event. However molecular studies of the origin of the extra chromosome in the trisomic cell line indicate it is of two types: meiotic and somatic (Kalousek, 2000). Hence aneuploid mosaicism is of two types. Meiotic mosaicism is where a loss of the trisomic chromosome occurs in a trisomic fetus producing a diploid cell line as well as the trisomic cell line, whereas somatic mosaicism is where a trisomic/monosomic cell line arises in a normal diploid embryo, giving two or three cell lines.

Munne *et al* (1997) analysed 138 normally fertilised human cleavage-stage embryos and found that 14% were chaotic suggesting that there maybe a relationship between embryo abnormalities and different drug regimes and embryo culture conditions. Furthermore, the same group reported that mosaicism due to aneuploid cells interfered with embryo development for some unknown reason (Sandalinas *et al*, 2000), since aneuploidy combined with extensive mosaicism had a stronger effect in that study resulting in none of the human embryos developing to blastocysts. In Gonzalez-Merino *et al* (2003) it was found that all types of blastocysts (eiher pre-expanded or expanded) were all diploid/mosaic with >70% of the cells being diploid and the author suggested that this finding might be a normal finding in *in-vitro* embryos. Delhanty *et al* (1997) observed 19% of human cleavage-stage embryos were diploid mosaic, however relatively high amounts of chaotic embryos (26% out of 93 embryos) were found (Table 3.3). It was proposed that the mechanism of mosaic aneuploidy were probably mitotic non-disjunction, which causes a reciprocal loss or gain in addition to anaphase lag (Delhanty *et al,* 1997). Moreover, Delhanty and Handyside (1995) suggested that due to the absence of cell-cycle checkpoints mosaic aneuploid embryos could form, which may be specific to the cleavage stage of development. The latter was also postulated by Harrison *et al* (2000), which observed a mirror-image distribution about the plane of attachment of the signals in each sequential hybridisation, indicating premature decondensation during anaphase, which is consistent with lack of checkpoint control. The possibility that cell-cycle checkpoints do not fully operate during cleavage of the human embryo may also

explain the relatively high incidence of various nuclear abnormalities which have been observed (Winston *et al,* 1991; Hardy *et al,* 1993) For example binucleate blastomeres are seen in 15% of human embryos, and are frequently associated with chromosomal abnormalities and appear to result from failure of cytokinesis (Winston *et al,* 1991; Hardy *et al,* 1993; Kligman *et al,* 1996; Staessen and Van Steirteghem, 1998). Most recently, work on inbred mouse strains with elevated levels of Y chromosome nondisjunction has shown that malsegregation in this system is largely restricted to the earliest mitotic divisions (Bean *et al,* 2001). This suggests that mammalian embryos are indeed susceptible to mitotic nondisjunction in early cleavage stages (Bean *et al,* 2001; 2002) and fits the lack of checkpoint control model.

Another study which analysed 161 embryos for sex determination using FISH, observed examples of three different mechanisms in aneuploid mosaic embryos: (i) involvement of an aneuploid gamete which finally lead to an aneuploid mosaic embryo; (ii) reciprocal mitotic non-disjunction leading to mosaic embryos with monosomic, disomic and trisomic blastomeres for the chromosomes involved; and (iii) chromosomal loss leading to a combination of monosomic and disomic blastomeres (Staessen *et al,* 1999) confirming the previous reports by Delhanty *et al* (1997)

Veiga *et al* (1999) revealed a high prevalence of mosaicism in both blastocyst and arrested embryos showing 87.5% and 62.5% respectively. Moreover, Ruangvutilert *et al* (2000a) showed that 30% of the day 5 arrested embryos and 21% of the blastocysts were aneuploid mosaics, which supports the hypothesis of the selection against chromosomal abnormalities through culture to the blastocyst stage proposed by Sandalinas and co-workers (2001). Coonen *et al* (2004) analysed 295 blastocysts and after finding high levels of mosaicism (57%) and chaos (17%) concluded that anaphase lagging appeared to be the major mechanism through which human embryos acquire a mosaic aneuploid pattern.

1.6.4.2 Ploidy Mosaicsim

Ploidy mosaics have also been frequently reported in cleavage stage embryos, blastocysts (Harper *et al*, 1995; Delhanty *et al*, 1997; Munne *et al*, 1997; Staessen *et*

al, 1999; Ruangvutilert *et al*, 2000a; Sandalinas *et al*, 2001) and those of other animal species (Long and Williams, 1982; Murray *et al,* 1986). Ploidy mosaics may well play a role in normal early development particularly when associated with TE lineages (Angell *et al,* 1987) with tetraploid or haploid predominating. It has been proposed that probably tetraploid cells may be a normal feature in the development of the trophectoderm (Angell *et al,* 1987). Tetraploid trophectoderm cells may arise as a result of endoreduplication or endomitosis and possibly play a role in embryo implantation (Drury *et al*, 1998). Although other studies have linked mosaic tetraploidy to poor quality arrested embryos (Wells and Delhanty, 2000). Laverge *et al* (1997) while studying 97 human cleavage-stage embryos reported that possible mechanisms for polyploid mosaics could involve endoreduplication of mononucleated blastomeres, or formation of a mitotic spindle during division of a binucleate cell which would subsequently form two mononucleate daughter cells with polyploid nuclei. The fusion of nuclei in binucleate blastomeres or less frequently blastomere fusion may also lead to polyploidy (Balakier *et al*, 2000).

Less common mosaicism findings includes haploid and triploid nuclei. The presence of a haploid cell in a mosaic embryo is difficult to explain, however the underlying mechanism maybe associated with binucleate cell production with a meiotic type of segregation (Delhanty *et al*, 1997) or maybe an incorporation of a polar body into the embryo (Staessen *et al*, 1999). Haploid/diploid mosaics can be found from pronuclear zygotes and are believed to arise due to the activation of the oocyte (Staessen and Van Steirteghem, 1998). Haploid and triploid cells have been characterised as less viable and less actively dividing than tetraploid cells (Ruangvutilert *et al*, 2000a); with the exception of some triploid cells persisting until later in development as in cases of mosaic diploid/triploid (Edwards *et al*, 1994).

The origin of diploid/triploid mosaicism is not clear. The underlying mechanism that leads to diploid/triploid mosaics may be due to an incorporation of another gamete or its genome into one of the daughter cells derived after the first mitotic division or later. The extra gamete might be a polar body (Mueller *et al*, 1993). Kuo *et al* (1998) also suggested that diploid/triploid mosaicism could result from fusion of a diploid zygotic nucleus with an extra sperm nucleus or the extrusion and degeneration of a haploid nucleus to produce a diploid cell line in a triploid embryo. In a recent prenatal

study of four cases of triploid/diploid mosaics, the three different mechanisms of origin for these apparent mosaics were detected: i) chimaerism with karyotypes from two separate zygotes developing into a single individual, ii) delayed digyny, by incorporation of a pronucleus from a 2nd PB into the embryonic blastomere and iii) delayed dispermy, similarly by incorporation of a 2nd sperm pronucleus into one embryonic blastomere (Daniel *et al*, 2003).

1.6.4.3 Chaotic Mosaicism

Chaotic embryos have been reported in many studies and involve embryos where all nuclei show a different chromosome complement. Chaotic embryos have been found in cleavage-stage embryos (Harper *et al*, 1995) as well as at the blastocyst stage (Evsikov and Verlinsky, 1998). It has been hypothesised that they originate due to uncontrolled "chaotic" division, which is possibly related to centriole or spindle deficiencies and disturbance of pronuclear syngamy (Klingman *et al*, 1996). During FISH analysis of human cleavage-stage embryos it was suggested by Delhanty and Handyside (1995) that chaotic embryos may result form the absence of cell cycle checkpoints leading to chaotic segregation of chromosomes. Furthermore, the frequency of chaotic embryos appears to be a patient related phenomenon (Delhanty *et al*, 1997).

1.6.4.4 Multinucleation

A normal human embryo should have a single nucleus in each blastomere (only visible during interphase when the nuclear membrane is present). The presence of multinuclear blastomeres has been reported for both *in vivo* (Hertig *et al*, 1954) and *in vitro* (Winston *et al,* 1991) developing embryos. Its frequency ranges from 17-69% in human embryos (Plachot *et al*, 1987; Hardy *et al,* 1993; Munne *et al,* 1994a) however, it might be significantly higher when associated with other morphological abnormalities (Munne *et al*, 1995a). Recently, Meriano et al (2004) found 24% mulitnucleation in 770 embryos derived from ICSI. The author suggested – after time-lapse photography – that multinucleated blastomeres were subject to dissolution of their nuclear membrane suggesting an asynchrony the nuclei and a possible interruption in proper nuclear and cell division (Meriano *et al,* 2004). Multinucleation first occurs at the 2-cell stage, but it has been shown to occur most frequently at the 8-

cell stage (Hardy *et al*, 1993). Kligman *et al* (1996) suggested that if multinucleation occurs at the first embryonic division all the embryonic cells might be affected. Laverge *et al* (1997) observed multinucleation in 15 out of 39 normal diploid embryos. This is in agreement with Munne and Cohen (1993) who concluded that multinucleation occurs frequently in arrested as well as normally developing embryos. In a study of 1885 embryos by Balakier and Cadesky (1997) it was observed that 44% of patients possessed embryos with at least one multinucleated blastomere (MNB) and 15% of embryos contained MNB, however, there was no correlation with maternal age. It has been proposed that asynchrony between karyokinesis and cytokinesis leads to fragmentation and production of multinucleate blastomeres (Lopata *et al,* 1983; Evsikov and Verlinsky, 1998). Furthermore, partial fragmentation of nuclei or defective migration of chromosomes during mitotic anaphase have also been linked as mechanisms leading to MNB (Tessarik *et al,* 1987; Wiston *et al*, 1991; Pickering *et al,* 1995). However, Staessen and co-workers (1998) after analysing 101 3- to 8-cell embryos developing from 2-cell embryos (where both blastomeres were bi- or multinucleate), reported that the genetic constitution of binucleate and multinucleate blastomeres and the daughter cells developing from them are not always abnormal.

1.6.4.5 Mosaicism and Translocations

A study carried out by Iwarsson *et al* (2000) in preimplantation embryos from translocation carriers during PGD cycles revealed a high degree of mosaicism for the chromosomes involved in the translocations (65%), compared to control chromosomes (35%). However, the degree of mosaicism within each embryo differed between the chromosomes involved in the translocation and the control probes. In order to explain these relatively high frequency of mosaicism Iwarsson *et al* (2000) proposed three hypothetical explanations: (i) Acrocentric chromosomes (13, 14, 15, 21 and 22) have a higher tendency to malsegregate during meiosis as well as mitosis; (ii) The translocation itself may predispose to malsegregation and; (iii) The difference between the chromosomes involved in the translocations with the control chromosomes could be methodological. Although it was concluded that this last hypothesis cannot be regarded as a major cause.

1.7 Aims of the Study

This study is comprised of three parts employing molecular and cytogenetic approaches to the analysis of human chromosomes in human preimplantation embryos.

The aim of the first part was to devise novel fluorescent PCR protocols for PGD acting as well as attempting to develop a universal-like protocol i.e. methodology applicable to different DM patients requiring little optimisation, for PGD for DM. Initially, several F-PCR polymorphic markers, linked or unlinked, were investigated to assess their efficiency at the single cell level and test whether they are informative for two couples. PGD was carried out for both couples using two different single step multiplex F-PCR protocols. A third protocol was devised and tested on single cells using whole genome amplification in addition to F-PCR. The purpose of employing whole genome amplification in this study was primarily to examine whether carrying out DOP-PCR on a single cell was able to amplify regions within the genome that would match the regions where specific F-PCR polymorphic primers hybridise. As a consequence this would eliminate the need for multiplexing and one would be able to carry out several single F-PCR procedures with different F-PCR markers using the DOP-PCR product as a template. Hence, during a PGD case of a single gene disorder such as DM, there would be no need to optimise for a multiplex PCR protocol. If a DOP-PCR of the single blastomere was carried out and then separate but simultaneous singleplex PCR reactions with different F-PCR markers could be performed informative for the parents involved. Also, enough DNA would be available to perform CGH analysis which would allow chromosomal examination of the blastomere. Therefore, from one blastomere the single gene defect as well as its chromosomal status could be identified.

The second part of this study was to develop a reliable FISH-based protocol for the analysis of chromosome abnormalities in day 5 human embryos in order to reveal the level of chromosomal mosaicism for the five chromosomes studied (1, 11, 18, X and Y). Furthermore, it would allow the study of the underlying mechanisms of mosaicism and the high rate seen *in vitro* human preimplantation embryos. Optimising conditions and investigating the possibility of different probe

combinations, therefore allowing the detection of all the possible mechanisms that lead to mosaic and chaotic embryos played an intricate role of this study. With the aid of two probes per chromosome in sequential rounds of FISH, problems of the FISH technique such as FISH artefacts e.g. monosomies, probe overlappings and failure of probe hybridisations were overcome. Such artificial findings would be detected and thus separated from the true findings enabling the study of the mechanisms of mosaicism in human blastomeres. This type of methodology has never been attempted before and would allow a innovative approach of investigating the phenomenon of mosaicism. Furthermore, the effects of the IVF culture media were examined. Interphase FISH was applied to spare/untransferred embryos derived from clinical treatment IVF or ICSI cycles

During the last part of this thesis, the use of CGH was investigated as an alternative to FISH for investigating the prevalence of mosaicism in day 3 and day 5 embryos. This involved assessing the efficiency of CGH, improving the protocol for optimised use on single cells, and its application as a research tool on human preimplantation embryos. The aim of this study was to assess the full chromosomal status of 1-2 blastomeres biopsied at day 3, and then confirm whether the abnormality persists until day 5 using FISH for the chromosome(s) involved. This study would allow the detection of mosaicism in a full karyotype at the cleavage stage and further enquire if the mechanisms causing mosaicism continue to exist until day 5.

CHAPTER 2

Materials and Methods

2.1 Materials

General laboratory chemicals and reagents were obtained from BDH Chemicals UK, Sigma UK and were of Analar or biochemical grade unless otherwise stated. The materials and stocks that have been used for this study are separated into '*FISH Materials*', '*PCR Materials*' *and* '*CGH Materials*' and are listed below:

2.1.1 FISH Materials

2.1.1.1 Materials for Lymphocyte culture

Cell culture flasks, glass pipettes and microscope slides were obtained from BDH whilst all microcapillaries (internal diameters 75-200μm) for embryo, oocyte and single cell handling were from Laser (UK). Nunc Nucleon 50x9mm Petri dishes were used for single cell isolation and purchased from Gibco BRL (UK). Reagents used for media preparation were listed below:

- GPS (Glutamine 200mM, penicillin 300mg/ml, streptomycin 500mg/ml; Gibco, UK)
- Iscoves modified Dulbeccos medium (Sigma, UK)
- Phytohaemogglutinin (PHA; Gibco, UK)
- Thymidine (Sigma, UK)
- Deoxycytosine (Sigma, UK)
- Colcemid (Gibco, UK)

2.1.1.2 Materials for Embryo Spreading

- Poly-l-lysine (Sigma, UK)
- Tween-20 (Sigma, UK)

2.1.1.3 Materials for Nick Translation (commercial kit)

- Nick translation enzyme mix (Vysis, UK)
- 10x nick translation buffer (Vysis, UK)
- dATP, dCTP, dGTP and dTTP (0.3mM each; Vysis, UK)
- Nuclease-free water (Vysis, UK)

- Fluorescent-labelled dUTP's used with the kit were SpectrumAqua-dUTP from

2.1.1.4 Materials for the FISH procedure

- Probes (commercial and non-commercial; Vysis, UK)
- Pepsin (Sigma, UK)
- Hydrochloric Acid (0.01N; Sigma, UK)
- Paraformaldehyde [37% formaldehyde (Sigma, UK) was saturated with $NAHCO_3$ (BDH, UK) and stored in the dark
- Ethanol (99.7%; Sigma, UK)
- Formamide (BDH, UK)
- 20xSSC (Vysis, UK)
- NP-40 (Vysis, UK)
- Vectashield (Vector laboratories, USA)
- 4',6-diamino-2-phenylindole (DAPI; Sigma, UK)

2.1.2 PCR Materials

2.1.2.1 Materials for Single-cell isolation

- **PCR Lysis Buffer** [125μg/ml Proteinase K (Sigma, UK), 17μM Sodium dodecyl sulphate (SDS; BDH chemicals, UK), Nuclease-Free Water (H_2O; Promega, UK)]
- Phosphate Buffer Saline (PBS; Sigma, UK)
- Polyvinyl Alcohol (PVA; Sigma, UK)
- Mineral oil (Sigma, UK)

2.1.2.2 Materials for DNA extraction

- **TKM1** (low concentration salt buffer): 10mM Tris-HCl pH 8.0, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA (BDH, UK)
- **TKM2** (high concentration salt buffer): 10mM Tris-HCl pH 8.0, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA, 0.4M NaCl (BDH, UK)
- **10xTE buffer**: 10mM Tris-HCl pH 8.0, 1M EDTA (BDH, UK)
- Igepal CA-630 (Sigma, USA)

- SDS (Sigma, UK)
- 6M NaCl (BDH, UK)
- 70% Ethanol (BDH, UK)

2.1.2.3 Materials for PCR procedure

- Primers (Oswel, UK) – See Table 2.2
- AmpliTaq Gold Polymerase (Applied Biosystems, UK)
- AmpliTaq Buffer (10x; 1.5mg $MgCl_2$; Applied Biosystems, UK)
- dNTP mix (10mM; Promega, UK)
- Nuclease-Free Water (H_2O; Promega, UK)

2.1.2.4 Materials for gel electrophoresis

- Agarose (Sigma, UK)
- Ethidium Bromide (500mg/ml; Sigma, UK)\
- 10xTBE (0.89M Tris Base, 0.89M Boric acid, 2.5M EDTA; Sigma, UK)
- Loading buffer (10x; 40% sucrose, 0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol) (Supplied by Bioline, UK)
- 1 Kb DNA ladder (Bioline, UK)

2.1.2.5 Materials for ABI Prism^{TM} 310 and 3100

- Deionised Formamide (Sigma, UK)
- Size Standard (Genescan 500-TAMRA; Applied Biosystems, UK
- Size Standard (Genescan 500-ROX; Applied Biosystems, UK)
- 0.5ml Sample Tubes (Applied Biosystems, UK)
- Septa for 0.5ml Samples Tubes (Applied Biosystems, UK)
- Plate for 3100 (Applied Biosystems, UK)
- Capillary on array for 310 and 3100 ABI Prism (Applied Biosystems, UK)
- Buffer for 310 and 3100 ABI Prism (Applied Biosystems, UK)
- Polymer for 310 and 3100 ABI Prism (Applied Biosystems, UK)

2.1.3 CGH Materials

Single buccal cell, single blastomere as well as clump isolation was performed using the same materials as in 2.1.5. Furthermore, all genomic DNA from whole blood or fibroblasts was carried out using the same material as in 2.1.6.

2.1.3.1 Materials for DNA Extraction

- Hank's medium (Sigma, UK)
- **DNA extraction lysis buffer** (In 100ml 1.21g Tris, 0.19g/100ml EDTA, 0.2gSDS, 1.17g NaCl., 10mg/ml of proteinase K added after autoclaving)
- Isopropanol (BDH, UK)

2.1.3.2 DOP-PCR Materials

- Primer (Oswel, UK) – See Table 5.3
- SuperTaq Plus (HT Biotechnology, UK)
- SuperTaq Buffer (10x; 1mg $MgCL_2$; HT Biotechnology, UK)
- dNTP mix (10mM; Promega, UK)
- Nuclease-Free Water (H_2O; Promega, UK)

2.1.3.3 Nick-Translation Materials

- Nick Translation kit (Vysis, UK)
- 0.2mM Spectrum Green or Spectrum Red dUTP's (Vysis, UK)
- Human Cot-1 DNA (GibCo BRL, UK)
- NaAc (3M; Sigma, UK)

2.1.3.4 CGH Procedure

- Hybridization mix (50% Formamide, 2xSSC, 10%Dextran Sulphate; Sigma, UK)
- CGH Metaphase Target Slides (Vysis, UK)
- Triton-X (Sigma, UK)
- Formamide (BDH, UK)
- 20xSSC (Vysis, UK)

2.1.4 Embryo Materials

2.1.4.1 Ethical Approval

The work on surplus embryos and the clinical application of PGD (Chapter 3) were approved by the Research Ethics Committees of the University College London Hospital Trusts, and carried out under licence from the Human Fertilisation and Embryology Authority (HFEA). The surplus embryos for Chapters 4 and 5 were donated from normal patients undergoing routine IVF at the London Fertility Clinic which were authorised by the HFEA. Informed written consent was obtained from patients for surplus embryos to be used for research purposes

2.1.4.2 Grading Criteria for the Embryos

Preimplantation cleavage stage embryos were graded according to Bolton *et al*, (1989) as follows;

Grade 1 Embryo at the correct stage of *in vitro* development with perfect symmetrical and even-sized blastomeres with no fragmentation.

Grade 1^- Embryo at the correct stage of *in vitro* development with perfect symmetrical and even-sized blastomeres with less than 10% fragmentation.

Grade 2^+ Development with unequally sized blastomeres with less than 20% fragmentation

Grade 2 Retarded development with unequally sized blastomeres with 25%-50% fragmentation.

Grade 3 Retarded development with unequally sized blastomeres with more than 50% fragmentation.

2.1.4.3 Categorisation of Chromosomal Abnormalities in Embryos.

Embryos analysed for the preliminary work and during PGD cycles were categorised after Delhanty *et al,* (1997) into four groups; normal, uniformly abnormal, mosaic (diploid mosaic or aneuploid mosaic) and chaotic (Table 1.1). Embryos were allocated where possible to each group on the basis of the chromosome constitution of the majority of cells present.

2.1.4.4 Embryo culture

Oocytes were retrieved using Flushing Medium (supplemented with sodium pyruvate, HAS, heparin 10IU/ml, penicillin 50,000IU/l, streptomycin 50mg/l, and HEPES; Medicult UK Ltd), incubated in 6%CO_2 in air, at 37°C, inseminated and cultured in 500μl of IVF Medium (Bicarbonate buffered medium containing human serum albumin, penicillin and sodium pyruvate; Vitrolife, Scandinavia). On day 1, oocytes were assessed for the number of pronuclei and transferred into 25μl microdroplets of Cleavage Medium (Bicarbonate buffered medium containing human serum albumin, penicillin-G, EDTA, glucose, inorganic salts and amino acids; G-1, Vitrolife, Scandinavia). On day 3, the best embryos were selected for transfer and suitable spare embryos were cryopreserved.

The embryos were divided into two groups depending on the culture medium. Group I embryos were cultured in standard IVF medium (6.1 Vitrolife, Scandinavia) from day 0 to day 5 and Group II embryos were cultured in standard IVF medium from day 0-3 and then in blastocyst medium (6.2 Vitrolife, Scandinavia) from day 3-5. Only embryos that arose from a bipronucleate zygote were included in the study.

2.1.4.5 Embryo Freezing

Good quality Day-1 embryos were considered for freezing when two pronuclei and two polar bodies were visible 16-18 hours after the oocytes were subjected to sperm either by IVF insemination or ICSI. Good quality Day-3 embryos were considered when they comprised 6-8 cells and were grade 2 and above.

All embryos (either day-1 or day-3 cell stage) were placed for a five-minute wash in Cryo-PBS medium (Vitrolife; Scandinavia) and then taken through consecutive washes of embryo freezing solutions 1 and 2 (EFS1 and EFS2; Vitrolife, Scandinavia) for ten minutes each at room temperature. The embryos were loaded into the freezing straw (maximum two embryos per straw) and placed into a cryobath. The cryobath is linked to a computer program which controls temperature until it reached -180°C..

2.1.4.6 Embryo Thawing

All thawed embryos (either day-1 or day 3-cell stage) were taken through consecutive washes of embryo thaw solutions 1, 2, and 3 (ETS1, ETS2 and ETS3; Vitrolife, Scandinivia) for five, five and ten minutes respectively at room temperature. A final wash was performed through Cryo-PBS medium for five minutes at room temperature followed by five minutes on a heated stage (38°C) (Nikon SMZ-U microscope stage).

Pronuclei (PN) stage embryos, after thaw, were initially cultured in G1 medium until day 3 (section 4.2.5) and subsequently in G2 medium until blastocyst stage, while day 3 thawed-embryos were directly cultured in G2 medium.

2.1.4.7 Embryo Spreading

Embryos were spread as described previously (Harper *et al*, 1994; Ruangvutilert *et al*, 2000a; section 2.2.1.2).

2.1.4.8 Embryo Classification and Scoring of Embryos

The criteria used for classifying the embryos were adopted from Delhanty *et al* (1997) and can be seen in Table 1.1. Whilst classifying an embryo all major cell lines should be mentioned e.g. if an embryo has 20 diploid cells, 4 tetraploid cells, 2 trisomy 18 cells, 1 monosomy 1 cell and 1 triploid cell, it should be classified as diploid/polyploid/aneuploid mosaic. Furthermore, if a blastomere displayed contradictory results in different rounds of FISH for the same chromosome e.g. one signal for 1p (1st round) but 2 signals for 1het (2nd round), this was considered as an "*inconsistent result*". When scoring the fluorescent signals (see section 2.2.1.5.5), signals must be a minimum of a signal's width apart in order to be scored as 2 individual signals (Hopman *et al*, 1991).

2.1.4.9 Mosaicism and Events

Diploid mosaic embryos with aneuploid cells were considered to have arisen through three different mechanisms: (a) when the embryo contained cells with monosomies, then the mechanism was classed as "chromosome loss" (CL), (b) when the embryo contained cells with trisomies, then the mechanism was classed as "chromosome

gain" (CG) and (c) when the embryo had monosomies and trisomies of the same chromosome(s) in different cells, this was classified as mitotic non-disjunction (MND). Nuclei with multiple abnormalities affecting at least three chromosomes were classed as chaotic and not included in the analysis. This included nullisomies and tetrasomies. During the last chapter chromosome breakage and partial mosaicism mechanisms were revealed, hence all three mechanisms (chromosome loss, chromosome gain and mitotic non-disjunction) can occur either partially (p) or in the whole (w) chromosome.

2.1.5 Probes and Primers

2.1.5.1 DNA Probes.

Details of DNA probes used in this study along with their sources are summarised in Table 2.1. The plasmid DNA clones for chromosome 1 was obtained from resource centres as agar stabs. Maxiprep of plasmid and cosmid DNA was carried out with Wizard maxiprep kit from Promega. Probe DNA was labelled via nick translation supplied in kit form (Nick Translation Kit Vysis UK). Commercially obtained labelled α-satellite and locus-specific probes were supplied by Vysis UK. All were stored at -20°C and protected from light.

Table 2.1. List of FISH DNA probes used in the FISH and CGH/FISH study

Probe	Type	Label	Source
1p	1pter	Spectrum Green	Vysis, UK
1q	1qter	Spectrum Orange	Vysis, UK
1het	Satellite-II/III	Spectrum Aqua	Lab-prepared
2q	2qter	Spectrum Orange	Vysis, UK
2cep	α-satellite	Spectrum Aqua	Lab-prepared
3cep	α-satellite 3	Spectrum Orange	Vysis, UK
4cep	α-satellite 4	Spectrum Aqua	Vysis, UK
5p	5pter	Spectrum Green	Vysis, UK
5p/5q (Cri-du-Chat microdeletion probe)	5p15.2 / 5q31	Spectrum Green /Orange	Vysis, UK
6cep	α-satellite 6	Spectrum Green	Vysis, UK
7p/7q (Willams microdeletion probe)	7p31 / 7q11.23	Spectrum Green/ Orange	Vysis, UK
8q	8qter	Spectrum Orange	Vysis, UK
8cep	α-satellite 8	Spectrum Orange	Vysis, UK
9cep	α-satellite 9	Spectrum Orange	Vysis, UK
10cep	α-satellite 10	Spectrum Green	Vysis, UK

11q	11qter	Spectrum Orange	Vysis, UK
11cep	α-satellite 11	Spectrum Green	Vysis, UK
11cep	α-satellite 11	Spectrum Aqua	Vysis, UK
13LSI	13q11	Spectrum Green	Vysis, UK
14q	14qter	Spectrum Orange	Vysis, UK
16p	16pter	Spectrum Green	Vysis, UK
16qter	16qter	Spectrum Orange	Vysis, UK
16cep	α-satellite 16	Spectrum Aqua	Vysis, UK
18q	18qter	Spectrum Orange	Vysis, UK
18cep	α-satellite 18	Spectrum Aqua	Vysis, UK
22LSI	22q11.2	Spectrum Green	Vysis, UK
Xcep	α-satellite X	Spectrum Green	Vysis, UK
Ycep	Satellite II/III	Spectrum Aqua	Vysis, UK
X / Y / 18	Probe-cocktail of heterochromatic probes	Spectrum Green / Orange / Aqua	Vysis, UK

2.1.5.2 PCR Primers

Several STR markers were used in different preliminary work carried out for couples undergoing PGD treatment for myotonic dystrophy. These markers were either linked or unlinked and varied in their size and fluorescent dyes. All primers for the myotonic dystrophy workup can be seen in Table 2.2 below

Table 2.2. List of PCR primers used in the PCR study

Type of Marker	Marker (F=forward) (R=reverse)	Sequence of primers (5'-3')	Chromosome location of primers	Size of the PCR product (bp)	Label of primer (at 5' end of forward primer)	Reference
STR (Dinucleotide)	DM (F)	5'-cttcccaggcctgcagtttgcccatc-3'	19q13.3	128-203	5'-FAM DYE	Brook *et al,* 1992
	DM (R)	5'-gaacggggctcgaagggtccttgtagc-3'			-	
STR (Dinucleotide)	APOC2 (F)	5'-ggctacatagcgagactccatctcc-3'	19q12 – 19q13.2	134-170	5'-HEX DYE	-
	APOC2 (R)	5'-gggagagggcaaagatcgataaagc-3'			-	
STR (Dinucleotide)	D19S207 (F)	5'-tgcggtgtttgaaccctcgctg-3'		118-160	5'-HEX DYE	-
	D19S207 (R)	5'-actgcactgcagcctgagtgac-3'			-	
STR (Dinucleotide)	D19S112 (F)	5'-ctgaaagacacgtcacactggt-3'		115-140	5'-HEX DYE	Jansen *et al,* 1992
	D19S112 (R)	5'-gccagccattcagtcatttgaag-3'			-	
STR (Tetranuclaotide)	D19S393 (F)	5'-gcaatgagccgagatagaa-3'			5'-HEX DYE	-
	D19S393 (R)	5'-tggctagcccattactcta-3'			-	
STR (Tetranucleotide)	D21S11 (F)	5'-tatgtgagtcaattccccaagtga-3'	21q21	200-260	5'HEX DYE	Sharma and Litt, 1992
	D21S11 (R)	5'-gttgtattagtcaatgttctccag-3'			-	
STR (Tetranucleotide)	D21S1414 (F)	5'-aaattagtgtctggcacccagta-3'	21q21	330-370	5'-HEX DYE	Sherlock *et al,* 1998
	D21S1414 (R)	5'-caattccccaagtgaattgccttc-3'			-	
STR (Tetranucleotide)	D18S535 (F)	5'-cagcaaacttcatgtgacaaaagc-3'	18q12.2 – 18q12.3	455-500	5'-HEX DYE	Lareu *et al,* 1998
	D18S535 (R)	5'-caatggtaacctactatttacgtc-3'			-	
STR (Tetranucleotide)	D13S305 (F)	5'-gcctgtttgaggacctgtcgtta-3'	13q12.1 – 12q14.1	430-465	5'-TET DYE	-
	D13S305 (R)	5'-tggttatagagcagttaaggcac-3'			-	
DOP (WGA)	DOP-50	5'-ccgactcgagnnnnnnatgtgg-3'	Random	-	-	Telenius *et al,* 1992

2.1.6 Equipment

2.1.6.1 FISH and CGH Equipment

Dissecting microscopes from Nikon and inverted microscopes from Olympus were used for embryo and oocyte handling as well as slide preparation. Fluorescence microscopy was carried out with the following microscope systems; Reichert Jung Polyvar microscope with single filters for TRITC, FITC and DAPI, Nikon optiphot microscope with Omega dual band-pass TRITC/FITC filter and Zeiss Axioskop microscope with Chroma multi-band pass TRITC/FITC/DAPI filter and single SpectrumAqua filter. Image capture and analysis was carried out using a Zeiss Axioskop microscope equipped with a Photometrics KAF 1400 cooled CCD (charged coupled device) camera controlled by Smartcapture software from Vysis, UK.

2.1.6.2 PCR and CGH Equipment

Three models of thermal cyclers were used for PCR amplification:

1. Hybaid Omnigene was manufactured by Hybaid Middlessex, UK.
2. ABI-9700 PCR System was manufactured and serviced by Applied Biosystems, UK.
3. Mastercycler Gradient was from Eppendorf-Netheler-Hinz GmbH, Cambridge, UK.

The analysis of fluorescent PCR products was performed on the ABI Prism 310 and 3100 using GeneScan analysis software (version 2.0.2). A Nikon dissecting microscope used for single cell isolation and oocyte spreading and a Nikon phase contrast microscope was employed to check slide preparations.

2.2 METHODS

2.2.1 FISH Methods

FISH was used in the mosaicism study (Chapters 4 and 5). FISH consists of:

- Sample (embryo) or control lymphocyte cell suspension
- Probe preparation which consists of:
  - DNA preparation
  - Nick Translation or use of commercial probes
- FISH procedure and analysis

- Slide preparation and pre-treatment
- Probe preparation
- Denaturation conditions
- Post-hybridisation washes
- Analysis (capturing and scoring)
- Sequential FISH procedure

2.2.1.1 Control Lymphocyte Preparation

Male lymphocyte cells were used as control samples to record the efficiency of the probe combination employed for each FISH procedure. A sample of blood was provided by a male donor and was cultured according to the following procedure. The blood sample was collected in a lithium heparin tube. Iscoves modified Dulbeccos medium (Sigma, UK), and Fetal calf serum-heat inactivated (FCS) (Gibco, UK), were warmed to 37° C. 2 ml of GPS {Glutamin (200 mM), Penicillin (300 mg/ml), Streptomycin (500 mg/ml)}, which was stored at – 20°C, was added to the medium in order to ensure antibiotic resistance.

Under aseptic conditions, the following were added to a 50 ml culture flask: 17ml Iscoves, 2 ml FCS, 200 μl of Phytohaemagglutinin (PHA) (Gibco, UK) which stimulates the mitotic process, and 1 ml of blood. These were mixed and incubated at 37° C for 48 hours or 72 hours. The flasks were gently shaken twice a day to re-suspend the cells. On day 3 at 4pm, 200 μl of thymidine (30 mg/ml stock) (Sigma, UK) was added to achieve synchronisation of mitosis. On day 4 at 10 am, 200 μl of deoxycytosine (0.227 mg/ml stock) (Sigma, UK) were added which enforced the effect of thymidine on the cell culture in terms of synchronisation of mitosis. Finally, at 2 pm, on day 4, 200 μl of colcemid (10 μg/ml stock) (Gibco, UK) was added in order to arrest the cells at metaphase prior to harvesting.

2.2.1.1.1 Harvesting

The flasks were shaken, their contents emptied into two 10 ml tubes and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded leaving only a small amount for pellet resuspension. An aliquot of 10 ml of 0.075 KCl was added slowly to each tube and left at room temperature for 15-20 minutes, in order for the KCl to cause the

cells to swell. The tubes were again centrifuged at 1000rpm for 5 minutes and the supernatant was discarded, leaving a small amount to re-suspend the pellet.

Approximately 1-2 drops of the fixative solution (3:1 methanol/ acetic acid), which was made fresh, were added to re-suspend the pellet by tapping the tube sharply until its contents turned brown and frothy. A few more drops of fix were added slowly, whilst the solution was agitated and this was repeated. When the solution stopped frothing, a few mls of fix were added at a time until the tube was full (approximately 10ml). The tubes were centrifuged at 1000 rpm for 5 minutes and the supernatant was discarded, leaving a small amount for pellet re-suspension. The fixation process was repeated as described above but fix was added a few mls at a time. After the final centrifugation the remaining pellet had a pale whitish colour. The lymphocyte suspension was stored at -20°C and left overnight before being used for control slide preparation.

2.2.1.1.2 Preparation of control lymphocytes

Whenever the FISH procedure was performed, it was of great importance to process a control slide as a reference to ensure the efficiency of the procedure. Fixative solution (3:1 methanol: acetic acid) and 70% acetic acid were prepared each time. The standard cytogenetic preparations of male control lymphocytes which were stored in 3:1 methanol: acetic acid at -20^{0}C, were centrifuged at 400 *g* for 5 minutes and re-suspended in fresh fixative (the volume depended on the pellet size, so as to ensure a good concentration of lymphocytes). A microscope slide was cleaned with a tissue and breathed on. A small drop of lymphocyte solution was placed on the centre of the slide, which was immediately warmed on the back of the hand, to ensure the nuclei were spread out. The slide was left to totally dry, while other slides were prepared. Subsequently, the slides were flooded with fixative for 10 seconds, which was poured off and the slides were left to dry, then they were flooded again with 70% acetic acid for 10 seconds. Once the slides were totally dry, they were checked under a phase microscope to ensure the presence of nuclei. Finally, they were dehydrated successively in 70%, 90%, and 100% ethanol at room temperature for 5 minutes each.

2.2.1.2 Day-5 Embryo Spreading

Poly-l-lysine coated slides were used to enhance the fixation of nuclei on slides. They were prepared by placing glass slides in a mixture of 50ml of 100% methanol and 0.5ml of 1N HCl for 3-5min. The slides were left to air-dry and subsequently rinsed in Poly-l-lysine (Sigma, UK) for 5min. They were left at room temperature overnight and stored at 4°C until required.

The embryos were spread as described previously by Harper *et al* (1994). The embryo was washed in a drop of PBS to remove excess culture medium. Then, the embryo was transferred to a small drop of spreading solution (0.01N HCl, 0.1% Tween 20) on a poly-l-lysine slide. The embryo was constantly observed under an inverted microscope. Most of the spreading solution was removed and replaced with fresh spreading solution in order to dissolve the zona pellucida and excess cytoplasm. The nuclei were further washed by gentle agitation of the spreading solution until clear of cytoplasm.

The slides were left to dry completely and washed for 5min in PBS and dehydrated through an ethanol series for 3min (70%, 90% and 100% respectively). The nuclei were located and mapped using an England Finder (Graticules Ltd, UK) under a phase contrast microscope. The slides were left at RT for subsequent FISH. Most of the embryos were spread by embryologists in the London Fertility Centre (LFC) after being trained by the author on spare IVF human embryos.

2.2.1.3 Plasmid Probe Preparation

The DNA for the laboratory-prepared (lab-prepared) probe for the α-satellite of chromosome 1 was isolated from an *E. coli* strain carrying the pZ20 vector. Initially, *E. coli* cells from stabs were inoculated into 4 ml of 2xTY medium (16g/l Bactotryptone, 10g/l Bacto-yeast extract, 5g/l NaCl), also containing 4 μl of ampicillin (100mg/ml). The culture was left overnight in a shaking incubator at 37°C. The following day this culture was re-inoculated into 200 ml of 2xTY medium and 200 μl ampicillin (100 mg/ml), and was again incubated overnight in a shaking incubator at 37°C. The DNA was extracted using a commercial maxiprep kit (Promega, UK). The procedure included the following: First the cells were pelleted by

centrifugation at 4,000 rpm for 10 min. The supernatant was discarded and the cells re-suspended into 15 ml of cell re-suspension solution (50 mM Tris-HCL pH 7.5, 10 mM EDTA, 100 μg/ml, RNase A). Approximately 15ml of cell lysis solution (0.2 NaOH, 1% SDS) were added and mixed gently but thoroughly, by stirring or inverting. When the cellular mixture became clear, 15 ml of neutralization solution (1.32M potassium acetate pH 4.8) were added, and immediately mixed by gently inverting the centrifuge bottle several times. The suspension was centrifuged at 9,000 rpm for 15 minutes at 22-25°C in a room temperature rotor.

The supernatant was filtered through blotting paper and transferred into a 100 ml graduated cylinder. After its volume was measured, the supernatant was transferred to a new centrifuge bottle. Half a volume of isopropanol was added to this supernatant, which was mixed by inversion. The suspension was centrifuged at 9,000 rpm for 15 minutes as above. The supernatant was discarded and the DNA pellet was re-suspended in 2 ml TE buffer (10 mM Tris-HCL, 1mM EDTA pH 7.5). The DNA purification was achieved with the use of the Wizard resin and vacuum pump. Finally, the concentration of the DNA was measured by a fluorometer, and the latter was stored at -20°C.

2.2.1.4 Probe Labelling

The plasmid probes were labelled using a nick translation kit (Vysis, UK). However, the labels that this kit contains (spectrum green or spectrum orange direct-labelled dUTP) were not used. The label used was diethylaminocoumarin (DEAC)-5-dUTP (Perkin Elmer, USA), which emits aqua fluorescence and incorporates into loci-specific identifier DNA probes for its use in FISH. Half the amount of the dTTP was substituted with labelled dUTP. The latter diluted the label incorporation, and increased the DNA Polymerase I efficiency. Hence, the procedure enabled the incorporation of about 20% of the fluorescent-labelled nucleotide into the DNA, generating in this way a clear bright signal during hybridization. Ethanol precipitation removed the unincorporated nucleotides.

2.2.1.4.1 Nick Translation

Initially a DEAC-5-dUTP (Perkin Elmer, USA) 50nmol concentration was prepared at 0.2 mM by adding 10 µl of 0.1 mM dUTP to 40 µl nuclease-free water. dTTP was prepared in a concentration of 0.1 mM with the addition of 10 µl of 0.3 mM dTTP to 20 µl nuclease-free water. A concentration of 0.1 mM dNTP mix was achieved by mixing together 10 µl each of 0.3 mM dATP, 0.3 mM dCTP, and 0.3 mM dGTP (all three from Vysis, UK).

The nick translation reaction contained the following: 17.5-x nuclease free water mixed with 1 µg of DNA (volume x, varied depending on DNA concentration), 2.5 µl of 0.2 mM spectrum green and spectrum orange, 5 µl of 0.1 mM dTTP, 10 µl of dNTP mix, 5 µl of 10X nick translation buffer, and, 10 µl of nick translation enzyme. The latter consisted of DNA polymerase I, DNase I in 50% glycerol, 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 DTT, and 0.5 mg/ml nuclease free BSA.

The Eppendorf tube was briefly centrifuged and vortexed to mix the reaction components and incubated for 2 hours at 15°C. This temperature was crucial for probe efficiency and the success of the labelling method. The nick translation reaction was stopped by the addition of 5 µl of 0.5 mm EDTA pH 8 (BDH, UK). Then, the following components were added to the tube: 5 µl of herring sperm DNA (Sigma, UK), 6 µl of 3M sodium acetate, and 1 ml of 100% ice cold ethanol. The probe was incubated at -70°C for 1 hour, and centrifuged for 10 min at 14,000 rpm. The supernatant was removed, and the pellet was air-dried by leaving the tubes open in the dark. Finally the probes were re-suspended in 100 µl of hybridisation buffer (2x SSC, 60% deionized formamide, 10% dextran sulphate), and stored in the dark at -4°C.

2.2.1.5 FISH Procedure

The probes used in this study can be viewed in Table 2.1. All the probes were commercial (Vysis, UK) apart from one (1cep in spectrum Aqua), which was lab-prepared. The probe combinations during the sequential rounds of FISH for Chapter 4 are summarised in Table 2.3

2.2.1.5.1 Slide Pre-treatment

The method of FISH performed was described previously by Harper *et al* (1994). The slides were incubated in 0.01N HCl containing 10mg/ml pepsin at 37^0C for 20 minutes, in order for any remaining protein to be removed and to make the nuclei accessible to the probes. The slides were briefly washed in bidistilled (FISH) water and PBS and incubated for 10 minutes at 4^0C in 1% paraformaldehyde in PBS to re-fix the nuclei. The slides were then washed in PBS and a further two washes in water. The slides were finally dehydrated through an ethanol series (70%, 90% and 100% for three minutes each) and left to dry.

2.2.1.5.2 Probe Preparation

A 5μl probe mix was prepared according to the source and the type of the probe and added to the nuclei under a 13mm diameter coverslip. The probe mix consisted of different volumes of probes and CEP hybridisation buffer.

2.2.1.5.3 Separate Denaturation

The FISH protocol and embryo analysis was more efficient whilst carrying out separate denaturation. Separate denaturation was performed for all three rounds of FISH. During separate denaturation both the slide and the probes were treated separately. The slide pre-treatment was followed as described above (Section 2.2.1.5.1) until the last dehydration. After the washes with PBS and twice with water, 100μl of denaturation solution (70% formamide in 2xSSC) was added to the slide. The slide was immediately denatured at 75°C for 5 min and incubated in 50ml of 70% ice-cold ethanol for 5 min to stop the denaturation. Subsequently, the slide was dehydrated through an ethanol series (70%, 90% and 100% for 3 min each) and left to dry. All probe combinations were sealed with fixo-gum (Qbiogene, UK) and incubated overnight in a moist chamber at 37^0C.

2.2.1.5.4 Post-Hybridisation Washes

The stringency of probe binding was controlled by the formamide concentration, salt concentration and temperature during the post-hybridisation washes. Therefore conditions were dependent on probe type. All washes were carried out in 50ml volume coplin jars with those containing formamide restricted to a laminar flow

cabinet and slides were protected from light at all stages. After hybridisation any rubber cement was discarded and coverslips were gently removed by immersing briefly in the first wash solution. For combinations of probes including locus-specific probes, slides were treated at 45°C with 3 x 3 minutes washes in 50% formamide in 2xSSC and then 3 x 3 minutes washes in 2xSSC followed by a 10 minute wash in SSCT [4xSSC; 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate), Sigma, UK] at room temperature. When only repetitive probes were used the stringency was raised by increasing the formamide concentration to 60% in the 2xSSC solution. All other washes were as above.

According to the *'long washes'* protocol from Vysis (UK) any unbound probe was removed by washing the slides for 10 minutes at 45^0C in 70% formamide/2xSSC pH 5.3 (three times), 10 minutes in 2xSSC pH 7.0 at 45^0C and then 5 minutes in 0.05% 2xSSC/ 0.1% NP40 at 45^0C.

2.2.1.5.5 Detection, Capturing and Scoring of the Probes

The examination and scoring of the slides was carried out using a fluorescent Olympus (BX-40) microscope, which was fitted with a photometrics cooled CCD camera utilising pathVysion software (Digital Scientific, Cambridge, UK). Two hundred interphase nuclei were scored in order to calculate probe efficiency as part of the FISH study. All embryonic blastomeres were located using an England Finder. FISH probe signals in interphase nuclei were scored following Hopman *et al.,* (1988) such that two signals closer than a signals diameter apart were considered a single split signal and those further apart were considered as two separate signals.

2.2.1.5.6 Sequential FISH Procedure

After the detection and capturing of the 1st (or 2nd) round probe combination (Table 2.4), co-ordinates of three interphase nuclei and three metaphase spreads were recorded in the control lymphocyte slide using an England Finder in order to assess the efficiency of the probes in future rounds. The coverslip was removed with care so as not to disturb the nuclei. The slide was washed twice in 4xSSC/0.05% Tween 20 (while shaking) for 10 min each time. The slide was incubated in PBS, washed again

for 10 min and then dehydrated through a 70, 90, and 100% ethanol series. The probe mix was prepared for the second round of FISH.

The probes were separately denatured from the slide (section 2.2.4.3) for 5 min in 75°C and the slide incubated at 37°C overnight. Any unbound probe was removed by washing the slides for 10 minutes at 45°C in 70% formamide/2xSSC pH 5.3 (three times), 10 minutes in 2xSSC pH 7.0 at 45°C and then 5 minutes in 0.05% 2xSSC/ 0.1% NP40 while at 45°C. The slides were left to dry in the dark and mounted in Vectarsheild (Vector Laboratories, USA) containing 5µl of 0.2mg/ml 4',6'-diaminidino-2-phenyolindole (DAPI). The DAPI volume was lowered to reduce its intensity and thus avoid bleaching the aqua probe signals. The FISH signals were analysed as described in section 2.2.1.5.5

2.2.1.6 FISH Protocol for Chapter 4

Initially the study was carried out on control lymphocytes to select and optimise the FISH protocol. Subsequently, the study was performed on embryos that were not transferred and not suitable for freezing and were donated with written consent from patients undergoing routine IVF treatment at the London Fertility Centre.

2.2.1.6.1 FISH Protocol Selection and Optimisation

The FISH protocol was carried out as described by Harper *et al* (1994) above. However, some of the steps were altered in order to obtain high efficiency in all three sequential rounds of FISH. Several probe combinations were tested during the initial stages of this study (Table 2.3).

Table 2.3. Multi-colour FISH-probe combinations

Chromosome combinations	Probes used in the 1st round	Probes used in the 2nd round
1 dual / 8 dual	1het / 1p	8cep / 8q
4 / 16 dual & 4 / 11 dual	4cep / 16p / 16q	4cep / 11cep / 11q
7 dual# / 18 & 11 dual / 18	7q / 7q / 18cep	11cep / 11q / 18cep
2 / 4 / 5	2q / 4cep / 5p	5p&5q */ 2cep
1 / 11 / 18	1p / 11q / 18cep	1het / 11cep / 18q
X / Y / 18 (cocktail)	Xcep / Ycep / 18cep	Xcep / Ycep / 18cep

The probe is the 7 (7q11.23 and 7p31) Willams microdeletion probe
* The probe is the 5 (5q31 and 5p15.2) Cri-du-Chat microdeletion probe

All FISH protocols were tested on lymphocytes and their efficiency was measured on 200 interphase nuclei for each probe. Once each probe by itself was efficient, all the probes were combined and adjustments to salt concentrations, temperature, formamide concentration were carried out in various FISH experiments to achieve increased efficiency values and clear, sharp, large signals for each probe. The final three different combinations of 3-colour FISH in the three rounds were optimised. Probe combinations as well as stringency conditions were altered and tailored to produce the best possible fluorescent signals for all the chromosomes tested.

2.2.1.6.2 Final FISH Protocol

FISH was performed in three sequential rounds (section 2.2.1.5). The 1st and 2nd rounds were performed with probes for chromosomes 1, 11 and 18, whilst the 3rd round used probes for the sex chromosomes and chromosome 18 (Table 2.2). All probes except one (the probe 1staII/III in spectrum aqua) were obtained from Vysis (UK) Ltd. The first round included the following probes: 1p SpectrumGreen (telomere CEB108/T7), which hybridises to the sub-telomere region of the short arm of chromosome 1; 11q SpectrumOrange (telomere VIJyRM2072), which hybridises to the sub-telomere region of the long arm of chromosome 11; CEP18 SpectrumAqua (alpha satellite D18Z1), which hybridises to the centromere region of the chromosome 18. The second round included the following probes: 1het (satellite II/III), which hybridises to the heterochromatic region of chromosome 1 (laboratory-prepared); CEP11 SpectrumGreen (satellite D11Z1), which hybridises to the centromere region of the chromosome 11; 18q SpectrumOrange (telomere VIJyRM2050), which hybridises to the sub-telomere region of the long arm of chromosome 18. The third round included the following probes: repeated use of 18 SpectrumAqua (as an internal control); CEPX SpectrumGreen (alpha satellite DXZ1), which hybridises to the centromere region of chromosome X; and CEPY SpectrumOrange (alpha satellite DYZ3), which hybridizes to the centromere region of chromosome Y (Table 2.2).

2.2.1.6.3 Stringency Conditions

Each probe combination reacts differently with respect to stringency conditions. The telomeric (labelled with a "*p*" or a "*q*") and locus specific probes (labelled *LSI*)

required an LSI buffer solution to hybridise more efficiently onto the DNA. However, centromeric probes (labelled "*CEP*" or "*satII/III*" or "*het*") required a CEP hybridisation buffer solution. During the post-hybridisation washes (see section 2.2.5.4) telomeric and locus specific probes needed a lower formamide concentration of 50% whereas centromeric probes need a 60% formamide concentration. In all three rounds of FISH, CEP buffer and 50% formamide was used. The denaturation time/temperature factor was modified since all the telomeric and LSI probes require 5min at 73°C, whereas CEP probes need 3min at 75°C. Hence, in all three rounds the probe combinations were denatured for 5min at 75°C. Also, further optimisation of the protocol was carried out whilst working on human embryos. It was found that if the denaturation step was carried out in a 75°C waterbath, rather than a 75°C oven, higher efficiencies were obtained. The final stringency conditions can be seen in Table 2.4. During this study, all the post hybridization washes were carried out according to the *'long washes'* protocol from Vysis (UK).

Table 2.4. Probe combinations and conditions for all three rounds of sequential FISH

Round of FISH	Probe combination	Volume of probe (μl)	Denaturation temperature/ time	Hybridisation time
1st Round	1p	0.6	75°C / 5min	Overnight
	11q	0.6		
	18cep	0.5		
	CEP buffer	3.3		
2nd Round	1het	1.0	75°C / 5min	Overnight
	11cep	0.5		
	18q	0.6		
	CEP buffer	2.8		
3rd Round	X / Y / 18	2.0	75°C / 5min	2 hours
	CEP buffer	2.0		

cep = centromeric probe, het = heterochromatic probe
p = sub-telomeric probe for the small arm, q = sub-telomeric probe for the big arm

2.2.2 PCR Methods

PCR was carried out in the CGH (Chapter 5) as well as the PCR-PGD (Chapter 3) study. Blood from normal individuals was collected and prepared for PCR to be used as control. Specimens for each case (Chapter 3) consisted of maternal and paternal blood (as well as relative blood when applicable) collected in ethylene-di-amine-tetra-

acetic acid (EDTA) blood tubes. Both maternal and paternal bloods were prepared for PCR.

2.2.2.1 DNA Extraction from Blood

The method described by Lahiri and Nurnberger (1991) was employed for DNA extraction from blood.

Blood samples of 5ml were collected into Falcon centrifuge tubes (Falcon, UK) containing 400mM EDTA. Whole blood was transferred into centrifuge tubes and 5ml of low salt buffer TKM1 and 125µl of Igepal CA-630 (Sigma, UK) were added to lyse the red blood cells. The mixture was mixed well by inversion and shaking and centrifuged at 2,200rpm for 10 minutes. The supernatant was slowly removed and the pellet was washed in 5ml of TKM1 and 125µl of Igepal CA-630 as previously. The washing and spinning were repeated until redness of the pellet was diminished. The pellet was then re-suspended in 100µl TKM1. A total of 800µl of TKM2 and 50µl of 10% (w/v) sodium dodecyl sulphate (SDS) were added to the suspended pellet to lyse the white blood cells. The solution was mixed thoroughly by pipetting up and down. The tube was placed in a 55^0C water-bath for >30min until the lumps disappeared completely. 300µl of 6M NaCl were added to ensure that all cells had lysed, and the tube was centrifuged at 10,000rpm for 10 minutes. The supernatant was transferred to a new centrifuge tube and the precipitated protein pellets were discarded. Two volumes of 100% ice-cold ethanol were added to the supernatant and the tube was inverted until the DNA strands precipitated. The sample was spun at 10,000rpm for 5 minutes and the supernatant was poured off. The remaining precipitated DNA was washed in 1ml of ice-cold 70% ethanol and spun at 10,000rpm for 5 minutes. The supernatant was discarded and the DNA pellet was left to dry for 5 minutes. The DNA pellet was dissolved in 300µl of 1xTE and stored at -20^0C until further use.

2.2.2.2 Single Cell Isolation

2.2.2.2.1 Preparation for Single-cell Isolation

All single cell isolations and single cell PCR procedures were carried out in a dedicated room (termed single cell room) which was free of PCR amplified samples. The 'single cell' room was a room with positive pressure which was fitted with a

Class II Laminar flow hood were all preparations took place which was cleaned before and after each use with 100% ethanol. Dedicated bench microfuge, a set of Eppendorf pipettes, sterile Eppendorf tips (Eppendorf, UK), sterile gloves and laboratory coats were all separate from the main lab. Approximately 3μl lysis buffer consisting of 2μl of 125μg/ml Proteinase K (PK) and 1μl of 17mM sodium dodecyl sulphate (SDS) was pipetted into each of the microcentrifuge tubes before adding the single buccal cells, single human blastomeres and cell clumps (El-Hashemite and Delhanty, 1997). Once the single cell was transferred into the lysis buffer, the mixture was covered with a drop of light mineral oil to prevent contamination and evaporation before closing the lid. The lysis buffer was activated at 37°C for 1h, and inactivated by incubating at 99°C for 15 min. After lysis, the DNA from the single cells or cell clumps was ready for PCR or storage at –80°C.

2.2.2.2.2 Single Buccal Cells

50μl of the concentrated cell suspension was transferred to a 5cm petridish in a laminar flow cabinet. Approximately ten 30μl drops of PBS containing 0.1% PVA were spotted onto the petridish. A drop of cell suspension (about 500μl) was transferred to an adjacent drop of PBS using a pulled glass micropipette, while visualising under a dissecting microscope. The transfer of the cells in different PBS/0.1% PVA drops was repeated 2-3 times to dilute the cell concentration until the isolation of a single cell was achieved. The single cells, once isolated, were transferred in and out of at least three fresh PBS drops to wash away any contaminants. Then the single cells were transferred into an individual thin-wall microcentrifuge tubes (0.2μl Eppendorf tubes, Eppendorf, UK) containing lysis buffer. A clump of 3-5 cells was taken as a positive control for each PCR. Also, 2μl of the last drop was taken as a blank (negative control) for each single cell.

2.2.2.2.3 Isolation and Tubing of Single Human Blastomeres

Blastomeres were used as part of the PGD procedure carried out in Chapter 3 in a clinical setting. Furthermore, surplus blastomeres were used for Chapters 5 and 3 for research purposes.

Human blastomeres were provided following informed patient consent, from day 3 or day 4 donated spare embryos from standard IVF/ICSI cycles. The blastomeres biopsied were selected based on good morphological appearance. The single blastomeres derived from biopsy were subsequently washed through several drops of PBS/0.1% PVA, to remove excess contaminants such as cumulus cells or sperm, before they were transferred into individual thin-wall microcentrifuge tubes (0.2μl Eppendorf tubes, Eppendorf, UK) containing 3μl PCR lysis buffer containing proteinase K (2.1.2.1.1). Each tube was checked under the microscope in order to visualise the cell within the tube. 2μl of the last wash drop was taken as a blank for each single blastomere. The lysed cells were stored at -70°C.

2.2.2.3 PCR Procedure

2.2.2.3.1 DOP-PCR

The procedure for the DOP-PCR was carried out according to Wells *et al* (1999) (Chapters 3 and 5). The DOP50 PCR mixture consisted of 0.2mM deoxynucleoside triphosphates (dNTP's: dATP, dTTP, dCTP and dGTP), 2μM DOP primer (Table 5.2) and 2 units of SuperTaq® (HT Biotechnology, UK) DNA polymerase with 10xSuperTaq buffer and was made up to a total volume of 50μl with nuclease-free, distilled, deionised sterile water (Promega, UK). The reaction mix was added to 200-300ng of genomic DNA or extracted DNA from single cells; one extra tube was taken as PCR-mix-only negative control for each reaction or blanks from each single cell. 25μl of lightweight mineral oil was added to prevent contamination and evaporation. The PCR was set up on ice, in a laminar flow cabinet using dedicated pipettes and sterile filtered tips.

The thermal cycling conditions were as follows: 94°C for 9 min, 30°C for 1 min, 72°C for 3 min (eight cycles); 94°C for 1 min, 62°C for 1 min and 72°C for 1.5 min (thirty five cycles); 72°C for 8 min.

2.2.2.3.2 Fluorescent PCR

Full details of the oligonucleotides used as primers for the fluorescent PCR can be seen in Table 2.2. The annealing (melting) temperature for the oligonucleotide primers was estimated by the following 'the-rule-of –thumb' calculation:

$$Tm = 2x(A+T) + 4x(G+C)$$

Where A, T, C and G are the nucleotides adenine, thymine, cytosine and guanine respectively. The working optimal annealing temperatures were determined empirically by experiments using the Mastercycler Gradient® thermal cycler. A temperature gradient of ±5°C from the calculated temperature across the block was carried out. The temperatures giving the most intense amplified products were considered as the optimal annealing temperature.

The PCR procedure was carried out as previously described by Piyamongkol *et al* (2001). The PCR mixture consisted of 0.2μM of each primer, 200μM deoxynucleoside triphosphates (dNTP's: dATP, dTTP, dCTP and dGTP) and 1 unit of SuperTaq® (Cambridge Technologies, UK) DNA polymerase with 1xSuperTaq buffer and was made up to a total volume of 25μl with nuclease-free, distilled, deionised sterile water (Promega, UK). The reaction was added to the genomic DNA or extracted DNA from single cells; one extra tube was taken as PCR-mix-only negative control for each reaction. 25μl of lightweight mineral oil was added to prevent contamination and evaporation. The PCR was set up on ice, in a laminar flow cabinet using dedicated pipettes and sterile filtered tips. For single cell PCR, 1.5 units of the polymerase enzyme were used in the reaction mixture. When AmpliTaq Gold™ polymerase with 1xGeneAmp® buffer were used instead of SuperTaq®, the primary denaturation step was set to 12 minutes to activate the enzyme. Table 2.5 reveals the conditions of the thermal cycler for all the cases.

Table 2.5 Conditions for the thermal cycler using different polymerase enzymes

Enzyme Polymerase / **PCR steps**	**SuperTaq® Plus**	**AmpliTaq Gold™**	**Number of cycles**
Primary denaturation	94°C 4min	94°C 12min	1
Denaturation	94°C 30sec	94°C 45sec	30 for genomic DNA 40 for single cell
Annealing	60°C 30sec	60°C 45sec	
Extension	72°C 45sec	72°C 1min	
Final Extension	72°C 10min	72°C 10min	1

2.2.2.4 PCR Analysis

2.2.2.4.1 Gel Electrophoresis

This type of analysis was only used during the early stages where it was required to test the DOP-PCR product as well as the Fluorescent-PCR (F-PCR) product to ensure amplification.

A 2% agarose gel containing 0.1 μl/ml ethidium bromide was prepared by mixing agarose in 1xTBE. The mixture was heated (approx 2min) gently in a microwave until the agarose dissolved completely. Ethidium bromide (5μl) was mixed into the agarose mixture and the mixture was poured into a mini-gel tank with an 8 or 16 well-forming-comb and left to set at room temperature (approx 20 min).

The comb was removed and the gel was immersed in 50ml of 1xTBE. Each PCR (8μl) product was mixed with a one tenth volume of the loading buffer and was loaded into each well. One Kb of ladder was used as a reference. Electrophoresis was performed for 30-60 min after which the gel was viewed under ultra-violet trans-illumination.

2.2.2.4.2 Automated laser DNA analyser (ABI Prism^{TM} 310 and 3100)

Fluorescent PCR products were separated and analysed using an automated laser DNA analyser (ABI Prism^{TM} 310 or 3100) and the appropriate software (GeneScan version 2.0.2; Applied Biosystems, UK). Each F-PCR product (1μl) was mixed with 12μl of deionised formamide and 0.5μl of the size standard (Genescan 500-TAMRA or Genescan 500-ROX, Applied Biosystem, UK). The mixture was run through a capillary (12kVolts, 26 min at 60°C) after 5 min denaturation at 95°C. Each PCR product was sized and distinguished illustrated as a peak using the GeneScan 2.0.2 version analysis software (Applied Biosystems, UK). Each peak represented the allele size of the product and the peak height the amount of the product.

2.2.2.4.3 Optimisation of PCR protocol

Each PCR protocol was optimised so the final product showed sharp, high peaks when analysed using the GeneScan 2.0.2 version analysis software (Applied Biosystems, UK). Efficiency of single cell PCR protocols was carried out by

calculating the number of single cells which showed amplification of at least one correct size allele (either homozygote or heterozygote samples). The allele dropout (ADO) rate was calculated only for heterozygote samples. The ADO rate was determined from the cells that did show amplification. e.g. if 9/10 cells showed amplification, then the ADO rate would be calculated from the 9 cells.

2.2.2.5 PGD

2.2.2.5.1 PGD Consultation

Patients were referred to the UCL centre for PGD. In most cases referral was from clinical genetics departments and the patients had already had genetic counselling. Prior to commencing treatment all patients were fully informed regarding the limitations of PGD. The requirement for IVF treatment, the risk of misdiagnosis, the problems caused by mosaicism, the expected implantation and pregnancy rates, were all outlined during two thorough IVF/PGD consultations. Before commencement of treatment, patients had full gynaecological investigations and the male partners sperm was assessed and a specific single cell diagnoses was developed for each couple.

2.2.2.5.2 IVF Treatment and Manipulation of Embryos.

The patients underwent routine IVF procedures as described previously (Ranieri *et al,* 2001). Following ovarian stimulation follicles were aspirated and fertilisation was evaluated 24h after insemination. Oocytes and embryos were cultured in IVF medium (Cook, Australia).

2.2.2.5.3 Embryo Biopsy (UCL)

PGD was performed for two patients 'G' and 'H'. On day 3 embryos were biopsied by the senior embryologist in Ca^{2+} Mg^{2+} -free embryo biopsy medium (Medicult, UK), using Research Instrument micromanipulators. Zona drilling was performed using acid Tyrode's solution as described previously (Piyamongkol *et al,* 2001a). One or two blastomeres were aspirated according to the developmental stage and morphology of the embryos. In general two cells were biopsied from embryos consisting of 6 or more cells. Biopsied cells were washed and tubed for analysis (section 2.2.2.2.3). The untransferred (spare) embryos were donated for research with the patient consent for

confirmatory diagnosis. All the cells from the spare embryos were biopsied and were tubed for molecular analysis (section 2.2.2.2.3).

2.2.2.5.4 Embryo Biopsy (LFC)

The embryo biopsy method carried out for this study was different to that used in chapter 3 carried out in the UCL Assisted Conception Unit (section 2.2.2.5.3). The embryos were incubated for a 1-2 minutes in Ca^{+2}/Mg^{+2}-free biopsy medium. The zona pellucida surrounding the embryo was drilled with a laser system as described by Veiga *et al* (1997). 1-2 cells were removed gently from the embryo and the embryo was subsequently washed in IVF medium. The embryos were then placed in blastocyst medium (6.2 Vitrolife, Scandinavia) from day 3-5.

2.2.2.6 PGD Strategy for DM Using F-PCR

The diagnosis of a normal embryo was based on the observation of two normal DM alleles, one from each parent. The mutant DM expanded allele cannot be detected since fragment sizes above 500bp are refractory to PCR, hence an affected embryo would only show one normal allele. Different polymorphic markers (STR's) linked and unlinked, were tried for each family to test whether they were informative (Table 5.2). A linked marker, such as the D19S112 which is located approximately 0.3cM upstream to the DMPK, would provide further information about the genetic state of the embryo i.e. if the embryo carries the mutation or not. An unlinked marker, such as the D21S1414, would only allow the detection of contamination (Piyamongkol *et al*, 2001a).

2.2.2.6.1 Genomic DNA Workup

Each family was tested for a variety of STR's from genomic DNA extracted from their blood (section 2.2.2.1), which were classed as informative depending on their allele size. For example, a tetranucleotide STR would be rendered informative if all four alleles from the two parents were of different size and at least one base pair apart from each other i.e. 120/130 for the mother and 126/142 for the father. If a family was informative for a linked marker, another affected member was asked to produce a DNA sample in order to obtain the 'phase'. Table 2.2 summarises the different STR's tested for all the couples' extracted genomic DNA (see Tables 3.1-3.10 for results)

2.2.2.6.2 Single Cell Workup

If a couple had at least one informative linked or unlinked polymorphic marker including the DM mutation marker, testing on single cells was initiated for each couple individually. Fifty single buccal cells along with 10-20 clumps of buccal cells were tested for each multliplex reaction on buccal cells from a normal heterozygous individual to test the protocol at the single cell level.

The protocol for family G was the multiplex reaction of DM+D21S1414 (Protocol 1), whereas for family H was the multiplex reaction of DM+D19S112 (Protocol 2). Subsequently, both protocols were performed on at least 100 single cells isolated from each partner of each family as well as spare single human blastomeres for assessing the amplification efficiency and ADO rates. The ADO rates were calculated from the number of single cells that were amplified, when only one allele was visible as a peak.

For the optimal protocol in both cases, the PCR mixture consisted of 0.1μM of the DM primer and 2.5μM for the D21S1414 primer (Protocol 1) and 3.0μM for the D19S112 primer (Protocol 2), 200μM dNTP's, 10xGeneAmp® Buffer and 1.5U AmpliTaq Gold™ and was made up to a final volume of 25μl with double-distilled, nuclease-free, de-ionised water. The amplifications were performed with the conditions 94°C, 45sec (96°C for the first ten cycles), annealing at 60°C, 45sec for family G (Protocol 1) and 61°C 45sec for family H (Protocol 2) and extension of 72°C, 1min for 42 cycles. These were preceded by denaturation at 94°C for 12 min to activate the AmpliTaq Gold™ enzyme.

Each marker was analysed on the 3100ABI Prism™ as wells as backup analysis on the 310 ABI Prism™. Since each marker was fluorescently labelled differently (see Table 5.2), analysis was possible in different dyes.

2.2.2.6.3 Clinical DM PGD Cases

Following ICSI treatment, one PGD cycle for each family (G and H) was performed. The single biopsied blastomeres were analysed using the optimal PGD protocols 1 and 2. Normal embryos (if any) were chosen for transfer on day 4 post-fertilisation. All the cells from the untransferred embryos were biopsied and analysed using the same protocols for each family for confirmatory results.

2.2.2.7 PGD Strategy for DM Using WGA

A universal protocol for PGD for DM was attempted using DOP-PCR as the method of whole genome amplification and then subsequent amplification with five different markers. The markers that were selected were DM (mutation marker), D19S112 (linked polymorphic marker) as well as D13S305, D18S535 and D21S1414 (unlinked polymorphic markers) (Table 5.2). These unlinked polymorphic markers were chosen as they were on different chromosomes in order to observe the coverage of the genome when amplified using DOP-PCR.

2.2.2.7.1 Procedure on Genomic DNA

The DOP-PCR procedure was carried out as described by Wells *et al* (1999) (section 2.2.2.3.1) initially on genomic DNA. The F-PCR procedure was carried out as previously described by Piyamongkol *et al* (2001) (section 2.2.2.3.2). However, not all the primers produced efficient yields when analysed on the automated laser DNA analyser (ABI Prism™ 3100). Therefore, the F-PCR protocols were adjusted, at the genomic DNA level, in order to generate sufficient quantities of PCR product when carried out at the single cell level. Several annealing temperatures were tested using a Gradient Thermal Cycler as well as different primer concentrations, to produce an efficient PCR protocol. The final annealing temperatures and concentrations of the primers can be seen in Table 2.6.

Table 2.6. Annealing temperatures and primer concentrations for the singleplex reactions for each polymorphic marker

Type of Marker	Marker	**Annealing Temperature (°C)**	**Primer Concentration**
STR (Dinucleotide)	DM	60 °C	0.1 µM
STR (Dinucleotide)	D19S112	61 °C	0.6 µM
STR (Tetranucleotide)	D21S1414	62 °C	1.0 µM
STR (Tetranucleotide)	D18S535	60 °C	0.6 µM
STR (Tetranucleotide)	D13S305	56 °C	0.8 µM

2.2.2.7.2 Procedure on Single Cells

The single cell DOP-PCR procedure was optimised during the CGH study (section 4.2.6). Once the singleplex F-PCR protocols were optimised on single cells, two individuals were chosen ('X' and 'Z') from which ten single cells and two clumps from each were amplified using DOP-PCR. Subsequently, a 1.0µl aliquot from each single cell DOP-PCR product was used as a template for each singleplex reaction with each of the markers (Table 5.2). The final product was run on the 3100ABI Prism™ for analysis. Genomic DNA from each individual was amplified to act as positive control.

2.2.2.7.3 CGH on DOP-PCR Amplified Single Cells

From a 50µl DOP-PCR reaction, only 10µl were used. The rest of the DOP-PCR product was utilised for CGH analysis. The full CGH protocol can be seen in section 2.2.3.4, which was performed as described by Wells *et al* (2002). The optimisation of the CGH protocol was performed as part of the study in Chapter 5.

2.2.3 CGH Methods

CGH was used for the study summarised in Chapter 5 (as well as Chapter 3). Some sections of the CGH Methods are identical to PCR; hence similar methods will be referred to the appropriate sections.

2.2.3.1 DNA Extraction from Frozen Fibroblasts

Several cell pellets (trisomy 13, 18, 21, 22, XXX and normal) were subjected to DNA extraction to be subsequently used as positive controls for preliminary studies to assess efficiency of the genomic CGH protocol. Cell suspensions were centrifuged at 1000rpm for 5 minutes, and the supernatant containing glycerol was removed. The cells were washed with Hank's medium (Sigma, UK) and centrifuged at 6,000rpm for 5 minutes. The supernatant was removed and 2.5ml of DNA extraction lysis buffer (section 2.1.3.1.2) warmed at 37°C was added and the cells were left at 37°C for 30 minutes. An equal volume of isopropanol was added in order to precipitate the DNA which was "hooked out" and dissolved in water.

For DNA Extraction from Whole Blood refer to section 2.2.2.1.

2.2.3.2 Single Cell isolation

Refer to section 2.2.2.2

2.2.3.3 DOP-PCR

Refer to previous section 2.2.2.3.1

2.2.3.4 CGH Procedure

2.2.3.4.1 Labelling of Probe DNA

The DNA product of DOP-50 was ethanol precipitated, redissolved in water and labelled by nick translation. The 50µl reaction contained 5µl of 10X reaction buffer mix, 10µl 0.1mM dNTP Mix, 5µl 0.1 mM dTTP mix, 2.5µl of 0.2mM Hapten/fluorochrome-dUTP, 10µl DNA Polymerase (10U/µl) and 17.5 µl of Nuclease-free water (Promega, UK). The time for the nick translation was between 60-120 minutes at 15°C. The reaction was stopped with a 10 minute incubation at 72°C

2.2.3.4.2 Preparation of Labelled DNA

A 1% agarose gel was run to check the fragment sizes (see section 2.2.2.4.1). Optimal sizes for CGH were estimated between 300-2000bp. The reference DNA was labelled with Spectrum Red (Vysis, UK) and the test DNA was labelled with spectrum Green (Vysis, UK). The appropriate DNA to be hybridised against the single blastomere was a group of 3 buccal cells that had undergone the same treatment as the single blastomeres and were amplified during the same DOP50-PCR experiment. Labelled reference and test DNA were mixed with 30µg Cot-1 DNA and ethanol precipitated and washed with 70% ethanol and air-dried. The pellet was resuspended in 6µl Hybridisation mix (50% formamide; 2xSSC; 10% dextran dulphate) and dissolved by 20 minutes incubation at 37°C. The probe was denatured at 75°C for 10 minutes and cooled at 37°C by incubation for 2 minutes at room temperature in the dark before being applied to the denatured normal chromosome spreads as described below.

2.2.3.4.3 Denaturation of Metaphase Chromosome Spreads and Probe Hybridisation

Denaturation of male lymphocyte slides was performed by a 5 minute incubation of the slides in a coplin jar with denaturation solution (70% formamide; 2xSSC pH 7.5) pre-warmed at 73°C. Immediately after denaturation the slides were put through an ice-cold (chilled at -20°C) ethanol series (70%, 85%, 100%) and dried. The probe was applied to the slide and covered with a coverslip sealed with rubber cement and hybridised in a moist chamber at 37°C for 72 hours.

2.2.3.4.4 Post-Hybridisation Washing

The post hybridisation washes consisted of 5 minutes in 2x SSC at 72°C, 5 minutes in 4xSSC at 37°C, 5 minutes in 4xSSC + 0.1% Triton-X (Sigma, UK) at 37°C, 5 minutes in 4xSSC at 37°C, and 5 minutes in 2xSSC at room temperature followed by dipping of the slides in double-distilled water. The slides were put through an ethanol series 3 minutes each, air-dried and mounted in anti-fade medium (Vector Labs, Peterborough, UK) containing diamidinophenylindole (DAPI) to counterstain the chromosomes and nuclei.

2.2.3.4.5 Microscopy and Image Analysis

Metaphase chromosome preparations were captured using a Zeiss Axioscope microscope equipped with a Photometrics KAFF 1400 cooled CCD camera, and SmartCapture software (Vysis Richmond, UK). Image analysis was performed using Vysis Quips CGH software. Green:Red fluorescence ratios of >1.2:1 indicated gain of genetic material, while ratios of <0.8:1 was indicative of deletions.

2.2.3.5 CGH Procedure for Chapter 5

This study was performed on frozen embryos which were subsequently thawed (frozen-thawed embryos) generated from patients undergoing IVF and ICSI at the London Fertility Centre. These embryos were suitable for freezing, hence were considered good quality embryos when assessed morphologically. All embryos were donated with written consent from patients and the study was licensed by the Human Fertilisation and Embryology Authority.

Quality control was performed for every step of this study. Prior to commencing this study a preliminary study was performed to ensure the optimal method for analysing single blastomeres by CGH. Single cell isolation of 100 buccal cells was carried out. Subsequent tubing of the buccal cells was followed by DOP–PCR. The final amplified product was run on a 2% agarose gel electrophoresis carried out at 50V for 30 minutes after which gels were viewed via ultra-violet trans-illumination to assess the efficiency of the tubing technique.

2.2.3.5.1 WGA Protocol

DOP-PCR was employed to amplify the whole genome of the cell uniformly as described by Wells *et al* (2002) using the Eppendorf PCR machine (See section 2.2.3.3)

2.2.3.5.2 Optimisation of WGA Protocol

For the WGA technique, namely DOP-PCR, gel electrophoresis provided the only means of assessing whether the PCR conditions were optimal. A non-intense smear should be visible for each sample; genomic DNA, clump of buccal cells or single cell. Furthermore, in each experiment a negative control was added for the DOP-PCR amplification to assess each reaction for contamination. All negative controls were tested by gel electrophoresis and by carrying out CGH experiments where the negative controls acted as *'test'* samples.

Two different thermal cyclers were tested: the Eppendorf and the Hybaid Omnigene (see section 2.1.5.2) in order to examine which provided the best amplification results. The DOP-PCR products were analysed on a 2% agarose gel

2.2.3.5.3 CGH Protocol

The full CGH protocol is detailed in section 2.2.3.4, which was performed as described by Wells *et al* (2002). Each Nick-Translation kit (Vysis, UK) was tested before use, since each aliquot of the Nick-translation enzyme (contained in each kit) behaved differently and required slightly different times of incubation. Hence, a trial Nick-Translation CGH experiment was set up after purchase of a new kit, and it was assessed by carrying out DOP on single buccal cells, labelling the sample and testing the fragment sizes by gel electrophoresis. The optimal sizes for CGH were estimated to be between 300-2000bp (Wells *et al,* 1999).

Six to twelve metaphases were captured using a Zeiss Axioscope microscope equipped with a Photometrics KAFF 1400 CCD camera. Each metaphase was karyotyped and assessed using Vysis Quips CGH software.

2.2.3.5.4 Optimisation of CGH Protocol

The optimisation of the CGH protocol was achieved by testing initially with high quality genomic DNA, which had been extracted 'in house', from 'healthy' individuals. Furthermore, genomic DNA from frozen fibroblast cell lines (cultured in-house) with a defined chromosomal abnormality such as trisomy 13, trisomy 18, trisomy 21, trisomy 22 and triploidy 69 XXX was employed as a positive control in assessing genomic CGH protocol efficiency. Single cell isolation was performed for buccal cells and fibroblasts to provide practice, and the positive controls in assessing single cell CGH protocol accuracy. A set of embryos were thawed with patient consent and were biopsied in order to establish the biopsy technique as well as the CGH technique applicable on single blastomeres. This set of embryos were used as a learning curve for both biopsy practitioner and the author.

2.2.3.5.5 FISH Protocol

The FISH protocol was carried out as described by Harper *et al* (1994). However, some of the steps were altered in order to obtain high efficiency in all the sequential rounds of FISH. All day 5 embryos were subjected to two sequential rounds of FISH (section 2.2.1.5). The first round of FISH was always carried out using the probe cocktail for chromosomes X, Y and 18, which acted partly as a control for the CGH experiment since the sex of the biopsied cells should match the sex of the rest of the

embryo (unless mosaic). The second round of FISH was a combination of 9cep/16cep/22LSI if the CGH showed euploid results for the biopsied blastomeres, or a combination of probes matching the abnormalities observed by CGH in as many chromosomes as possible. Hence, there were a variety of FISH protocols carried out for this study. The different combinations can be viewed in Table 2.7.

Table 2.7. FISH probe combinations and stringency conditions.

Chromosomes	Probe Combination	Stringency Conditions		
		Probe Vol (μl)	Denaturation	P-H[a] washes
X, Y, 18	Xcep/Ycep/18cep	2μl[b]	Co[c], 75° for 5min	60% FA
9, 16, 22	9cep/16cep/22LSI	0.5μl/0.5μl/0.7μl	Sep[d], 75° for 5min	Vysis[e]
3, 11, 13	3cep/11cep/13LSI	0.5μl/0.5μl/0.7μl	Sep, 75° for 5min	Vysis[e]
10, 14	10cep/14q	0.5μl/0.6μl	Co, 75° for 5min	60% FA
1, 16	1p/1q/16cep	0.6μl/0.5μl/0.5μl	Sep, 75° for 5min	50% FA
3, 6, 18	3cep/6cep/18q	0.5μl/0.5μl/0.6μl	Sep, 73° for 5min	60% FA
Y, 4	Ycep/4cep	0.5μl/0.5μl	Co, 75° for 5min	60% FA
X, Y, 16	Xcep/Ycep/16q	0.6μl/0.5μl/0.6μl	Co, 75° for 3min	50% FA

[a]Post-Hybridisation washes
[b]This was a probe cocktail, where 2μl from the cocktail were added into the probe mixture
[c] Co-denaturation
[d]Separate Denaturation (section 2.2.1.5.3)
[e]The post-hybridisation washes termed *'long washes'* proposed by Vysis (UK) (section 2.2.1.5.3)

For probe information refer to section 2.1.5.1 in Table 2.1. Most of the probe combinations required co-denaturation with the slide. However, there were also probe mixtures where diffused signals and cross-hybridisation was observed, therefore separate denaturation of the probes and the slide was performed (see section 2.2.1.5.3).

2.2.3.5.6 Optimisation of FISH Protocol

During each FISH experiment on the embryos a control slide of normal male lymphocytes was also included to assess the efficiency of the FISH protocol. 50-100 interphase nuclei were scored and the efficiency of each probe combination was examined.

2.2.3.6 Patient Details for Chapter 3

A total of ten couples were initially referred to our centre for PGD of DM. PGD was performed for two couples: G and H (Table 2.8). The rest are awaiting PGD for various reasons.

Table 2.8. Patient details for couples undergoing PGD for DM

Family	Patient with DM expansion	Maternal Age	Reproductive History
A	Mother	42	6 pregnancies, 2 affected sons, 4 spontaneous miscarriages
B	Mother	39	Infertile (Gave birth to healthy female after PGD)
C	Father	38	2 pregnancies, 1 normal birth, 1 affected (TOP)
D	Mother	40	4 pregnancies (x2 with IVF), 3 TOP, 1 affected son, 1 PGD cycle where all were affected
E	Mother	36	1 pregnancy, 1 affected female which died at weeks old
F	Mother	39	4 pregnancies, 1 normal son, 3 TOP
G	Mother	36	1 affected son 4 affected natural pregnancies
H	Mother	41	4 pregnancies, 4 TOP's
I	Mother	40	2 pregnancies, 1 affected son, 1 TOP
J	Father	36	3 pregnancies, 1 normal female, 1 affected male, 1TOP

2.2.3.7 Patient Details for Chapter 4

All patients were healthy individuals that were referred to the London Fertility Centre for fertility problems. Patients were either IVF or ICSI patients. Table 2.9 reveals the background information of all the patients and their embryos. All patients gave consent for their embryos to be used for research. The first three patients' embryos (n=10) were used for preliminary optimisation of the protocol (data not shown).

Table 2.9. Patient details for Chapter 4

Patient No.	Age of patient	Date of treatment	IVF or ICSI	No. of eggs collected	No. of embryos (no. of nuclei)	Embryo Type
4	40	5/5/02	ICSI	8	2 (4)	Arrested
5	35	17/5/02	IVF	20	5 (65)	Arrested
6	47 (35-O.D.)	17/5/02	ODOR/ IVF	13	1 (22)	Arrested
7	36 (33-O.D.)	28/6/02	ODOR/ ICSI	17	2 (35)	Arrested
8	41	05/07/02	ICSI	9	1 (6)	Arrested
9	27	05/07/02	IVF	11	1 (8)	Arrested
10	41	08/07/02	ICSI	12	1 (5)	Arrested
11	34	09/07/02	IVF	37	1 (1)	Lost
12	35	09/07/02	IVF	34	2 (31)	Arrested
13	37	30/08/02	ICSI	12	3 (110)	Blastocysts
14	19	30/08/02	IVF	13	3 (90)	Blastocysts
15	26	03/10/02	ICSI	19	4 (236)	Expanded Blastocysts
16	33	04/10/02	ICSI	19	1 (58)	Expanded Blastocyst
17	31	09/01/03	IVF	11	2 (58)	Arrested
18	38	21/01/03	IVF	7	1 (55)	Hatched Blastocyst
19	30	31/01/03	ICSI	14	2 (175)	Expanded Blastocysts
20	32	03/02/03	ICSI	16	1 (43)	Expanded Blastocyst
21	39	04/02/03	IVF	8	2 (57)	Arrested
22	39	25/03/03	IVF	21	2 (143)	Expanded Blastocysts
23	49 (20-O.D.)	28/03/03	ODOR/ IVF	23	1 (100)	Hatched Blastocyst
24	41	04/04/03	ICSI	6	1 (63)	Expanded Blastocyst
25	36	07/04/03	IVF	11	2 (94)	Expanded Blastocysts
26	34	14/05/03	IVF	9	2 (61)	Expanded Blastocysts

27	46 (33-O.D.)	04/06/03	ODOR/ IVF	11	2 (59)	Arrested

IVF = In vitro fertilisation, ICSI = Intracytoplasmic sperm injection, ODOR = oocyte donation cycle, O.D. = oocyte donor

2.2.3.8 Patient Details for Chapter 5

All patients were healthy individuals that were referred to the London Fertility Centre for fertility problems. Patients were either IVF or ICSI patients. Table 2.10 below reveals the background information of all the frozen embryos. All patients gave consent for their embryos to be thawed and used for research.

Table 2.10. Patient details regarding their IVF/ICSI cycle and their embryo details

Patient No.	**Age of patient**	**Date of Egg Collection**	**IVF or ICSI**	**No. of eggs collected**	**Frozen embryos donated for research**	**Embryo Type (time of thaw) (Grade)** 1	2	3	4
1	38	19/03/03	IVF	21	3	7c (2-3)	6c (2-3)	6c (1-2)	
2	29	20/04/03	ICSI	30	3	7c (3)	7c (2)	5c (2)	
3	35	05/02/97	IVF	16	1	6c (2)			
4	35	23/02/99	ICSI	16	4	5c (3)	8c (2)	7c (2-3)	6c (2-3)
6	30	18/05/02	IVF	18	3	5c (2-3)	8c (1-2)	9c (1-2)	
7	39	06/09/01	IVF	14	3	5c (2)	10c (1-2)	7c (2)	
8	31	02/03/03	IVF	19	2	6c (2-3)	5c (2)		
9	33 (OD)	14/05/02	IVF	22	4	6c (2-3)	8c (1-2)	5c (3)	6c (2-3)
9	33 (OD)	14/05/02	IVF	22	4	5c (2-3)	6c (2)	7c (2-3)	8c 1-2)
10	32	23/02/01	IVF	12	1	6c (1-2)			
11	35	7/10/02	IVF	15	1	5c (2)			
12	30	19/03/99	IVF	14	3	5c (2)	8c (1-2)	7c (2-3)	
13	36	12/10/00	ICSI	24	3	5c (1-2)	8c (1-2)	7c (1-2)	
14	28 (OD)	12/06/02	IVF	18	3	6c (1)	6c (1)	5c (1-2)	

IVF = In vitro fertilisation, ICSI = Intracytoplasmic sperm injection, OD = oocyte donor, OR = oocyte recipient

CHAPTER 3

Novel approaches for the preimplantation genetic diagnosis of Myotonic Dystrophy (DM1)

3.1 Aims

There were two parts to this study. The first was to develop a novel protocol for PGD for DM1 for a number of patients using a single, multiplex PCR and the second was to determine if WGA followed by a number of singleplex PCRs could be used for PGD of DM1 with the potential to also carry out CGH.

PGD for DM1 requires inclusion of highly polymorphic markers, the primary function of which is to confirm the affected embryos and reveal when contamination has occurred. Several polymorphic markers, linked and unlinked, were tested for a variety of families. Two couples underwent PGD for DM1 employing two different protocols. The first protocol included the mutation marker and a contamination marker whereas the second novel protocol included the mutation marker and a linked marker which was able to provide information regarding the mutation as well as presence of contamination.

Due to the complexity of developing specific protocols depending on the patients being informative for different markers and optimising the multiplex PCR, the second part of the project was attempted. A whole genome amplification method, namely DOP-PCR, was employed initially on genomic DNA and subsequently on single cells to investigate whether specific regions of the genome were amplified. Five different markers were tested to see if they were adequately amplified, a mutation marker (DM1), a linked marker (D19S112) and three highly polymorphic markers on different chromosomes (D21S1414, D18S535 and D13S305). The combination of WGA and F-PCR would provide a universal-like protocol able to tackle the time-consuming predicament of devising patient-specific single cell multiplex protocols, since after DOP-PCR of a single cell, sufficient amounts of DNA would be available for many singleplex reactions. Furthermore, the ability to carry out other assays from one single cell such as CGH could be performed.

3.2 Results

3.2.1 Preliminary Work-up of Families

Each family was referred to our centre (UCL Centre for Preimplantation Genetic Diagnosis) where bloods were taken from each prospective parent or DNA aliquots were sent from cytogenenetic laboratories where original investigations were carried out. The extracted DNA (section 2.2.2.1) from both partners was tested with a variety of markers to determine for which markers they are informative in order to devise a multiplex F-PCR protocol (Tables 3.1-3.10). If a linked marker was informative for each patient (i.e. parents share no allele in common), if possible, an affected family member was asked to provide a DNA sample in order to obtain the 'phase' (Underlined allele sizes in Tables 3.1-3.10). Although for some families the same F-PCR protocols can be devised and applied e.g. the DM/APOC2 duplex protocol for families B, E, F and I; each family was informative for different combinations of markers. Furthermore, there were some families where the DM1 primers were semi-informative since they share one allele (B, E, F and I), hence the diagnosis could only be carried out by multiplexing with a linked marker (Tables 3.2, 3.5, 3.6 and 3.9). For family C although there were four informative markers (APOC2, D19S112, D21S1414 and D21S11) because their allele sizes were very close, stutter peaks can create problems during diagnosis (Table 3.3).

Table 3.1. List of polymorphic markers tested on genomic DNA for family A and their allele sizes

Primer	Marker	Types	Maternal allele sizes	Paternal allele sizes	Comments
			Am	***Af***	
DM1			*- /146*	122 (hm)	Informative
APOC2	Linked	di-	*149/151*	126/149	Not informative
D19S207	Linked	di-	*145/145*	140/143	Not informative
D19S112	Linked	di-	*130/132*	130/136	Not informative
D19S393	Linked	Tetra-	*285/285*	285/ 285	Not informative
D21S1414	Unlinked	tetra-	*349/349*	345/349	Not informative
D21S11	Unlinked	tetra-	*227/227)*	223/227	Not informative
D18S535	Unlinked	tetra-	*485/485*	481/485	Not informative

Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.2. List of polymorphic markers tested on genomic DNA for family B and their allele sizes

Primer	Marker	Types	Maternal allele sizes	Paternal allele sizes	Mother's father allele sizes	Comments
			Bm	***Bf***	***Bfm***	
DM1			*120/-*	120/138	*170/-*	Semi-informative
APOC2	Linked	di-	*126/153*	142/157	*150/153*	Informative
D19S207	Linked	di-	*125/141*	144/144	*125/125*	Informative[&]
D19S112	Linked	di-	*128/130*	116/123	*128/130*	Informative
D19S393	Linked	tetra-	*285/296*	296/296	*289/296*	Not informative
D21S1414	Unlinked	tetra-	*343/348*	339/343	*335/348*	Not informative
D21S11	Unlinked	tetra-	*223/228*	223/229	*225/227*	Not informative
D18S535	Unlinked	tetra-	*478/486*	478/482	*477/490*	Not informative

[&]Practically difficult since close allele sizes would cause difficulties in interpretation due to stutter peaks
Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.3. List of polymorphic markers tested on genomic DNA for family C and their allele sizes

Primer	Marker	Types	Paternal allele sizes	Maternal allele sizes	Comments
			Cf	**Cm**	
DM1			*146/-*	140/149	Informative
APOC2	Linked	di-	*134/153*	149/155	Informative[&]
D19S207	Linked	di-	*141/145*	144/144	Not informative
D19S112	Linked	di-	*130/132*	124/134	Informative[&]
D21S1414	Unlinked	tetra-	*337/355*	341/349	Informative
D21S11	Unlinked	tetra-	*215/233*	219/237	Informative
D18S535	Unlinked	tetra-	*485/485)*	477/482	Not informative

[&]Practically difficult since close allele sizes would cause difficulties in interpretation due to stutter peaks
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.4. List of polymorphic markers tested on genomic DNA for family D and their allele sizes

Primer	Marker	Types	Maternal allele sizes	Paternal allele sizes	Mother's sister allele sizes	Comments
			Dm	**Df**	***Dms***	
DM1			*139/-*	146/184	*139/-*	Informative
APOC2	Linked	di-	*128/150*	128/152	*135/150*	Not informative
D19S207	Linked	di-	*125/143*	142/142	*125/143*	Not informative
D19S112	Linked	di-	*123/128*	128/132	*124/128*	Not informative
D19S393	Linked	tetra-	*281/289*	285/289	*285/289*	Not informative
D21S1414	Unlinked	tetra-	*337/337*	343/348	*338/361*	Not informative
D18S535	Unlinked	tetra-	*482/486*	478/490	*486/490*	Informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.5. List of polymorphic markers tested on genomic DNA for family E and their allele sizes

Primer	Marker	Type	Maternal allele sizes	Paternal allele sizes	Mother's brother allele sizes	Comments
			Em	**Ef**	***Emb***	
DM1			*122/-*	122/149	*122/-*	Semi-Informative
APOC2	Linked	di-	*142/156*	126/152	*152/156*	Informative
D19S207	Linked	di-	*125/143*	142 (hm)	*127/143*	Not Informative
D19S112	Linked	di-	*132/136*	130/136	*128/132*	Not Informative
D19S393	Linked	tetra-	*289/297*	290/298	-	Not Informative
D21S1414	Unlinked	tetra-	344/*348*	344/360	344/*369*	Not Informative
D21S11	Unlinked	tetra-	*224/228*	224/240	*224/228*	Not Informative
D18S535	Unlinked	tetra-	*466/478*	482/486	*478/490*	Informative
D13S305	Unlinked	Tetra-	*452/452*	446/456	-	Not Informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.6. List of polymorphic markers tested on genomic DNA for family F and their allele sizes

Primer	Marker	Type	Maternal allele sizes	Paternal allele sizes	Comments
			Fm	**Ff**	
DM11			*139/-*	122/139	Semi-Informative
APOC2	Linked	di-	148/*158*	150/152	Informative
D19S112	Linked	di-	*129/129*	127/127	Not Informative
D19S207	Linked	di-	127/*144*	123/141	Informative
D19S393	Linked	tetra-	*289/293*	293/301	Not Informative
D21S1414	Unlinked	tetra-	*349/363*	345/351	Informative
D21S11	Unlinked	tetra-	*227/242*	223/230	Informative
D18S535	Unlinked	tetra-	*466/486*	489/489	Not Informative
D13S305	Unlinked	tetra-	*443/455*	443/455	Not Informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
\- indicates the affected allele that is refractory to PCR

Table 3.7. List of polymorphic markers tested on genomic DNA for family G and their allele sizes

Primer	Marker	Types	Maternal allele sizes	Paternal allele sizes	Affected son allele sizes	Comments
			Gm	**Gf**	***Gas***	
DM11			*142/-*	122/122	*122/-*	Informative
APOC2	Linked	di-	*152/<u>154</u>*	135/150	*150/<u>154</u>*	Informative[&]
D19S207	Linked	di-	*<u>142</u>/142*	141/141	*<u>142</u>/142*	Not informative
D19S112	Linked	di-	*128/130*	128/130	*128/130*	Not informative
D21S1414	Unlinked	tetra-	*346/355*	335/350	*350/354*	Informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
[&]Practically difficult since close allele sizes would cause difficulties in interpretation due to stutter peaks
\- indicates the affected allele that is refractory to PCR

Table 3.8. List of polymorphic markers tested on genomic DNA for family H and their allele sizes

Primer	**Marker**	**Types**	**Maternal allele sizes**	**Paternal allele sizes**	**Mother's sister allele sizes**	**Comments**
			Hm	**Hf**	***Hms***	
DM1			*146/ -*	122/143	*- /146*	Informative
APOC2	Linked	di-	*155/155*	149/155	*132/155*	Not informative
D19S207	Linked	di-	*127/142*	144/146	*127/142*	Not informative
D19S112	Linked	di-	*130/132*	117/117	*130/134*	Informative
D21S1414	Unlinked	tetra-	*359/359*	345/349	*340/355*	Not informative
D21S11	Unlinked	tetra-	244/249	259/259	-	Not Informative
D18S535	Unlinked	tetra-	478/482	481/481	-	Not informative
D13S305	Unlinked	tetra-	440/448	438/448	-	Not informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.9. List of polymorphic markers tested on genomic DNA for family I and their allele sizes

Primer	**Marker**	**Type**	**Maternal allele sizes**	**Paternal allele sizes**	**Mother's sister allele sizes**	**Comments**
			Im	**If**	***Imf***	
DM1			*122/-*	122/145	*122/-*	Semi-informative
APOC2	Linked	di-	*136/150*	127/147	*136/150*	Informative
D19S207	Linked	di-	*144/146*	141/145	*142/144*	Not informative
D19S393	Linked	tetra-	*285/297*	289/293	-	Informative
D19S112	Linked	di-	*117/128*	117/134	*128/132*	Not informative
D21S1414	Unlinked	tetra-	*359/359*	345/349	*359/359*	Not informative
D21S11	Unlinked	tetra-	*238/359*	224/228	*238/359*	Not informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
- indicates the affected allele that is refractory to PCR

Table 3.10. List of polymorphic markers tested on genomic DNA for family J and their allele sizes

Primer	Marker	Type	Paternal allele sizes	Maternal allele sizes	Affected fetus allele sizes	Comments
			Jf	Jm	*Jaf*	
DM1			*141/-*	154/177	*-/178*	Informative
APOC2	Linked	di-	*152/154*	126/143	*143/152*	Informative
D19S207	Linked	di-	*142/142*	125/143	*125/143*	Not informative
D19S112	Linked	di-	*128/130*	126/128	*128/130*	Not informative
D19S393	Linked	tetra-	*293/293*	289/293	-	Not informative
D21S1414	Unlinked	tetra-	*335/352*	339/347	*335/339*	Informative
D21S11	Unlinked	tetra-	*220/227*	216/232	*216/227*	Informative
D18S535	Unlinked	tetra-	*481/481*	490/490	*481/490*	Not informative

Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles would be classed as affected
Patient in *italics* indicates the affected partner
&Practically difficult since close allele sizes would cause difficulties in interpretation due to stutter peaks
- indicates the affected allele that is refractory to PCR

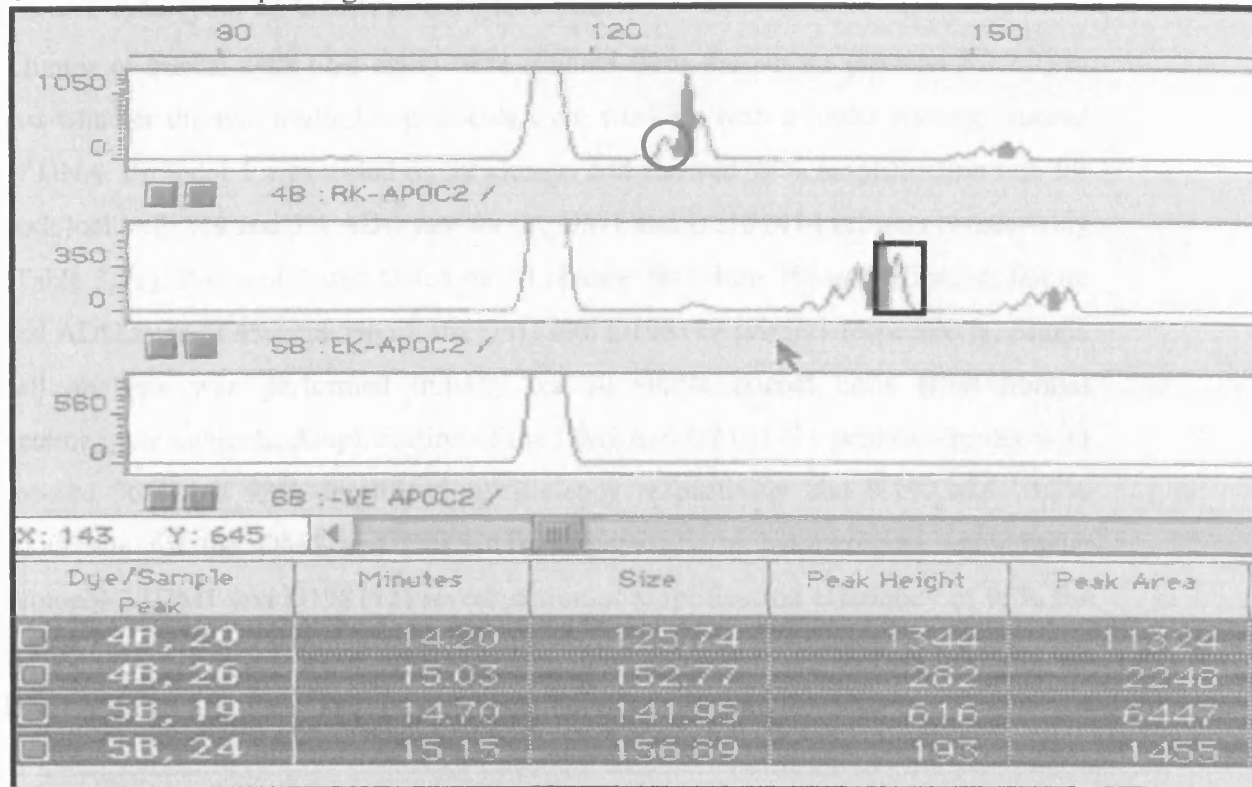
3.2.2 PGD Workup

Workup for a total of ten families was performed during this study (Tables 3.1-3.10). PGD was carried out for two families only (families G and H). For family A apart for the DM1 marker, no other marker was found to be informative hence further investigations of different markers should be carried out. For families B, E, F and I the DM1 mutation marker was found to be 'Semi-informative' and at least two other markers, linked or unlinked, were informative for each couple. The DM1 marker was termed 'Semi-informative' when the affected partner's allele that could be sized was the same as one of the alleles from the other e.g. for family B, for '*Bm*' it was (120/-), which was the same for 'Bf' (120/138). The DM marker could be semi-informative in the case where the embryo would show the 120/138 genotype, meaning that the 120 was inherited from the mother and the 138 from the father. However, it is always necessary to perform a multiplex protocol for these families with a linked marker since the 120/138 genotype can also arise from paternal contamination. Families C and J were awaiting PGD to be performed due to personal reasons, since the protocol for both families was ready. For family D, the DM and the D18S535 markers were informative, which required optimisation at the single cell DNA level.

Two families opted to come through for a PGD cycle (families G and H). Two different multiplex F-PCR protocols for the detection of DM1 affected embryos were devised for this study for the two families. In addition to amplifying the triplet repeat region within the DMPK gene, the PGD protocols for DM1 were designed to incorporate either an unlinked polymorphic marker on chromosome 21 (Protocol 1) or a linked polymorphic marker flanking the DM1 gene (Protocol 2). The CTG repeat region of DM1 is also very polymorphic; therefore it was usually possible to distinguish both alleles of normal heterozygote subjects on an automated sequencer.

For both protocols the markers could be simultaneously analysed on the 3100ABI Prism™ using GeneScan® analysis software. The peak area of the F-PCR analysis was indicative of the amount of amplified product, which was similar to the intensity of a band when analysing a sample after gel electrophoresis. However, F-PCR allows for accurate sizing of fragments and precise quantification of the amount of the PCR product. In some instances, the DNA samples revealed large differences between their allele sizes (for all three markers) e.g. for Ef (father of family E) the DM1 allele sizes were 122/149 indicating a 27bp difference. In those cases, there was significant preferential amplification of the smaller allele and also increased incidence ADO of the larger allele during single cell amplification. However, this was partly overcome by decreasing the temperature by 1-2°C of the annealing temperature. The incidence of stutter peaks was also encountered, especially at the single cell level (Figure 3.1). Stutter peaks were mostly observed when dinucleotide repeat regions where amplified and did not cause diagnostic error since they were always one repeat unit smaller than the true allele and relatively under-amplified. However, they were taken into account in individuals where their allele sizes differed in size by just one repeat and were regarded as practically difficult.

Figure 3.1. Illustration of preferential amplification, stutter peaks and the +A artefact on a dinucleotide repeat region



The GeneScan® analysis software shows the amplification fragment for each marker in different colours (here the APOC2 is in blue). Below the blue graph there is a table which shows the allele size (heading 'Size') and the quantification of each fragment (heading 'Peak Height'). Samples 4B and 5B represent single cells from the father and mother of family E respectively. Sample 6B is the negative control. In both parents the smaller allele shows preferential amplification over the larger allele. For example in sample 4B the smaller allele (126) has a peak height of 1344, whereas the larger allele (153) has a peak height of 282.
The **red circle** represents the stutter peak that is caused due to polymerase slippage during the PCR extension step. The **black square** represents the +A effect

Furthermore, the Plus-A (+A) artefact was also encountered during the optimisation process of single cell F-PCR. This was due to the addition of a nucleotide, usually adenosine, from the DNA polymerase enzyme to the 3'ends of the amplified fragments, which lead to PCR products one bp longer than the expected fragment size (Figure 3.1). The +A effect would not cause misdiagnosis, however, it may confuse the peak area calculation. However, after subsequent optimisation experiments, this problem was reduced by omitting the final 10min extension step typically used in PCR protocols.

3.2.2.1 Analysis of Control Single Cells

Clumps of buccal cells (2-4 cells) were isolated from the author (section 2.2.2.2) to test whether the two multiplex protocols were working with a lower starting amount of DNA. Protocol 1 was tested on 30 clumps and showed 98% amplification rate for both loci with 3% and 5% ADO rate for the DM1 and D21S1414 primers respectively (Table 3.11). Protocol 2 was tested on 30 clumps revealing 3% amplification failure and ADO rates of 4% and 5% for the DM1 and D19S112 primers respectively. Single cell analysis was performed initially on 50 single buccal cells from normal heterozygote subjects. Amplification of the DM1 and D21S1414 primers (protocol 1) showed 96% and 93% amplification efficiency respectively and 9.1% and 10.2% ADO rate for the DM1 and D21S1414 respectively in heterozygote individuals. Protocol 2 (DM1 and D19S112) revealed similar amplification efficiency of 95% for the DM primers and 91% for the D19S112 primers. The ADO rates were 8.8% for the DM 12.9% and for the D19S112 primers in heterozygote individuals.

Table 3.11. Results of amplification efficiency and ADO in control clumps and single cells

Type of cells	Rates	Protocol 1		Protocol 2	
		DM 1	D21S1414	DM	D19S112
Clumps (n=30)	Amplification efficiency	98%	98%	98%	97%
	Allele Dropout	3%	5%	4%	5%
Single cells (n=50)	Amplification efficiency	96%	93%	95%	91%
	Allele Dropout	9.1%	10.2%	8.8%	12.9%

3.2.2.2 Analysis of Patients Single Cells

The single buccal cells used in optimising and testing the PGD protocol were derived from members of both families, including 80 cells from each father ('Gf' and 'Hf') and 80 cells from each mother ('Gm' and 'Hm') and 50 cells from 'Hs' (mother's sister; Table 3.7). The data from all 160 single buccal cells of the members of family G displayed an amplification rate of 90.9% and 85.7% and an ADO rate of 14.1% for the D21S1414 primer (Table 3.12).

Table 3.12. DM1 and D21S1414 amplification results on single buccal cells from family G (Protocol 1)

Results	'Gf' (n=80)	'Gm' (n=80)	Total (n=160)
DM1			
Amplification rate	**89.8%**	**92%**	90.9%
Amplification failure	**10.2%**	**8%**	9.1%
ADO	-	-	-
Contamination	**1.2%**	-	0.6%
D21S1414			
Amplification rate	**84.4%**	**87%**	85.7%
Amplification failure	**15.6%**	**13%**	14.3%
ADO	**15%**	**13.2%**	14.1%
Contamination	-	-	-

'Gf'= father of G family and 'Gm' = mother of G family

The total amplification rate from 210 single buccal cells for family H was 91.3% and 83.7% for the DM1 and D19S112 respectively, whilst the ADO rate was 7.9% and 15.6% for the DM1 (from 80 cells for Hf) and D19S112 primers respectively (Table 3.13 The ADO rates for 'Hms' single buccal cells were markedly higher compared to those of 'Hm' for the D19S112 primer. The underlying reason might be the prolonged time from the sample collection until the time of single cell isolation, since the cells were sent at room temperature during transit, hence, many cells might have been dead or degenerating.

Table 3.13. DM1 and D19S112 amplification results on single buccal cells from family H (Protocol 2)

Results	'Hf' (n=80)	'Hm' (n=80)	'Hms' (n=50)	Total (n=210)
DM1				
Amplification rate	**92%**	**92%**	**90%**	91.3%
Amplification failure	**8.1%**	**8%**	**10%**	8.7%
ADO	**7.9%**	-	-	7.9%
Contamination	**2.5%**	-	**4%**	2.9%
D19S112				
Amplification rate	**85.7%**	**87%**	**78.3%**	83.7%
Amplification failure	**14.3%**	**13%**	**21.7%**	16.3%
ADO	-	**12.8%**	**23.2%**	15.6%
Contamination	-	**5%**	**4%**	3.8%

'Hf'= father of H family, 'Hm' = mother of H family and 'Hms' = mother's sister of H family

A negative control was included in every ten single buccal cells from the last wash drop for both protocols. No contamination was observed in any of the final wash drop blanks, lysis-buffer only negative controls or PCR mixture only negative controls. During blastomere analysis a wash drop blank was used for every single blastomere. All personnel involved in the diagnosis were genotyped to allow detection of contamination. No contamination was found by one or more of the personnel.

3.2.3 Clinical PGD Results

The patients underwent routine IVF procedures and ICSI was employed for both families to avoid sperm contamination. The embryos were biopsied on day 3 and the single blastomeres were prepared for the diagnosis (section 2.2.2.2.3).

3.2.3.1 Family G

During the PGD cycle for family 'G', 7 oocytes were collected from which 5 underwent ICSI. Five embryos were of sufficient quality for biopsy on day 3 post-fertilisation; however overall embryonic morphology was not good since from all embryos only one cell was biopsied (Table 3.14). Molecular analysis revealed 2 embryos to be affected, 2 with incomplete results (one of which was suspected to be abnormal) and one with no results for either marker. No contamination was detected and no allele dropout.

Table 3.14. PGD results of family 'G'

Embryo No.	No. of cells (time of biopsy)	Grade	Cell No.	Notes	DM1	D21S1414	Result
G1	5	2	a	-	122/-	335/346	Affected
G2	5-6	2+	a	Cell lysing	AF	335/355	Incomplete
G3	5	2+	a	Degenerating embryo	122/-	Inconsistent*	Affected
G4	5	2-	a	No nucleus seen	AF	AF	No result
G5	6	2	a	-	122/-	346/350	Affected
+ve ctrl ♀	-	-	-	-	*142/-*	*346/355*	-
+ve ctrl ♂	-	-	-	-	*122/-*	*335/350*	-

DM=myotonic dystrophy mutation marker; D21S1414=contamination STR marker; AF=amplification failure
*Allele sizes that did not correspond to any of the parents nor the people involved in the diagnosis

Full information was achieved for embryo G1a and embryo G5a (Figure 3.2). In two instances (G2a and G4a) the DM1 primer failed to give any kind of results. However, cell G2a was lysing during biopsy and some material might have been lost during transfer since the contamination marker was able to give a result. Furthermore, in cell G4a no nucleus could be seen during biopsy and washing procedures.

Table 3.15. Confirmatory results from spare embryos after PGD for family 'G'

Embryo No.	**Cell No.**	**DM**	**D21S1414**	**Result**
Gs1	A	122/ -	346/351	Affected
	B	AF	AF	No Result
Gs2	A	122/ -	335/346	Affected
Gs4	A	122/ -	AF	Affected
	b	122/ -	351/355	Affected
	c	AF	AF	No Result
Gs5	a	122/ -	AF	Affected
	b	122/ -	/351 ADO	Affected
	c	122/ -	346/351	Affected
	d	122/ -	346/351	Affected
	e	122/ -	/351 ADO	Affected

DM=myotonic dystrophy mutation marker; D21S1414=contamination STR marker; AF=amplification failure; ADO=allele dropout

Overall, from 16 blastomeres the DM1 locus revealed a 75% amplification rate whereas the D21S1414 showed a 68.75% amplification rate and 12.5% allele dropout.

3.2.3.2 Family H

During the PGD cycle for family 'H', 9 oocytes were collected from which 8 underwent ICSI. Five embryos were of sufficient quality for biopsy on day 3 post-fertilisation and from all embryos two cells were biopsied (Table 3.16). One embryo (embryo H1) was normal and was chosen for embryo transfer. Two embryos were considered to be affected and two revealed inconclusive results.

Table 3.16. PGD results of family 'H'

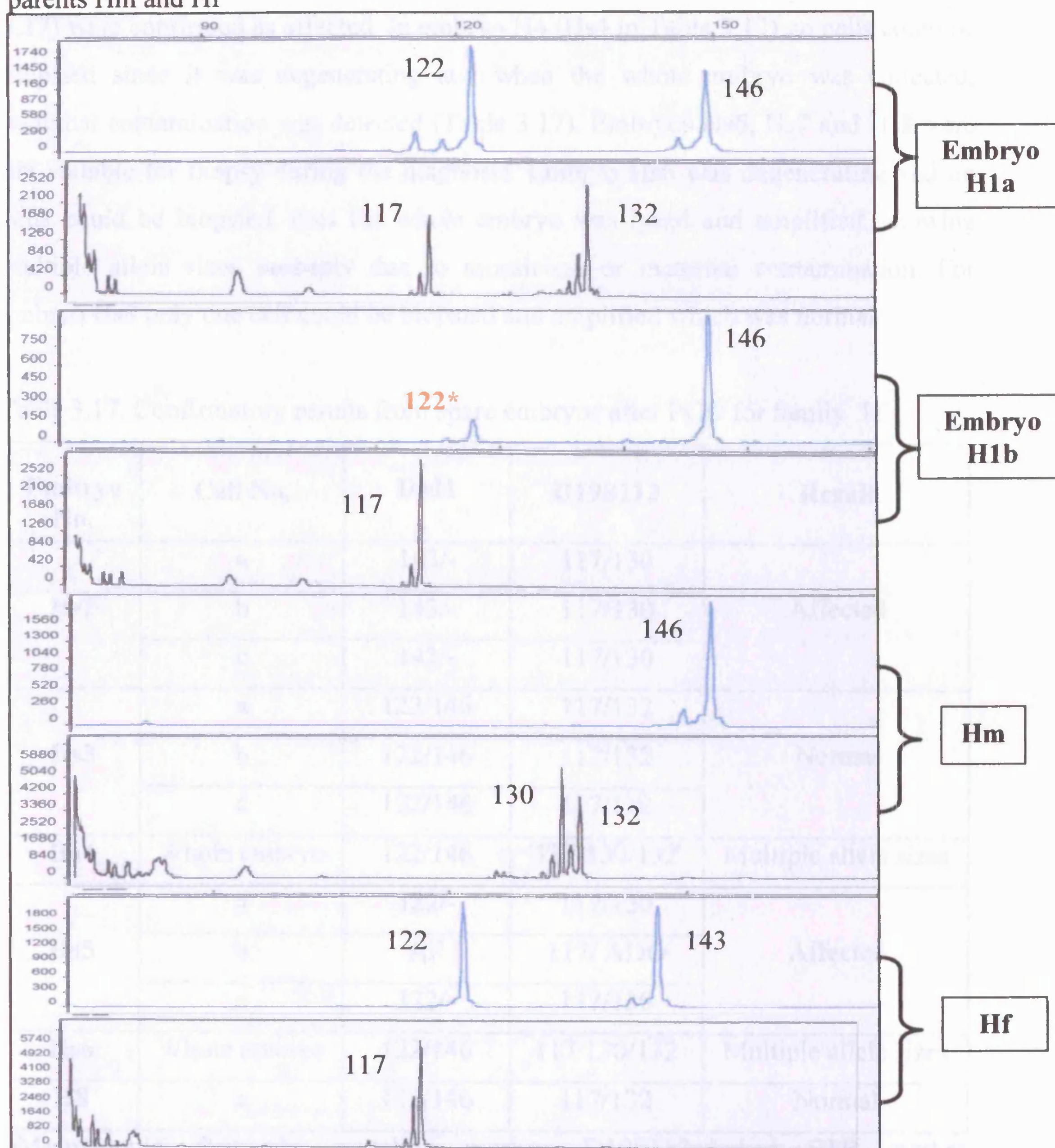
Embryo No.	No. of cells (time of biopsy)	Grade	Cell No.	Notes	DM1	D19S112	Result
H1	**8**	**1-**	**1a**	**-**	**122/146**	**117/132**	Normal
			1b	**-**	**-*[1]/146**	**117/ ADO**	
H2	7	2	**2a**	**-**	**AF**	**117/130**	Affected (incomplete)
			2b	**No nucleus seen**	**AF**	**AF**	
H3	6	2+	**3a**	**-**	**122/-*[2]**	**ADO /132**	Inconclusive
			3b	**-**	**122/-*[2]**	**AF**	
H4	**8**	**1-**	**4a**	**-**	**122/-**	**117/130**	Affected
			4b	**-**	**122/-**	**117/ ADO**	
H5	**8**	**1-**	**5a**	**Cell lysing**	**AF**	**AF**	Affected
			5b	**-**	**143/-**	**117/130**	
***+ve ctrl* ♀**	**-**	**-**	**-**	**-**	***146/-***	***130/132*** (130 underlined)	**-**
***+ve ctrl* ♂**	**-**	**-**	**-**	**-**	***122/143***	***117/- (hm)***	**-**

*[1]Extreme cases of preferential amplification of the large allele (v.v low amplification of the 122 allele), which at the time of diagnosis were considered as allele dropout to avoid misdiagnosis.
*[2]Extreme cases of preferential amplification of the small allele (v.v low amplification of the 146 allele), which at the time of diagnosis were considered as allele dropout to avoid misdiagnosis
Underlined numbers indicate the phase i.e. those alleles are linked to the mutation hence if the embryo carries these alleles it would be classed as affected
DM=myotonic dystrophy mutation marker; D19S112=linked STR marker; AF=amplification failure; ADO=allele dropout; mh=homozygous

In embryo H1b, there was extreme preferential amplification of the larger allele (122) for the DM1 marker (Figure 3.3). However, since the larger allele was the mother's unaffected allele, the whole embryo was considered normal (Figure 3.3). Embryo H2 was thought to be affected since only the linked marker (D19S112) amplified in one of two cells (117/130) containing the phase allele (130). In embryo H3 the linked marker showed inconclusive results and the mutation marker (DM) showed extreme

preferential amplification of the 146 allele inherited from the mother but it was probably normal (Table 3.16). However, to reduce the chance of misdiagnosis it was considered as affected at the time of diagnosis.

Figure 3.3. Analysis results for embryo H1 (blastomeres H1a and H1b) and the parents Hm and Hf



The DM1 (Blue) and D19S112 (Black) genotypes of blastomeres H1a and H1b from embryos H1 can be seen during the clinical case. Furthermore, the prospective couples' DM1 and D19S112 genotypes (Hm=mother and Hf=father) can be seen which were used as positive controls. Cell H1a showed conclusive results indicating a normal cell. Cell H1b revealed extreme preferential amplification of the smaller allele of the DM1 genotype (122) and ADO of the larger allele for the D19S112 marker.

However, since the mother's normal allele was inherited, it was concluded that the embryo was normal.

The rest of the embryos were donated for confirmation diagnosis. The results from the spare embryos revealed that embryo H3 (Hs3 in Table 3.17) was actually normal and could have been transferred (Table 3.17). Embryos H2 and H5 (Hs2 and Hs5 in Table 3.17) were confirmed as affected. In embryo H4 (Hs4 in Table 3.17) no cells could be biopsied since it was degenerating and when the whole embryo was collected, maternal contamination was detected (Table 3.17). Embryos Hs6, Hs7 and Hs8 were not suitable for biopsy during the diagnosis. Embryo Hs6 was degenerating and no cells could be biopsied, thus the whole embryo was lysed and amplified, showing multiple allele sizes probably due to mosaicism or maternal contamination. For embryo Hs8 only one cell could be biopsied and amplified which was normal.

Table 3.17. Confirmatory results from spare embryos after PGD for family 'H'

Embryo No.	Cell No.	DM1	D19S112	Result
Hs2	a	143/-	117/130	Affected
	b	143/-	117/130	
	c	143/-	117/130	
Hs3	a	122/146	117/132	Normal
	b	122/146	117/132	
	c	122/146	117/132	
Hs4	Whole embryo	122/146	117/130/132	Multiple allele sizes
Hs5	a	122/-	117/130	Affected
	b	AF	117/ ADO	
	c	122/-	117/130	
Hs6	Whole embryo	122/146	117/130/132	Multiple allele sizes
H8	a	122/146	117/132	Normal

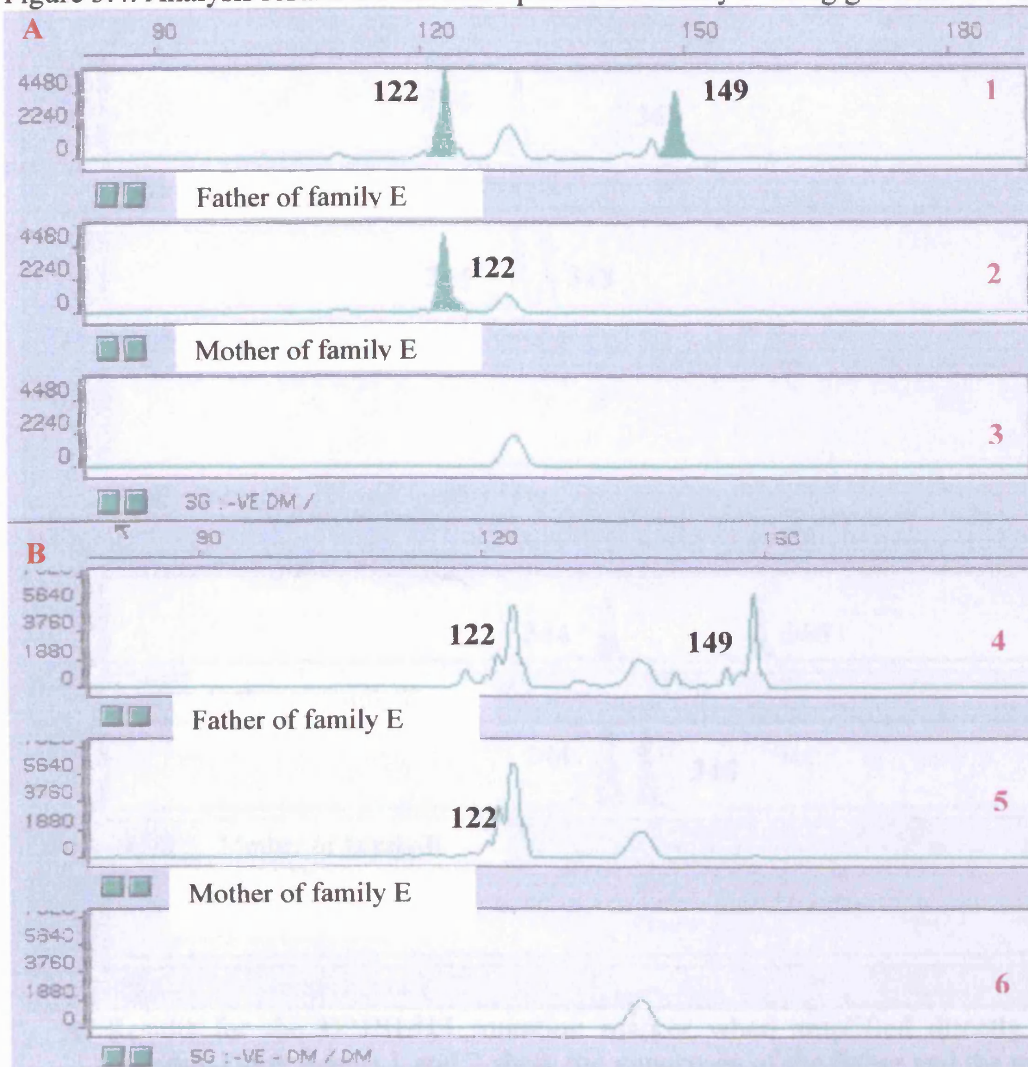
DM=myotonic dystrophy mutation marker; D19S112=linked STR marker; AF=amplification failure; ADO=allele dropout

Overall, from 20 blastomeres the DM1 locus revealed an 80% amplification rate and no ADO, 15% of cases showed extreme preferential amplification. The D19S112 showed an 85% amplification rate and a 15% allele dropout.

3.2.4 DOP-PCR on Genomic DNA

Initially, DOP-PCR was carried out on genomic DNA which was subsequently used as template for singleplex reactions of each primer from the mother and father of family E as well as individuals X and Z (Table 2.8). However, due to lack of single cell material from family E, single cell results were only obtained from individuals X and Z. All the F-PCR primers where successfully amplified when genomic DNA (100-300ng approximately) was used as template. The amplification rate was 98% for all primers except for the D13S305 which was 96%. There was no incidence of allele dropout, which was expected due to the nature of the starting template. The similarity of amplifications when a singleplex reaction was carried out from genomic DNA compared to a singleplex reaction when it was carried out using a DOP-PCR product as template is depicted in Figures 3.4, 3.5, 3.6 and 3.7. The peaks in all lanes (except the negative control lanes) are high indicating sufficient PCR product, with no ADO and little or no stutter effects. However, more importantly the genotypes for both primers can be easily interpreted in the amplifications which used the DOP-PCR product as starting template (Figures 3.4B, 3.5B, 3.6B and 3.7B).

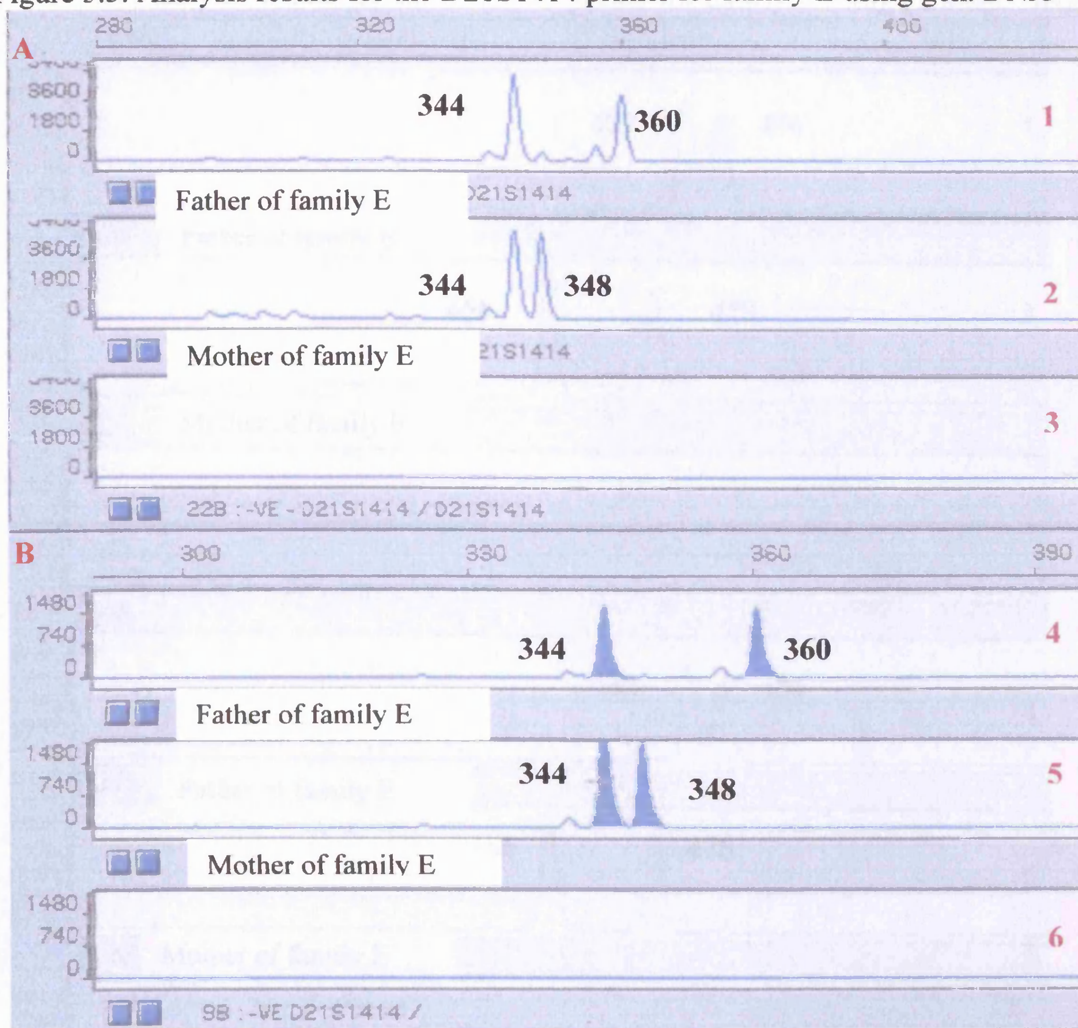
Figure 3.4. Analysis results for the DM1 primer for family E using genomic DNA



A. Results for the DM1 mutation marker when amplified directly from genomic DNA. Lanes 1 and 2 show the genotypes of the father and the mother of family E respectively (Table 3.5) and lane 3 is the negative control.
B. Results for the DM1 mutation marker when amplified from a DOP-PCR product. Lanes 4 and 5 show the genotypes of the father and the mother of family E respectively and lane 6 is the negative control.

Each peak represents the alleles of the individual. In both sets of results the peak were easily distinguished with no +A artefacts and little or no stutter effects. However, there was a slight difference appearance of 'B' compared to 'A', since the latter showed more distinct and sharper peaks.

Figure 3.5. Analysis results for the D21S1414 primer for family E using gen. DNA

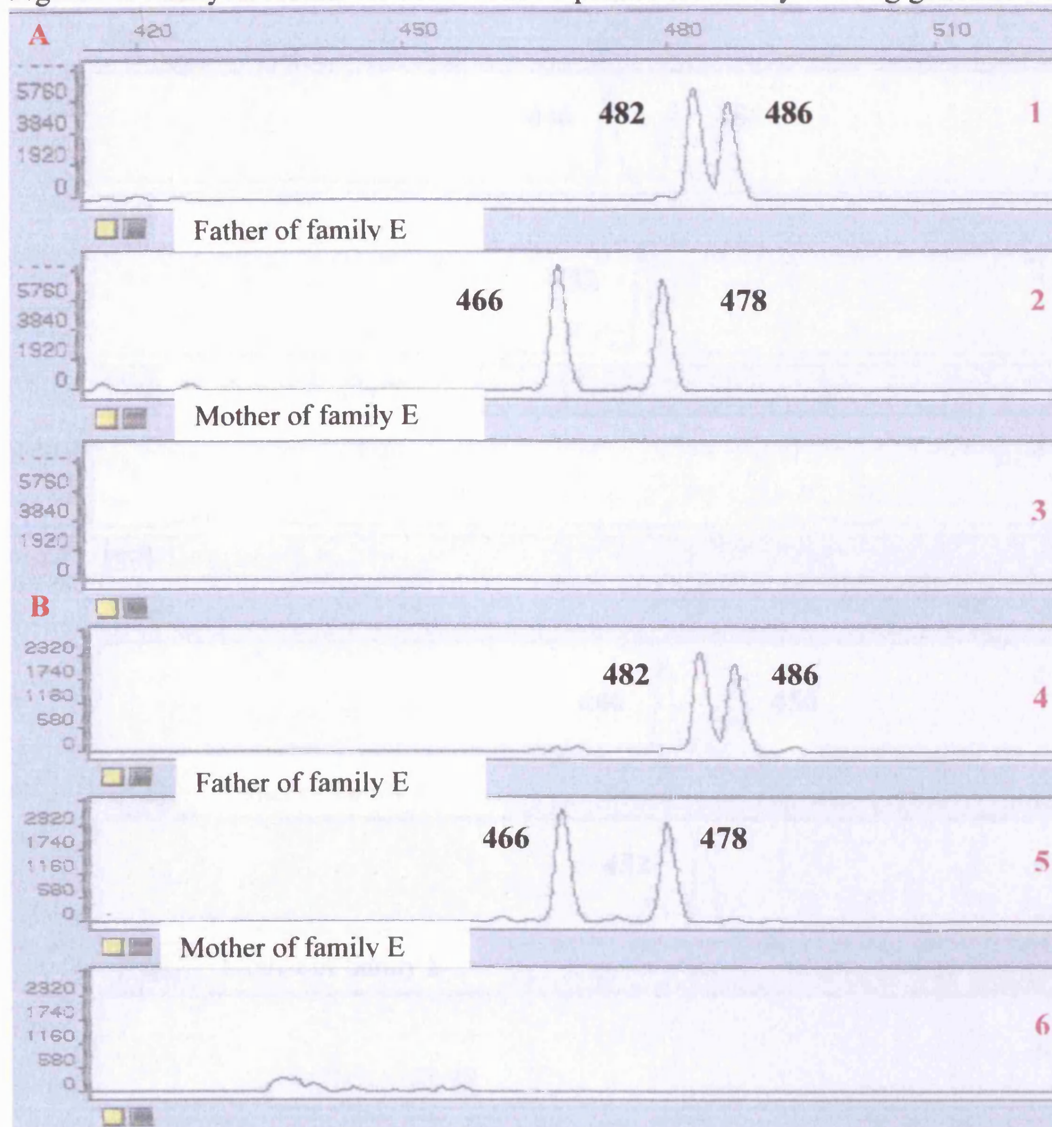


A. Results for the D21S1414 mutation marker when amplified directly from genomic DNA. Lanes 1 and 2 show the genotypes of the father and the mother of family E respectively (Table 3.5) and lane 3 is the negative control.

B. Results for the D21S1414 mutation marker when amplified from a DOP-PCR product. Lanes 4 and 5 show the genotypes of the father and the mother of family E respectively and lane 6 is the negative control.

Each peak represents the alleles of the individual. In both sets of results the peak was easily distinguished with no +A artefacts and no stutter effects. However, there was a slight difference in appearance of 'B' compared to 'A', since the latter showed increased peak heights of more than 3600 in comparison to 'B' which showed peak heights of around 1500. Such results indicated that amplification products in 'B' were half the amount of 'A'.

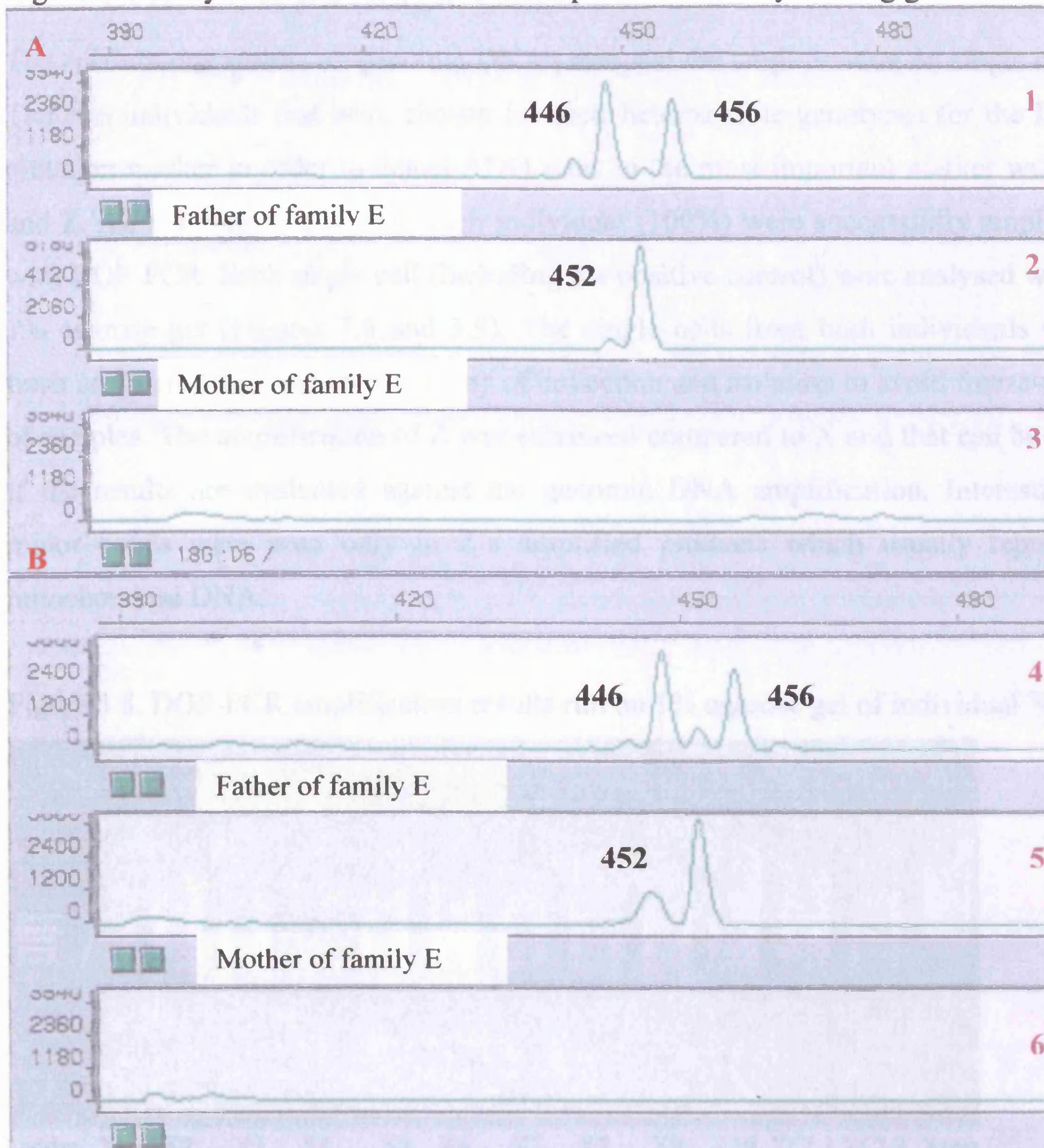
Figure 3.6 Analysis results for the D18S535 primer for family E using genomic DNA



A. Results for the D18S535 unlinked marker when amplified directly from genomic DNA. Lanes 1 and 2 show the genotypes of the father and the mother of family E respectively (Table 3.5) and lane 3 is the negative control.
B. Results for the D18S535 unlinked marker when amplified from a DOP-PCR product. Lanes 4 and 5 show the genotypes of the father and the mother of family E respectively and lane 6 is the negative control.

Each peak represents the alleles of the individual. In both sets of results the peak were easily distinguished with no +A artefacts and little or no stutter effects. However, in 'B' the peak heights were nearly half the size compared to 'A'. Such results indicated that amplification products in 'B' were half the amount of 'A'

Figure 3.7 Analysis results for the D13S305 primer for family E using genomic DNA



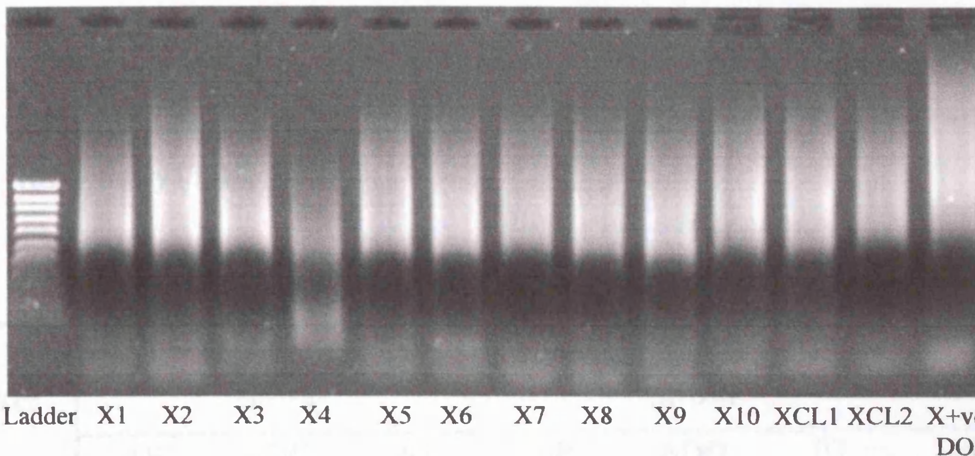
A. Results for the D13S305 unlinked marker when amplified directly from genomic DNA. Lanes 1 and 2 show the genotypes of the father and the mother of family E respectively (Table 3.5) and lane 3 is the negative control.
B. Results for the D13S305 unlinked marker when amplified from a DOP-PCR product. Lanes 4 and 5 show the genotypes of the father and the mother of family E respectively and lane 6 is the negative control.

Each peak represents the alleles of the individual. In both sets of results the peak were easily distinguished with no +A artefacts and little or no stutter effects. There ass however, slightly increased stutter band effect in 'B' which could be seen by comparing lanes 1 and 4.

3.2.5 DOP-PCR on Single Cells

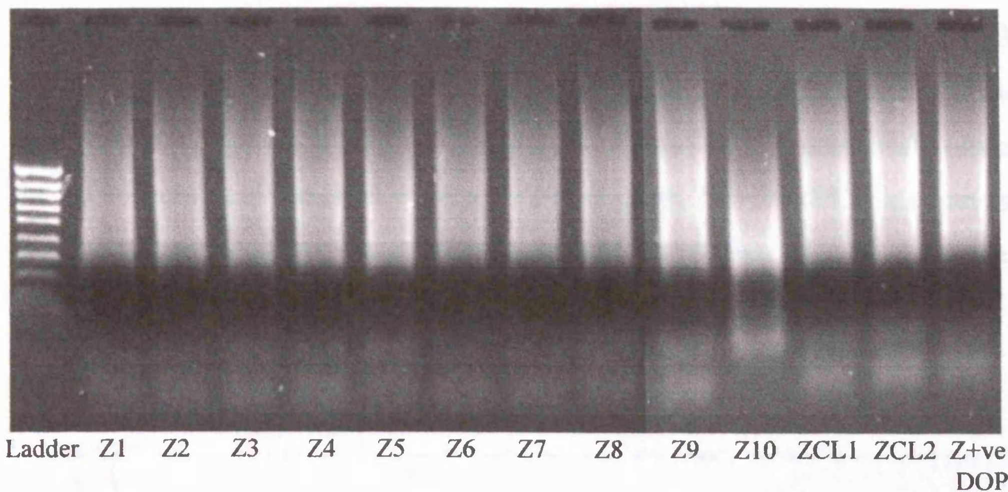
The encouraging results on genomic DNA prompted the amplification on single cells. The two individuals that were chosen for their heterozygote genotypes for the DM1 mutation marker in order to detect ADO rates in the most important marker were X and Z. All ten single cells from each individual (100%) were successfully amplified with DOP-PCR. Each single cell (including the positive control) were analysed with a 1% agarose gel (Figures 3.8 and 3.9). The single cells from both individuals were fresh and were amplified the same day of collection and isolation to avoid freeze-thaw of samples. The amplification of Z was enhanced compared to X and that can be seen if the results are evaluated against the genomic DNA amplification. Interestingly minor bands were seen only in Z's amplified products which usually represent mitochondrial DNA.

Figure 3.8. DOP-PCR amplification results run on 1% agarose gel of individual X



The first lane displays the 1Kb ladder. Lines X1-X10 depict each single cell whereas XCL1 and XCL2 represent the positive controls from clumps and X+ve DOP shows the positive control from genomic DNA. All single cells showed a smear of results with no distinct bands except for C4 where the smear was faint and reduced in size. The smear from the genomic DNA was more intense and increased in size which suggested better amplification of the genome.

Figure 3.9. DOP-PCR amplification results run on 1% agarose gel of individual Z



The first lane displays the ladder. Lines Z1-Z10 depict each single cell whereas ZCL1 and ZCL2 represent the positive controls from clumps and Z+ve DOP shows the positive control from genomic DNA. All single cells showed a smear of results with no distinct bands except for Z10 where the smear was reduced in size, however it was very intense.

From the 20 single cells (10 from each individual) the amplification rate was 85%, 55%, 65%, 60% and 65% for DM, D19S112, D21S1414, D18S535 and D13S305 respectively (Table 3.18).

Table 3.18. Amplification results for two different individuals

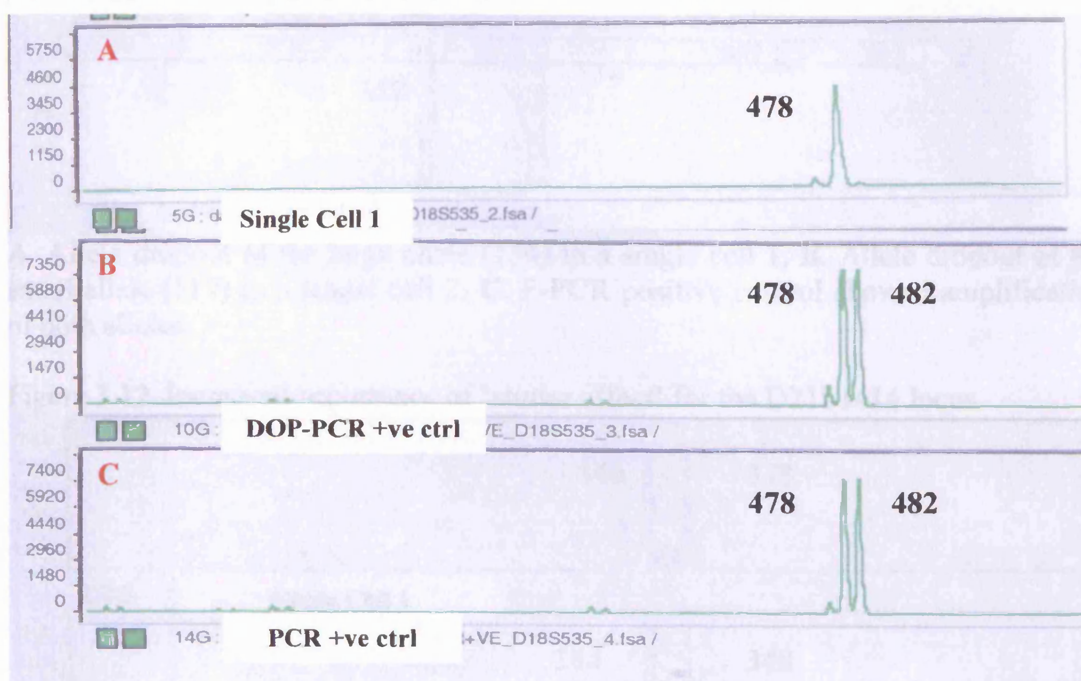
Marker	Individual Z (n=10)			Individual X (n=10)			**Total**	
	AR	ADO	IG	AR	ADO	IG	**AR**	**ADO**
DM1	90%	0%	0%	80%	20%	0%	**85%**	**10%**
D19S112	70%	50%	10%	40%	30%	20%	**55%**	**40%**
D21S1414	50%	20%	10%	80%	40%	10%	**65%**	**30%**
D18S535	60%	60%	10%	60%	-	10%	**60%**	**60%**
D13S305	70%	-	10%	60%	40%	0%	**65%**	**40%**

AR = amplification rate, ADO = allele dropout, IG = incorrect genotype.

Overall, there was a high incidence of ADO for all STR's ranging from 10-60%. The highest allele dropout rates were observed for the D18S535 primers, showing 60% ADO (Table 3.18 and 3.9), whilst the DM1 locus displayed the lowest rate of allele

dropout. This was probably due to the size of the products that the D18S535 primers amplify, which is >430bp, whereas the DM1 are considerably lower (Table 3.2 From Materials and Methods). Moreover, there was occurrence of incorrect genotype in all the markers except for the DM1 (Table 3.18, termed IG). It was rarely observed and the differences in allele sizes were of 1-2 base pairs. The D18S535 also revealed high ADO rates (60%), however, it showed that ADO affected equally the large and the small allele (Figure 3.10).

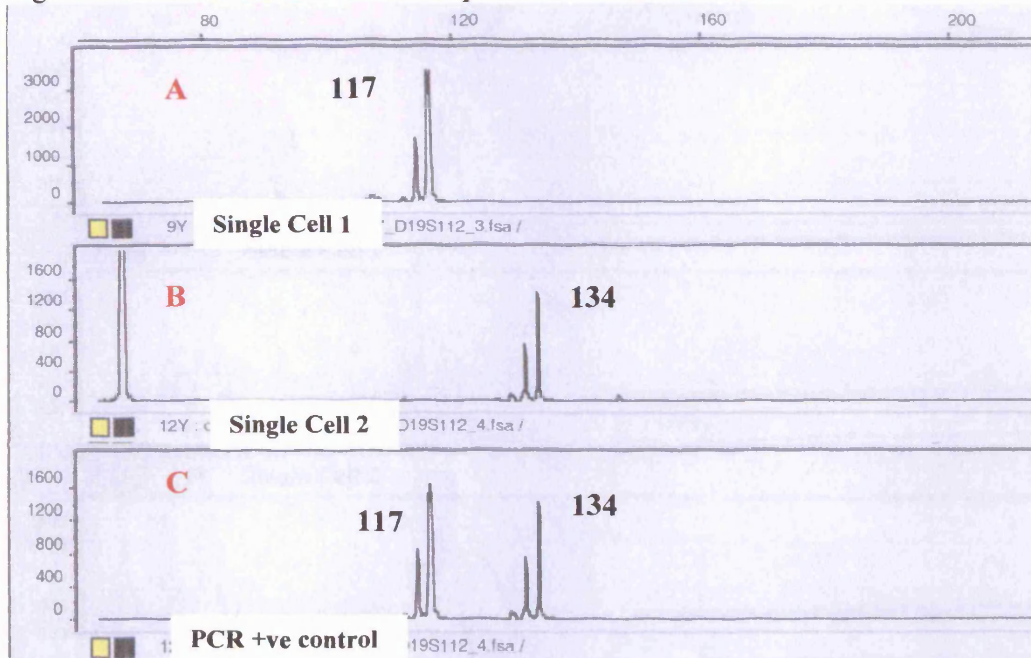
Figure 3.10. Illustration of allele dropout of the D18S535 locus



A. Allele dropout of the large allele (482) in a single cell. **B.** DOP-PCR positive control showed amplification of both alleles. **C.** F-PCR positive control showed amplification of both alleles

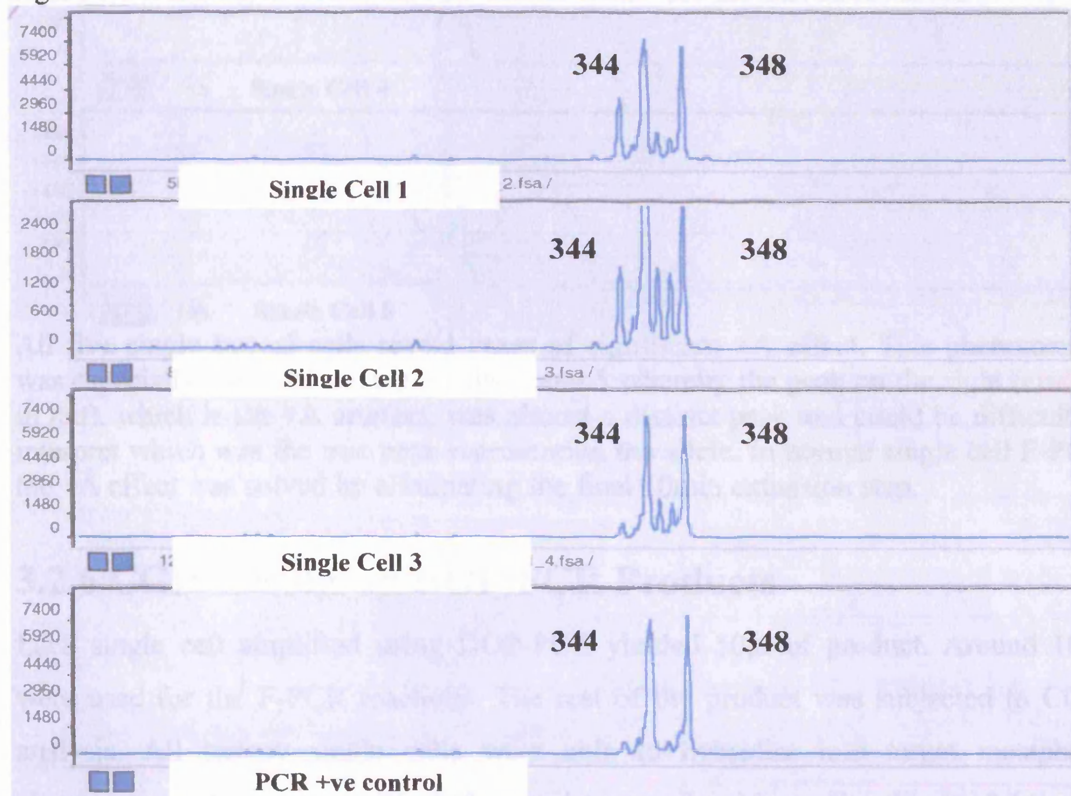
The D19S112 and D21S1414 showed increased stutter effect as well as random peaks outside the region of the amplified products (Figure 3.11 and 3.12). Furthermore, the DM marker showed high +A artefact incidence compared to when they were amplified as singleplex reactions from single cell DNA (Figure 3.13).

Figure 3.11. Illustration of allele dropout of the D19S112 locus



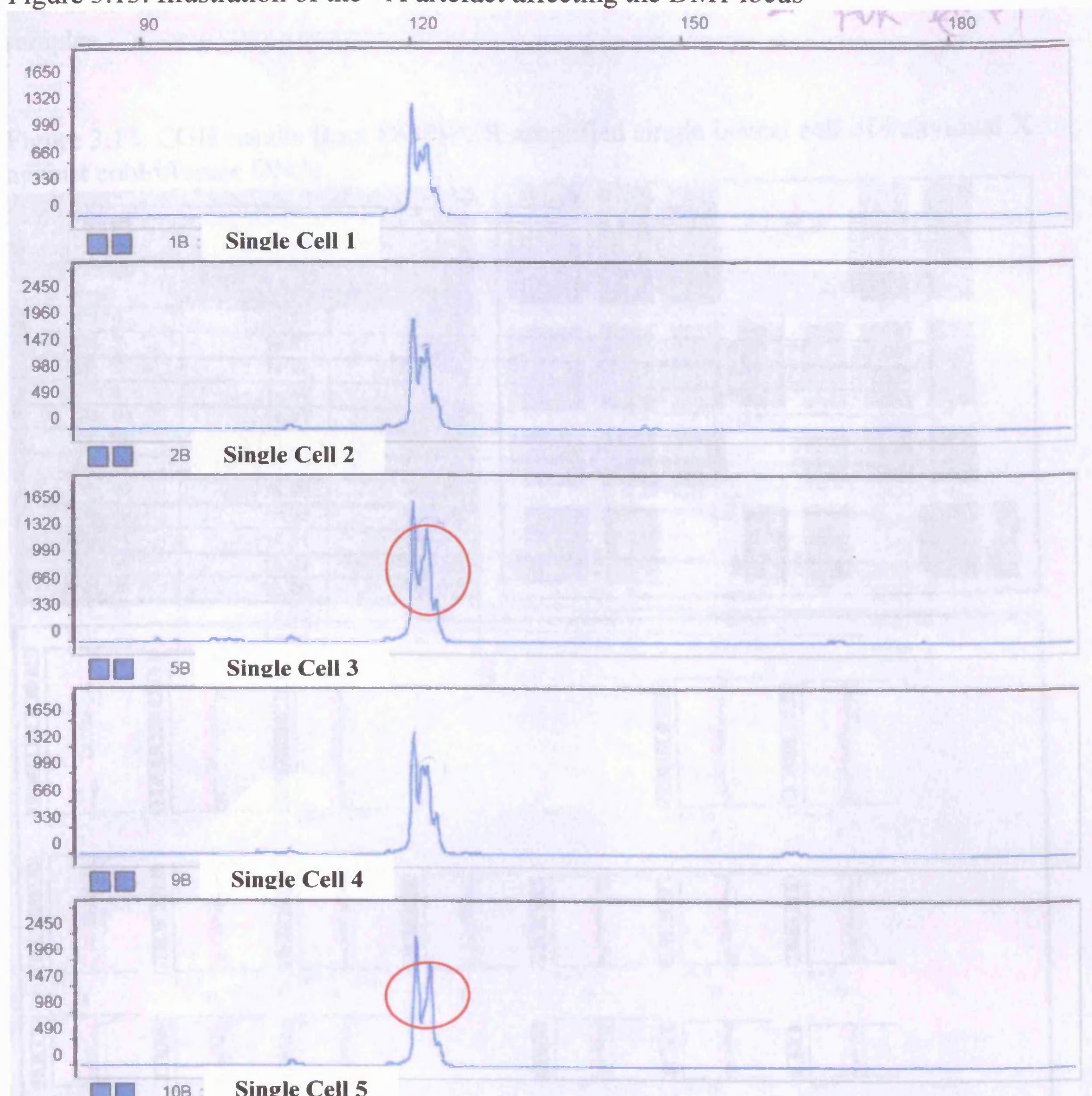
A. Allele dropout of the large allele (134) in a single cell 1. **B.** Allele dropout of the small allele (117) in a single cell 2. **C.** F-PCR positive control showed amplification of both alleles

Figure 3.12. Increased occurrence of 'stutter effect' for the D21S1414 locus



All three single cells showed increased stutter effect of both alleles. However, in single cell 2 there was increased stutter effect. Such results were only observed on single cells and never at genomic DNA level.

Figure 3.13. Illustration of the +A artefact affecting the DM1 locus



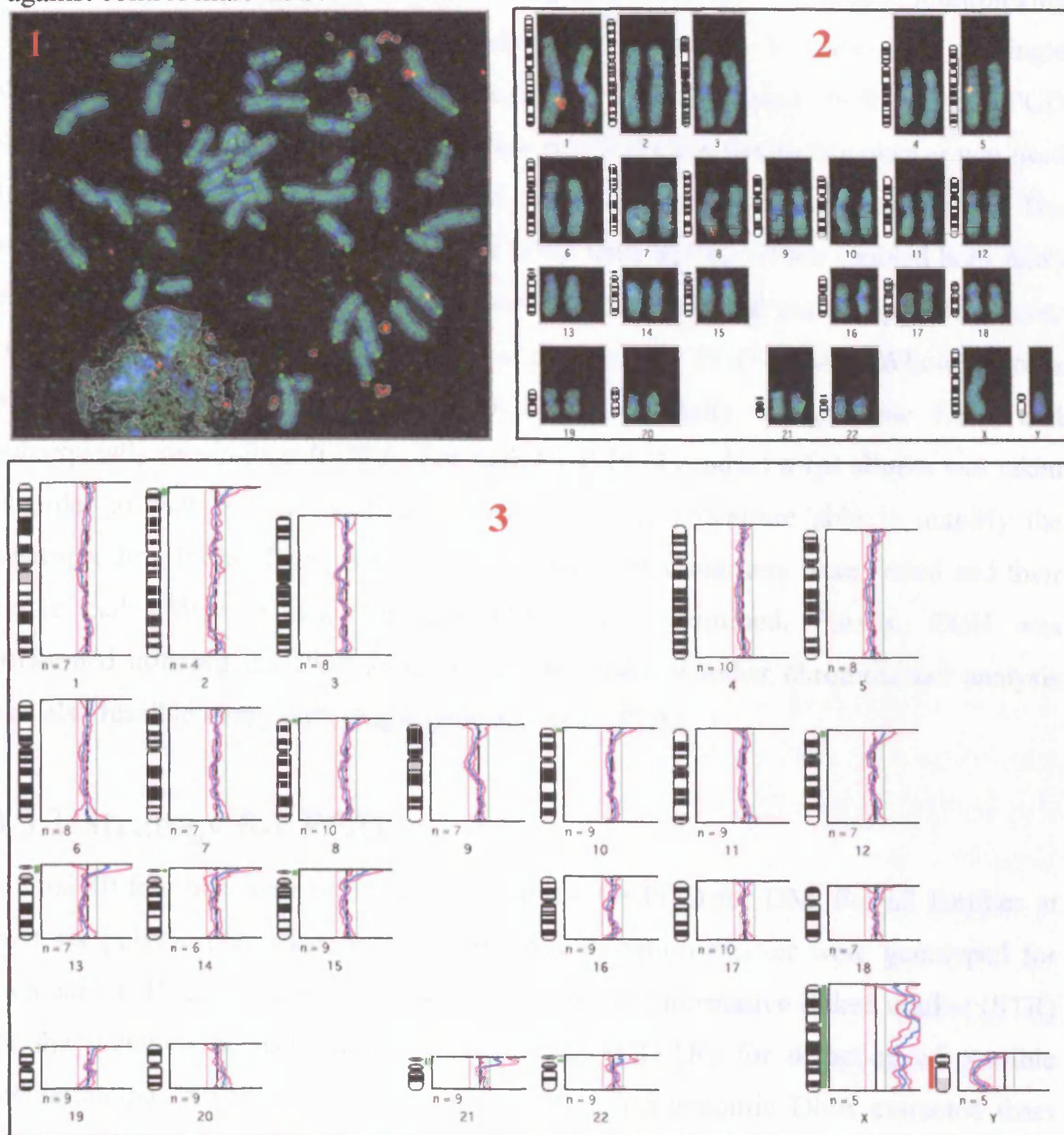
All five single buccal cells reveal cases of significant +A effect. This phenomenon was especially apparent in single cells 3 and 5 whereby the peak on the right (circled in red), which is the +A artefact, was almost a distinct peak and could be difficult to interpret which was the true peak representing the allele. In normal single cell F-PCR the +A effect was solved by eliminating the final 10min extension step.

3.2.6 CGH Results on DOP-PCR Products

Each single cell amplified using DOP-PCR yielded 50µl of product. Around 10µl were used for the F-PCR reactions. The rest of the product was subjected to CGH analysis. All twenty single cells were able to hybridise into target metaphase chromosomes; however, 17/20 (85%) produced analysable results (Figure 3.14). All

analysable cells showed normal chromosome results and the sex was confirmed in all samples.

Figure 3.14. CGH results from DOP-PCR amplified single buccal cell of individual X against control male DNA.



For this CGH experiment 11 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. 1. Capturing of metaphase spread. 2. Karyotype of the captured metaphase 3. This shows the cumulative analysis of eleven metaphases, which was the basis of the interpretation. There was a shift in fluorescence only towards the green in chromosome X and towards the red in chromosome Y showing that the test sample was a normal female. Overall, there was dynamic hybridisation with little background fluorescence demonstrating that the DOP-PCR amplified single cell can be used for PGD of DM as well as further chromosomal analysis.

3.3 Discussion

This study aimed to develop two multiplex PGD F-PCR protocols for DM1 and furthermore to determine if WGA could be used for PGD of DM. The PGD protocols were developed by exploring informative STR markers for the parents, multiplexing them on genomic DNA and finally testing and perfecting the procedure on single cells. Two multiplex F-PCR protocols were devised and carried out for clinical PGD cases of two families (G and H). In the first protocol a contamination marker was used (similar to that used by Piyamongkol *et al,* 2004). No embryo was transferred. The second protocol entailed a linked marker never used before, which enabled both ADO and contamination to be tackled. However, this laborious and patient-specific process prompted the development of a universal protocol for PGD of DM. Whole genome amplification was employed using DOP-PCR, initially on genomic DNA and subsequently on single cell DNA. For each DOP-PCR product a 1µl aliquot was taken in order to carry out a separate singleplex F-PCR procedure able to amplify the corresponding locus. Thus, five different polymorphic markers were tested and their single cell efficiency and reproducibility was determined. Finally, CGH was performed utilising the DOP-PCR product to assess whether chromosomal analysis was also feasible along with single gene analysis of DM.

3.3.1 Strategy for PGD

In total 10 families were referred to our centre for PGD for DM. For all families at least six polymorphic markers plus the DM1 mutation marker were genotyped for each parent. This was carried out in order to find an informative linked marker (STR) for the mutation as well as an unlinked marker (STR) for detection of possible contamination. All this work was carried out using genomic DNA extracted from whole blood from each parent and some relatives. All primers used were fluorescently labelled and analysed in an automated sequencer (ABI Prism™ 3100). Once a strategy was devised, the protocol was tested and optimised on single buccal cells and single blastomeres.

Initially accurate molecular analysis of the DM1 repeat expansion was (and is still) performed by Southern Blotting (Brook *et al,* 1992). However, this method requires a high copy number of the DNA template and thus cannot be applied to PGD. The use

of PCR can provide a fast and efficient means for the molecular diagnosis of DM1. However, the interpretation is based on the exclusion principle as the amplification of the fragment size larger than 500 bases is inefficient, hence only normal alleles can be examined. The first report of PGD for DM DM1 was from Sermon and colleagues (1997) who performed traditional PCR and analysis by gel electrophoresis. The DM DM1 mutation marker is polymorphic and provides the advantage of identifying the presence of both normal alleles in the normal sample (unless homozygous) and in some cases contamination. However, in the case of an affected cell or abnormal cell with ADO where only one allele is present, the occurrence of contamination may be concealed and may lead to misdiagnosis. The first PGD case using F-PCR was also performed by the same group (Sermon *et al,* 1998a), however, a misdiagnosis occurred which was attributed to maternal DNA contamination (Vandervors *et al,* 2000). This provoked Piyamongkol *et al* (2001a) to develop a multiplex F-PCR protocol able to tackle contamination, which is one of the most significantcan be a problems affecting single cell PCR. Hence, the DM DM1 marker was amplified along with another polymorphic marker. An addition of a fluorescent polymorphic marker, such as the D21S1414, in the F-PCR protocol would eliminate the need for nested PCR since the F-PCR technique is sensitive without the need of carrying out two2 sets of PCR amplifications and the addition of a contamination marker wouldand would provide information on whether a sample is contaminated. The D21S1414 locus is unlinked to the DMPK gene, thus providing no information on inheritance of the DM DM1 mutation, although itis able to serves as a very basic form of DNA fingerprint. The compound heterozygoscity of the D21S1414 locus is 0.88 (Sherlock *et al,* 1998). During this study, a similar protocol to Piyamongkol *et al* (2001a) was employed (Protocol 1) for family G. For family H a linked marker was used, namely D19S112, in addition to the DM DM1 marker (Protocol 2). The polymorphic characteristic of the D19S112 locus offers solution to both contamination and ADO problems encountered during single cell PCR. In family H the father was homozygous (117) and the mother was heterozygous (130/132) for the D19S112 locus. Hence, the parents had different allele sizes between them and were quite far apart rendering the marker informative. The mother's sister gave a samples of blood sample so thatand the phase was distinguished. The phase is the allele of the linked locus (here the D19S112) which is transmitted with the disease. If that allele (in this case the phase is the 130 allele) is present in an embryo it will be considered to be affected. In total, the

linkage information of the D19S112 marker can predict the presence of the mutant DM1 expanded allele in the embryo, which cannot be obtained by direct PCR analysis of the DM DM1 gene (due to the over-expanded allele). This allows differentiation between the affected embryo and normal embryo whose results are complicated by ADO. Therefore, complete diagnosis can be drawn from more normal embryos, giving rise to a large number of embryos for transfer and a better chance of pregnancy. Moreover, using the DM1/D19S112 multiplex F-PCR protocol reduces the case of misdiagnosis since the D19S112 marker acts aslike a safety net for the DM DM1 mutation marker (Ao *et al,* 1998).

The analysis was performed using the automated laser sequencers ABI Prism™ 3100 and ABI Prism™ 310. The use of F-PCR in comparison to traditional analyses provides protocols with further sensitivity since even tiny amounts of amplified fluorescent product can be detected as a small peak. The accuracy of the sequencers even allows differentiation of single base pair differences making the protocol more specific than those using traditional gel analysis (Sermon *et al,* 1998a). In the context of PGD, the most striking advantage of using the automated laser sequencers is time. Both the ABI Prism™ 310 and the ABI Prism™ 3100 can analyse up to three genes immediately and simultaneously after one multiplex PCR. However, the added advantage of the ABI Prism™ 3100 is that it can analyse 4 samples per hour (in comparison to one sample per hour for the ABI Prism™ 310), reducing the time of the analysis. During the PGD case the ABI Prism™ 310 was used as a backup analysis for all the samples.

During optimisation of the PCR protocols the phenomenon of stutter bands and the +A artefact was encountered (Figure 5.1). Stutter bands usually cause problems during allele scoring of heterozygote individuals whose alleles are close in size especially whilst amplifying mono- and dinucleotide repeats. This could be observed in many of the families when the allele sizes were different for the parents e.g. for family C the APOC2 marker showed 149/155 for the mother and 134/153 for the father, however, some of the alleles are only one two base pair apart (155 from the mother and 153 from the father) (Table 5.63). Stutter bands are usually one repeat unit length shorter than the main allele (Ellegren, 2004), hence during analysis of the APOC2 locus for family C the stutter band for the 155 allele would coincide with the

153 allele. The solution to stutter bands was to choose in our multiplex protocols STR's which displayed alleles between the parents of more than one base pair apart.

The +A artefact was caused by the addition of an extra nucleotide, usually adenosine, to the 3' ends of the amplified fragment during the amplification reaction. The +A products are mostly depicted as an extra peak or as a split peak one base pair longer than the expected PCR products. Such a problem would confuse the interpretation of the results but would not cause misdiagnosis. The split peak pattern can be minimised by either encouraging or suppressing the nucleotide addition. During the whole study it was decided to minimise the +A artefact by omitting the final extension step.

Both protocols showed high amplification efficiencies in clumps of 98% for both DM DM1 and D21S1414 and 97% for D19S112 (Table 5.1411). The ADO rates in clumps were also below 5% for both protocols. In the control single cells the ADO rates increased as expected to 8.8%-9.1% for the DM1, 10.2% for the D21S1414 and 12.9% for the D19S112 respectively. These results are within range of previously reported rates in studies of different genes (Ray and Handyside, 1996; Piyamongkol *et al*, 2001a). The optimised protocols 1 and 2 were tested in single buccal cells from the members of both families (Tables 5.15 12 and 5.1613). The amplification results slightly decreased which was expected due to the fact that the samples were not fresh and some were frozen. This was exaggerated in the case of 'Hms' (mother's sister of H family) which was due to the prolonged time from the sample collection until the time of single cell isolation causing the cells to die or degenerate (Table 5.1613). However, the amplification efficiencies were high with ADO rates remaining low.

3.3.2 Clinical DM PGD Cases

One cycle for each of the two families was carried out during this study. For family G five embryos were suitable for biopsy however, the embryos were of poor quality. Hence, only one blastomere was biopsied from each embryo. This was possibly due to the advanced maternal age (42 years of age) at the time of egg collection. Three embryos were affected and two embryos had either incomplete or no results, therefore no embryos were transferred and all were donated for confirmation of diagnosis (Table 5.1714). There were no cases of contamination observed in any of the negative

control samples. The low quality of the embryos was also reflected in the number of blastomeres that were biopsied for confirmation of the diagnosis. Embryo no. 3 had completely degenerated and no cells could be retrieved. During confirmation analysis, all embryos were found to be affected (Table 5.1815). In total the amplification rates for the DM DM1 and D21S1414 markers were 75% and 68.75%. The reduced amplification rate for both markers when compared to the workup single cell rate has been widely reported. Sermon *et al* (1997) revealed 100% amplification during single cell workup which was reduced to 78% whilst performing the clinical cases. In a study by Dean and co-workers (2001) a decrease in amplification was also found when tested on single lymphocytes (92%) and single blastomeres (84%). No problems occurred regarding stutter bands or the +A effect in any of the blastomeres. However, in embryo Gs2 there were peaks, outside the region which the D21S1414 product produces a peak, which were disregarded and were thought to be artefacts.

For family H the DM/D19S112 (Protocol 2) was also used on five embryos. Three embryos were affected, one was normal and one showed incomplete results (Table 5.1916). The normal embryo was transferred but no pregnancy was achieved. In embryo H2 the DM DM1 locus failed to amplify in either of the cells biopsied and the D19S112 showed results from one blastomere which was considered affected. However, in the case of embryo H3 the results displayed a normal embryo, but, due to extreme cases of preferential amplification for both blastomeres regarding the DM DM1 allele and ADO of the D19S112 locus it was not considered for transfer to avoid misdiagnosis. Carrying out confirmation of the diagnosis in the untransferred (spare) embryos revealed that embryo H3 was found to be normal and embryo H2 was affected (Table 5.2017). The phenomenon of mosaicism or maternal contamination in the spare embryos Hs4 and Hs6 was due to more than one cell being present in the tube since some embryos were compacted and could not be either biopsied or disaggregated therefore some cumulus cells attached to the ZP might have given such results. During the clinical case no incidence of maternal contamination was found in any of the 16 blanks (one final wash drop blank for each blastomere; three for lysis-buffer-only blanks; and three PCR reaction-mixture-only negative controls). Overall, from 20 blastomeres the DM DM1 locus revealed an 80% amplification, no ADO but 15% of extreme preferential amplification, whereas the D19S112 showed an 85% amplification rate and a 15% allele dropout.

ADO is a major concern of single cell PCR and although no single protocol has managed to eliminate ADO completely, the use of F-PCR used in this study can increase 1000-fold the sensitivity of PCR (Hattori *et al,* 1992). Findlay and co-workers (1995) were able to demonstrate the advantages of F-PCR over conventional PCR and concluded that a number of cases of ADO are in fact preferential amplification of one of the alleles and that the other weakly amplified product is not detected by conventional analysis. Such results were seen during the clinical case for family G H when the DM DM1 locus presented in three blastomeres (15%) extreme cases of preferential amplification, which at the time of diagnosis were considered as ADO. However, upon reanalysis of the data it was observed that the larger allele (146) was not able to amplify in the blastomeres biopsied on the day of diagnosis. Normal samples with markedly preferential amplification of the father's DM DM1 allele can resemble an affected sample. Unless the mother's unaffected allele was present at an analysable level above the base line (peak height = 150) it was not considered safe to transfer the embryo. This was the case for embryo H3. It has been noted during our experience with single cell F-PCR analysis that it is more common for the large allele to be affected by preferential amplification or allele dropout. Similar findings were reported from Piyamongkol *et al* (2003) whilst trying to identify the reasons behind allele dropout.

The use of the STR markers on chromosome 21 (D21S1414) and 19 (D19S112) could also have the added benefit of providing copy number information for these chromosomes in the cells sampled. Sherlock *et al* (1997) utilised QF-PCR to detect small numbers of trisomic fetal cells isolated from the transcervical canal. However, when the same group was able to test the D21S1414 on single cells the method produced precise results for 75% of amplifications (Sherlock *et al,* 1998). This technique was based on the fact that the alleles of a heterozygous STR locus in a normal individual should amplify equally as long as the PCR is in the exponential phase of amplification (Mansfield, 1993). At an STR locus a trisomic subject has three alleles each amplified to the same extent (i.e. three different copies of the trisomic chromosome) or alternatively two alleles with one amplified twice as much as the other (i.e. two identical copies of the trisomic chromosome and one non-identical copy). Dean and colleagues (2001) were able to distinguish between an

embryo being monosomic (or mosaic) for chromosome 19 from an embryo being affected from ADO, by including an unlinked STR. During analysis the group observed only the affected parent's normal allele being amplified for the DM DM1 marker for both biopsied blastomeres, whilst the unlinked STR showed both alleles amplified, which was later confirmed from the spare embryos (Dean *et al,* 2001). Therefore, additional STR's during multiplex analysis can provide further genetic information and maximise the number of embryos in which a genotype can be assigned.

For both clinical cases the genotypes matched the expected genotypes that were known from the workup of control DNA and patient single cells. There was rarely a base pair difference (123 instead of 122) in the allele sizes. Apart from the unexpected fragments seen in embryo Gs2, there was no other unexplained amplification indicating that the results obtained were true genotypes of the embryos and DNA contamination was unlikely. Similar observations were observed from Dean *et al* (2000), however, their analysis was performed using an ALF automated sequencer (Pharmacia Biotech). Maximising the number of diagnosed embryos is particularly important in the case of dominant diseases since only 50% of all generated embryos will be expected to be normal and thus available for transfer.

It has been postulated that two cells per embryo should be biopsied in order to reduce misdiagnosis (Sermon *et al,* 1998; Ao *et al,* 1998; Ray *et al,* 1998). During this study it was appreciated that biopsy of two cells per embryo is a necessity in order to avoid misdiagnosis. Two cells are biopsied (in the UCL centre for PGD) from embryos with ≥6 cells. However, for family G only one blastomere was biopsied from each embryo due to reduced embryo development. This lead to attaining a complete result from only 60% of the embryos which were biopsied. An embryo that does not reach the 7-cell stage by 72h of development is less likely to progress to the expanded blastocyst stage (8.1% for ≤6 cells compared to 43.3% for ≥7 cells) (Shapiro *et al,* 2000). Although, no misdiagnosis occurred, the results for family H were enhanced due to the addition of the second cell.

In conclusion a comprehensive PGD protocol for DM DM1 has been developed using single step multiplex analysis of the normal DM DM1 triplet region and either a

polymorphic linked marker (D19S112) or a polymorphic unlinked marker (D21S1414). The linked marker will provide backup linkage analysis as well as contamination identification and when the linked marker is partially informative for a family, the unlinked D21S1414 marker can be used for the detection of contamination. In an ideal world triplex analysis would be performed for a PGD protocol by employing the mutation marker, a linked marker and an unlinked marker, in order to have backup linkage analysis as well as backup contamination exposure.

3.3.3 Universal PGD Protocol for DM

During the second part of this study the development of a PGD protocol for DM1 was attempted using a whole genome amplification method, namely DOP-PCR. DOP-PCR amplified genomic and single cell DNA was tested for genome coverage through amplification of the DM1 mutation marker along with another four polymorphic linked (D19S112) and unlinked markers (D21S1414, D18S535 and D13S305). Thus, the DOP-PCR coverage would be tested in at least four chromosomes (13, 18, 19 and 21). Such a protocol would eliminate the time consuming need for multiplexing and optimising different F-PCR protocols in single cells for different patients.

3.3.3.1 DOP-PCR on Genomic DNA

To be useful, a whole genome amplification procedure should have the following features: generation of long DNA fragments, successful amplification of starting DNA template, high amplification fidelity and yield, and good coverage of the genome. Due to the increased amount of information gathered from the workup of all the families (section 3.2.1) initially genomic DNA was amplified using DOP-PCR from family E (mother and father). The genomic DNA was approximately 100-300ng and was able to produce very distinct smears when run on agarose gel. All microsatellite markers showed similar amplification results when they were amplified from genomic DNA or from genomic DNA amplified using DOP-PCR (Figures 3.2, 3.3, 3.4 and 3.5). When the DOP-amplified DNA was used as a template for the further amplification of the five polymorphic markers, there was a slight increase of stutter bands especially for the DM1 marker and the D19S112 marker. This was not unexpected as mono- and dinucleotide repeats are well known to produce prominent stutter bands which might complicate the genotyping analysis (Ellegren, 2004).

Furthermore, all markers amplified using the DOP-PCR as template showed reduced amount of product, especially for the D21S1414 and D18S535 (Figures 3.3 and 3.4 respectively). However, all DOP-PCR amplified genomic DNA displayed the correct genotypes which was in accordance with the findings of Cheung and Nelson (1996) which analysed microsatellite repeats of DOP-PCR amplified genomic DNA (1ng concentration). There was no ADO present, although there were instances of mild preferential amplification of the smaller allele. Similar results have been reported from Cheung and Nelson (1996) who achieved 100% amplification of the markers and correct assignments of genotypes, though it was noticed that there was some preferential amplification of the shorter allele. Recently, Struan and colleagues (2002) managed to use DOP-PCR to amplify genomic DNA (1-40ng) and subsequently carry out single nucleotide polymorphism (SNP) genotyping. In the latter study DOP-PCR yielded satisfactory results but, displayed loss in accuracy and quality of the genotype assignments. Furthermore, a new method of DOP-PCR (LL-DOP-PCR) has been reported which is able to generate long fragments from pg quantities of genomic DNA (Kittler *et al,* 2002). It has been shown that by using LL-DOP-PCR, products of up to 10kb can be produced from less than one ng template genomic DNA, thus providing better coverage for microsatellite and unique sequences compared to the conventional DOP-PCR method. During this study, the overall amplification rate was 98% for all primers except for the D13S305 which was 96%.

3.3.3.2 DOP-PCR on Single Cells

Two individuals (X and Z) were chosen to donate 10 single cells each as well as genomic DNA. This was due to the fact that they were found to have heterozygous alleles for almost all the markers and especially for the DM DM1 mutation marker. The underlying reason was to be able to detect any allele dropout present in the most important polymorphic markers in this study, since a diagnosis would certainly involve this marker as well as the availability of fresh single cells. 100%Hundred percent amplification (20/20) was achieved for the DOP-PCR part of the study (Figures 5.6 and 5.7). Moreover, the single cells from individual G Z showed better amplification results compared to individual X. However, all single cells from both individuals were collected, isolated and amplified the same day in order to avoid freezing and thawing of the samples which might lead to dead or degenerating cells

and increase the levels of ADO (Piyamongkol *et al,* 2003). Only one cell from each individual (X4 and Z10) displayed reduced amplification with a smaller and fainter smear. This was expected since those particular buccal cells might have been in the process of degeneration or the lysis protocol was unable to completely lyse them. Furthermore, only in individual G Z the DOP amplified product showed some distinct bands in the 450bp region, which has previously been observed from Voullaire *et al,* (2000). For each individual two clumps of buccal cells (3-4 cells) and one sample of genomic DNA was also added to observe the differences between the amounts of starting DNA and act as a positive control. The negative controls for all single cells and of the DOP reaction displayed no amplification and were free of contaminants.

During the DOP-PCR amplified single cell DNA all microsatellite loci revealed decreased amplification, increased ADO and the incidence of incorrect genotype of about 10% (Table 5.2118). As seen in the DOP results, the genotyping results for the microsatellite loci regarding individual G Z showed better overall results. The combined amplification efficiency for all markers was 66% which was lower compared to the previously reported 85% amplification rate achieved from DOP-PCR amplified single cell DNA (Wells *et al,* 1999). The DM DM1 locus exhibited the highest amplification rate of 85% and the most reduced ADO rate of 10%. Overall, during this entire study it had been observed that the primer for the DM DM1 locus achieves high amplification rates, with minimal ADO and was not affected by the presence of other primers (e.g. during multiplex PCR). It was postulated during the study by Wells *et al* (1999), that unique sequences (such as the DM DM1 mutation marker) have a higher fidelity for replication compared to microsatellite loci (linked and unlinked STR's), which might account for the higher amplification results. However, it was noticed in this study that during amplification of the DM DM1 locus using DOP-PCR amplified single cell DNA, there was a greater increase of the +A artefact (section 5.4.1; Figure 5.10). The complete elimination of the final ten minute extension step and the reduction of the extension step during each cycle of amplification from one minute to 30 seconds were not found to improve the fidelity of the primer. However, these artefacts would not cause misdiagnosis since the true allele was easily distinguishable.

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The linked marker to the DM DM1 mutation, the D19S112, showed the loweeast amplification rate (55%) with one of the highest ADO rates (40%). However, this result was partly due to the low amplification seen in individual CX. Such reduced amplification was in contrast to the amplification seen for the D19S112 marker when amplified directly from single cell DNA (78.3%; Table 5.1613). Furthermore, the ADO rates nearly tripled compared to direct amplification (15%; Figure 5.8). It has been observed in previous studies that microsatellite loci show less reliability for replication (Wells *et al,* 1999) which is in accordance with this study. The D21S1414 microsatellite locus showed good amplification (65%), but, lower compared with direct amplification. A significant number of PCR artefacts were seen to affect the D21S1414 and D19S112 repetitive markers particularly small deletions and insertions. Allele sizes were increased or decreased by a number of base pairs equivalent to one repeat length (Figure 5.9). This phenomenon was locus specific but was shown to be present in all the markers though with decreased incidence. Similar findings were observed from Wells *et al* (1999), which suggested that this locus specificity is due to variation in factors such as chromatin structure, GC content and whether the repeat is perfect or disrupted by other sequences. Focault *et al* (1996) also reported such results when amplifying $(CA)_n$ repeats from less than ten single cells and concluded that this phenomena are due to replication slippage.

The highest incidence of allele dropout was exhibited from the D18S535 locus (50%; Table 5.2118). Allele dropout and preferential amplification was found to affect 60-83% of the heterozygous samples when amplified with repetitive microsatellite loci (Wells *et al,* 1999). ADO however, was rarely observed (3-5%) in unique sequences such as CFTR ΔF508 carrier cells in the same study. Preferential amplification was not often seen in this study however, it was shown to mainly affect the D18S535 and D13S305 loci (data not shown). This might be due to the fact that both microsatellite loci produce large products of more than 430bp. Piyamongkol and colleagues (2003) have noticed that the larger the amplified product the more prone to preferential amplification and ADO it will be. However, both loci showed the best results regarding sharp peaks, limited stutter peaks and +A artefact. Such results are in accordance with tetranucleotide microsatellite alleles being amplified directly from single cells.

During the analysis of the five markers after whole genome amplification of single cells, the incidence of incorrect genotyping was observed (Table 5.2118). Although a small number of single cells were tested, it was found that around 10-20% of the loci showed incorrect genotypes i.e. 1-2bp difference from true allele size and rarely 3-4bp difference e.g. for the D18S535 marker the correct genotype was 478/482, but, the analysis revealed 473/477. However, more single cells need to be tested and more loci investigated.

The poor fidelity of amplification seems to be attributable to the WGA reaction, namely the DOP-PCR. Firstly, whilst devising a PGD protocol for DM DM1 using F-PCR in the first part of this study, it was found that ADO was present in less than 20% when directly genotyping microsatellite markers from single cell DNA. This is in accordance with other studies that have amplified single cells (Sherlock *et al,* 1998; Sermon *et al,* 1998). When DOP-PCR amplified single cell DNA was genotyped with STR's, during this study, the overall ADO rate was 35%, suggesting that probably the DOP-PCR part was not reliable. Furthermore, different aliquots from the same DOP-PCR amplified product showed similar PCR artefacts and the increased failure of amplification of the five markers was random during this study. Wells *et al* (1999) noticed similar behaviour. However, when genomic DNA was amplified using DOP-PCR, the results were excellent and there were no increase in ADO and PCR artefacts. Hence, the success of the DOP-PCR reaction depends on the starting template DNA and if it is limited, the DOP-PCR enhances the problems of single cell PCR.

3.3.3.3 CGH and DM

In the final part of this study, the remaining DOP-PCR product was subjected to CGH analysis. It has been shown (Chapter 4) that DOP-PCR provides reliable and reproducible results for CGH analysis after amplification of single cell DNA. A total of 17/20 (85%) DOP-PCR amplified single cell DNA produced dynamic fluorescence and specific hybridisation, thus yielding analysable results. Both individuals (C and G) were female, hence, their amplified single cells were hybridised against control male DNA. In all 17 cells the sex status of the test samples was confirmed (Figure 3.11). Wells *et al* (1999) was also able to show reproducible CGH results when using as template DOP-PCR amplified single cell DNA. This additional test from the DOP-

PCR amplified single cells provides the opportunity in the context of PGD of investigating a specific single gene disorder as well as the chromosomal status of the embryos. Therefore, the phenomenon of mosaicism (Chapter 3) can be explored even in single gene analysis and a greater amount of information can be attained from a single cell. During a PGD case of myotonic dystrophy the affected embryos as well as the overall chromosomal status of the embryo can be distinguished, which will lead to better chances of a normal embryo being transferred.

3.3.4 Conclusions and Future Work

It was shown during this study that the development of multiplex F-PCR protocols for clinical cases of PGD are efficient and can provide accurate diagnosis. Two protocols were used for two families. Protocol 1 has been previously reported (Piyamongkol *et al,* 2001a) however it was found that certain modifications needed to be carried out to increase the fidelity of the amplifications. Unfortunately, only 5 embryos were biopsied and all were found to be unsuitable for transfer. The multiplex reaction for protocol 2 was devised specifically for family H and included a linked marker (D19S112), which could detect contamination and ADO. One embryo was found to be unaffected and was transferred but no pregnancy was achieved.

An alternative protocol was tested which would allow the detection of DM affected embryos as well as any whole chromosome abnormalities with the use of WGA. DOP-PCR amplified genomic and single cell DNA were used as templates for subsequent F-PCR reactions using five different markers. This would alleviate the problem of multiplexing and optimising protocols as well as creating patient-specific protocols. DOP-PCR amplified genomic DNA showed excellent results of amplifications from four individuals (mother and father of family E and individuals X and Z). DOP-PCR amplified single cell DNA showed reduced amplification efficiencies and increased ADO rates compared to direct amplification of the five markers from single cells. Although it was shown that in addition to single gene analysis, whole chromosome analysis using CGH can be carried out.

Future work should include analysis of more single cells (>1000) from more individuals, in order to assess the fidelity of amplification. Even though, DOP-PCR

amplified single cell DNA showed efficient CGH analysis, it did not provide acceptable coverage of the genome. Hence, the testing of novel WGA techniques such as multiple displacement amplification should be performed (sections 1.3.3.1.3 and 1.5.3.3).

Recently, the scope of PGD of single gene disorders using micro-arrays has been explored. Cystic fibrosis (CF), and specifically the ΔF508 mutation, has been used as a model disease to prove the diagnostic capability of micro-arrays for PGD (Salvado *et al,* 2004). A single micro-array platform was constructed using oligonucleotide probes for both the normal and ΔF508 disease alleles and the target DNA was fluorescently labelled which was hybridised to the array and the ΔF508 genotypes assigned from the fluorescence bound to ach allelic probe (Salvado *et al,* 2004). In a mix of samples (homozygous normal, homozygous affected, heterozygous, samples from previous PGD case for CF) strong binding of the target DNA to the probes was observed and all samples were correctly genotyped (Salvado *et al,* 2004).

CHAPTER 4

Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy

4.1 Aims

In this study the level of mosaicism was explored using sequential rounds of FISH in two groups of day 5 human embryos (mainly blastocysts). The chromosomes studied were 1, 11 and 18, X and Y. Three rounds of FISH were performed. The aim of this study was to develop a FISH protocol that can accurately and precisely determine the true level of abnormalities and mosaicism. This unique protocol employed two probes for each chromosome at different loci, which were analysed in sequential rounds of FISH. Such protocol technique would allow the direct distinction of FISH artefacts, failure of hybridisation that can occur during FISH analysis on human embryos. Any contradictive results between the two probes for the same chromosome were excluded from the analysis therefore revealing the true level of chromosomal abnormalities and specifically aneuploidies. So far previous studies have utilised only one probe per chromosome and have been hurdled with FISH failure and unexplained findings. However, with this novel protocol the FISH procedure is more robust and can aid in understanding the extent of mosaicism and most significantly the mechanisms leading to mosaicism.

4.2 RESULTS

4.2.1 Preliminary Work

Initial work included developing three FISH probe combinations that would yield high efficiencies whilst performing 3-round sequential FISH procedures. The various combinations that were attempted and their efficiencies are shown in Table 4.1. Most probes were commercially obtained however, probes e.g. 1het and 2cep were laboratory grown (Table 2.1). From the combinations, the 1/11/18 chromosome combination was picked since it produced high efficiencies and was able to test 3 chromosomes of different size. A 3rd round of FISH was included using the probe-cocktail for chromosomes X, Y and 18. This last round of FISH had a dual purpose. First, the probe for chromosome 18 was included in all rounds acting as a control and the presence of gonosome abnormalities can be investigated in blastocyst and their involvement in mosaic patterns.

Table 4.1. Various probe combinations that were attempted in order to find the most efficient 3-colour FISH in both rounds.

Chromosome combinations	Probes used in 1st round	Probe efficiency	Probes used in 2nd round	Probe efficiency
1 dual / 8 dual	1het / 1p	95% / 93%	8cep / 8q	94% / 87%
4 / 16 dual & 4 / 11 dual	4cep / 16p / 16q	97% / 95% / 97%	4cep / 11cep / 11q	89% / 94% / 90%
7 dual# / 18 & 11 dual / 18	7q / 7q / 18cep	91% / 92% / 95%	11cep / 11q / 18cep	91% / 89% / 94%
2 / 4 / 5	2q / 4cep / 5p	95% / 98% / 94%	5p&5q */ 2cep	80%&78% / 67%&61%
1 / 11 / 18	1p / 11q / 18cep	94% / 97% / 96%	1het / 11cep / 18q	88% / 89% / 90%
X / Y / 18 (cocktail)	Xcep / Ycep / 18cep	96% / 95% / 98%	Xcep / Ycep / 18cep (3rd Round results)	94% / 90% / 93% (3rd Round results)

The probe was the 7 (7q11.23 and 7p31) Willams microdeletion probe
* The probe was the 5 (5q31 and 5p15.2) Cri-du-Chat microdeletion probe
The colours show the corresponding fluorescent labels i.e. orange-spectrum red, green-spectrum green, blue-spectrum aqua. cep = centromeric or heterochromatic probe, p = sub-telomeric probe for the small arm, q = sub-telomeric probe for the big arm

The chosen combination was then applied to 10 arrested day 5 human embryos (118 blastomeres) donated from 3 patients (data not shown). This was performed to assess the spreading technique in order to reduce loss of cells and more significantly to evaluate the FISH protocol. The preliminary results showed high FISH efficiencies with bright and sharp signals in all three rounds of FISH and minimal cell loss. Overall, 101 blastomeres were FISHed for all sequential rounds out of 118 blastomeres counted before spreading (86%). 98/101 blastomeres (97%) produced visible and foremost interpretable results for all FISH rounds. The embryos were analysed in two separate FISH procedures (five embryos each time). In each of the two procedures a control male lymphocyte slide was also included in the FISH

procedure and 200 interphase nuclei were counted to calculate the probes' efficiency. The yields were 97%, 91% and 88% for the 1st, 2nd and 3rd round respectively.

4.2.2 FISH Study

Twenty-seven couples donated a total of 47 embryos for this research project (Table 2.9). The overall mean maternal age was 33.7 (range 19-41) years. The mean maternal age for Group I embryos was 35.2 (range 27-41) and for Group II was 32.1 (range 19-41) years. Five arrested embryos were not included, since the signals were not analysable due to loss of most nuclei during sequential rounds of FISH and poor quality of the nuclei. Embryos from both groups included nuclei which showed contradicting information for the two probes used for each autosome in the different rounds of FISH. These cells were classed as *inconsistent results* and excluded from the analysis.

4.2.2.1 FISH Analysis of Controls

Each FISH experiment included a control male lymphocyte slide with mapped nuclei in order to assess efficiency of probe hybridisation in the sequential rounds. Overall, 87.1% (range 78-96) of the control nuclei showed normal signals for all 8 probes used. Sub-telomeric probes for chromosomes 1p, 11q and 18q, showed a higher incidence of one signal per chromosome per nucleus, 7.8% (range 4-11%), 3.1% (range 1.2-4.5%) and 3.9% (range 2-5%) respectively. Heterochromatic region or centromeric probes, 1het, 11CEP and 18CEP (in both 1st and 3rd rounds) displayed a lower incidence of one signal per chromosome per nucleus, 6.2% (range 4.4-7.5%), 2.4% (range 0.9-3.3%) and 2.2% (1.5-3.4%) respectively (Table 4.2). Nuclei with one signal for the X chromosome and no signal for chromosome Y and nuclei with three or more signals for autosomes comprised less than 1%. Furthermore, all three sub-telomeric probes, 1p, 11q and 18q showed the occurrence of split signals (i.e. replicated DNA) in 2.2%, 1.4% and 1.9% of nuclei respectively, which was considered normal due to the position of the probes. The difference in the efficiency of the 18cep probe (used in the 1st and 3rd round of FISH) was not statistically significant ($p<0.05$) and can be attributed to the fact that FISH efficiency decreases with sequential rounds of hybridisation due to the DNA being over-processed.

Table 4.2. Probe efficiencies scored in 200 interphase nuclei of each control slide whilst carrying sequential 3-round FISH.

Round of FISH	**Probe**	% of missing a signal		Split signals	
		Mean	Range	Mean	Range
1st	**1p**	7.8	4-11	2.2	1.1-2.8
	11q	3.1	1.2-4.5	1.4	0.4-1.9
	18cep	2.2	1.5-3.4	0.3	0.1-0.4
2nd	**1het**	6.2	4.4-7.5	0.4	0.3-0.7
	11cep	2.4	0.9-3.3	-	-
	18q	3.9	2-5	1.9	0.8-2.4
3rd	**Xcep**	0.9	0.5-1.1	-	-
	Ycep	0.5	0.1-0.9	-	-
	18cep	3.4	2.1-4.3	0.5	0.4-0.8

The results for the probes were calculated during sequential rounds of FISH, hence each control slide was subjected to 3 rounds of FISH. The table lists only the percentages where a signal was missing, hence 1 signal was seen for the autosomes and no signal for the gonosomes. The incidence of extra and complete lack of signals was minute (<0.4%) for all the probes tested.

4.2.2.2 FISH Analysis of Embryos

Twenty-one embryos were analysed for each group. FISH results for Group I and Group II embryos are shown in Tables 4.3 and 4.6 respectively. In Group I the total number of nuclei analysed was 401/410 (9 lost during sequential rounds of FISH). Thus, the mean number of nuclei analysed was 20 (range 4-45). In all, 72% (±20 SD) of the nuclei analysed were diploid for the chromosomes tested (Figure 4.1) however only a single embryo was uniformly diploid for the tested chromosomes (embryo 9.1). One embryo was aneuploid mosaic, and 14 were diploid mosaics (4.3). Three embryos contained chaotic complements (embryos 8.1, 12.2 and 17.1) and thus a mechanism of mosaicism could not be suggested for those embryos. Since 19/21 embryos were considered arrested for Group I (<30 cells), four embryos contained polyploidy cells and predominantly tetraploidy cells, however, 17.2 had hexaploid and octaploid cells. One embryo only (20.1) contained a haploid chromosome complement for all five chromosomes tested. Although, this is quite low compared to other studies, haploid cells have been characterised as less viable and less actively dividing than tetraploid cells (Ruangvutilert *et al,* 2000a)

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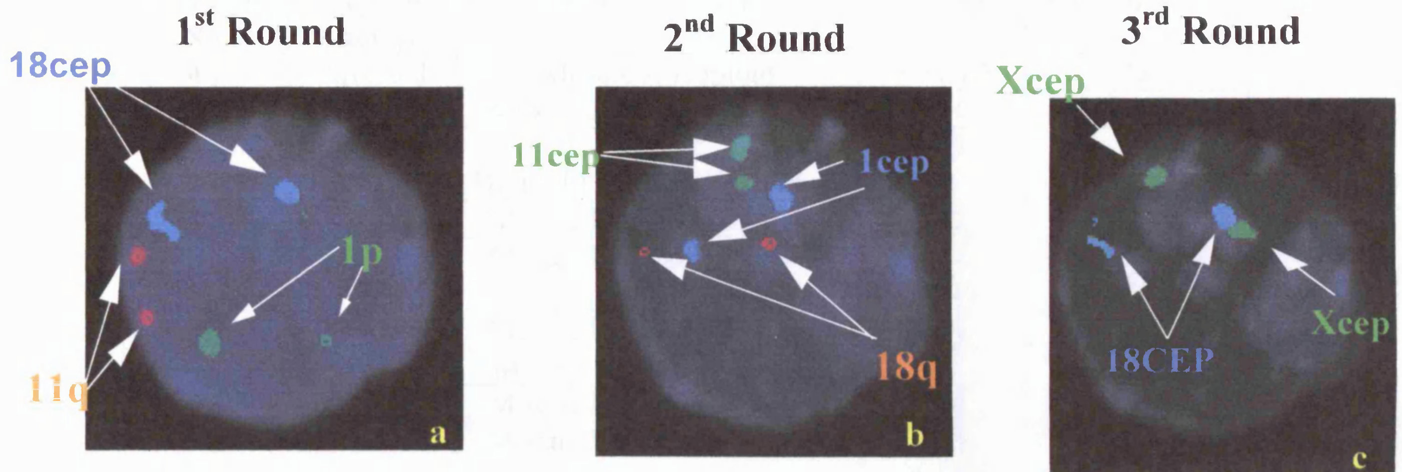
Table 4.3. Results of FISH analysis of Group I embryos after three sequential rounds of hybridisation with probes for chromosomes 1, 11, 18, X and Y

Embryo No.	Cells analysed	Sex	Chromosome constitution of cells (number)	Diploid (%)	Classification
4.1	4	F	-18 (3) / +18 (1)	0	Mosaic Aneuploid
5.1	9	M	Dip (7) / +X, +Y (1) / *(1)	77.8	Mosaic Diploid/Aneuploid
5.2	10	F	Dip (6) / tet (3) / -1 (1)	60	Mosaic Diploid/Polyploid/Aneuploid
5.3	20	F	Dip (15) / -1 (1) /-X (1) / *(3)	75	Mosaic Diploid/Aneuploid
5.4	22	F	Dip (18) / -1 (2) /+18 (1) / *(1)	81.8	Mosaic Diploid/Aneuploid
5.5	4	F	Dip (3) / -1 (1)	75	Mosaic Diploid/Aneuploid
6.1	22	M	Dip (17) / tet (2) / *(3)	77.2	Mosaic Diploid/Polyploid
7.1	26	F	Dip (24) / -1 (1) / -11 (1)	92	Mosaic Diploid/Aneuploid
7.2	9	M	Dip (7) / -1 (1) / -1, +X (1)	77.8	Mosaic Diploid/Aneuploid
8.1	6	M	Dip (4) / +X, +Y (1) / chaotic (1)	66.7	Mosaic Diploid/Aneuploid/Chaotic
9.1	8	F	Dip (7)/ *(1)	87.5	Uniformly Diploid
10.1	5	F	Dip (4) / -11 (1)	80	Mosaic Diploid/Aneuploid
12.1	13	M	Dip (6) / -18, -Y (3) / -Y (1) / -18, +Y (1) / -18 (1) / -1 (1) / *(1)	46.2	Mosaic Diploid/Aneuploid
12.2	30	F	Dip (22) / -11 (1) / -18 (1) / -X (1) / -1, -18 (1) / -11, -18 (1) / chaotic (3)/ *(1)	73.3	Mosaic Diploid/Aneuploid/Chaotic
17.1	45	M	Dip (40) / +Y (2) / chaotic (2) / *(1)	88.9	Mosaic Diploid/Aneuploid/Chaotic
17.2	12	F	Dip (9) /tet (3)	75	Mosaic Diploid/Polyploid
20.1	43	F	Dip (34) / -18 (4) / +11 (4) / hap (1) / *(3)	79	Mosaic Diploid/Aneuploid/Haploid
21.1	27	M	Dip (22) / hex (3) / oct (1) / *(1)	81.5	Mosaic Diploid/Polyploid
21.2	29	F	Dip (25)/ +11 (1) / +1, +11 (1) / *(2)	86.2	Mosaic Diploid/Aneuploid
27.1	24	M	Dip (16) / +Y (2) / +18 (1) / +X (1) / *(4)	66.6	Mosaic Diploid/Aneuploid
27.2	33	M	Dip (28) / +Y (1) / *(4)	84.8	Mosaic Diploid/Aneuploid

* Inconsistent results that were observed during scoring. **dip** = diploid, **tet** = tetraploid, **trip** = triploidy, **hex** = hexaploidy, **oct** = octaploidy.
The - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

Figure 4.1. Results of three sequential rounds of FISH analysis on a single female embryonic nucleus from embryo 9.1.



a) Using probes 1p, 11q and 18cep, b) using probes 1het, 11cep and 18q and c) using probes for Xcep, Ycep and 18cep. In all three rounds the cell appears to be diploid. The probe 18CEP is present in rounds 1 and 3 and shows identical results.

In Group I there were 32 post-zygotic errors (16 chromosome losses, 14 chromosome gains and 2 instances of mitotic non-disjunction) (Table 4.4). There was no statistical significance ($p<0.05$) between chromosome loss and chromosome gain. For the chaotic blastomeres the mechanism causing mosaicism was not included due to the nature of chaotic cells. Embryo 12.2 showed the highest incidence of aneuploid mosaicism involving 4 chromosomes (chaotic cells not included). The two instances of MND were for chromosomes 18 and Y in embryos 4.1 and 12.1.

Table 4.4. Aneuploidy mosaicism mechanisms for Group I embryos

Embryo No.	Type of embryo	Classification	Events	Chromosome
4.1	Arrested	Mosaic Aneuploid	1 MND	18
5.1	Arrested	Mosaic Diploid/Aneuploid	2 CG	X, Y
5.2	Arrested	Mosaic Diploid/Polyploid/Aneuploid	1 CL	1
5.3	Arrested	Mosaic Diploid/Aneuploid	2 CL	1, X
5.4	Arrested	Mosaic Diploid/Aneuploid	1 CL 1 CG	1 18
5.5	Arrested	Mosaic Diploid/Aneuploid	1 CL	1
6.1	Arrested	Mosaic Diploid/Polyploid	-	-
7.1	Arrested	Mosaic Diploid/Aneuploid	2 CL	1, 11
7.2	Arrested	Mosaic Diploid/Aneuploid	1 CL 1 CG	1 X
8.1	Arrested	Mosaic Diploid/Aneuploid/Chaotic	2 CG	X, Y
9.1	Arrested	Uniformly Diploid	None	-
10.1	Arrested	Mosaic Diploid/Aneuploid	1 CL	11
12.1	Arrested	Mosaic Diploid/Aneuploid	2 CL 1 MND	1, 18 Y
12.2	Arrested	Mosaic Diploid/Aneuploid	4CL	1, 11, 18, X
17.1	Arrested	Mosaic Diploid/Chaotic/Aneuploid	1 CG	Y
17.2	Arrested	Mosaic Diploid/Polyploid	-	-
20.1	Expanded Blastocyst	Mosaic Diploid/Aneuploid/Haploid	1 CL 1 CG	18 11
21.1	Arrested	Mosaic Diploid/Polyploid	-	-
21.2	Arrested	Mosaic Diploid/Aneuploid	2 CG	1, 11
27.1	Arrested	Mosaic Diploid/Aneuploid	3 CG	18, X, Y
27.2	Arrested	Mosaic Diploid/Aneuploid	1 CG	Y
Total 21		-	**16 CL (50%)** **14 CG (44%)** **2 MND (6%)**	

CL = chromosome loss, CG = chromosome gain, MND = mitotic non-disjunction

Table 4.5 summaries the breakdown by chromosome. Chromosome 1 was mostly affected by chromosome loss (7 events) whereas the sex chromosomes showed the highest incidence of chromosome gain. The Y chromosome did not display any chromosome loss events, although it was affected greatly by chromosome gain and

mitotic non-disjunction. Inconsistent results affected 26/401 (6.5%) nuclei and were considered to be FISH artefacts

Table 4.5. Mechanisms of mosaicism by chromosome for Group I embryos

Event / Chromosome	Chromosome Loss	Chromosome Gain	Mitotic Non-Disjunction
1	7	1	0
11	3	2	0
18	4	2	1
X	2	4	0
Y	0	5	1
Total	16	14	2

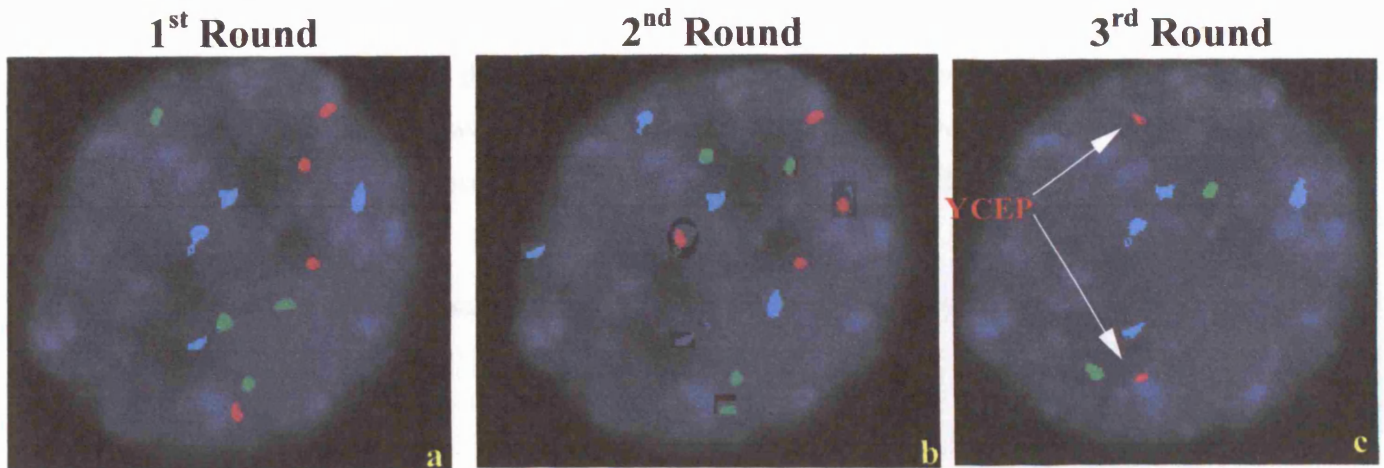
Table 4.6 shows the results of Group II embryos. The total number of nuclei analysed was 1143/1171 (28 nuclei lost during sequential rounds of FISH). The mean number of nuclei per embryo was 55, ranging from 20-100. 78% (±15 SD) of the nuclei analysed were found to be diploid, although only two embryos were found to be uniformly diploid (16.1 and 23.1) as tested. The majority (16/21) of embryos were found to have some tetraploid cells (confirmed in all rounds), which totalled approximately 7% of the abnormal cells analysed (Figure 4.2). Six out of twenty-one embryos included a chaotic complement. Those embryos included at least one cell, which had more than three abnormalities per cell, including nullisomies and tetrasomies. Furthermore, there was one embryo (15.4), which was a diploid/aneuploid/haploid/chaotic mosaic. This embryo contained only one cell which was haploid, with the results being consistent in all rounds, and three aneuploid cells affecting chromosomes 18 and X (Figure 4.3). 20/21 embryos in Group II were dividing embryos and only one was arrested (13.1). Ten embryos were male and eleven embryos were female, hence no difference was observed in abnormalities regarding the sex, although both uniformly diploid (for the chromosomes tested) embryos were male.

Table 4.6. Results of FISH analysis of Group II embryos after three sequential rounds of hybridisation with probes for chromosomes 1, 11, 18, X and Y

Embryo No.	Cells analysed	Sex	Chromosome constitution of cells (number)	Diploid (%)	Classification
13.1	29	F	Dip(13) / trip(7) / tet(5) / -18(1) / +1, +11(1) / chaotic (1) / *(1)	44	Mosaic Diploid/Polyploid/Aneuploid/Chaotic
13.2	38	F	Dip(18) / tet(8) / trip(4) / hex(1) / enn(1) / +1, +11(1) / -1(1) / chaotic (2) / *(1)	47.4	Mosaic Diploid/Polyploid/Aneuploid/Chaotic
13.3	43	F	Dip(33) / tet(6) / trip(1) / chaotic (1) / *(2)	76.7	Mosaic Diploid /Polyploid/Chaotic
14.2	20	F	Dip(12) / -X(3) / +X(2) / +11(1) / tet(1) / chaotic (1)	60	Mosaic Diploid/Aneuploid/Polyploid/Chaotic
14.3	43	M	Dip(36) / tet(2) / chaotic (3) / *(2)	85.7	Mosaic Diploid/Polyploid/Chaotic
15.1	34	F	Dip (31) / tet (3)	91.7	Mosaic Diploid/Polyploid
15.2	100	M	Dip(67) /tet(22) / trip(5) / -18(1) / -11, -18(1) / *(4)	67	Mosaic Diploid /Polyploid/Aneuploid
15.3	62	F	Dip(57) / tet(1) / trip(1) / +11(1) / *(2)	91.9	Mosaic Diploid /Polyploid/Aneuploid
15.4	33	F	Dip(26) / -X(2) / hap(1) / -18(1) / chaotic (1) / *(2)	72.2	Mosaic Diploid/Aneuploid/Haploid/Chaotic
16.1	57	M	Diploid (57)	100	Uniformly Normal
18.1	57	M	Dip (43) / +X(2) / +Y(2) / -11(2) / -X(1) / -Y(1) / *(6)	75.4	Mosaic Diploid/Aneuploid
19.1	76	F	Dip(66) / -X(3) / -18(2) / tet(1) / -11(1) / *(3)	86.8	Mosaic Diploid/Aneuploid/Polyploid
19.2	94	M	Dip(81) / tet(5) / trip(1) / +18(2) / -Y(1)/ +X, +Y (1) / *(3)	86.1	Mosaic Diploid/Polyploid/Aneuploid
22.1	75	F	Dip(67) / +X(2) / tet(1) / -18(1) / -X(1) / *(3)	89.3	Mosaic Diploid/Aneuploid/Polyploid
22.2	67	F	Dip(55) / tet(3) / -X(3) / +1, +X(2) / +11, -18(1)/ +X(1) / *(2)	83.3	Mosaic Diploid/Aneuploid/Polyploid
23.1	100	M	Diploid(94) / *(6)	94	Uniformly Normal
24.1	63	M	Dip(55) / tet(4) / *(4)	87.3	Mosaic Diploid/Polyploid
25.1	62	F	Dip(53) / tet(3) / -18(3) / *(3)	85.5	Mosaic Diploid/Polyploid/Aneuploid
25.2	31	M	Dip(24) / +X(2) / tet(1) / -18(1) / -18, -X(1) / *(2)	77.4	Mosaic Diploid/Aneuploid/Polyploid
26.1	30	M	Dip(20) / +1, +11(2)/ -1(1)/ +X(1)/ +X, +Y(1)/ chaotic (2)/ *(3)	66.6	Mosaic Diploid/Aneuploid/Chaotic
26.2	31	M	Dip(19) / tet(5)/ trip(1)/ -11, -18(1)/ +X(1)/ chaotic (2)/ *(2)	61.3	Mosaic Diploid/Polyploid/Aneuploid/Chaotic

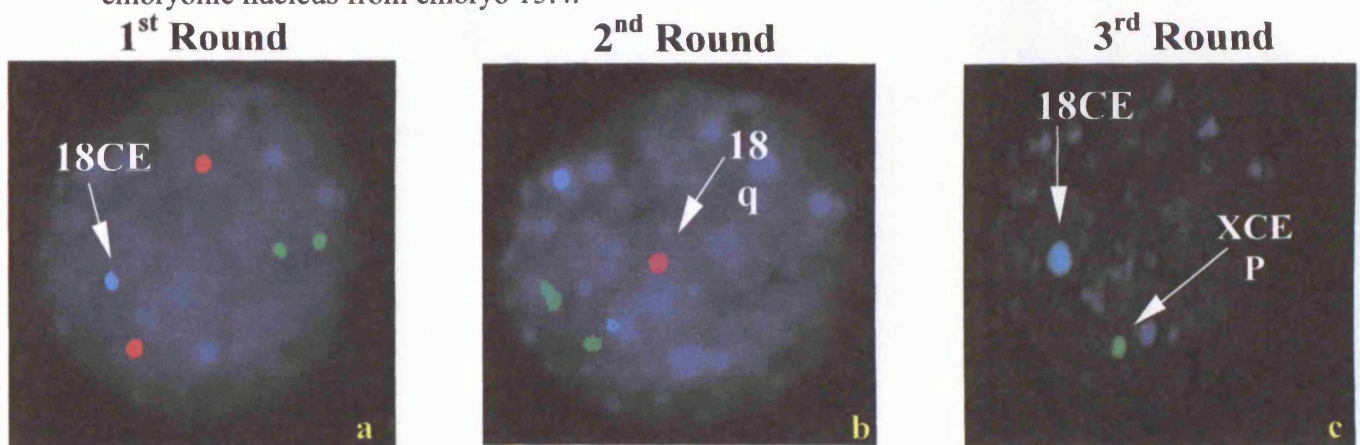
* Inconsistent results that were observed during scoring. **dip** = diploid, **tet** = tetraploid, **trip** = triploidy, **hex** = hexaploidy, **oct** = octaploidy, **enn** = enniaploidy. The - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

Figure 4.2. Results of three sequential rounds of FISH analysis on a single male embryonic nucleus from embryo 13.2.



a) Using probes 1p, 11q and 18cep, b) using probes 1het, 11cep and 18q and c) using probes for Xcep, Ycep and 18cep. This nucleus displayed a tetraploid chromosome complement in all three rounds of FISH. In the 3rd round there are 2 signals for X and 2 signals for Y.

Figure 4.3. Results of three sequential rounds of FISH analysis on a single female embryonic nucleus from embryo 15.4.



a) Using probes 1p, 11q and 18cep, b) using probes 1het, 11cep and 18q and c) using probes for Xcep, Ycep and 18cep. This nucleus displayed monosomy 18 in all three rounds and XO in the final round of FISH. Therefore, two events of chromosome loss were reported for this nucleus.

In Group II 37 post-zygotic errors took place, which gave rise to 14 mosaic aneuploid embryos (Table 4.7). Some of those embryos also contained polyploid, chaotic and haploid cells.

Embryos 22.2 and 26.1 displayed the most chromosomal events affecting four chromosomes, sharing between them four chromosome losses, two chromosome gains and two mitotic non-disjunctions as mechanisms leading to aneuploid mosaicism.

Table 4.7. Aneuploidy mosaicism mechanisms for Group II embryo

CL = chromosome loss, CG = chromosome gain, MND = mitotic non-disjunction

Embryo No.	Classification	Number of Events	Chromosome
13.1	Mosaic Diploid/Polyploid/Aneuploid/Chaotic	1 CL 2 CG	18 1, 11
13.2	Mosaic Diploid/Polyploid/Chaotic	1 CG 1 MND	11 1
13.3	Mosaic Diploid /Polyploid/Chaotic	-	-
14.2	Mosaic Diploid/Aneuploid/Polyploid	1 CG 1 MND	11 X
14.3	Mosaic Diploid/Polyploid/Chaotic	-	-
15.1	Mosaic Diploid/Polyploid	-	-
15.2	Mosaic Diploid /Polyploid/Aneuploid	2 CL	11, 18
15.3	Mosaic Diploid /Polyploid/Aneuploid	1 CG	11
15.4	Mosaic Diploid/Aneuploid/Haploid/Chaotic	2 CL	18, X
16.1	Uniformly Normal	None	-
18.1	Mosaic Diploid/Aneuploid	1 CL 2 MND	11 X, Y
19.1	Mosaic Diploid/Aneuploid/Polyploid	3 CL	11, 18, X
19.2	Mosaic Diploid/Polyploid/Aneuploid	2 CG 1 MND	18, X Y
22.1	Mosaic Diploid/Aneuploid/Polyploid	1 CL 1 MND	18 X
22.2	Mosaic Diploid/Aneuploid/Polyploid	1 CL 2 CG 1 MND	18 1, 11 X
23.1	Uniformly Normal	None	-
24.1	Mosaic Diploid/Polyploid	-	-
25.1	Mosaic Diploid/Polyploid/Aneuploid	1 CL	18
25.2	Mosaic Diploid/Aneuploid/Polyploid	1 CL 1 MND	18 X
26.1	Mosaic Diploid/Aneuploid/Chaotic	3 CG 1 MND	11, X, Y 1
26.2	Mosaic Diploid/Polyploid/Aneuploid/Chaotic	2 CL 1 CG	11, 18 X
TOTAL 21 embryos	-	**15 CL (40.5%)** **13 CG (35.1%)** **9 MND (24.4%)**	

Moreover, in Group II there were nine MND events, mostly affecting chromosome X, occurring in comparison to two in Group I embryos (Table 4.5). Once again, chromosome loss was the predominant mechanism leading to mosaic aneuploidy (Table 4.8), but in this group it involved mainly chromosome 18, while an additional copy of chromosome 11 was the most frequent gain. FISH artefacts (inconsistent results) affected 50/1143 (4.4%) of nuclei.

Table 4.8. Mechanisms of mosaicism by chromosome for Group II embryos

Event / Chromosome	Chromosome Loss	Chromosome Gain	Mitotic Non-Disjunction
1	0	2	2
11	4	6	0
18	9	1	0
X	2	3	5
Y	0	1	2
Total	**15**	**13**	**9**

Of the nuclei showing inconsistent results, 34/76 (45%) were due to failure of hybridisation of the sub-telomeric probe for chromosome 18 (18q). The probe for the satellite II/III region of chromosome 1, exhibited the highest failure rate of the centromeric probes (14.5%) (Table 4.9). The underlying reason for the high rate of artefacts for the heterochromatic probe for chromosome 1 can be attributed to the fact that it was the only laboratory prepared probe. Group II embryos showed increased number of FISH artefacts (67%), termed '*inconsistent results*' (highlighted in red colour in Tables 4.3 and 4.6), compared to Group I (33%). This finding, however, might due to the difference in the number of cells analysed for each group.

Table 4.9. Failure rates for each probe in the embryonic nuclei for both Groups.

Probe	1p	11q	18q	1HET	11CEP	18CEP	**Total**
No. of FISH artefacts	12	6	34	11	7	6	**76**
Percentages	15.8%	7.9%	45%	14.5%	9%	7.9%	

4.3 Discussion

During this study the levels and mechanisms leading to aneuploid mosaicism in human embryos were investigated. This was achieved by developing a triple colour FISH protocol carried out in three sequential rounds of hybridisation. The use of two probes per autosome and the dual use of a chromosome 18 centromere probe allowed the distinction between true single cell anomalies and artefacts, which showed up as inconsistent results. This study was able to accurately define the true extent of chromosomal mosaicism in human embryos without the use of statistical aids like previous studies have done. Of the 42 day 5 embryos, three only were uniformly diploid for the tested chromosomes, a single embryo was the product of a meiotic error and the remainder were mosaic. Furthermore, it was found that chromosome loss was the main mechanism leading to mosaic embryos.

4.3.1 Preliminary Work Assessment

Early work to develop interphase cytogenetics using FISH was hampered in part by the lack of access to a reliable panel of locus-specific DNA probes and as such was limited in scope (Griffin *et al,* 1991; Griffin *et al,* 1992). Methods of screening DNA clones for use as FISH probes can be labour intensive and time-consuming as many will be chimaeric (large BAC/YAC clones etc), map to another location (cross-hybridize) or produce weak FISH signals and so need to be discarded. The plasmid clone for the 1het centromeric probe used in this study was found to be suitable, producing discrete, easily scored FISH signals in lymphocyte interphase nuclei. Fortunately, the increasing availability of a wide range of commercial probes has made the method of producing 'laboratory prepared' diagnostic probes largely redundant. These commercial probes have revolutionised the use of interphase cytogenetics particularly in clinical laboratories where FISH is now used routinely to complement standard karyotyping for a wide range of pre- and postnatal applications (Blennow *et al,* 1995; Knight and Flint, 2000; Quilter *et al,* 2001). In addition the introduction of multi-probe cocktails specifically designed for polar bodies and blastomere analysis have contributed to the increase in the number of groups carrying out PGD and related research. Finally, the introduction and subsequent general accessibility to subtelomeric probes for all chromosomes has greatly simplified the

strategies for FISH protocols. The majority of the probes used in this study were commercially available.

Whilst carrying out FISH in order to analyse chromosome constitutions several difficulties emerge such as scoring errors, cross-hybridisation, background fluorescence as well as low quality fluorescent signals (Griffin *et al*, 1994). Therefore, during this study several FISH protocols were carried out using different probe combinations and changing the stringency conditions (salt and formamide concentration, temperature and pH), on control male blood lymphocytes. The most efficient protocol was found to be a sequential FISH protocol involving chromosomes 1, 11, 18, X and Y. It has been postulated from Bielanska *et al* (2002b) that similar rates of mosaic imbalances can be detected from different probe combinations either for three or five chromosomes. The first three chromosomes were examined in both the first two rounds, using probes for different loci for each chromosome. Similar double loci analysis for just one chromosome was concluded as a preferable method of avoiding false monosomies whilst carrying out PGD for aneuploidy (Magli *et al*, 2001). Furthermore, the use of two differentially labelled probes to detect a single chromosome increased the accuracy of detection, by reducing scoring errors and confirming that the mosaicism observed in previous preimplantation diagnosis cycles is not a FISH artefact owing to hybridisation failure or overlapping signals, even when just one cell differs and no cell resulting from a reciprocal non-disjunction event is found (Conn *et al*, 1999). This study was able to detect any failure of hybridisation or FISH artefacts since the three major chromosomes studied (1, 11 and 18) were analysed in two sequential rounds using differentially labelled probes at different loci. Where differences occurred they were defined as "*inconsistent results*". These inconsistent results have not been included in the interpretation of the results. Other studies have compared levels of aneuploidy present in embryos and only if that proportion was higher than the value obtained from the control lymphocytes, its presence was considered true (Ruangvutilert *et al,* 2000b). However, in this study such comparison was not needed and the genuine level of aneuploidy and mosaicism could be detected due to the safety net provided by the two probes per chromosome analysis.

As a three sequential round of FISH protocol was used in this study it was possible to assess the efficiency of each FISH experiment in each round. A control slide from male blood lymphocytes was used that was FISHed at the same time as the embryos and scored. 200 interphase nuclei were scored for all probes in all the rounds. This step enabled observation of whether the FISH technique worked efficiently without the presence of cross-hybridisation and background fluorescence and assessed the efficiency of a third-round of hybridisation. All three rounds showed high efficiencies when the same slide was FISHed sequentially (Table 4.2). However, the efficiency of the 3rd hybridisation decreased by 9% (to 88%) in comparison with the 1st round of FISH. This seems to be due to the decrease in the quality of the nuclei DNA and has been confirmed by other studies (Ruangvutilert *et al*, 2000a). Also, the image quality of the nuclei and fluorescent signals decreased in intensity during sequential hybridisations. Hence, during the denaturation step the slides were incubated in a 75°C waterbath containing 70% formamide solution instead of incubation on a hot plate in an oven. This step showed an increase the intensity of the signals and nuclei quality (data not shown).

4.3.2 Distinction between Artefacts and True Results

The use of two probes per autosome allowed this study to detect an error rate of 5% per nucleus and to exclude those artefacts from the analysed results. For both Groups a true chromosomal error in a total of 76 nuclei would have been missed if only one probe per chromosome had been used. Therefore, by adopting our chosen strategy we were able to detect the true levels of mosaicism for the three autosomes studied. The probe that showed the highest rate of failure of hybridisation on embryonic material was the sub-telomere probe for chromosome 18 (Table 4.9). Sub-telomeric probes generally have a lower hybridisation efficiency than probes that detect repeat sequences, but in this case the situation was possibly exacerbated because the probe was used in the second treatment round. However, in the lymphocyte control nuclei, the probes for chromosome 1, both for the sub-telomeric (used in the first round) and heterochromatic regions, showed the highest failure rates.

Many of the aneuploidies detected in our study were of a single chromosome and confined to a single cell. Other studies would have dismissed these aneuploidies as FISH artefacts due to their lack of probe backup. Low level aneuploidy is rarely considered to be a mosaicism phenomenon and it is usually classed as normal (Group I embryos in Table 1.2 from Munne and Weier, 1996). Bielanska and co-workers (2002a) postulated that minor aneuploid cell lines indicated that the aneuploid cells did not persist from early cleavage, but were formed at, or shortly before, blastulation, and suggest that most cleavage stage embryos with a high degree of mosaic aneuploidy do not complete preimplantation development. It is clear from CGH data on full analysis of individual blastomeres that single cell anomalies affecting one chromosome are a common feature at cleavage stages (Voullaire *et al*, 2000; Wells and Delhanty 2000). In these two small series, nine of the 24 embryos fully analysed showed such single cell anomalies. Furthermore, unlike that which has been observed in diploid-tetraploid mouse aggregation chimeras (James and West, 1994), recent studies of human blastocysts did not show evidence of preferential allocation of aneuploid cells into the trophectoderm lineage (Evsikov and Verlinsky, 1998; Magli *et al*, 2000)

4.3.3 Group I Embryos

The embryos that were left to grow in the basic media showed a decreased rate of growth. From the 21 embryos spread, the mean number of blastomeres present was 20, which was low considering that normal developing day 5 embryos should have more than 30-40 cells on day 5 of development. Hence, all embryos, apart from two, from Group I were considered arrested (less than 30 cells). The fact that almost all embryos were arrested in this group was due to the media they were grown in. Group I embryos were left into the medium which contains lactate and pyruvate which are considered to be adverse for healthy embryonic development after genome activation (Gardner and Sutherland, 1996; section 1.1.3).

The FISH results showed a high prevalence of mosaicism in this group of embryos (95%, 20/21 embryos). Veiga *et al* (1999) observed a lower rate of mosaicism (62.5%) in arrested embryos however, most mosaic embryos included a chaotic complement. The prevalent type of mosaicism was diploid/aneuploid mosaics (76%)

and the predominant mechanism that lead to mosaicism was chromosome loss (50%) which is in contrast with other studies (Munne, 2002) that showed that mitotic non-disjunction was the most common type of mosaicism. This is due to the fact that most studies rely on one probe per chromosome in order to analyse the highest number of chromosomes simultaneously, thus sacrificing the reliability of their results. Monosomies, when carrying out FISH using one probe per chromosome, are considered false based on the fact that it was failure of hybridisation or a FISH artefact (if they are found at low percentages). However, this diminishes the ability to determine the true values from the false ones. This study was able to confirm whether a monosomy was false or true and classified as an inconsistent result or as true monosomy respectively.

Some mosaic embryos containing aneuploid cell lines arose due to chromosome loss and gain such as embryo 5.4, which had a cell monosomic for chromosome 1 and a cell trisomic for chromosome 18 (Table 4.4). Therefore, two separate mechanisms lead to the occurrence of such mosaic embryos. Diploid/aneuploid mosaic embryos resulting from mitotic non-disjunction were only found in two embryos "4.1" and "12.1" for chromosomes 18 and X respectively (Table 4.4). In mosaic diploid/aneuploid embryos arising from MND, there was a reciprocal loss and gain of the same chromosome in different embryonic nuclei. Three embryos (14%) were classified as diploid/aneuploid/chaotic (8.1, 12.2 and 17.1). There was no apparent mechanism that can identify the reason of the chaotic lines. Although, in these embryos there was a high percentage of diploid nuclei (67-89%), the remaining cells were either aneuploid or chaotic. Veiga *et al* (1999) described an increased rate of chaotic complement of 37.5% in arrested embryos in comparison to the 0% observed in blastocysts. This can be partly explained by the ability of chaotic complements in embryos to block further development. Sandalinas and co-workers (2001) found that some chaotic mosaic embryos developed further but never more than 50 cells and were considered to be developmentally compromised. Moreover, it has been suggested that there is a strong developmental block at compaction of chromosomally abnormal embryos compared to normal embryos (difference was statistically significant) (Sandalinas *et al*, 2001).

4.3.4 Group II Embryos

None of Group II embryos were considered arrested since they contained more than thirty cells per embryo at the time of spreading. 90% (19/21) of the blastocysts analysed were mosaic and only 2 embryos were uniformly diploid for the chromosomes tested (Table 3.7). Studies where blastocysts were karyotyped also found increased levels of mosaicism (Clouston *et al*, 1997, 2002). Veiga *et al* (1999) also found 7/8 (87.5%) blastocysts analysed to be mosaic and Ruangvutilert *et al* (2000a) reported similar figures of mosaicism to this study in blastocysts (89.5%). Evsikov and Verlinsky (1998) also found high levels of mosaicism whilst performing FISH on blastocysts (86%), although they did not distinguish between normal and diploid mosaics. Ten out of 21 embryos (47%) were diploid/aneuploid mosaics (including haploid or polyploidy cell lines). Embryo "14.2" was a diploid/aneuploid/polyploid mosaic embryo, which was generated due to three different mechanisms, chromosome loss for chromosomes 1 and 18, chromosome gain for chromosome 11 and mitotic non-disjunction for chromosome X (Table 4.5).

Group II embryos showed a larger variety of cell lines within each embryo compared to Group I, which might have been attributed to the increased number of cells analysed (1123 over 401 cells in Group I) or the marked presence of polyploidy cell lines. 80% of the embryos analysed contained relatively high levels of tetraploidy ranging from 2-22%. This has been also been reported by Verlinsky and Evsikov (1998), Veiga *et al* (1999) and Ruangvutilert *et al* (2000a). Ruangvutilert *et al* (2000a) suggested that mosaic embryos with a tetraploid cell line might have more viability that those with other non-diploid cell lines. The increased number of cells present in Group II embryos was expected since embryos in this group were transferred to blastocyst medium. The blastocyst medium contains the required nutrients that an embryo needs after day 3 of development i.e. glucose.

Compared to studies carried out thus far, a relatively high percentage of triploid cells have been found during this study. For 4% (19/458) of the cells scored were found to

be triploid for the five chromosomes tested. Ruangvutilert *et al* (2000a) found only 0.3% (4/1272) of triploid cells present in the blastocyst group. However, similar findings of haploid cells were observed between this study and other studies of human blastocysts, since only 0.2% (1/458) of the cells were haploid. Triploid and haploid nuclei have been found more frequently in cleavage stage embryos.

4.3.5 Types of Mosaicism

Aneuploid mosaics arise from post-zygotic mitotic errors. In Group I almost all mosaic embryos were diploid/aneuploid mosaics. These embryos arose mostly due to post-zygotic chromosome loss, followed by chromosome gain, with only a few examples of mitotic non-disjunction. Other studies that found aneuploid mosaic embryos suggested that the main mechanism leading to mosaicism was mitotic non-disjunction (Munne *et al*, 1998c; 2003a). The basis of this observation lies in the fact that those studies did not include two probes (at a different locus) for each chromosome, thus accounting monosomy findings as failure of hybridisation or signals overlapping, hence, reducing the incidence of aneuploid mosaics due to chromosome loss. Although most studies are able to test a higher number of chromosomes, they are not able to be positive whether their findings are 100% accurate. By using two probes per chromosome at different loci in different rounds of FISH can accurately determine the chromosome abnormalities present and suggest a mechanism. Magli *et al* (2001) also came to a similar conclusion, when two probes were applied for chromosome 21 on a PGS case so that the misdiagnosis of normal embryos as monosomies were reduced. It has been hypothesised that the transition from the morula to the blastocyst stage is critical in terms of starting a negative selection against aneuploid cells (Evsikov and Verlinsky, 1998), since a high degree of mosaicism was observed up until the morula stage in comparison to that of blastocysts. However, this hypothesis was not shared with Sandalinas *et al* (2001), which showed that extended culture to blastocyst stage is not a reliable selection tool to screen against those chromosomally abnormal embryos that may survive after implantation. Monosomies were observed for both groups for all the chromosomes tested. Although studies by Sandalinas *et al* (2001) and Clouston *et al* (2002) retrieved low numbers or were unable to find monosomies respectively, in this study monosomies were present, especially in embryos grown in the basic medium. 15%

(29/188) of the cells scored in Group I were monosomic for all chromosomes tested (1, 11, 18 and X), hence, the increased incidence of chromosome loss was a mechanism for the aneuploid mosaics. But, in the blastocyst medium group only 3% (14/458) of the cells scored included monosomies.
The embryos grown in the blastocyst medium (Group II) showed an increased rate of tetraploid cells. It has been suggested that tetraploidy is a normal feature of the trophectoderm (Angell *et al*, 1987) and may be associated with invasion of the maternal deciduas (Drury *et al*, 1998). The significant increase of polyploidy cells, and especially tetraploidy, from day 4 to day 6 of development has been suggested to be, in human embryos as in other mammalian species, a hallmark of trophoblast differentiation (Bielanska *et al,* 2002a). Harper *et al* (1995) suggested that the underlying mechanism leading to tetraploid cells might be failure of cytokinesis after the chromosomes divide. Furthermore, endoreduplication might cause cells to become tetraploid by doubling of the chromosomes and failure to divide. Also, tetraploid cells might originate from cell fusion (Benkhalifa *et al*, 1993). Fusion of cells of different ploidies, such as 2N plus 4N and 4N plus 4N, may also explain the origins of 6N and 8N complements found in both Group I and II embryos. Similar findings of mixoploidy have been identified in 109 blastocysts analysed with FISH by Bielanska *et al* (2002c). It has been postulated that not all types of mosaicism and the proportion of abnormal cells have the same impact on embryo development (Sandalinas *et al,* 2001), and a high proportion of tetraploid cells may be detrimental in embryo development. In the study by Sandalinas *et al* (2001) 2n/4n mosaics with <38% abnormal cells developed 78% of the time to blastocyst stage compared with only 33% of those with >38% abnormal cells (the difference was shown to be statistically significant).

Slightly higher levels of triploid cells (in comparison to similar studies carried out so far) were interestingly observed in the Group II embryos. Seven out of the twenty one embryos analysed contained at least one triploid cell line. The origin of diploid/triploid cells is not clear, however the presence of an extra haploid set might be derived from an extra gamete, such as a second sperm (in a dispermy event where the second sperm remains unincorporated into the formation of the zygote) or a polar body (Mueller *et al,* 1993). Triploid cells were more often seen in cleavage-stage

embryos (Delhanty *et al,* 1997), nevertheless, in some instances triploid cells may persist until later in development as in cases of mosaic diploid/triploid have been reported postnatally (Edwards *et al,* 1994). Haploid cells were rarely found and it seems that they are less viable or less actively dividing in comparison to tetraploid cells. Delhanty *et al* (1997) suggested that they maybe associated with binucleate cell production with a meiotic type of segregation. Staessen and co-workers (1999) hypothesised that haploid cells might be due to incorporation of a polar body into the embryo. In this study only one embryo from each Group revealed a haploid cell line displaying an incidence of 0.1%. It has been suggested that 1N cells have a proliferative disadvantage among 2N cells (Harper *et al,* 1995; Bahce *et al,* 1999; Bielanska *et al,* 2002a). In contrast to diploid/triploid mosaics, diploid/haploid mosaicism has not been documented in fetal tissues, hence such embryos must become eliminated at, or shortly after, implantation.

Chaotic embryos were first described in cleavage-stage embryos (Harper *et al,* 1995; Delhanty *et al*, 1997). Three embryos were chaotic in Group I and eight in Group II; however, all 11/42 embryos included diploid cells as the major cell line. Evsikov and Verlinsky (1998) found three chaotic embryos out of 91 in their series, Ruangvutilert *et al* (2000a) observed five chaotic embryos out 40 after FISH analysis whereas Baart *et al* (2004), found only one chaotic embryo out of 22 analysed. Delhanty and Handyside (1995) proposed that the absence of cell cycle checkpoints might cause such chaotic chromosome constitutions. Cell cycle checkpoints were first identified in yeast and would normally protect cells from genetic damage by ensuring that each cycle phase is completed before the initiation of the next (Hartwell and Weinert, 1989). In mammalian embryos the absence of cell cycle checkpoints may relate more generally to the absence of embryonic gene transcription and dependence on maternal products inherited in the oocyte (Delhanty and Handyside, 1995). Hardy *et al* (1993) suggested that the presence of cells with nuclear abnormalities or highly abnormal chromosome complements may reflect both lack of co-ordination of the different processes of the cell cycle. Other studies have proposed that chaotic cell lines arise from a group of events such as a chromosome misalignment on a disorganised spindle in combination with a non-functional metaphase/anaphase checkpoint control (LeMarie-Adkins *et al,* 1997; Harrison *et al,* 2000). The abnormalities of the mitotic

spindle could be related to suboptimal *in vitro* culture environment (Pickering *et al*, 1990). Munne *et al* (2004) has suggested that chaotic mosaicism might occur due to differences in hormonal stimulation as well as imperfect culture conditions.

4.3.6 Mechanisms of Aneuploidy Mosaicism

Compared to other species, humans display a low fecundity. Of all human conceptions, only ~30% progress successfully to delivery (Hassold *et al,* 1986). To a large extent, embryonic death is caused by chromosomal abnormalities that are primarily the result of chromosomal errors during female gamete formation. Some errors will arise at the time of fertilisation but most are the recently discovered mitotic errors which take place during early cleavage division. These errors in cell divisions, namely non-disjunction and anaphase lagging lead to mosaicism and chaotic imbalances. Kalousek (2000) highlighted the significance of the chromosome centromere in the process of cell division. It was suggested that the centromere is involved in: i) sister chromatid pairing, ii) mitotic and meiotic spindle attachment, iii) chromosome movement, iv) cell cycle control i.e. cell cycle checkpoint control and v) marshalling of passenger proteins. This was taken into account in our study and five out of the nine probes used were centromeric probes.

In the current study chromosome loss was the predominant mechanism leading to mosaicism in both groups of embryos, being responsible for 50% of aneuploid cells in Group I and 40.5% in Group II. Chromosome gain followed with 44%, in Group I and 35.1% in Group II. However only 6% of aneuploid cells occurred due to mitotic non-disjunction in Group I, while four times this percentage arose by this mechanism in Group II. The differential involvement of chromosomes in MND (predominantly chromosome X in Group II which was not involved at all in Group I) is interesting but may well be due to chance.

Similarly, it is of interest that in Group I embryos, in which growth had slowed or arrested, chromosome 1 showed a high incidence of loss but in Group II embryos, which had continued dividing, chromosome 1 was not affected by loss at all. This might possibly be due to the fact that the presence of cells with monosomies of such a large chromosome would have a detrimental effect on development and had been selected against in the more rapidly dividing Group II embryos. Trisomy 18 caused by

chromosome gain was seldom found in either Group, which is a similar finding to that reported recently by Coonen *et al* (2004). The occurrence of monosomy 18 was shown to be high (especially in Group II embryos), indicating that chromosome 18 might be more prone to chromosome loss compared to the other autosomes and gonosomes tested.

The current study shows that chromosome loss is the most common mechanism that leads to mosaicism detected in human day 5 embryos. This reinforces data on day 3 embryos obtained earlier by our group using dual locus specific YAC and plasmid probe combinations for various autosomes (C. Conn & JDA Delhanty, unpublished observations). Chromosome loss is presumed to occur via anaphase lag, in this case during mitosis. Coonen *et al* (2004) concluded in their study on a much larger number of blastocysts that anaphase lagging is the major cause of chromosomal mosaicism. However, since they were using a single probe for each chromosome they were only able to count as valid abnormalities affecting at least two cells. In relation to the findings in these two studies, it is of considerable interest that aneuploidy screening of cleavage stage embryos has shown that chromosome loss is more common than chromosome gain as a cause of constitutional aneuploidy arising during meiosis (Munné *et al*, 2004). This is in contrast with all previous findings from the same group which supported that mitotic non-disjunction was the most common type of mosaicism mechanism (Munne *et al,* 1994; 1995a; 1998c; Munne and Cohen 1998, Munne, 2002). However, all the studies above were limited to one probe per chromosome and were attributing abnormalities confined to low number of cells as signal overlap, probe failures, whereas in the recent study by Munne *et al* (2004) all samples were re-analysed with probes binding to a different locus.

In contrast to the chromosomal abnormalities seen in cleavage stage embryos, mosaic chromosome patterns observed in blastocysts are derived from mitotic division errors. A meiotic division error would render all embryonic cells chromosomally aneuploid. In this study only one embryo (from Group I) out of 42 rose due to a meiotic error. Embryo 4.1 was aneuploid mosaic and must have originated due to a non-disjunction event during meiosis. Coonen and co-workers (2004) also reported only one out of 299 blastocysts analysed by FISH had occurred from meiotic division errors. Other

studies have shown no or only a few blastocysts presenting with aneuploid cells only (Magli *et al*, 2000b; Ruangvutilert *et al,* 2000a; Bielanska *et al,* 2002a; Baart *et al,* 2004)

No difference in embryonic sex ratio between Group I and Group II was observed in this series. In Group I there were 12 female and 9 male embryos whereas in Group II there were 11 female and 10 male embryos (Tables 4.3 and 4.6). It has been shown in a recent study that more male than female babies have been born after blastocyst transfer (Menezzo *et al,* 1999), which might reflect a greater viability of male embryos after implantation. However, no other study has confirmed these results.

During this study the average maternal age was 34 years but still there was a high prevalence of mosaicism. The three embryos that were uniformly diploid for the tested chromosomes were donated from three women less than 30 years of age. Whereas six women whose age was above 38 donated nine embryos, which were either diploid/aneuploid mosaic or diploid/aneuploid/chaotic mosaic. While these observations may indicate a trend, clearly the numbers in our study are too small to enable analysis of a maternal age effect. However, a report of a study of large number of cleavage stage embryos from women in an older age group showed a significant association of mitotic aneuploidy with advanced maternal age (Munné *et al*, 2002). The association was particularly marked for the MND category. In that study chromosome loss was only a third as frequent as MND and consequently mitotic anaphase lag failed to show a significant increase with maternal age. The increased frequency of MND may be due to the age of the group studied but since only a single probe per chromosome was used a cut off point of 10% was used to avoid 'FISH error'. That is, embryos with fewer than 10% abnormal cells were considered 'normal', almost certainly leading to an underestimate of anomalies affecting a single cell. Possibly a study of a large number of embryos using two probes per chromosome might show a significant association of mitotic anaphase lag with increasing maternal age.

4.3.7 Conclusions and Future Work

In conclusion, during this project it was revealed that three rounds of sequential FISH can provide efficient results in human preimplantation embryos in order to study the effect of chromosomal mosaicism. High proportion of mosaicism was found during this study in both groups of embryos which were mostly affected by aneuploidy. The double-loci analysis of each chromosome in different rounds of FISH enabled the determination of true level of mosaicism in day 5 human embryos, by detecting the FISH artefacts. The major mechanism behind aneuploidy mosaicism for both groups of embryos was found to be chromosome loss, which was postulated to be due to anaphase lagging. These findings were found to be in agreement with similar studies which used different protocols (Coonen *et al,* 2004; Baart *et al*, 2004; Munner *et al,* 2004). Furthermore, the effects of the two different media used were observed and it was found that the nutrients in IVF media are very important to the development of human blastocysts.

Examination of a larger cohort of embryos would enable extensive information of the chromosomal mosaicism and would further allow statistical analysis to be performed in order to find statistical significance, if any, between the mechanisms of mosaicism i.e. chromosome loss or gain or mitotic non-disjunction. Analysis of all the chromosomes of all the blastomeres would reveal the true extent of mosaicism in day 5 human embryos. Such task could be carried out using CGH (Wells *et al,* 1999; Voullaire *et al,* 1999) or using micro-arrays (Schaeffer *et al,* 2004; Shaffer *et al,* 2004). CGH analysis of all the blastomeres on normally developing day 5 embryos or blastocysts has not been carried out and might demonstrate results that might explain whether mosaicism affects blastocysts more than day 3 embryos. Micro-arrays can provide comprehensive (genome-wide), high resolution, amenable to automation, rapid and sensitive detection. Moreover, molecular analysis of genes responsible for embryo genome activation could be performed on embryos in order to observe the effect of IVF media in the development of the embryos.

CHAPTER 5

Analysis of the development of chromosome abnormalities in human embryos from day 3 to day 5 using CGH and FISH

5.1 Aims of the Study

The aim of this study was to assess the level of chromosomal abnormalities in human embryos at different times of development. A novel protocol was devised were the chromosomal status of the embryos was investigated at the cleavage stage and furthermore at the blastocyst stage. Frozen-thawed embryos were biopsied on day 3 and 1 or 2 cells were extracted for CGH analysis in order to examine the full chromosomal status of those blastomeres. The rest of the embryo was left to grow until the blastocyst stage (day 5) were it was spread on the whole to carry out sequential FISH analysis. The first combination of probes included the X/Y/18 which would allow confirmation of the sex when compared to the CGH results from the biopsies blastomeres as well as any abnormalities for the corresponding chromosomes. However, the second round involved probe combinations according to the CGH results i.e. any abnormalities seen in the CGH results would be tested on the spread blastomeres using FISH. This would allow the monitoring of abnormalities during the different stages of development.

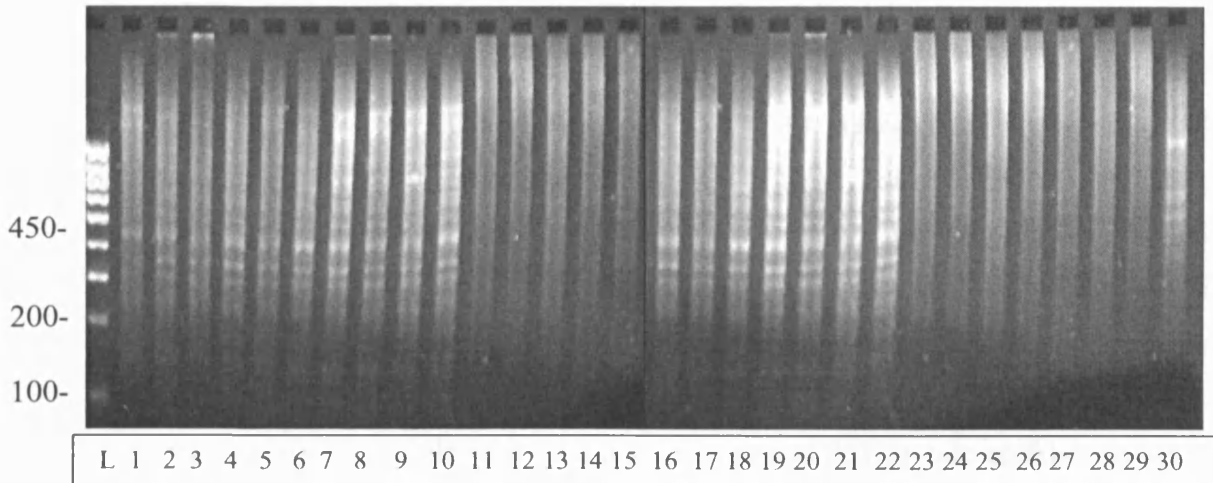
5.2 Results

5.2.1 WGA Optimisation

The efficiency of the tubing technique was found to be 97% (section 2.2.2.2.3). Efficient tubing was confirmed by successful amplification, which in turn was revealed by a smear on the gel. Bands indicating amplification were observed corresponding to a range of fragment sizes of DNA of approximately 1550, 1200, 600 and 450 bp in length, while the average fragment size was approximately 600 bp in length (Figure 5.1). Positive and negative controls were always included in the tubing process, to ensure reliability of results. The frequency of amplification in negative controls was <5%, while amplification in positive controls was successful with a frequency of 98%. During optimisation of the WGA protocol two thermal cyclers were tested and the results were run on a 2% agarose gel (Figure 5.2). As shown in Figure 5.2B the Omnigene Thermal cycler proved to be superior compared to the Eppendorf since the smears from the samples were more intense and more similar to the genomic DNA samples revealing a better coverage of the genome. Furthermore,

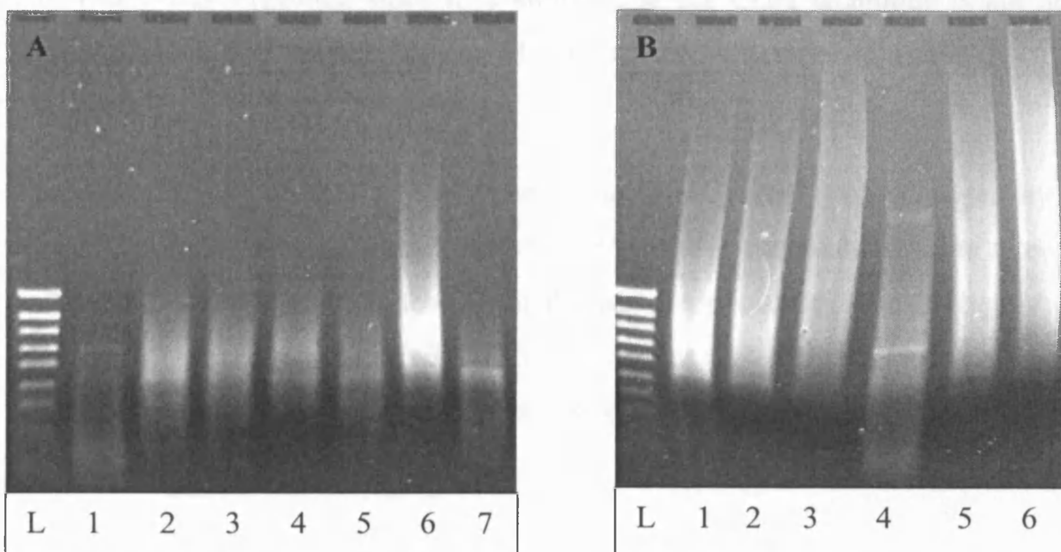
the size of the smears was definitely improved when using the Omnigene, since the smears were expanded over the 1Kb Ladder.

Figure 5.1. DOP-PCR amplification results from single cells, clumps of 3-4 buccal cells and genomic DNA on an agarose gel.



In lane L the 1kb Ladder can be seen. Lanes 1-10 were amplified products from single buccal cells. Lanes 11-15 a clump of 3-4 buccal cells were run and in lanes 16-23 single blastomeres were amplified and analysed. Finally, in lanes 24, 25, 26, 27 and 28 DNA from frozen fibroblasts of known abnormality (trisomy 13, 18, 21 and 22 and triploidy XXX respectively) was run. Lanes 29 and 30 represent amplification results from normal genomic DNA.

Figure 5.2. Agarose gel results from (A) Eppendorf and (B) Omnigene thermal cyclers



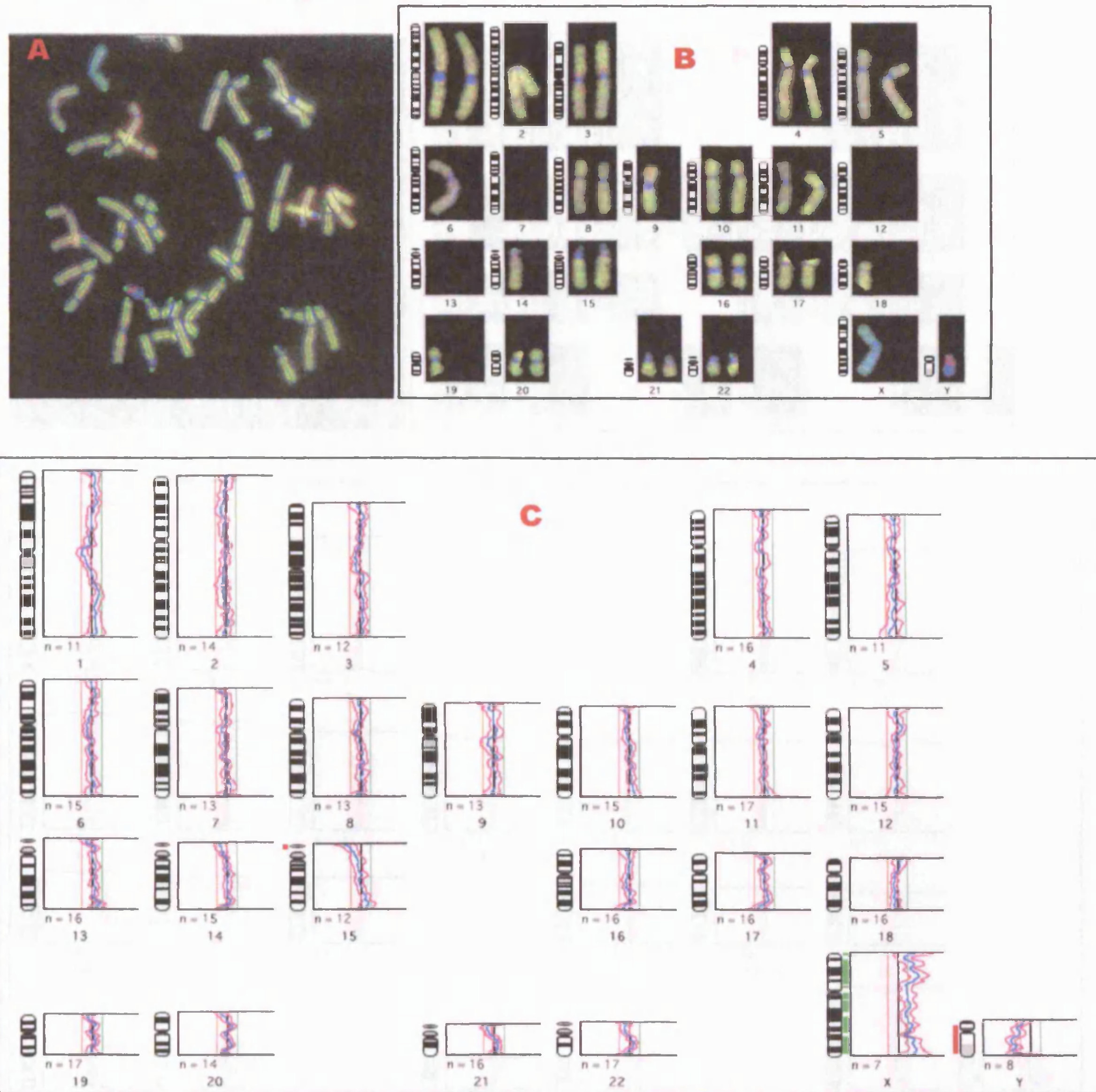
A. In lanes 2-5 and 7 the amplified products from single buccal cells were run. In Lane 6 the amplified product from genomic DNA was analysed and in lane 1 the negative control can be viewed. B. Lanes 1, 2, 3 and 5 show the final amplified product from single buccal cells. In lanes 4 and 6 the negative control and genomic DNA was run. In both Figures, A and B, the lane L represents the 1kb ladder.

5.2.2 CGH Optimisation

CGH analysis of both control lymphocytes and trisomic cells was carried out to optimise the protocol. All control and embryo CGH experiments were carried out on control male lymphocyte slides. Figure 5.3 shows an initial CGH experiment carried out on DOP-PCR amplified genomic DNA (section 2.2.2.1) between a normal male and female individual. The interpretation of 8-12 metaphases was performed for each sample. In Figure 4.3 the CGH software was able to distinguish between the male and female status of the control (red) and test (green) samples. Five different chromosomally abnormal fibroblast cell cultures (trisomy 13, 18, 21, and 22 and triploidy XXX) were made available for this study acting as positive controls in the identification of aneuploidy in single cells. Figure 5.1 illustrates the DOP-PCR results from the amplification of the trisomic and triploidy samples. Aneuploidy was correctly identified in single cells with a success rate of 96%, while no false positives were recorded. Figures 5.4, 5.5, 5.6, 5.7 and 5.8 show the CGH results which acted as positive controls for trisomy 13, 18, 21, 22 and triploidy XXX respectively once the CGH protocol was optimised. In all positive control experiments, the CGH interpretation was able to distinguish the type of abnormality as well as the sex of each trisomic line. However, when the triploid cell line was tested against a single male buccal cell, the CGH was not able to distinguish the ploidy status (Figure 5.8). This result was expected, since it is known that the CGH technique is not able to detect differences in the ploidy status of a tissue (Kallioniemi *et al*, 1994).

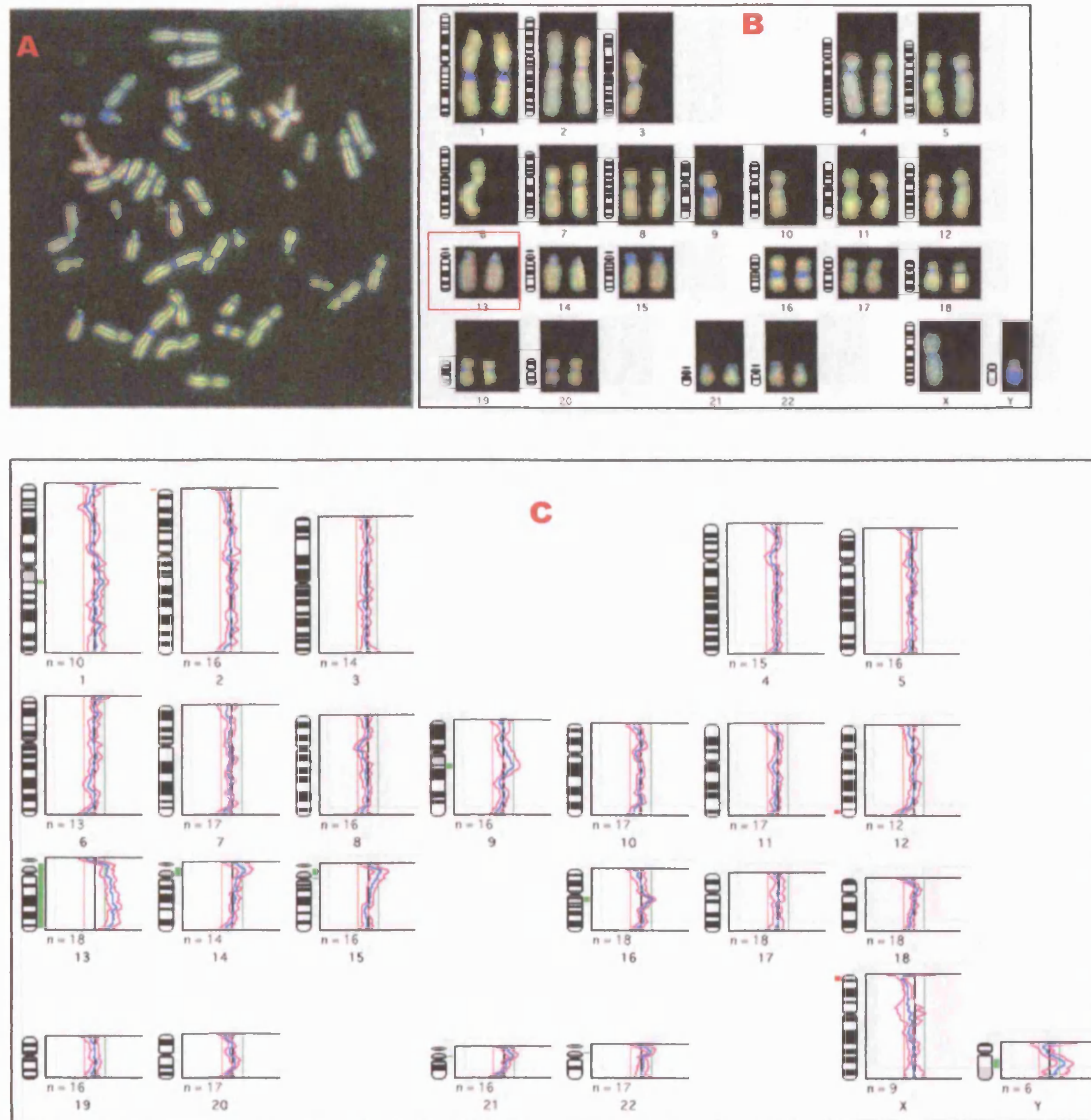
Thirty single cells (including buccal cells and blastomeres) were analysed by CGH once the protocol was optimised. 93.3% (28/30) gave analysable results after CGH and in 83.3% (25/30) the difference in the sex chromosomes was distinguished. In 10% (2/30) the fluorescence was of low intensity and with excess of background fluorescence indicating that the single cell was either anucleate or degenerating during isolation.

Figure 5.3. Illustration of a control CGH experiment between male genomic DNA (red) acting as control against female genomic DNA (green) acting as test



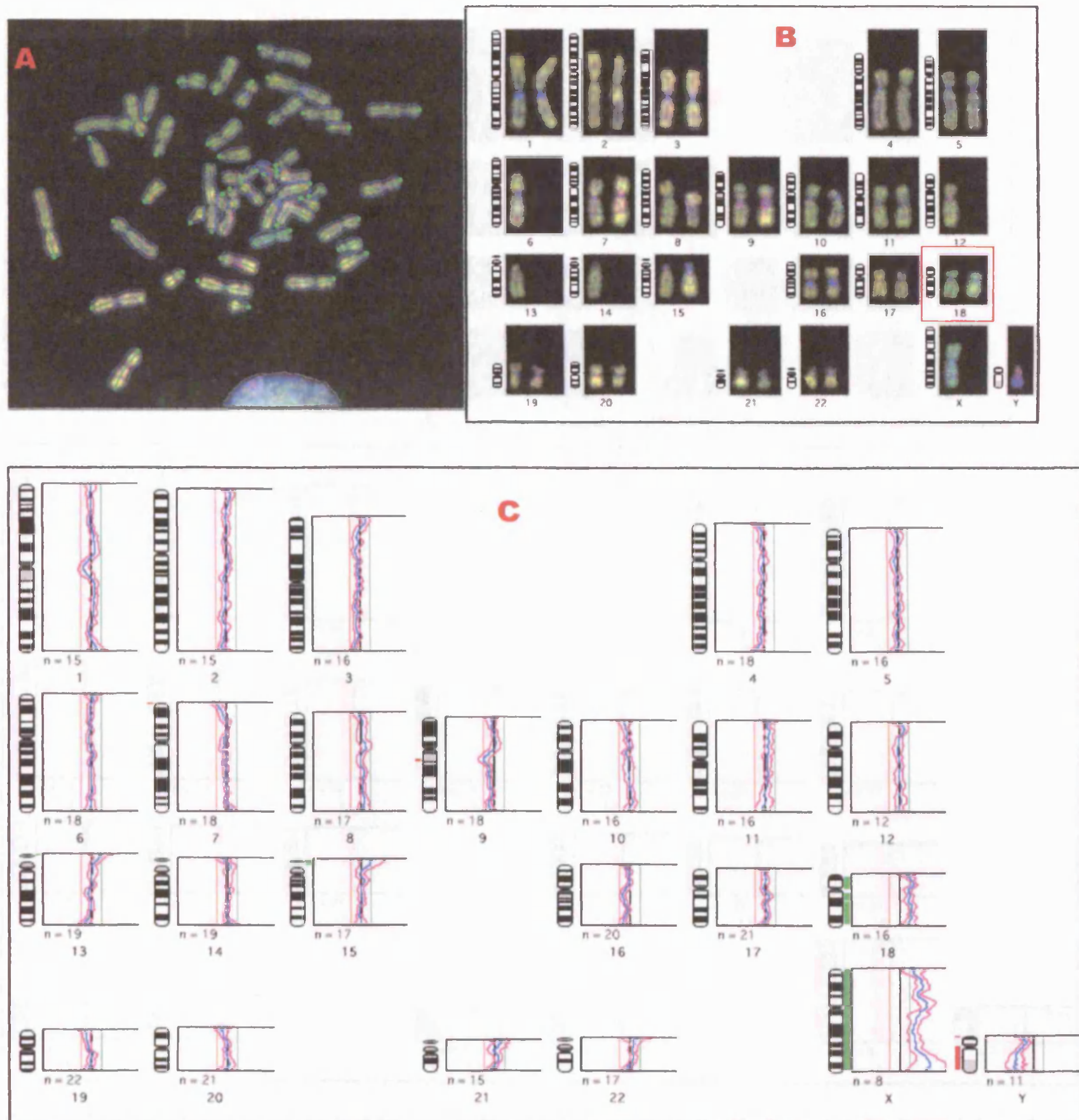
For each CGH experiment 8-12 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of metaphase spread. B. In the karyotype of the captured metaphase the X chromosome has a distinct green fluorescence indicating excess in the test sample and the Y chromosome shows red fluorescence indicating deficiency in the test sample. C. This shows the cumulative analysis of ten metaphases, which was the basis of the interpretation. There was a shift in fluorescence only towards the green (excess) in chromosome X and towards the red (deficiency) in chromosome Y showing that the test sample was a normal female

Figure 5.4. Illustration of a positive control CGH experiment between male single cell (red) acting as control against DNA trisomic for 13 (green) acting as test



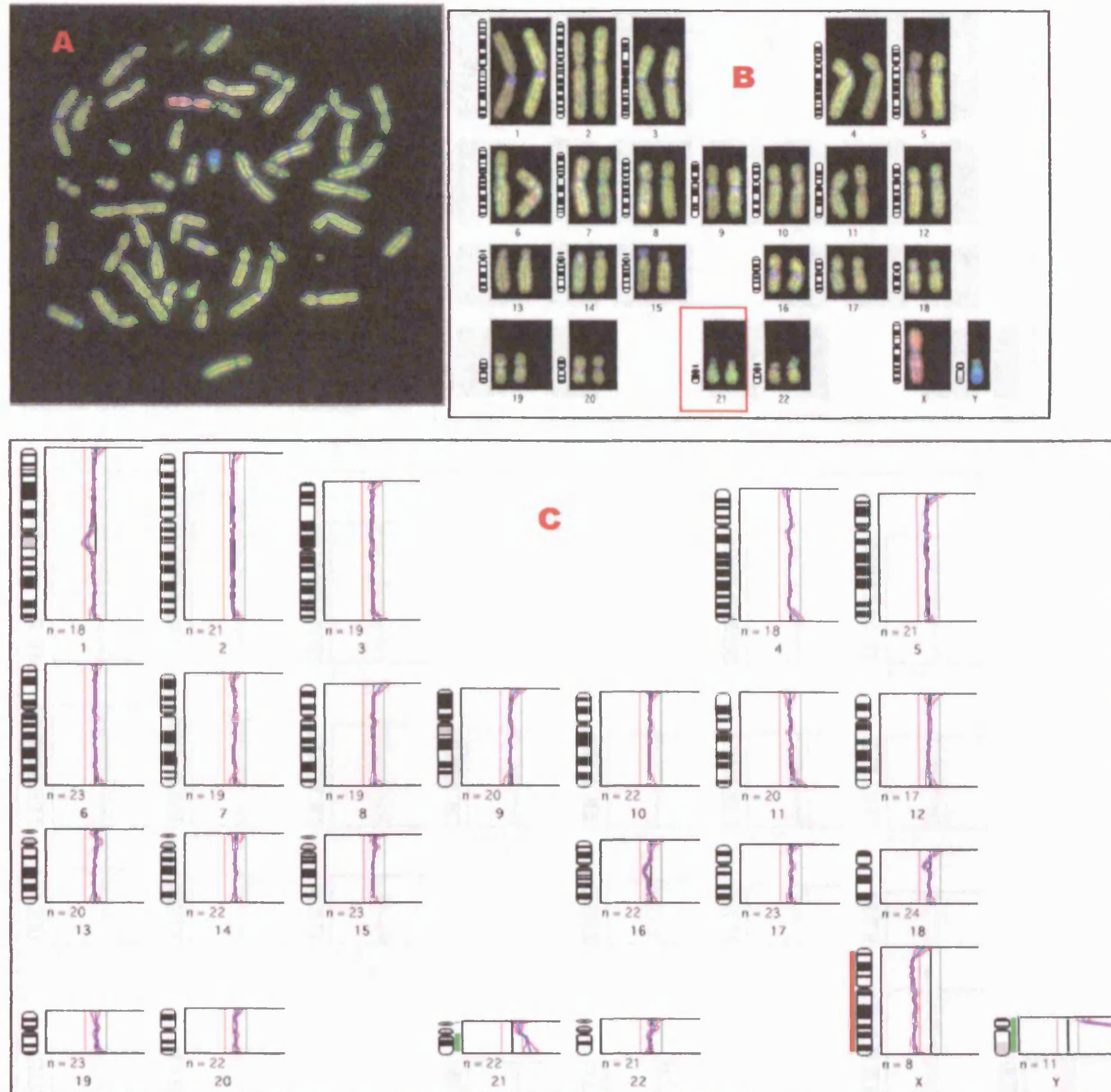
For this CGH experiment 9 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of a metaphase spread. B. In the karyotype of the captured metaphase chromosome 13 has a distinct green fluorescence indicating excess in the test sample. C. This shows the cumulative analysis of the nine metaphases, which was the basis of the interpretation. There was a shift in fluorescence only towards the green in chromosome 13 showing that the test sample was male, trisomic for chromosome 13.

Figure 5.5. Illustration of a positive control CGH experiment between male single cell (red) acting as control against DNA trisomic for 18 (green) acting as test



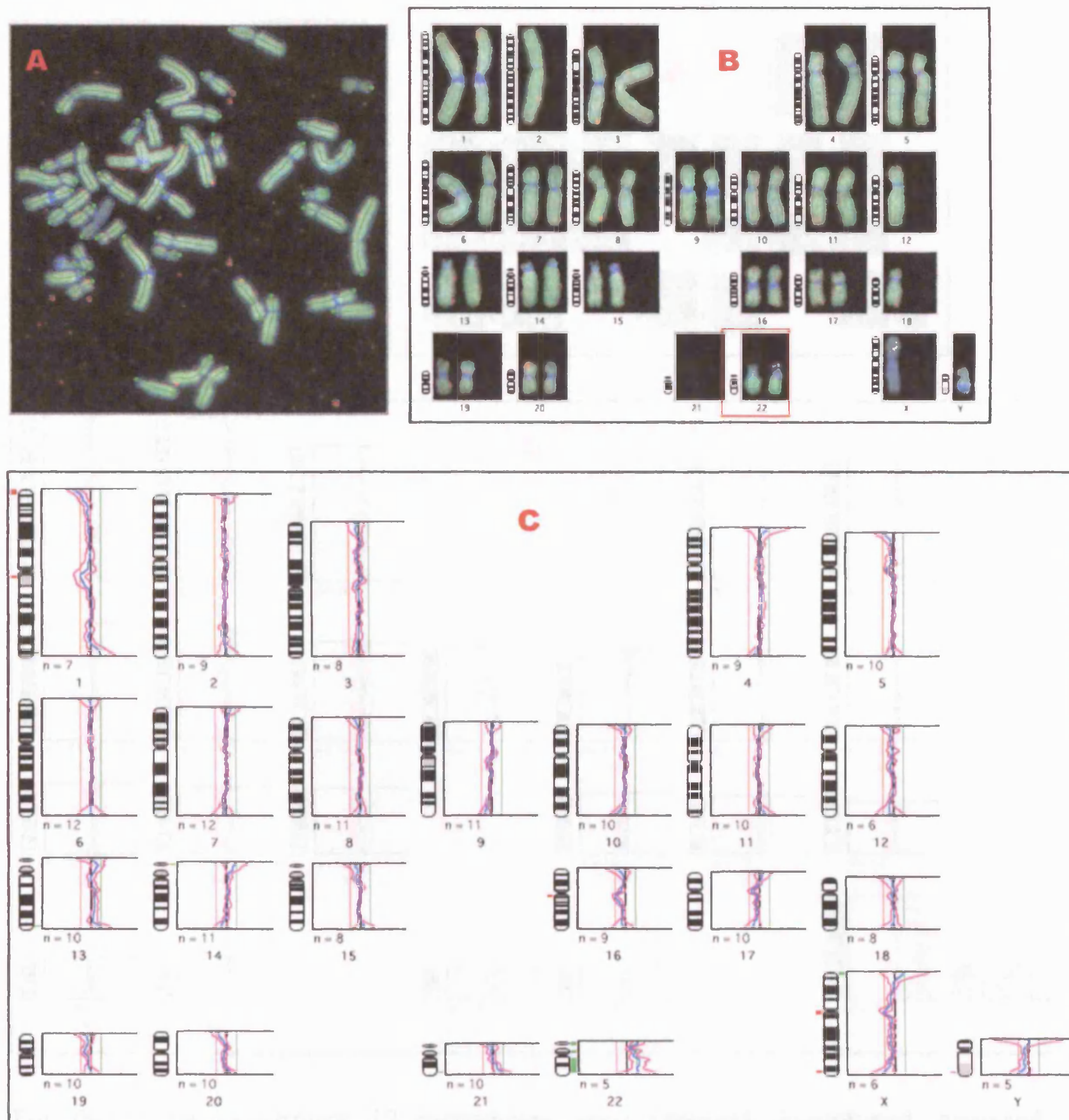
For this CGH experiment 11 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of a metaphase spread. B. In the karyotype of the captured metaphase chromosomes 18 and X have a distinct green fluorescence indicating excess and red fluorescence in the Y chromosome indicating deficiency in the test sample. C. The CGH interpretation successfully detected that the test sample was female, trisomic for chromosome 18.

Figure 5.6. Illustration of a positive control CGH experiment between female single cell (red) acting as control against DNA trisomic for 21 (green) acting as test



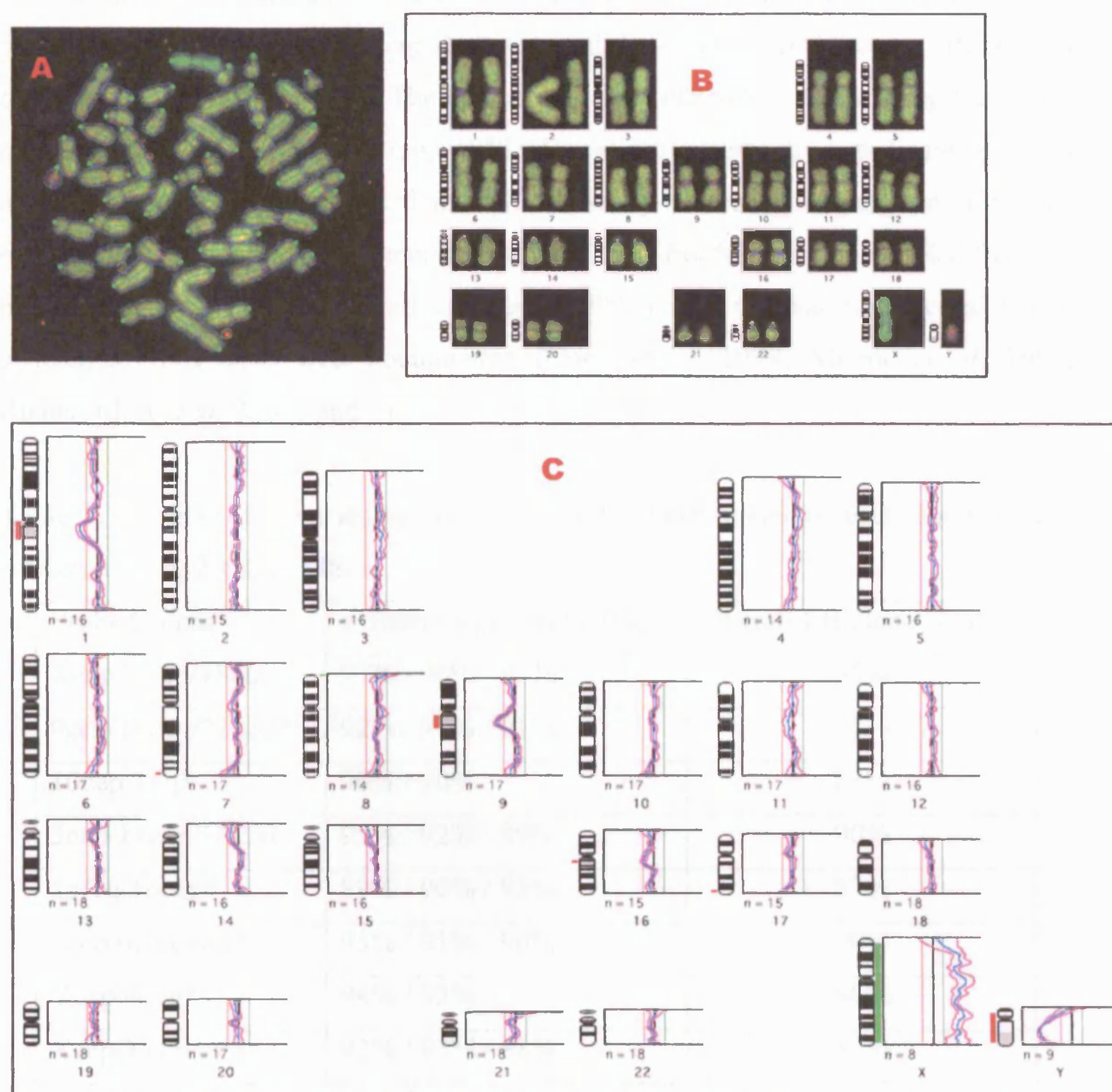
For this CGH experiment 11 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of a metaphase spread. B. In the karyotype of the captured metaphase chromosomes 21 and Y have a distinct green fluorescence indicating excess and red fluorescence in the X chromosome indicating deficiency in the test sample. C. The CGH interpretation successfully detected that the test sample was male, trisomic for chromosome 21.

Figure 5.7. Illustration of a positive control CGH experiment between male single cell (red) acting as control against DNA trisomic for 22 (green) acting as test



For this CGH experiment 10 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of a metaphase spread. B. In the karyotype of the captured metaphase chromosomes 22 has a distinct green fluorescence indicating excess. C. The CGH interpretation successfully detected that the test sample was male, trisomic for chromosome 22.

Figure 5.8. Illustration of a positive control CGH experiment between male single cell DNA (red) acting as control against triploid DNA (green) acting as test



For this CGH experiment 12 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Capturing of a metaphase spread. B. In the karyotype of the captured metaphase chromosome X has a distinct green fluorescence indicating excess and red fluorescence on chromosome Y indicating deficiency. C. The CGH interpretation successfully detected that the test sample is male, however, was unable to detect the triploidy status.

5.2.3 FISH Optimisation

Each FISH experiment included a control male lymphocyte slide with mapped nuclei in order to assess efficiency of probe hybridisation in the sequential rounds. Overall,

90% (range 87-97) of the control nuclei showed normal signals for all 14 probes used (Table 5.1). Sub-telomeric probes for chromosomes 1p and 16q showed the lowest hybridisation efficiencies of 89% and 88% respectively; whilst the α-satellite probes for chromosomes 16cep and Ycep demonstrated the highest hybridisation efficiencies of 94% and 93% respectively. The difference in the efficiency of the Xcep and Ycep probe (used in the 1st and 2nd round of FISH) was not statistically significant ($p<0.05$) and can be attributed to the fact that FISH efficiency decreases with sequential rounds of hybridisation due to the degeneration of the DNA. Furthermore, the ≥5% difference between the first round overall efficiency (95%) and the various second round efficiencies has been well documented (Conn *et al,* 1998; Munne *et al,* 1998; Ruanvutilert *et al,* 2000a and b).

Table 5.1. Probe efficiencies scored in 200 interphase nuclei of each control slide whilst carrying 2-round FISH

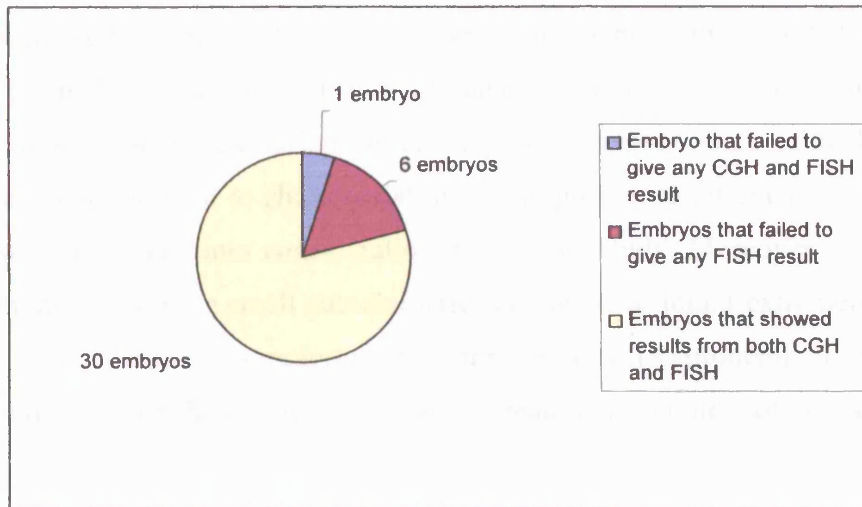
Probe Combinations	Efficiency per probe (%)	Overall Efficiency (%)
Xcep/Ycep/18cep	97% / 96% / 97%	95%
9cep/16cep/22LSI*	92% / 94% / 91%	90%
10cep/14q*	90% / 90%	88%
3cep/11cep/13LSI	92% / 92% / 89%	90%
1p/1q/16cep*	89% / 90% / 93%	87%
3cep/6cep/18q*	95% / 91% / 90%	90%
Ycep/4cep*	94% / 92%	90%
Xcep/Ycep/16q*	92% / 93% / 88%	87%

* Carried out in the second round

5.2.4 CGH Analysis of Embryos

Thirteen couples donated a total of 37 embryos for this research project (Appendix Table 8.1). The overall mean maternal age was 33.1 (range 28-39) years.

Graph 5.1. Cumulative results from the 37 embryos that were donated for research



One embryo was not included since it failed to provide any results during either CGH or FISH, which was not unexpected due to its low morphology grade (Graph 5.1). A total of fifty four blastomeres were biopsied on day 3 for CGH analysis and forty-eight (88.9%) provided an interpretable result. There are a variety of factors which may account for failure to provide a result. Blastomere 3.1b failed to give hybridisation from the test DNA, while the control DNA hybridised successfully. This could indicate either an anucleate blastomere (as visibility of the nucleus was not possible for all the blastomeres biopsied), or that the cell was mistaken for an anuclear fragment. Moreover, a poor slide preparation or premature lysis of the blastomere can be responsible. Apart from the non-specific fluorescence and the granular hybridisation effect (meaning that although hybridisation was successful it presents with a granular effect due to poor chromosome quality, failing to provide analysable fluorescence), the strength of the counterstain banding, essential for the identification of chromosomes, would also be negatively affected by this factor. These problems accounted for lack of analysable results for blastomeres 1.1a, 1.2b, 2.1b, 2.2b and 7.2b (as well as 3.1b mentioned above).

A total of 48 blastomeres obtained for this study provided good analysable results. All embryos were labelled in green and the control male DNA was labelled in red. Hybridisation that presented with no granulation, dynamic fluorescence (strength and consistency of fluorescent signals) was considered to be of high-quality and good chromosome morphology allowed strong counterstain banding essential for the analysis. If the sex chromosomes could be determined confidently this provided an

internal positive control regarding the efficiency and reliability of the CGH technique. All biopsied cells from each embryo were consistent with regard to the sex chromosome pattern as confirmed by FISH analysis. With regard to the autosomes, the literature suggests the exclusion for an analysis of certain regions of the karyotype as they have been proven to show variation in the profile, (Kallioniemi *et al*, 1994) and this was also taken into consideration during this study. Moreover, deletions or amplifications concerning small subtelomeric regions were found extremely difficult to detect and interpret, as reported by other groups (Kallionemi *et al,* 1994). Consequently abnormalities involving distal breakpoints could not be confidently detected.

Six out of 36 embryos failed to grow further to day 5 and degenerated, thus no cells were available for spreading (4.1, 4.3, 4.4, 13.1, 14.1, 14.3). However, all of these embryos showed, in at least one biopsied cell, analysable CGH results (Table 5.2).

Table 5.2. CGH results from six embryos that did not have blastomeres available on day 5 for FISH analysis. The CGH results are presented as excess of GR (green) or RD (red) fluorescence in part of the chromosome or in the whole chromosome.

Embryo No.	No. of cells prior to biopsy	No. of cells biopsied	CGH Result	Interpretation
4.1	5 cells	1	Normal	rev ish XY
4.3	7 cells	1 (+1)[a]	Normal	rev ish XY
4.4	6 cells	1 (+1)[a]	Excess GR in whole of X	rev ish XY, enh(X)
13.1	5 cells	1	Excess GR: 5p15.3-q11.2 & 5q14-q34	rev ish XY, enh(5)
14.1	6 cells	2	Excess GR: whole of X, 19q12-q13.4[b] Excess RD: whole of Y	rev ish XX
			Excess GR: whole of 2, 4, X, 9q (whole arm) Excess RD: whole of 1, 16, 21 and Y	rev ish XX, enh(2,4, 9qter), dim(1, 16, 21)
14.3	5 cells	1	Excess GR: whole of X Excess RD: whole of Y	rev ish XX

[a]The other cell lysed during the biopsy procedure

[b] Excluded from analysis since it was considered to be an artefact

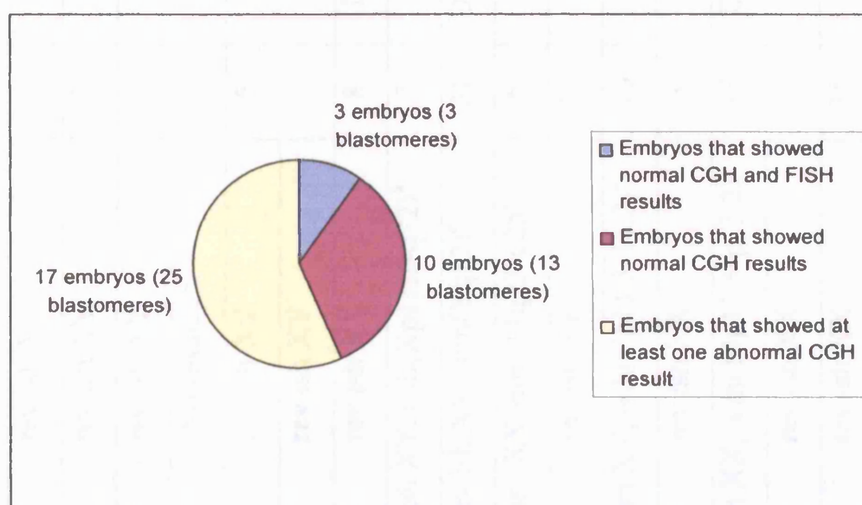
5.2.5 FISH Analysis of Embryos

FISH was able to achieve higher efficiencies of 93% overall (out of the thirty embryos), since 343/359 blastomeres from day 5 were analysable. The 7% of blastomeres that failed to return a result included blastomeres lost whilst spreading and blastomeres which could not provide an analysable result either on the first or second round of FISH. The first round of FISH was carried out using the probe cocktail for sex determination. This was performed in order to act as an internal control between the two techniques. FISH and CGH were in agreement regarding the sex in all of the embryos.

5.2.6 Interpretation of CGH and FISH Results

A total of thirty embryos (41 biopsied blastomeres) provided results from both techniques (Graph 5.2). Only three embryos (10%) showed normal results from at least one cell after CGH and normal results for all the blastomeres analysed by FISH for the chromosomes tested. The rest of the embryos demonstrated various levels of abnormality and mosaicism. Table 5.3 displays the embryos with completely normal CGH results whilst Table 5.4 shows the embryos where CGH revealed at least one cell with an abnormal karyotype.

Graph 5.2. Cumulative analysis results from 30 embryos (41 blastomeres)



CGH showed normal chromosome complements in thirteen embryos from which 18 blastomeres were biopsied (termed Group 1) (Table 5.3; Figure 5.9).

Table 5.3. Results from embryos showing normal CGH findings (Group 1) followed by sequential FISH analysis

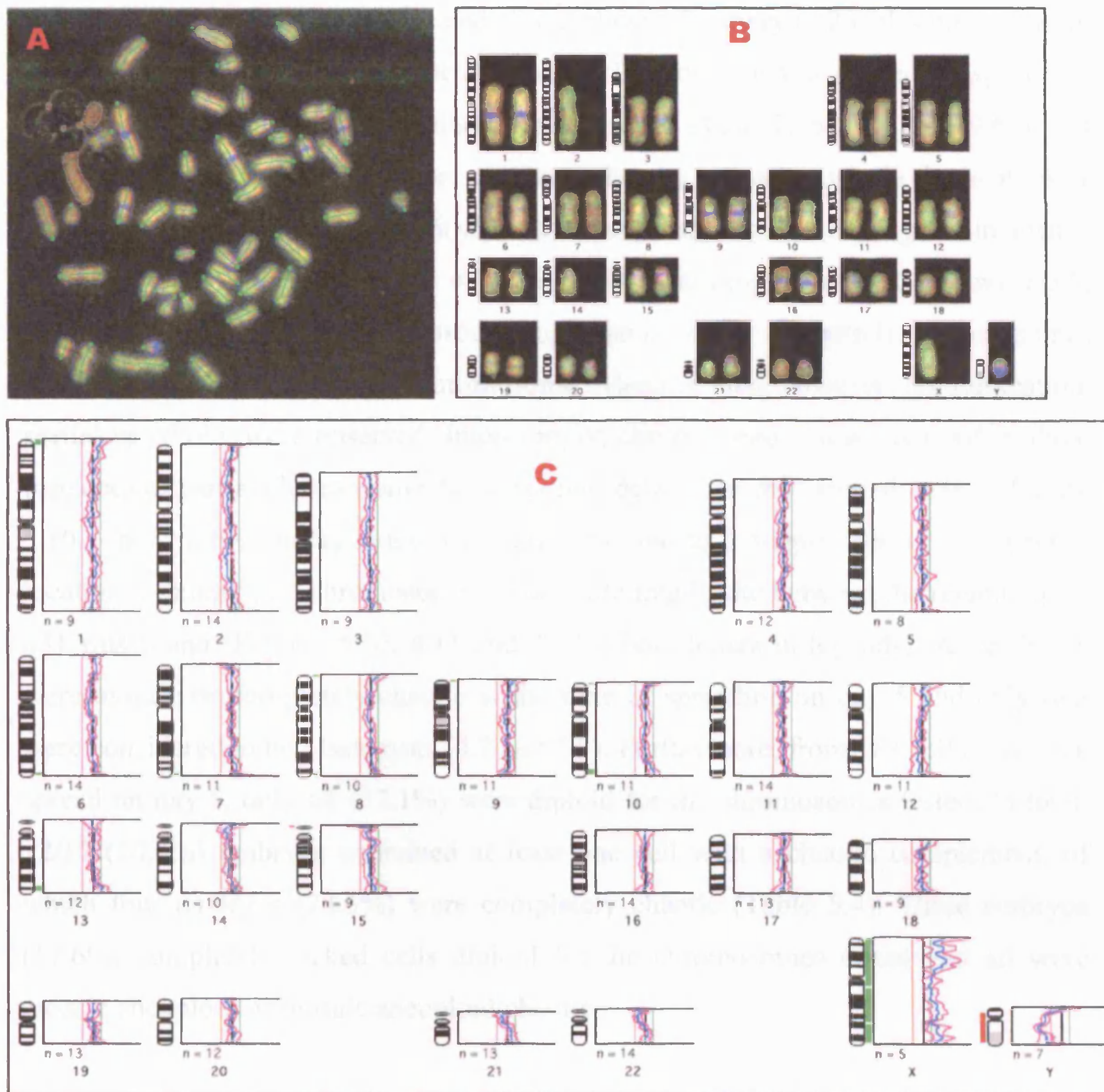
Embryo No.	Cell	CGH Result on day 3	FISH Result on day 5[b]		Interpretation
			Cells	Results (no. of cells)	
1.1	a	No result	13	Dip (7) / +X,+Y (2) / +22(2) / -18(2)	Mosaic Diploid/Aneuploid
	b	rev ish XY			
1.3	a	rev ish XY	2	Dip (1) / +X,+Y(1)	Mosaic Diploid/Aneuploid
2.1	a	rev ish XY	4	Dip (4)	Uniformly normal
	b	No result			
6.2	a	rev ish XY	5	Dip(3) / -22(2)	Mosaic Diploid/Aneuploid
	b	rev ish XY			
7.1	a	rev ish XX	8	Dip (3) / -18(2) / +18(1) / -22(1) / -16,-18(1)	Mosaic Aneuploid/Diploid
9.1	a	rev ish XY, enh(Xp11.2-q22)[a]	7	Dip (7)	Uniformly normal
9.2	a	rev ish XY, dim(Yq12)[a]	31	Dip (25)/ -18(1)/ -X(1) / -16,+22(1)/chaotic(3)	Mosaic Diploid/Aneuploid/Chaotic
9.4	a	rev ish XX, dim(11q23-q25)[a]	6	Dip (3) / tet(2) / chaotic(1)	Mosaic Diploid/Polyploid/Chaotic
9.5	a	rev ish XY	11	Dip(7) / -X(3) / chaotic(1)	Mosaic Diploid/Aneuploid/Chaotic
11.1	a	rev ish XY, enh(Yp11.3-11.2)[a]	10	Dip (10)	Uniformly normal
12.1	a	rev ish XX	3	Dip(1) / chaotic(2)	Mosaic Diploid/Chaotic
13.2	a	rev ish XX, dim(19p13.3-p13.2)[a]	15	Dip(7) / tet(1) / trip(1) / -9,+22(1) / chaotic(5)	Mosaic Diploid/Polyploid/Aneuploid/Chaotic
	b	rev ish XX			
13.3	a	rev ish XX	35	Dip(28) / trip(2) / +X(2) / chaotic(3)	Mosaic Diploid/ Polyploid/Aneuploid/ Chaotic
	b	rev ish XX			

[a]These findings were considered as artefacts. **dip** = diploid, **tet** = tetraploid, **trip** = triploidy. - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

[b]The 1st round of FISH was always performed with the X/Y/18 probe cocktail. The 2nd round was performed with the 9/16/22 probe combination

Two blastomeres (11%) failed to give interpretable results (1.1a and 2.1b). In the case where two cells were available (embryos 6.2, 13.1 and 13.3) for analysis, the sex was in agreement between the two cells. Furthermore, in 5 blastomeres (9.1a, 9.2a, 9.4a, 11.1a and 13.2a) there were some cases of excess of green or red fluorescence in small subtelomeric regions, which were considered as artefacts due to their position, which is an area characterised by repetitive DNA sequences and has been described as difficult to interpret via CGH (Kallioniemi *et al,* 1994). In three embryos (2.1, 9.1 and 11.1) the CGH and FISH results were normal and diploid respectively for the chromosomes tested (Figure 5.9). Ten embryos were mosaic with various levels of abnormalities. The probe combination for all of these embryos was Xcep/Ycep/18cep for the first round and 9cep/16cep/22LSI for the second round. FISH results regarding the gonosomes were concordant with the CGH results. Only two embryos were considered to be blastocysts (>30 cells) on day 5, whilst the rest were arrested. For the embryos that showed a normal karyotype on day 3, on day 5 in total 150 cells were spread and 106 (70.6%) were diploid for the chromosomes tested. However, six out of the ten mosaic embryos (60%) contained cells with chaotic chromosome complements including nullisomies, tetrasomies and cells with more that three abnormalities present in different chromosomes. Embryos 9.4, 13.2 and 13.3 contained polyploid cells (4% overall), either triploidy or tetraploidy.

Figure 5.9. Illustration of a control CGH experiment between a male single buccal cell (red) acting as control against blastomere 7.1a (in green)



For each single cell CGH experiment 10 metaphases were captured, karyotyped, analysed individually and then interpreted cumulatively. A. Captured metaphase spread. B. In the karyotype of the captured metaphase the X chromosome has a distinct green fluorescence indicating excess in the test sample and the Y chromosome shows red fluorescence indicating deficiency in the test sample. C. This shows the cumulative analysis of ten metaphases, which is the basis of interpretation. There was a shift in fluorescence only towards the green in chromosome X and towards the red in chromosome Y showing that a the test sample (blastomere 7.1a) was a normal female

Table 5.4 displays the embryos which showed an abnormal finding in at least one biopsied cell (Group 2). Therefore, the FISH combinations were tailored according to the abnormalities the CGH revealed. From these 17 embryos 28 blastomeres were biopsied and 3 (10.7%) were not analysable. In four embryos one cell displayed a normal karyotype whereas the other was abnormal (4.2a, 7.3b, 8.1a and 9.6b). All chromosomes where shown to be affected either by partial or whole duplication or deletion. Chromosomes 1(x4), 16(x5) and 22(x4) demonstrated the highest incidence of chromosome deletion (whole or partial). Chromosomes 1(x4), 2(x3) and Y(x5) revealed a higher rate of chromosome duplication (whole or partial). Chromosomes 13 and 15 showed three whole chromosome deletions each, however, no duplication, partial or whole, were observed. Interestingly, chromosome 1 was involved in three instances of partial chromosome duplication or deletion at the locus of 1p36.1 (Figure 5.10 – bold letters in legends). This might be due to a fragile site at that specific location. Similarly, on chromosome 2 a possible fragile site between the region 2q21-q31 was found (Figures 5.10, 4.11 and 4.12 – bold letters in legends). All embryos were mosaic or completely chaotic at the time of spreading on day 5 and only two were considered to be blastocysts (4.2 and 7.3). Furthermore, from 183 cells that were spread on day 5, only 68 (37.1%) were diploid for the chromosomes tested. In total, 12/17 (70.5%) embryos contained at least one cell with a chaotic complement, of which four embryos (23.5%) were completely chaotic (Table 5.4). Three embryos (17.6%) completely lacked cells diploid for the chromosomes tested and all were mosaic aneuploid or mosaic aneuploid/chaotic.

In four embryos it was demonstrated that one of two biopsied cells was abnormal whereas the other blastomere may carry a normal chromosome complement (4.2, 7.3, 8.1 and 9.6; Figures 5.12 and 5.13). The FISH results for such embryos varied with two embryos showing that diploid cells were the predominant cell line (7.3 and 9.6) and two embryos having principally aneuploid cell lines (4.2 and 8.1). In 15 out of the 17 embryos the abnormality seen in a biopsied cell after CGH was confirmed during FISH analysis on day 5 embryos. In embryo 1.2, CGH analysis showed *rev ish XY dim(22)* and the FISH results showed cells with tetrasomy 22 and monosomy 22 indicating reciprocal loss and gain however, was classed as chaotic due to additional complex abnormalities by FISH (Appendix Table 8.1). In embryo 2.2 the CGH result

was *rev ish XY, dim(18)* and the FISH results also revealed chromosome loss since monosomy and nullisomy 18 were observed. Interestingly, in embryo 8.2 from one biopsied cell the karyotype *rev ish XX, enh(9 and 16)* was seen, whereas during FISH analysis the chaotic cells were monosomic and/or nullisomic for chromosome 16 indicative of reciprocal loss and gain in the embryo (Appendix Table 8.1). In an attempt to see whether the phenomenon of chromosome breakage can be observed by FISH later in development, in embryo 9.7 where cell 9.7a displayed the following karyotype: *rev ish XY, enh(1p36.3-q21), dim(1q31-q44)*, the chosen FISH probe combination was 1p/1q/16cep (Table 5.4). Two cells showed whole chromosome 1 loss, one cell revealed partial loss of the *1p* arm and gain for the *1q* arm and the one other cell showed partial loss of the *1q* arm and gain for the *1p* arm. It can be argued that the numbers are small and FISH artefacts can be affecting the results, however, both probes are sub-telomeric locus specific probes and not repetitive. In embryo 12.2 the CGH results hinted of chromosome breakage at the short arm at chromosome 4 and FISH was able to detect the partial chromosome loss in 2/12 cells which were also classed as chaotic.

Table 5.4. Results from embryos showing abnormal CGH findings (Group 2) followed by sequential FISH analysis.

Embryo No.	Cell	CGH Result on day 3	FISH Result on day 5[b]		Interpretation
			Cells	Results (no. of cells)	
1.2	a	rev ish XY, dim(22)	2	Chaotic(2)	Chaotic
2.2	a	rev ish XY, dim(18)	3	Chaotic(3)	Chaotic
	b	No result			
3.1	a	rev ish XY, enh(5pter, 9qter, 17), dim(4,19)	2	Chaotic(2)	Chaotic
	b	No result			
4.2	a	rev ish XX	33	Dip(9) / -X(8) / -18(5) / -18,-22(2) / -X,-18(2) / +18,+16(1) / trip(1) / chaotic(5)	Mosaic Aneuploid/Diploid/ Polyploid/Chaotic
	b	rev ish XX, enh(1, 22), dim(16pter, 18)			
6.3	a	rev ish XY, enh(6p25-p21.1)[a]	8	Dip(2) / +X(2) / -16,-X(1) / -X,+22(1) / -X(1) / -22(1)	Mosaic Aneuploid/Diploid
	b	rev ish XY, enh(Y)			
7.2	a	rev ish XYY, enh(1p36.2-36.1, 2q31-p25, 5, 7, 8, 18, 19, 20, 21 and Y), dim(1p31-q44, 2q32-q37, 3, 6, 11, 13, 14, 15)	3	**3cep/11cep/13LSI**[b] Chaotic(3)	Chaotic
	b	No result			

[a]These findings were considered as artefacts.

[b]The 1st round of FISH was always performed with the X/Y/18 probe cocktail. The 2nd round was performed with the 9/16/22 probe combination unless stated in bold and underlined combinations.

dip = diploid, **tet** = tetraploid, **trip** = triploidy. The - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

Embryo No.	Cell	CGH Result on day 3	FISH Result on day 5[b]		Interpretation
			Cells	Results (no. of cells)	
7.3	a	rev ish XY, dim(22q11.1-q13)	30	Dip(13) / +22(5) / -16,-22(2) / -9(1) / tet(1) / chaotic (8)	Mosaic Diploid/Aneuploid/ Polyploid/Chaotic
	b	rev ish XY			
8.1	a	rev ish XX	4	**<u>10cep/14q[b]</u>**	Mosaic Aneuploid/Chaotic
	b	rev ish XX, enh(4, 6qter, 12p11.2-q24.3, 14q21-q32) dim(2q31-q37, 10)		-10(2) / -10,+14qter(1) / chaotic(1)	
8.2	a	rev ish XX, enh(9 and 16)	10	Dip(6) / chaotic(4)	Mosaic Diploid/Chaotic
9.3	a	rev ish XY, dim(15q15-q26, 16)	4	Dip(1) / -16, -16(2) / -16,+22(2)	Mosaic Aneuploid/Diploid
9.6	a	rev ish XX, enh(2q21-q33, 3q11.1-q25), dim(1p36.1-p31, 16, 19 and 22)	23	Dip(13) / -16,-22(3) / tet(2) / -22(2) / -18(1) / +X(1) / chaotic(1)	Mosaic Diploid/Aneuploid/ Polyploid/Chaotic
	b	rev ish XX			
9.7	a	rev ish XY, enh(1p36.3-q21), dim(1q31-q44)	5	**<u>1p/1q/16cep[b]</u>**	Mosaic Aneuploid/Chaotic
	b	rev ish XY, dim(1)		-1qter,-1pter,+X(1) / +1qter,-1pter,+Y(1) / +1qter,-1pter(1) / -16,-X(1) / chaotic(1)	

[a]These findings were considered as artefacts.

[b]The 1st round of FISH was always performed with the X/Y/18 probe cocktail. The 2nd round was performed with the 9/16/22 probe combination unless stated in bold and underlined combinations.

dip = diploid, **tet** = tetraploid, **trip** = triploidy. The - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

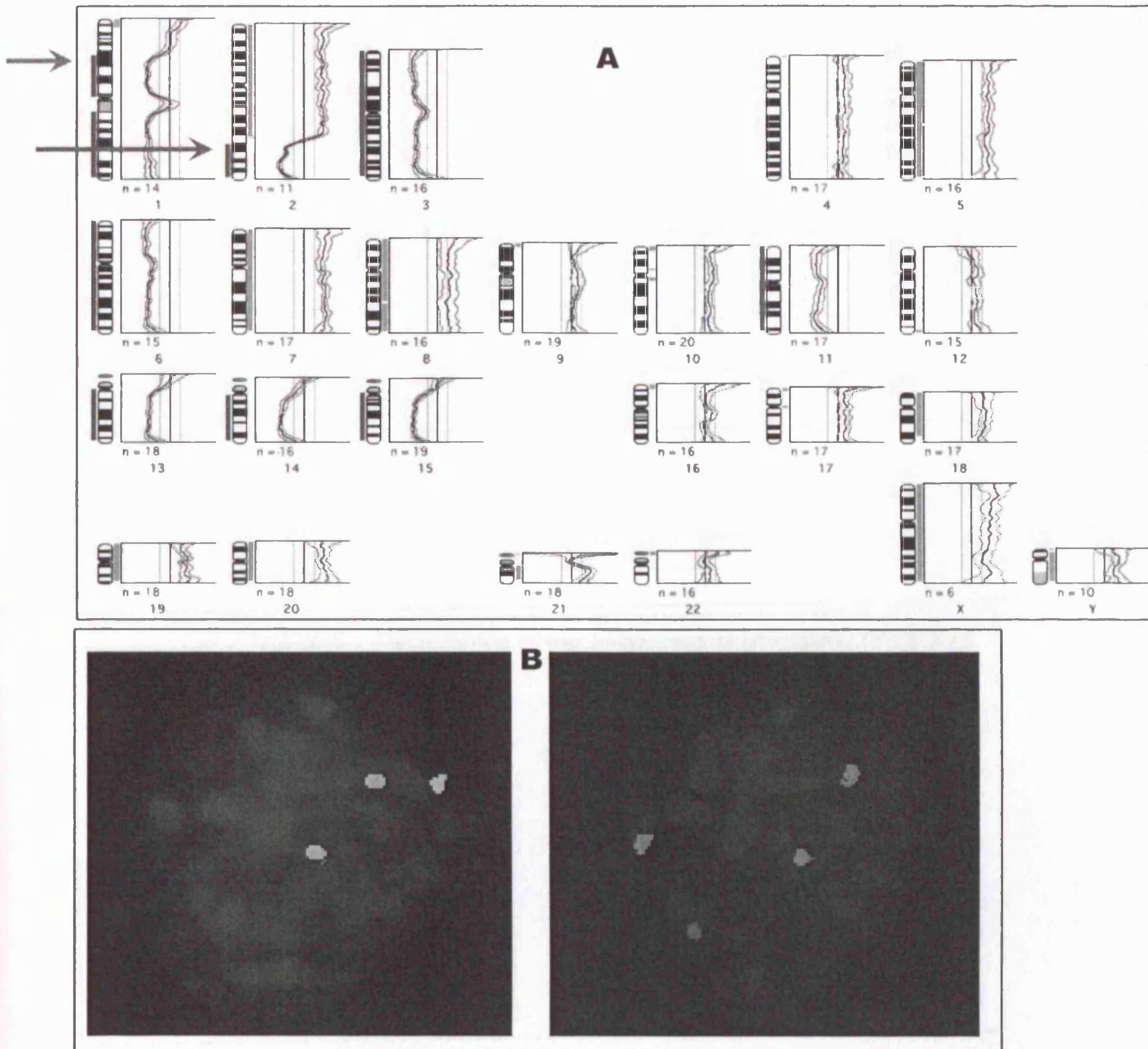
Embryo No.	Cell	CGH Result (day 3)	FISH Result (day 5)[b]		Interpretation
			Cells	Results	
9.8	a	rev ish XY, enh(17p13-q11, 18p11.3-q11.1), dim(3p26-p14)	17	**<u>3cep/6cep/18cep</u>**[b] Dip(11) / +6(3) / -6(1) / -18,+Y(1) / -X,+Y(1)	Mosaic Diploid/Aneuploid
	b	rev ish XY, enh(6)			
10.1	a	rev ish XX, enh(20)	19	Dip(6) / +X,+X(6) / +X(3) / -X(1) /-22(1) / tet(1) / trip(1) /	Mosaic Aneuploid/Diploid/Polyploid
12.2	a	rev ish XY, enh(Y), dim(4pter)	12	**<u>Ycep/4cep</u>**[b] Dip(6) / -18(2) / -Y(2) / chaotic(2)	Mosaic Diploid/Aneuploid/Chaotic
12.3	a	rev ish XY, enh(Yq11.1-q12), dim(16q21-q24)	2	**<u>Xcep/Ycep/16q</u>**[b] -16,-18(1) / -16(1)	Mosaic Aneuploid
14.2	a	rev ish XX, enh(1, 10, 16qter), dim(8, 13, 21, 22)	6	Dip(1) / -16(1) / -16,-22(1) / +16,+22(1) / chaotic(2)	Mosaic Aneuploid/Diploid/Chaotic
	b	rev ish XX, enh(2,6), dim(9qter,13, 15, 16qter, 17)			

[a]These findings were considered as artefacts.

[b]The 1st round of FISH was always performed with the X/Y/18 probe cocktail. The 2nd round was performed with the 9/16/22 probe combination unless stated in bold and underlined combinations.

dip = diploid, **tet** = tetraploid, **trip** = triploidy. The - indicates loss of chromosome and + indicates gain of chromosome e.g. +18 is trisomy 18 or -1 is monosomy 1.

Figure 5.10. CGH and FISH analysis results from embryo 7.2

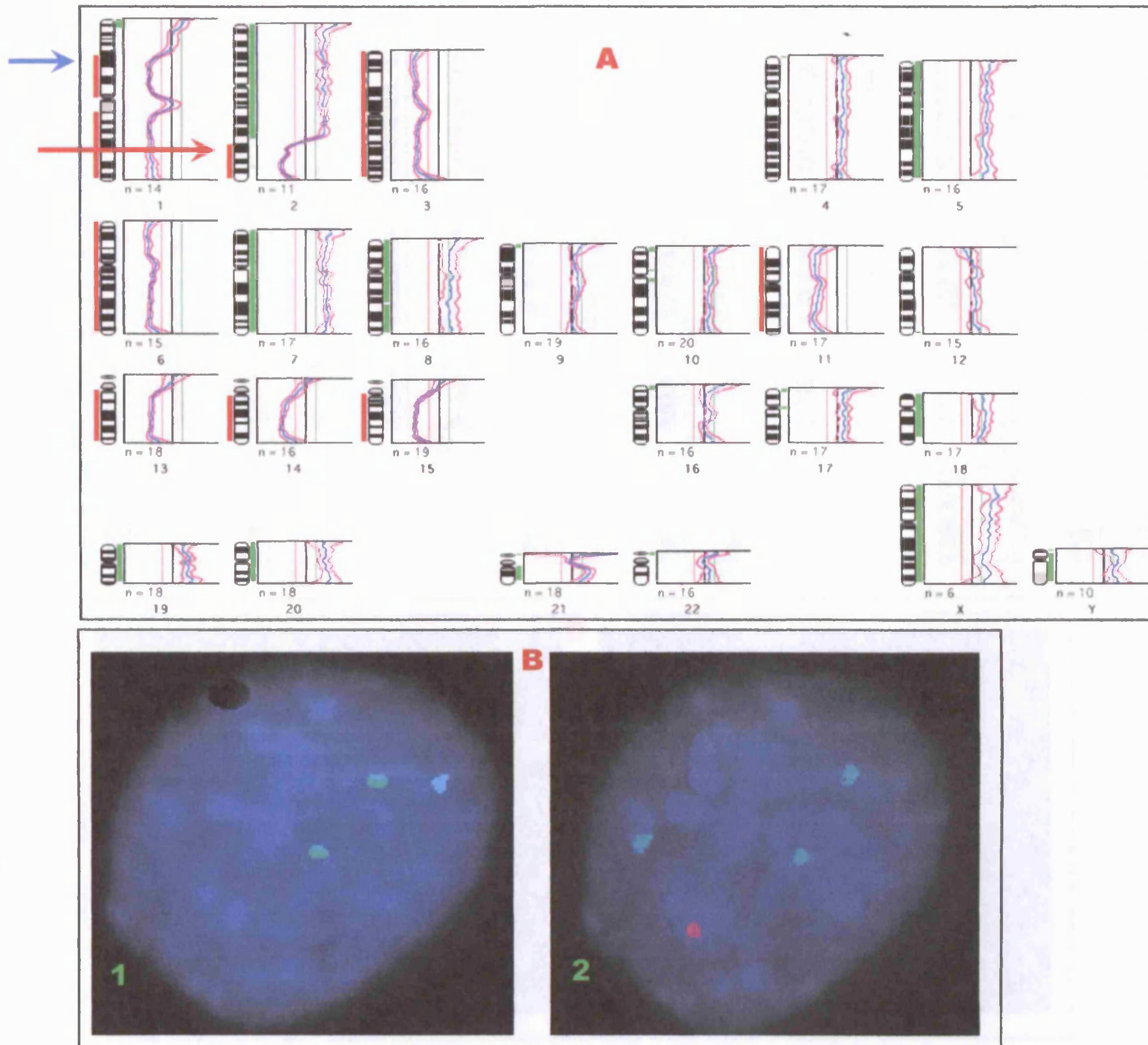


A. Interpretation of CGH experiment showing the cumulative analysis of ten metaphases from blastomere 7.2a. The test sample (blastomere 7.2a) is rev ish XYY, enh(1p36.2-36.1, **2p25-q31**, 5, 7, 8, 18, 19, 20, 21 and Y), dim(**1p31-q44**, **2q32-q37**, 3, 6, 11, 13, 14, 15). The blue arrow indicates the possible fragile site for chromosome 1 and the red arrow the fragile site for chromosome 2.

B. Results from the two sequential rounds of FISH in a cell from embryo 7.2. In B1 the cell was subjected to the X(green) / Y(red) / 18(aqua) probe cocktail where it showed female (XX) and monosomy 18. In B2 the cell was subjected to the 3(orange) / 11(aqua) / 13(green) probe combination which showed monosomy 3, nullisomy 11 and trisomy 13

Overall, the FISH results confirmed the chromosome loss events for chromosomes 3 and 11 and revealed reciprocal loss and gain (MND event) for chromosomes 13, 18 and Y.

Figure 5.10. CGH and FISH analysis results from embryo 7.2

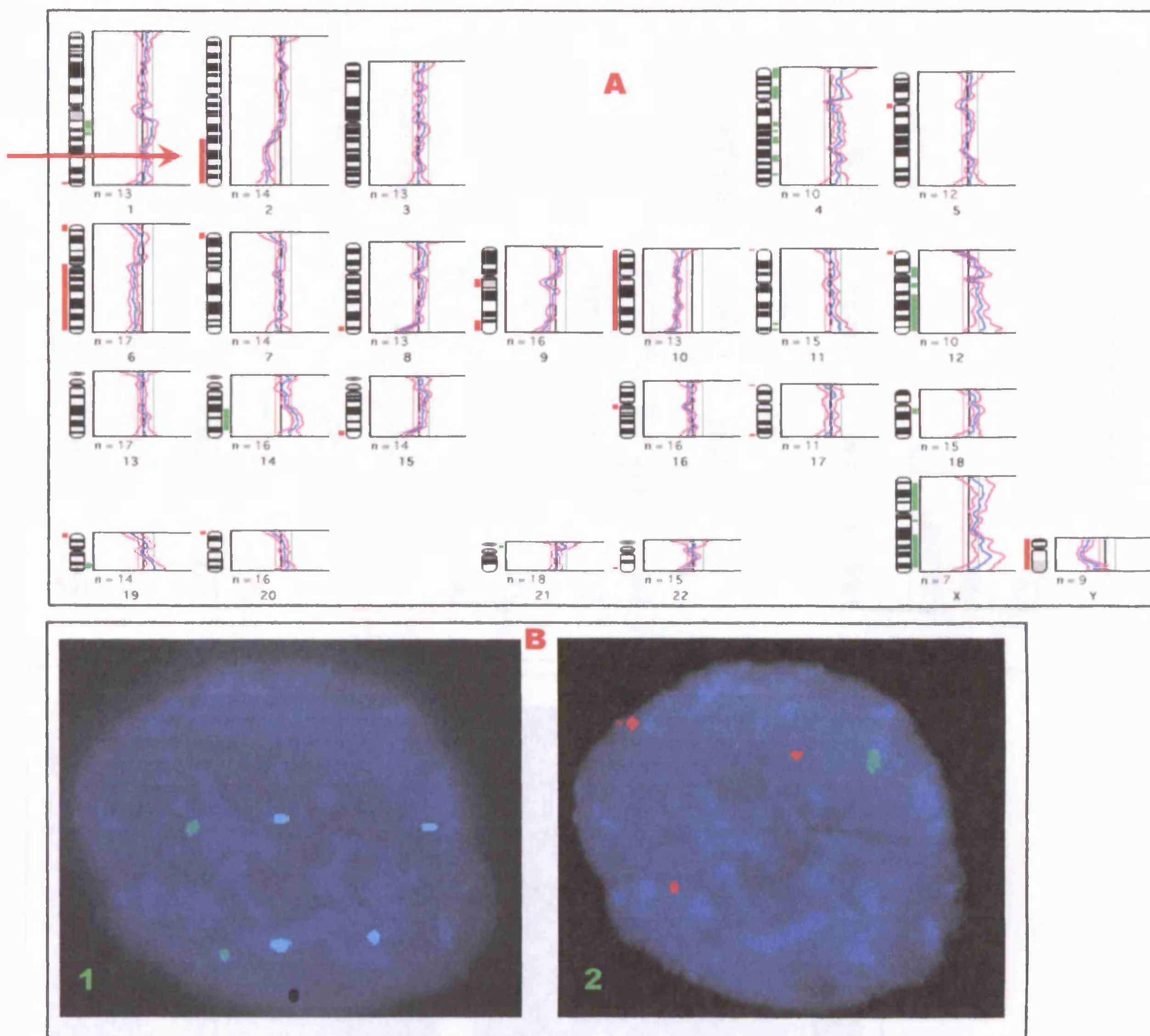


A. Interpretation of CGH experiment showing the cumulative analysis of ten metaphases from blastomere 7.2a. The test sample (blastomere 7.2a) is rev ish XYY, enh(1p36.2-36.1, 2p25-q31, 5, 7, 8, 18, 19, 20, 21 and Y), dim(1p31-q44, 2q32-q37, 3, 6, 11, 13, 14, 15). The blue arrow indicates the possible fragile site for chromosome 1 and the red arrow the fragile site for chromosome 2.

B. Results from the two sequential rounds of FISH in a cell from embryo 7.2. In B1 the cell was subjected to the X(green) / Y(red) / 18(aqua) probe cocktail where it showed female (XX) and monosomy 18. In B2 the cell was subjected to the 3(orange) / 11(aqua) / 13(green) probe combination which showed monosomy 3, nullisomy 11 and trisomy 13

Overall, the FISH results confirmed the chromosome loss events for chromosomes 3 and 11 and revealed reciprocal loss and gain (MND event) for chromosomes 13, 18 and Y.

Figure 5.11. CGH and FISH analysis results from embryo 8.1

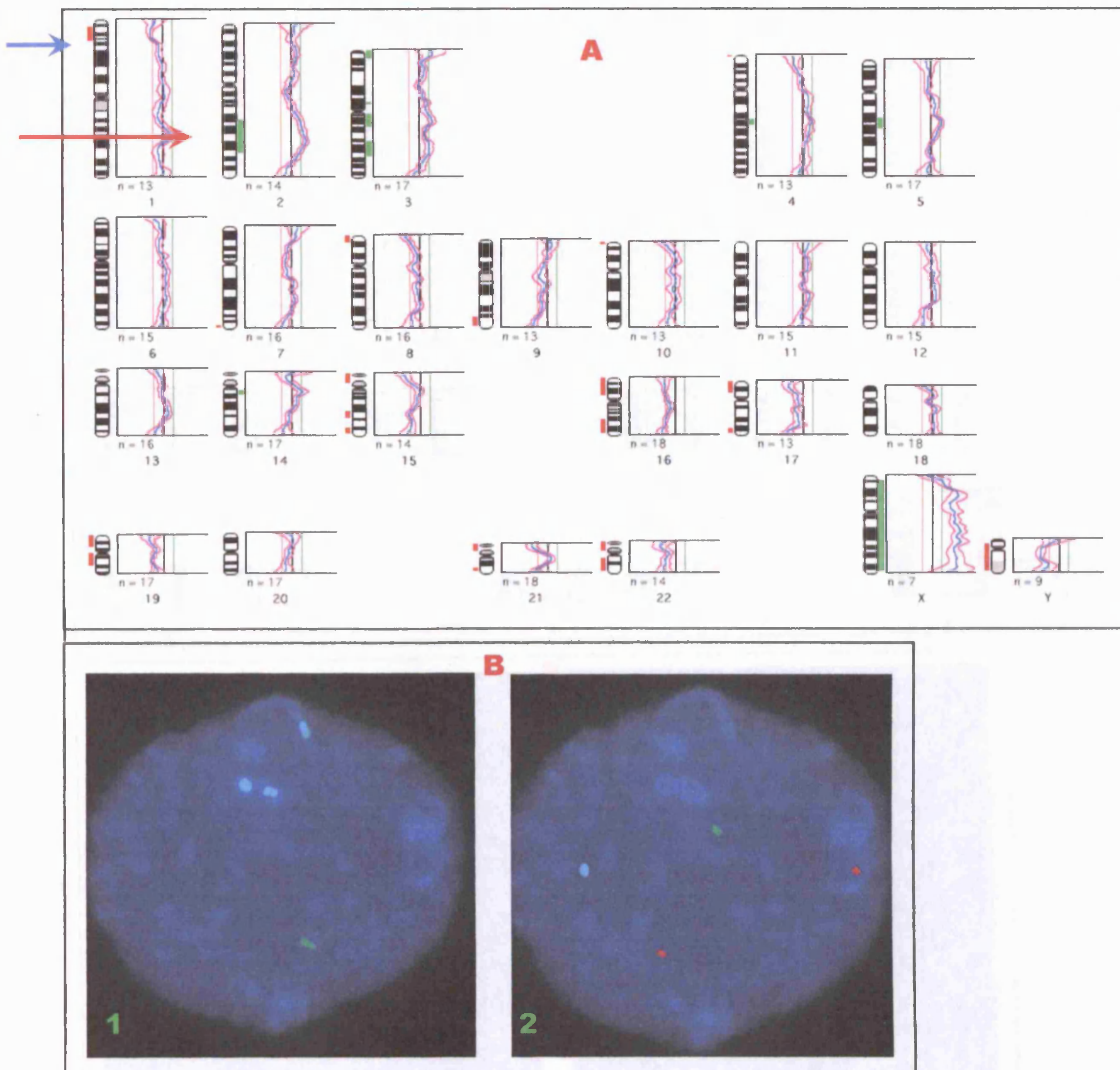


A. Interpretation of CGH experiment showing the cumulative analysis of ten metaphases from blastomere 8.1b. The test sample (blastomere 8.1b) was rev ish XX, enh(4, 6qter, 12p11.2-q24.3, 14q21-q32) dim(2q31-q37, 10). The red arrow indicates the possible fragile site for chromosome 2.

B. Results from the two sequential rounds of FISH in a cell from embryo 8.1. In B1 the cell was subjected to the X(green) / Y(red) / 18(aqua) probe cocktail where it showed female (XX) and tetrasomy 18. In B2 the cell was subjected to the 10(green) / 14(orange) probe combination which showed monosomy 10 and trisomy 14.

Overall, the FISH results confirmed the female status of the embryo, the whole chromosome loss event for chromosome 10 and the partial chromosome gain for the telomere of chromosome 14 (14qter).

Figure 5.12. CGH and FISH analysis results from embryo 9.6 (blastomere a)

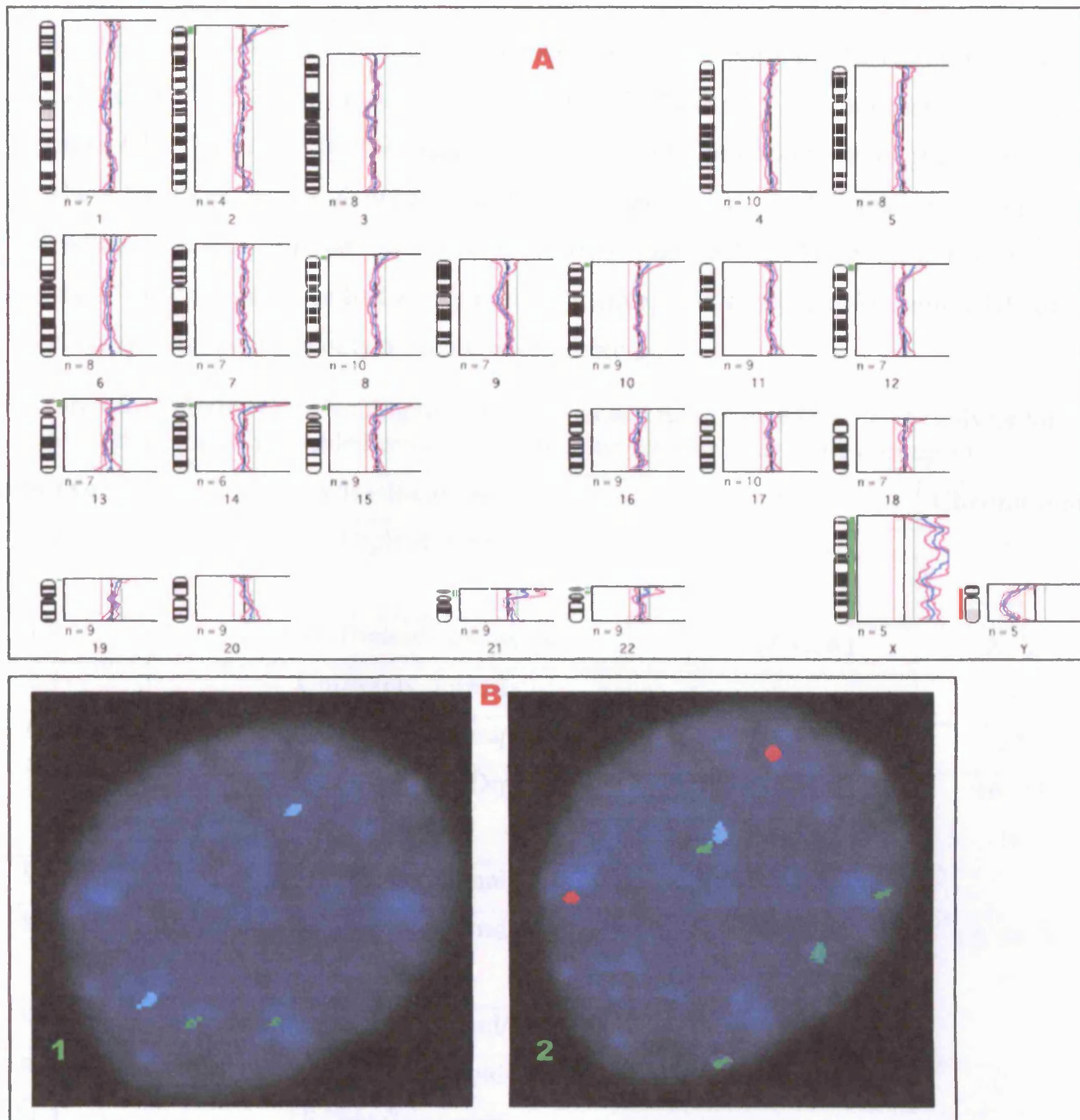


A. Interpretation of CGH experiment showing the cumulative analysis of eight metaphases. There is a shift in fluorescence towards the green in chromosome X and towards the red in chromosome Y showing that a the test sample (blastomere 9.6a) was rev ish XX, enh(2q31-q33, 3p25-p21), dim(1p36.1-p31, 16, 19 and 22). The red arrow indicates the possible fragile site for chromosome 2.

B. Results from the two sequential rounds of FISH in a cell from embryo 9.6. In B1 the cell was subjected to the X(green) / Y(red) / 18(aqua) probe cocktail where it showed female (XX) and diploid for chromosome 18. In B2 the cell was subjected to the 9(green) / 16(aqua) / 22(orange) probe combination which showed monosomy for chromosomes 16 and 22 and diploid for chromosome 9.

Overall, FISH analysis confirmed the sex status of the embryo as well as the presence of monosomy for chromosomes 16 and 22.

Figure 5.13. CGH and FISH analysis results from embryo 9.6 (blastomere b)



A. Interpretation of CGH experiment showing the cumulative analysis of twelve metaphases. There is a shift in fluorescence towards the green in chromosome X and towards the red in chromosome Y showing that a the test sample (blastomere 9.6b) was rev ish XX. The test sample (blastomere 9.6b) was female with a normal chromosome complement.

B. Results from the two sequential rounds of FISH in a cell from embryo 9.6. In B1 the cell was subjected to the X(green) / Y(red) / 18(aqua) probe cocktail where it showed female (XX) and disomy 18. In B2 the cell was subjected to the 9(green) / 16(aqua) / 22(orange) probe combination which showed disomy 9, 16 and 22.

Overall, the FISH results confirmed the sex status of the embryos and the presence of normal (diploid) cells in embryo 9.6

5.2.6.1 Cytogenetic Events Leading to Mosaicism

In total, only 10% (3/30) of the embryos were uniformly normal. The rest were either mosaic 76.7% (23/30) or completely chaotic 13.3% (4/30). The mechanisms were either CL, CG or MND. All events involved whole chromosomes for the embryos where the CGH results showed normal karyotypes (Group 1). The main mechanism leading to mosaicism was whole chromosome loss (50%) (Table 5.5). However, whole chromosome gain had also a high incidence with 44.5% (8/18), with MND of whole chromosomes in only 5.5% of cases (Table 5.5).

Table 5.5. Mechanisms leading to aneuploid mosaicism detected by FISH analysis for the embryos which revealed cells with normal karyotypes after CGH (Group 1).

Embryo	Classification	Event	Chromosome
1.1	Mosaic Diploid/Aneuploid	1CL(w) 3 CG(w)	18 22, X, Y
1.3	Mosaic Diploid/Aneuploid	2CG(w)	X, Y
2.1	Uniformly normal	-	-
6.2	Mosaic Diploid/Aneuploid	1CL(w)	22
7.1	Mosaic Aneuploid/Diploid	2CL(w) 1MND(w)	16, 22 18
9.1	Uniformly normal	-	-
9.2	Mosaic Diploid/Aneuploid/Chaotic	3CL(w) 1CG(w)	16, 18, X 22
9.4	Mosaic Diploid/Polyploid/Chaotic	-	-
9.5	Mosaic Diploid/Aneuploid/Chaotic	1CL(w)	X
11.1	Uniformly normal	-	-
12.1	Mosaic Diploid/Chaotic	-	-
13.2	Mosaic Diploid/Polyploid/Aneuploid/Chaotic	1CL(w) 1CG(w)	9 22
13.3	Mosaic Diploid/ Polyploid/Aneuploid/ Chaotic	1CG(w)	X
Total **13**		**9CL (50%)** **8CG (44.5%)** **1MND (5.5%)**	

'w' stands for whole chromosome event (either loss, gain or MND)

'p' stands for partial chromosome event (either loss, gain or MND)

It must be noted, that taking into account the previous study (Chapter 3), some losses can be considered as FISH artefacts when they are confined to just one cell.

In the embryos where there was at least one abnormal karyotype (Group 2) revealed during CGH interpretation from 57 events of aneuploid mosaicism, the main mechanism was whole CL (37%) followed by whole CG (19%) and whole MND (16%) (Table 5.6). In this group of results the incidence of events leading to mosaicism involving partial chromosomes was also considerable especially for partial chromosome loss which accounted for 14%. Chromosomes involved in partial chromosome events included chromosomes 1, 2, 3, 4, 9, 14, 15, 16, 17 and 18. All these chromosomes showed breakage in the short (p) or long (q) arms, however, chromosome 18 showed partial CG in the region of 18p11.3-q11.1 during CGH and partial CL in the same region in one cell during FISH, thus revealing reciprocal aneuploidy due to partial MND for the that region of chromosome 18 (Table 5.4 and Table 5.6)

Table 5.6. Mechanisms leading to aneuploid mosaicism for the embryos, which revealed a least one cell with abnormal karyotype after CGH (Group 2).

Embryo	**Classification**	**Event**	**Chromosome**
1.2	Chaotic	-	-
2.2	Chaotic	-	-
3.1	Chaotic	-	-
4.2	Mosaic Aneuploid/Diploid/Polyploid/Chaotic	2CL(w) 1CG(w) 1MND(w)	22, X 16 18
6.3	Mosaic Aneuploid/Diploid	1CL(w) 1CG(w) 2MND(w)	16 Y 22, X
7.2	Chaotic	-	-
7.3	Mosaic Diploid/Aneuploid/Polyploid/Chaotic	2CL(w) 1MND(w)	9, 16 22
8.1	Mosaic Aneuploid/Chaotic	1CL(p) / 1CL(w) 1CG(p) / 2CG(w)	2*qter* / 10 14*qter* / 4, 12

8.2	Mosaic Diploid/Chaotic	-	-
9.3	Mosaic Aneuploid/Chaotic	1CL(p) / 1CL(w) 1CG(w)	15*qter* / 16 22
9.6	Mosaic Diploid/Aneuploid/Polyploid/Chaotic	1CL(p) / 4CL(w) 2CG(p) / 1CG(w)	3*pter* / 16,18,19, 22 2*qter*, 3*qter* / X
9.7	Mosaic Aneuploid/Chaotic	3CL(w) 1CG(w) 2MND(p)	1, 16, X Y 1*pter*, 1*qter*
9.8	Mosaic Diploid/Aneuploid	1CL(p) / 1CL(w) 1CG(p) / 1CG(w) 1MND(p) / 1MND(w)	3*pter* / X 17*pter*/ Y 18*cep* / 6
10.1	Mosaic Aneuploid/Diploid/Polyploid	1CL(w) 1CG(w) 1MND(w)	22 20 X
12.2	Mosaic Diploid/Aneuploid/Chaotic	1CL(p) / 1CL(w) 1MND(w)	4*pter* / 18 Y
12.3	Mosaic Aneuploid	1CL(p) / 1CL(w) 1CG(w)	16*qter* / 18 Y
14.2	Mosaic Aneuploid/Diploid/Chaotic	2CL(p) / 3CL(w) 2CG(w) 2MND(w)	9*qter*, 16*qter* /13,15,17 2, 6 16, 22
Total **17**		**8CL(p) (14%)** **21CL(w) (37%)** **5CG(p) (9%)** **11CG(w) (19%)** **3MND(p) (5%)** **9MND(w) (16%)**	

'w' stands for whole chromosome event (either loss, gain or MND)

'p' stands for partial chromosome event (either loss, gain or MND)

5.3 Discussion

This study was aimed at assessing the CGH technique and examining its suitability as a research tool on human preimplantation embryos. Each step of the technique was

optimised for single cell use. Furthermore, CGH was tested against FISH in order to investigate whether by obtaining a full karyotype on 1-2 cells on day 3 of embryo development can reveal any surplus information about the mechanisms of mosaicism on day 5 of embryo development when the whole embryo was analysed by FISH.

5.3.1 WGA Optimisation

Initial work included assessing the handling of single buccal cells and tubing and amplifying each single cell. Amplification efficiency reached 97% (from 100 single cells) after DOP-PCR. DOP was selected as the preferred method of whole genome amplification since it has been postulated that it produces sufficient quantities of amplified product and provides sufficient coverage of the genome from a single cell (Wells *et al,* 1999; Voullaire *et al,* 1999). All DOP-PCR products were analysed using gel electrophoresis where contamination in the negative controls was present in less than 5% (Figure 5.1). All single buccal cells and blastomeres yielded a smear that contained a single distinct band at 450bp (Figure 5.1). Such bands have previously been suggested to be mitochondrial DNA, which is amplified by DOP-PCR although this does not interfere with the CGH profile, as it does not hybridise to the template chromosomes (Voullaire *et al,* 2000). It was essential to maintain stringent precautions against contamination throughout single cell isolation, lysis and amplification. The incidence of contamination was assessed regularly using numerous control blanks containing PCR reaction mixture but no DNA. A non-intensive smear in the single cell amplified products was visible thus displaying sufficient coverage of the whole genome. Such heterogeneous mixture of fragments generated by DOP-PCR and visualised as a smear has also been reported by Wells *et al* (1999). During amplification of single cells using DOP-PCR, two different thermal cyclers were assessed, the Omnigene™ and Eppendorf Master Gradient®. In each thermal cycler 20 single cells were amplified and their results were analysed on 1-2% agarose gel stained with ethidium bromide (Figure 5.2). During analysis it was observed that the Omnigene™ thermal cycler displayed a larger smear thus showing enhanced amplification of the genome. This considerable difference in smears between thermal cyclers was later reduced by adjusting the 'ramp' speed of the Eppendorf Master

Gradient®, however, the Omnigene™ remained the preferred choice of thermal cyclers during DOP-PCR amplification.

5.3.2 CGH Optimisation

Preliminary work on genomic DNA extracted 'in house' was utilised to optimise the CGH technique in order to be able to produce reliable results in single cells. Normal male genomic DNA (labelled in red which acted as the 'control' sample) against normal female DNA (labelled in green which acted as the 'test' sample) was amplified and analysed using CGH. CGH was able to detect the sex chromosomes in the samples with shift of fluorescence towards the green for the X chromosome indicating excess and towards the red for the Y chromosome indicating deficiency in the 'test' sample (Figure 5.3). The determination of the sex chromosome CGH pattern was a criterion for the reliability of the procedure. However, it must be taken into consideration that the euchromatic region of the Y chromosome is at the limit of size resolution for which aneuploidy can be detected by CGH (Voullaire *et al*, 1999). Subsequently CGH was performed on clumps of buccal cells from normal male and female individuals, which was successful. Thus, the remaining single cell DOP-PCR products (section 5.2.1) were put through CGH to assess its efficiency at the single cell level. The CGH efficiency on control single cell samples was 93.3%, with only two cells producing non-interpretable results. Furthermore, in a study of single fibroblasts the DOP-PCR amplification allowed reliable detection of trisomies 13, 18, 21 and 22 (Figures 5.4-5.7). The hybridisation of DOP-PCR products to normal metaphase chromosomes produced strong even signals with no obvious sites of amplification deficiency or excess. The fluorescence shifts on the trisomic chromosomes were later used as reference for shifts displayed by day 3 blastomeres. Similar results on single fibroblasts of trisomic samples have been shown by Wells *et al* (1999). Furthermore, the fluorescence intensity observed on the Y chromosome of the female 'test' samples revealed the extent of background fluorescence could be used 'roughly' as a reference to distinguish between monosomic and nullisomic samples. During the whole of this study homo-hybridisation (the hybridisation of samples containing similar amounts of DNA, each amplified and labelled using the same methods) was performed for all blastomeres, since it has been suggested that

hetero-hybridisation of DOP-PCR CGH displays high background, uneven hybridisation and is also associated with false deletions or amplifications (Huang *et al,* 2000)

CGH sensitivity is usually considered to be low in terms of the minimum size of detectable over/underrepresented chromosome fragments. Studies using CGH in a variety of samples have reported a resolution limit of 10-40Mbp (Kallioniemi *et al,* 1994; Daniely *et al,* 1998; Voullaire *et al,* 1999; Lestou *et al,* 2000; Malmgrem *et al,* 2002). However, Kirchhoff *et al* (1999; 2000; 2001) managed to develop the technique further to increase the sensitivity as well as the specificity in order to detect chromosomal aberrations as small as 3Mbp. Their modification was termed high resolution CGH (HR-CGH). During this study the CGH technique was not designed to detect small deletions such as the study which aimed and accomplished the detection of Prader Will/Angelman deletions (Kirchhoff *et al,* 1998), which are thought to be approximately 4Mbp (Christian *et al,* 1998) and thus are likely to be undetected by normal G-banding. However, during CGH interpretation of the captured images in some cases the presence of hybridisation artefacts was observed for the heterochromatic regions, and both the short and long arm telomeres of certain chromosomes, including 1, 9, 16, and Y and the satellite regions of the acrocentric chromosomes. These were caused due to the extreme suppression of these regions by the Cot-1 DNA and any low level fluorescence at these sites was attributable to background. Consequently, these regions were not considered during interpretation. In addition, in cases where abnormal results were obtained for chromosomes 19 and 22, they were interpreted with caution, due to the fact that these chromosomes are also known to be prone to labelling artefacts. Other similar observations regarding such artefacts on these chromosome regions have been reported from various studies in the literature (Tabet *et al,* 2001; Wilton *et al,* 2001).

5.3.3 CGH and FISH on Human Embryos

CGH is a technique with several difficulties due to the complexities of its nature. All cells from each embryo were consistent for the determination of the sex chromosomes and all were in agreement with the respective FISH results. However, it should be

noted that a possible case of mosaicism for a sex chromosome abnormality would negate this suggestion of an 'internal control'. In addition, the chromosomes present in a normal diploid copy number can be used as an internal negative control for assessment of efficiency of hybridisation (Malmgren *et al,* 2002). It has been reported that small deletions or amplifications of the telomeric regions are difficult to interpret by CGH analysis and might be missed (Malmgren *et al,* 2002).

A total of 54 blastomeres were biopsied on day 3 and 48 (88.9%) produced interpretable results. Failure of the CGH could indicate either an anucleate blastomere (as visibility of the nucleus was not possible for all the blastomeres biopsied), or that the cell was mistaken for an anuclear fragment which is a common finding in day three embryos (Voullaire *et al,* 2000). It has been shown that 5% of good quality embryos and 12% of poor quality embryos contain anucleated blastomeres (Hardy *et al,* 1993). Another explanation accounting for failure of results could be premature cell lysis or loss of a cell during transfer to the PCR tube. A cytoplasmic metaphase preparation of poor chromosome morphology would present a major obstacle to the successful hybridisation of both control and test DNA.

The efficiency of single blastomere CGH for this study was 88.9%, which is exactly the same as the single buccal cell rates. Wells and Delhanty, (2000) reported a CGH efficiency of 88% whilst Voullaire *et al,* (2000) revealed a slightly higher efficiency of 89%. Similarly to this study, both studies employed IVF embryos of good quality. However, during this study the embryos were frozen-thawed and not fresh which may explain the minor difference in efficiency. The efficiency of the study performed by Malmgren *et al,* (2002) using spare embryos from PGD cases was 70%, considerably lower than the one reported in this project. The fact that Malmgren *et al*, (2000) used a different type of WGA than the one used by Voullaire *et al,* (2000), Wells and Delhanty (2000) and this study could account for the lower efficiency seen in their study. The protocol used, based on linker-adapter mediated PCR, might be less reliable or amplify DNA less efficiently leading to their poorer results (D. Wells, personal communication). The FISH efficiency on embryos was higher than CGH with 343/354 (93%) producing analysable results. The FISH probe efficiency was

88.6% (range 81-97) for the 14 probes used, which was calculated on 200 interphase nuclei from a normal male lymphocyte slide.

Thirteen couples donated a total of 37 embryos for this research project with a mean maternal age of 33.1 (range 28-39) years. One embryo was not included since it failed to provide any kind of results during either CGH or FISH, which was expected due to their low morphology grade. A further six embryos failed to grow to day 5 and degenerated thus no cells could be spread. However, from all these embryos at least one cell was biopsied and the CGH results showed 3/7 blastomeres being of a normal karyotype, 2/7 were aneuploid and one was chaotic. Overall, from the thirty embryos where analysis was possible with both techniques the mean number of nuclei investigated on day 5 was 12. The low number of nuclei which was available for FISH analysis on day 5, where the embryos should have been blastocysts (at least 30 cells per embryo) can be attributed to several factors. Firstly, the culture media at the time might have been problematic hence, blastocyst formation was not promoted. Furthermore, the biopsy procedure was performed with a laser and might have had an adverse effect on the embryos. However, similar pregnancy rates have been obtained from studies comparing the laser biopsy technique to the widely used acid Tyrodes technique (De Vos and Van Steirteghem, 2001). It has been postulated that blastomere excision on day 3 could risk embryonic death by reducing the number of cells available for differentiation or by the excision of blastomeres essential to form a particular cell line (De Vos and Van Steirteghem, 2001). Technical expertise is required for a successful biopsy procedure and the person performing the technique was training and so was not adequately experienced. It has been suggested that the biopsy procedure can cause a strain on the embryo by disturbing tight junctions either by incubating the embryos in Ca^{+2}/Mg^{+2}-free medium prior to operating or by forceful aspiration (Munne and Cohen, 1998). Dumoulin and co-workers (1998) evaluated the embryo viability and implantation after exposing embryos for 45min to Ca^{+2}/Mg^{+2}-free medium and concluded that subsequent development to the blastocyst was not affected. Overall it has been shown from untransferred biopsied embryos in PGD cycles for sexing or aneuploidy screening that less than 30% show blastocyst formation after cleavage stage biopsy (Veiga *et al,* 1999; Magli *et al,* 2000; Sandalinas *et al,* 2000). A more recent study by Baart *et al* (2004) showed that around 50% of the embryos formed into blastocysts after biopsy of two blastomeres on day 3. Another factor that may have affected the blastocyst formation is the freeze-thaw

procedure, which might have rendered the embryos more fragile. It has been broadly suggested that the formation to blastocyst stage and consequent implantation potential of frozen-thawed embryos is probably compromised (Levran *et al*, 1990; Van Steirteghem *et al*, 1992; Van der Elst *et al*, 1995). Therefore, addition of the biopsy procedure on frozen-thawed embryos might have influenced the low numbers of blastomeres present during day 5 of spreading.

A total of thirty embryos provided results from both techniques. Only three embryos (10%) showed normal results both after CGH and for all the blastomeres analysed by FISH for the chromosomes tested. Compared to other studies the normality rate for this study is quite low since Wells and Delhanty (2000), Voullaire *et al* (2000) and Trussler *et al* (2004) showed rates of 25%, 25% and 42.5% respectively. In all three studies good quality fresh embryos were analysed of a grade 3-4/4 with grade 4 being the best quality embryos. Whereas in the present study though the embryos were considered of good quality, they had been frozen-thawed thus they might have been compromised. Laverge *et al* (1998) after analysing 63 frozen-thawed embryos by FISH concluded that embryos which do not grow further after freezing and thawing carry chromosomal abnormalities. Malmgrem *et al* (2002) found 0% normal embryos however the embryos used in that study were previously diagnosed as unbalanced and were considered not fit for transfer hence the higher degree of mosaicism. IVF culture conditions could also be responsible for the high frequency of mosaicism in this study. An example is a sudden decrease in temperature that could in turn affect cytokinesis, leading to the generation of diploid/polyploid embryos (Munne and Cohen, 1998). In addition, it has been suggested that embryos produced by different stimulation protocols and cultured under different conditions have very diverse mosaicism rates (Munne *et al*, 1997).

5.3.3.1 Group 1 Embryos

CGH showed a normal chromosome complement in thirteen embryos (Group 1). FISH analysis confirmed the sex of the embryo in all thirteen embryos. However, in five blastomeres (Table 5.3) there were sites on the sub-telomeric regions of chromosomes 19, X and Y that showed enhanced or diminished fluorescence. This set

of results was considered as artefacts. The underlying reason was that certain regions of the karyotype such as centromeres and sub-telomeric segments show variation in the profile and these regions are normally excluded from CGH (Kallionemi *et al,* 1994). Moreover, certain chromosomes (1p, 17, 19, 22 and Y) are prone to show frequent enhancement of the test signal and are also excluded from the analysis (Moore *et al,* 1997). Other chromosomes that have been reported to be prone to artefactual results in placental tissues include chromosomes 4, 13, and 18 (Lestou *et al,* 2000) however, these chromosomes did not present similar results in the current study. Embryo 13.2 displayed the only blastomere which was affected by an abnormality involving chromosome 9 after FISH analysis, whereas all other embryos had mixed aneuploid cells of monosomies or trisomies for chromosomes 16, 18, 22, X and Y, with aneuploidies for chromosomes 22 and X showing the highest incidence. Trisomy of the small autosomes and monosomy for the X chromosome, such as embryo 9.5 (Table 5.3) are the most common abnormalities detected in human pregnancy (Boue *et al,* 1985). Furthermore, embryos 13.2 and 13.3 displayed triploid cells the origin of which is unclear in the literature. The underlying mechanism that could lead to diploid/triploid mosaics may be due to an incorporation of another gamete or its genome into one of the daughter cells derived after the first mitotic division or later. The extra gamete might be a polar body (Mueller *et al*, 1993). It has also been suggested that diploid/triploid mosaicism could result from fusion of a diploid zygotic nucleus with an extra sperm nucleus or the extrusion and degeneration of a haploid nucleus to produce a diploid cell line in a triploid embryo (Kuo *et al,* 1998). Embryo 13.3 displayed mosaicism involving the sex chromosomes as well as triploidy. It has been postulated that delayed IVF might also cause triploid cells (Plachot *et al,* 1988) and more interestingly the embryo came from a couple which had ICSI treatment. Although this is an isolated phenomenon an increase of sex chromosome abnormality has been reported in children conceived using ICSI affecting at least 1% (Martin, 1996). Similar observations have been reported in a CGH study of human day 3 embryos where 2/12 embryos displayed mosaicism involving only sex chromosomes which were thought to have arisen from 47, XXX zygotes after ICSI treatment (Wells and Delhanty, 2000). Chaotic complements were also present in this group of embryos, which revealed normal CGH karyotypes from the biopsied blastomeres, in 6 embryos. Chaos has been reported in almost all the studies in embryos using either FISH or CGH.

5.3.3.2 Group 2 Embryos

In 17 embryos at least one cell showed an abnormal karyotype when analysed by CGH. Ultimately, all 17 embryos were classified as mosaic or chaotic (Group 2). Four embryos (23.5%) were completely chaotic containing cells with three or more abnormalities in different chromosomes. All four embryos when analysed by FISH on day 5 were arrested consisting of only 2-3 blastomeres each, which is consistent with suggestions that chaos is associated with impaired development (Delhanty and Handyside, 1995; Delhanty *et al,* 1997). All four embryos displayed abnormalities such as nullisomies, tetrasomies and completely abnormal gonosome karyotypes.

Except for embryo 4.2 all embryos were arrested (<30 blastomeres) and all embryos had a decreased number of blastomeres with a diploid cell line compared to Group 1. It has been implied from blastocyst studies that embryos with a lower proportion of diploid cell line (therefore higher degree of mosaicism) have a low developmental potential (Ruangvutilert *et al,* 2000a). However, this selection against embryos with a high degree of mosaicism does not operate perfectly.

Most of the evidence for different chromosome susceptibilities to aneuploidy has been obtained from clinically recognised pregnancies. Major studies reported that although trisomy 16 accounts for 20-35% of all trisomies, acrocentrics and chromosome 2 account for 5-10% each (Hassold *et al,* 1984; Warburton *et al,* 1986). In Group 2 embryos all chromosomes were involved in abnormal karyotypes either due to duplications or deletions (partial or whole). Chromosome 1 was shown to be most affected with four incidences of chromosome duplication (whole or partial) and four incidences of chromosome deletion (whole or partial). Similar data were obtained during our FISH study on day 5 embryos for the group of embryos (Group I), which were arrested (Chapter 3). However, in the case of these set of results it must be highlighted that some confined chromosome losses can be attributed to FISH artefacts since there was not two-loci per chromosome analysis during the current study. Therefore, FISH failure could not be detected and excluded from the results. Wells and Delhanty (2000) also found an increased incidence of chromosome 1 deletion however chromosome 2 was shown to be affected by chromosome deletion (partial or whole) on six different occasions. Baart *et al* (2004) also found chromosome 1

affecting 9/29 (31%) embryos when analysed by FISH. In our study chromosome 2 was mostly involved in chromosome duplications (partial or whole). In a recent CGH study nine instances of trisomy 22 were found (Trussler *et al,* 2004), which is in accordance with a FISH study investigating differences in chromosome susceptibility to aneuploidy that concluded that trisomy 22 is the most common aneuploidy (Munne *et al,* 2003b). In this study aneuploidy of chromosome 22 was also increased however, chromosome 22 deletions were more prominent than duplications.

In two embryos, 7.2 and 14.2, a total of 16 and 13 chromosomes were shown to be affected respectively. Embryo 7.2 displayed a complete breakdown of normal chromosome complement (Figure 5.10). The embryo was classed as chaotic and the FISH results were able to confirm the CGH abnormalities in six chromosomes. Different aneuploidies were seen including nullisomy for chromosomes 11, 13, 14 and 15, trisomy for chromosomes 3, 4, 5, 17, 18 and 19 as well as abnormalities in the sex chromosomes revealing karyotypes of XXYY, XXXO and XXXXXYY. Similar complete aneuploidy and embryos with random allocation of chromosomes to daughter cells have been previously observed by Wells and Delhanty (2000) in 2/12 embryos and by Trussler *et al* (2004) in 3/40 embryos. There is no pattern to the aneuploidy seen; chromosome losses and gains occur with similar frequency and there is no evidence of that any particular chromosome is involved more often than any other (Wells and Delhanty, 2000). Evsikov and Verlinsky (1998) postulated that embryos with chaotic chromosome segregation do survive to the blastocyst stage but will not progress further and would fail to implant.

FISH analysis was able to confirm the sex status of all seventeen embryos shown by CGH. Analysis of the CGH and FISH data demonstrated results 'in agreement' in 15/17 embryos (in the other two embryos there were no FISH probes available for the specific chromosome). The 'agreeing results' showed a similar abnormality on the chromosome in question or revealed a reciprocal abnormality for that chromosome. For example in embryo 8.1 CGH analysis revealed deletion of chromosome 10 and duplication of the 14qter. On day 5, the FISH protocol showed 4/4 cells with monosomy 10 and 2/4 cells with trisomy for the 14qter chromosome segment.

Furthermore, the CGH results in embryo 4.2 displayed duplication of chromosome 22 and deletion in chromosomes 16pter and 18. FISH analyses demonstrated two blastomeres with monosomy 22, nine blastomeres with monosomy 18 and one blastomere with trisomy 16 and 18 therefore revealing, overall, reciprocal loss and gain for chromosomes 16, 18 and 22.

The use of CGH to study human preimplantation embryos has enabled the detection of a specific type of error involving structural alteration of chromosomes, namely chromosome breakage. Embryos 7.2, 8.1, 9.6 and 9.7 displayed possible breakage in chromosomes 1 and 2. In embryo 9.7 the FISH protocol was chosen so that it can detect whether the breakage was an experimental artefact by having one sub-telomeric probe for each arm ('p' and 'q'). FISH analysis revealed 2/5 blastomeres showing reciprocal products of the breakage and 2/5 blastomeres displaying similar products of the breakage. In embryos 7.2 and 8.1 chromosome breakage was noted at the region of 2q31-2q37 where the acentric fragment was lost. Interestingly, in embryo 7.2 the remaining centric chromosome fragment (2p25-q31) was shown to be duplicated. Partial aneuploidy due to chromosome breakage is likely to result in an unstable karyotype through the formation of acentric and dicentric chromosomes (Voullaire *et al,* 2002).

5.3.4 Mosaicism and Chaos

Overall, 23/30 (76.6%) of the embryos analysed were mosaic, from which 14/23 and 9/23 were diploid as the major cell line and the aneuploid line respectively. In Group 1 embryos FISH results on 150 blastomeres showed 70.6% being diploid whereas from 183 cells analysed by FISH in Group 2 embryos only 37.1% were diploid for the chromosomes tested. The significant drop of the diploid cells was expected since Group 1 embryos would have been diagnosed as normal in a PGD setting due to their normal CGH results. All errors leading to aneuploid mosaic embryos were post-zygotic with no meiotic error present. A meiotic division error would render all embryonic cells chromosomally aneuploid. This is an interesting finding which is in accordance with a similar study performed by Baart *et al* (2004) which analysed two blastomeres by FISH on day 3 and re-analysed the whole embryos by FISH, using the

same probe combination on day 5. Their aim was to clarify whether it is more informative and less prone to misdiagnosis to carry out PGD for aneuploidy screening on day 3 or on day 5. However, in all embryo CGH studies a low percentage of meiotic errors (7.5-8.3%) has been reported (Wells and Delhanty, 2000; Voullaire *et al,* 2000; Trussler *et al,* 2004).

Furthermore, it was disturbing that in 18/30 (60%) embryos at least one cell was chaotic in its chromosome complement. Chaotic embryos are thought to arise due to absence of cell cycle checkpoints. A study performed on mouse oocytes demonstrated the absence of the metaphase-anaphase checkpoint (Le Maire-Adkins *et al,* 1997). This checkpoint is responsible for the correct alignment of chromosomes onto the mitotic spindle, and the situation could be similar for human oocytes. The abnormalities of the mitotic spindle could be related to the sub-optimal *in vitro* culture environment (Pickering *et al,* 1990). Moreover, maternal genome support could result in the survival of embryos with multiple aneuploidies up until the blastocyst stage. Elimination of maternal mRNAs could lead to the arrest that is frequently observed prior to blastocyst formation for highly abnormal embryos (Wells and Delhanty, 2000). Wilding and co-workers (2003) recently published data stating that if poor vascularization of follicles is the underlying cause for the loss of mitochondrial activity in maturing oocytes this would lead to chaotic development.

5.3.5 Mechanisms of Aneuploidy Mosaicism

Embryonic death is caused by chromosomal abnormalities that are primarily the result of chromosomal errors during female gamete formation. However, there are some errors that will arise during early cleavage divisions as a consequence of mitotic errors. These errors in cell divisions, namely mitotic non-disjunction and anaphase lag lead to mosaicism and chaotic imbalances.

In Group 1 embryos only whole chromosome mosaicism mechanisms were seen from which the predominant was chromosome loss (CL) (50%), followed by chromosome gain (CG) (44.5%) and just one instance of mitotic non-disjunction (MND) (5.5%).

These results are very similar to the 'Group I embryos' in our FISH study on day 5 embryos (Chapter 4 Table 4.3). All errors were post-zygotic for Group 1 embryos. In the group of embryos that the CGH analysis displayed at least one cell with an abnormal karyotype (Group 2) there was an increased incidence of partial chromosome errors (28% in total). The predominant mechanism of mosaicism was whole chromosome loss (37%). Whole CG gave rise to 19% of the aneuploid mosaic embryos and whole MND to 16%. In comparison to Group 1, in Group 2 embryos the incidence of whole CG was halved whereas the rate of whole MND was tripled. Chromosome 22 was mostly affected by reciprocal loss and gain of material. Anaphase lagging is the causative agent behind whole CL and CG. Anaphase lagging leads to mosaicism and chaotic chromosome distribution, most probably reflecting asynchrony between karyokinesis and cytokinesis. Coonen *et al* (2004) recently reported that anaphase lag is the main cause chromosomal mosaicism in embryos. This was in accordance with our findings in the FISH study (Chapter 3). Similarly by employing CGH and FISH it has been shown that chromosome loss and gain are the main causes of mosaicism which occur probably due to anaphase lag.

In the partial chromosome mechanisms, again, partial CL was found to be 14%, followed by partial CG (9%) and partial MND (5%). This is the first study that recognises partial chromosome mechanisms as an underlying reason causing chromosomal aneuploidy. It has been postulated that chromosome breakage and whole chromosome aneuploidy could be caused by different factors (Wells and Delhanty, 2000). In a recent study, 6% of the embryos analysed by CGH were found to be affected solely by partial aneuploidy (Voullaire *et al,* 2002). Wells and Delhanty (2000) have proposed that acentric fragments would not be stably transmitted to daughter cells and the resultant loss of material would leave the embryo with a potentially lethal monosomy for that chromosome region. Furthermore, both initial studies of embryos using CGH observed chromosome breakage at the point of 2q31 (Wells and Delhanty, 2000; Voullaire *et al,* 2000) which is similar to this study (Figures 5.10, 5.11 and 5.12) along with the breakpoint in chromosome 1 (1p31). Both sets of breakpoints map to defined chromosomal fragile sites (Sutherland, 2003), which are prone to chromosome breakage. Fragile sites are non-randomly located gaps or breaks in chromosomes that are induced to appear by specific culture

conditions (Sutherland, 1979) and are frequently involved in de-novo chromosome rearrangements (Warburton, 1991). They are classified according to their chemistry and of induction and by their frequency in the population (Richards, 2001). Rare (or heritable) fragile sites are found on the chromosomes of less than 5% of individuals, whereas common (or constitute) fragile sites are found in all individuals (Sutherland and Baker, 2000). The most common 'fragile' site in the genome is FRA3B at 3p14.2 which has occurred once in this study in embryo 9.8 where that part of the chromosome 3 region (3p14-p26) was lost. A fragile site is a region of chromatin that fails to compact normally for mitosis (Sutherland, 2003). Therefore, during this study CGH has demonstrated that chromosome breakage in human preimplantation embryos can be considered as a possible mechanism of aneuploidy. FISH can only reveals information about a small area of each chromosome to which they hybridise, thus rearrangements affecting chromosomal regions rather than whole chromosomes, are not detected. During this study, this FISH limitation was used to our advantage in order to establish chromosome breakage rather than regard it as a CGH artefact.

5.3.6 Conclusions

Throughout this study all three techniques used i.e. whole genome amplification, FISH and CGH were optimised through a series of control experiments in order to achieve high amplification rates and hybridisation rates respectively. After FISH and CGH analysis it was found that only 10% of the day 5 embryos were uniformly diploid, while the rest were mosaic or completely chaotic. The major mechanism of mosaicism leading to aneuploid cells was found to be chromosome loss from Group 1 and Group 2 embryos. However, the difference between chromosome loss with chromosome gain and mitotic non-disjunction was not statistically significant. Similar results were obtained in Chapter 4, adding to the conclusion that chromosome loss, which probably arises due to anaphase lag, is mostly responsible for the high level of mosaicism present in embryos.

5.3.7 Future Work

CGH and FISH were employed during this study and assessed as techniques. Each technique offered information on the process of understanding the mechanisms that lead to chromosomal mosaicism and chaos in human preimplantation embryos.

Initially the CGH technique should be made less laborious and more reliable in order to be used in a clinical setting e.g. for PGD of chromosomal abnormalities. This can be achieved by reducing the time of hybridisation from 72 hours to less than 24. Wells and co-workers (2002) were able to reduce the time to 30 hours during a PGD case for aneuploidy screening, however, the CGH was applied to polar bodies.

The use of DNA microarrays to act as hybridisation templates, instead of the target metaphase chromosomes that have been used so far could further reduce the period of hybridisation, and improve the analysis of the obtained results, increasing the simplicity of interpretation by avoiding the need to karyotype metaphase chromosomes. During CGH-array analysis, patient DNA and normal reference DNA are hybridised to arrays of genomic clones in order to detect unbalanced gains or losses of genetic material across the genome (Shaffer *et al,* 2004). In a recent study by Schaffer *et al (*2004) where production-of-conception samples were analysed with G-banding and CGH-array technology. It was found that the array technology detected all abnormalities as reported by G-banding analysis and revealed ~10% new abnormalities. Bermudez *et al* (2004) carried out microarray CGH to analyse 5 human oocytes and were able to identify 1361 transcripts expressed in oocytes. Chan *et al* (2002) designed a DNA disc chip array, based on comparative genomic hybridisation in order to study sperm and concluded that the technique was reliable however it was still prone to manual processing involving the fluorescent microscope and computer. In recent study carried out on single lymphocytes, Hu *et al* (2004), described the application of array-CGH for normal and trisomic (13, 15, 18) single cell lymphocytes. During that study the slides were arrayed with chromosome-specific DNA libraries and the expected karyotypes of all cells was analysed and confirmed whilst hybridisation took place for 30hrs (Hu *et al,* 2004). These results prompted the author to suggest the use of this technique for PGS (Hu *et al,* 2004).

The whole genome amplification procedure could be replaced and use MDA as an alternative method of WGA. This method uses a φ29 DNA polymerase and random exonuclease-resistant primers, whilst DNA amplification takes place at 30°C (Dean *et al.,* 2002). It has been shown that MDA provides a highly uniform representation of the genome, with the amplification bias being less than 3-fold among eight chromosomal loci, compared to the 4-6-fold observed with the application of the DOP-PCR (Dean *et al,* 2002). Spits *et al* (2006) developed a protocol using MDA and were able to amplify 22 loci in 60 single cells and concluded that it can be used for PGD for single gene disorders.

Chapter 6

Conclusion

6.1 Final Thoughts

The constant evolution of molecular, cytogenetic and combinatorial techniques aims to facilitate and enrich the study of human preimplantation embryos and the subsequent application of PGD. During this study new methods and protocols for different techniques were devised and tested.

The phenomenon of mosaicism was explored in human preimplantation embryos with a novel protocol using multi-colour FISH. Mosaicism can be explained by the presence of two or more cell line present in one embryo. Mosaicism has been the 'culprit' causing misdiagnosis during PGD using either FISH or PCR. Therefore, the need of biopsying two cells per embryo whilst carrying out PGD has been put forward. The decision as to whether one or two cells should be removed is controversial; removing two cells reduces the cellular mass and could potentially reduce its developmental capacity (Braude *et al,* 2002). Early studies to measure the effect of biopsy on human embryonic development showed that two cells could be removed from 8-cell embryos on day three post-insemination without reducing the number blastulating or disturbing cleavage rates (Hardy *et al,* 1990). Furthermore, two-cell biopsy procedures should only be carried out on day three post-insemination at the 6-8 cell stage, when up to a quarter of the blastomeres can be removed without disturbing subsequent development (Handyside *et al,* 1989; Handyside, 1991; Hardy *et al,* 1990), as the biopsied cells are still undifferentiated (Harper *et al,* 1996). The debate over whether taking two cells rather than one is detrimental to the embryo, is ongoing (De Vos and Van Steirteghem, 2001). However, it has been shown that the accuracy of the diagnosis is likely to be enhanced if embryos are replaced when results from both cells are concordant (Van de Velde *et al,* 2000).

Therefore, it is necessary to determine the level of mosaicism. Many studies have been carried out in search of the levels of mosaicism using FISH both in cleavage stage (Delhanty *et al,* 1993; Munne *et al,* 1994b; Harper *et al,* 1995; Delhanty *et al,* 1997; Laverge *et al,* 1997; Iwarsson *et al,* 1999; Magli *et al,* 2000; Coonen *et al,* 2004) as well as blastocysts (Evsikov and Verlinksy, 1998; Veiga *et al,* 1999; Ruangvutilert *et al,* 2000a). All of these studies tried to assess the level of mosaicism using probes for as many chromosomes as possible. However, all previous studies

conclude that the FISH efficiency ranges from 60-95% depending on the type of probe, the number of probes used simultaneously and the number of sequential rounds. Hence, up to 40% of abnormalities can be missed whilst studying mosaicism and its effects. The protocol devised in this study was based on the detection of mosaicism of three main chromosomes (1, 11 and 18) as well as the degree of mosaicism in the sex chromosomes (due to the near perfect efficiency results of the probes). The three main chromosomes were investigated during two sequential rounds of FISH by employing a different probe and at a different site for each chromosome e.g. a centromere and a sub-telomere for each chromosome. This allowed the detection of any FISH artefacts and any FISH failure that might occur, providing the true level of abnormality for the chromosomes investigated. A total of 76 abnormal results would have been missed unless the two probes per chromosome protocol was carried out.

One of the main aims of this study was to explore the mechanisms that give rise to aneuploid mosaic embryos in order to understand the high levels in *in vitro* human preimplantation embryos. It has been suggested that the high level of embryonic death is mainly due to chromosomal abnormalities (Wells and Delhanty, 2000). Chromosomal abnormalities and especially aneuploidy has been found to be extremely high after as >60% of spontaneous abortions at <12 weeks are aneuploid (Hassold *et al,* 1980). In our study chromosome loss was found to be the mechanism affecting most aneuploid lines. Chromosome loss probably occurs due to anaphase lagging. Other studies have found that mitotic non-disjunction was the main mechanism affecting mosaicism. This may be due to the fact that the FISH protocols used by other groups regard low level monosomy findings as FISH failure or artefacts since they are using one probe per chromosome. In a very recent study, it was suggested that anaphase lagging is the major mechanism behind aneuploid mosaic embryos (Coonen *et al,* 2004).

The two different groups of embryos analysed were distinguished based on the type of IVF medium that they were grown. Group I embryos were cultured until day 5 in non-sequential medium, which is considered sub-optimal (Jones *et al,* 1998), whereas Group II embryos were grown in sequential medium. Their main difference was the number of nuclei available on days which was ~20 cells per embryo for Group I and

~55 cells per embryo for Group II signifying the limited growth in Group I. Both Groups revealed chromosomal loss as the main mechanism behind aneuploid mosaic embryos, however, in Group II there was an increased number of tetraploid cells present compared to Group I. This can be explained by the findings of multiple studies, which have proposed that probably tetraploid cells may be a normal feature in the development of the trophectoderm. This would add more support to the presumption of limited growth for Group I embryos.

In order to achieve definitive proof of the underlying mechanisms of mosaicism more embryos need to be analysed as well as analysis of all the chromosomes. Although, the current study was performed on spare, not fit for transfer embryos, therefore morphologically the embryos were compromised, still the data obtained were useful. A larger cohort of embryos which are morphologically sound should be analysed with means that can provide information regarding all the chromosomes. Novel techniques such as interphase conversion allowing the entire karyotype to be screened in one step have been developed in the recent years and have found clinical application. Their principle involves fusion of polar bodies or blastomeres with enucleated human or bovine oocytes to induce mitosis (Verlinsky and Evsikov, 1999a; Willadsen *et al,* 1999). Interphase conversion methods for inducing metaphase in biopsied blastomeres have been successfully applied in a number of translocation cases to date with subsequent analysis by standard chromosome painting (Verlinsky and Evsikov, 1999a; Evsikov *et al,* 2000) or SKY FISH (Willadsen *et al,* 1999). For both these methods however there remains the difficulty inherent in working with single metaphase spreads of limiting artefactual chromosome loss.

Therefore, in an attempt to assess the level of mosaicism in all the chromosomes, by avoiding the problems encountered by FISH i.e. FISH failure and artefacts, CGH was used in the second part of this study. One or two cells were biopsied on day 3 in order to carry out CGH and the rest of the embryo was left to grow until day 5, when it was spread and FISHed. CGH has been found to be able to diagnose 25-38% more abnormalities during PGS in comparison to five- and nine-probe FISH protocols (Voullaire *et al,* 2002). Therefore, by employing CGH in embryos to investigate the whole genome, the entire level of mosaicism would be revealed. The set of embryos used for this study were frozen-thawed embryos of good quality, which at the time of

freezing were considered to be fit for transfer. Thus, good quality embryos (morphologically assessed) would be analysed using CGH. However, CGH has been considered to be laborious and not able to detect small deletions or insertions (Mamgrem *et al,* 2002), as well as responsible for artefacts regarding chromosomes 19, 22 and Y (Kallioniemi *et al,* 1994; Tabet *et al,* 2001; Wilton *et al,* 2001). Hence, FISH was carried out on day 5 to act as a safety net for CGH as well as provide confirmation results regarding the abnormalities present on day 3 and whether these abnormalities persist until day 5. CGH and FISH results were found to be in agreement by scoring the sex chromosomes, where it was found that both techniques showed the sex chromosome complement. Overall, CGH was found to be an efficient technique whilst analysing single embryonic blastomeres (83.3%). During CGH analysis it was found that on day 3 when 1-2 cells were biopsied, 13 embryos were found to be normal, however, when left to grow and spread until day 5 and FISHed with probes for six chromosomes (9, 16, 18, 22, X and Y), only three were uniformly normal. Overall, only 10% (3/30) of the embryos analysed were normal, whilst the rest were either mosaic or chaotic. This high level of mosaicism was also observed in the 1st study and has been reported from other CGH and FISH studies (Tables 3.3 and 4.1). Sandalinas and colleagues (2001) found similar percentages of chromosomal mosaicism (85%) during FISH analysis of human blastocysts. However, Baart and co-workers (2004) analysed embryos using FISH on day 3 (by 2-cell biopsy) and day 5 (whole embryos spreading) and found significantly decreased levels of abnormality and mosaicism (55%). However, the latter study was carried out as part of the PGS programme and was aiming to distinguish whether day 3 analysis of two biopsied cells is able to provide a reliable estimate of chromosomal mosaicism (Baart *et al,* 2004).

Similar to the 1st study, chromosome loss was found to be the most common mechanism of mosaicism leading to mosaic aneuploid cell lines. However, due to the CGH involvement, partial aneuploid events were also exposed with an incidence of 28% of events producing partial aneuploidy results. Partial chromosome loss was found to be the predominant mechanism leading to partial aneuploidy (14%). The phenomenon of partial aneuploidy has not been well documented during studies carried out on human preimplantation embryos. This is partly due to the fact that FISH is unable to distinguish between whole and partial aneuploidy since each FISH

probe span is relatively small and can only cover a limited stretch of the chromosome. For example, when one or three signals are observed during a FISH study employing the locus-specific probe for chromosome 21, whole monosomy or trisomy respectively is assumed rather than loss or gain of that specific part. However, studies on embryos employing CGH (Wells and Delhanty, 2000; Voullaire *et al,* 2000; Voullaire *et al,* 2002; Malmgrem *et al,* 2002; Trussler *et al,* 2004), have all documented chromosome breakage. It has been postulated that chromosome breakage and whole chromosome aneuploidy are caused by different factors (Wells and Delhanty, 2000). In our study, chromosome breakage was observed and documented and two chromosome sites were mostly affected, namely 1p31 and 2q31. Both locations have been found to be fragile sites within the chromosomes (Richards, 2001). Where possible the FISH protocol was devised in order to detect and confirm chromosome breakage by employing two probes per chromosome (embryo 9.7 Table 4.7). Therefore, during this study CGH has demonstrated that chromosome breakage in human preimplantation embryos can be considered as a possible mechanism of aneuploidy and mosaicism.

During the two studies on preimplantation embryos and especially at the blastocyst stage more than 80 embryos and >2200 cells were analysed either by CGH or FISH for at least five chromosomes. Overall, it was shown that chromosome loss, either whole or partial was the main mechanism leading to mosaic aneuploid cell lines. Both studies were developed to produce accurate and reliable results, either by employing a second probe for the same chromosome to confirm the status of the cell (Chapter 3) or by utilising two techniques to confirm the results. It has been shown that CGH can provide reliable analysis on single cells as well as blastomeres. However, it still remains laborious and time-consuming and will probably not be used for clinical purposes in this form. Initially the CGH technique should be made less laborious and more reliable in order to be used in a clinical setting e.g. for PGD of chromosomal abnormalities. This can be achieved by reducing the time of hybridisation from 72 hours to less than 24. Wells and co-workers (2002) were able to reduce the time to 30 hours during a PGD case for aneuploidy screening, however, the CGH was applied to polar bodies. The use of DNA microarrays to act as hybridisation templates, instead of the target metaphase chromosomes that have been used so far could further reduce the period of hybridisation, and improve the analysis of the obtained results,

increasing the simplicity of interpretation by avoiding the need to karyotype metaphase chromosomes. DNA microarray or microarray CGH (M-CGH) analysis is a rapid evolving method of molecular analysis that could find several potential uses in PGD (Maughan *et al,* 2001; Clarke *et al,* 2001). Following in the steps of CGH, microarrays could replace the metaphase spreads that are used now to assess chromosomal imbalance in CGH. It is likely that their versatility will make them an attractive option for PGD. However, at present, technical imitations such as paucity of material available for hybridisation, sensitivity and reliability of the data, and the cost of producing appropriate microarrays are likely to hinder their application in PGD for some time (Braude *et al,* 2002). Chan *et al* (2002) designed a DNA disc chip array, based on comparative genomic hybridisation in order to study sperm and concluded that the technique was reliable but it, was still prone to manual processing involving the fluorescent microscope and computer. Furthermore, the whole genome amplification procedure could be replaced and MDA can be tested as an alternative method of WGA. This method uses a φ29 DNA polymerase and random exonuclease-resistant primers, whilst DNA amplification takes place at 30°C (Dean *et al,* 2002). It has been shown that MDA provides a highly uniform representation of the genome, with the amplification bias being less than 3-fold among eight chromosomal loci, compared to the 4-6-fold observed with the application of DOP-PCR (Dean *et al,* 2002).

In the last part, two protocols were devised for carrying out PGD for a single gene disorder, namely myotonic dystrophy. These protocols were tested and carried out during clinical cases. Furthermore, a novel universal protocol was developed and tested on single cells for future application of clinical PGD cases for myotonic dystrophy. During the whole PGD protocol workup fluorescent PCR was utilised to provide the most sensitive and accurate results. An array of fluorescent polymorphic markers was tested initially for 10 different families at the genomic DNA level to observe which markers were informative for each family. Two different F-PCR protocols were tested and analysis on more than 700 single cells in order to optimise the protocols. Protocol 1 had been recently published for its efficiency (Piyamongkol *et al,* 2001a), because it employs a duplex method of detectionof the affected embryos as well as a contamination marker (D21S1414). Contamination is the most significant

problem during single cell PCR. Contamination can be caused by cumulus cells of maternal origin, sperm, culture media and the PCR products present in the laboratory environment (Delhanty, 1998; section 1.6.3.4.1). Protocol 2 was devised for family H and was clinically applied for the first time during this study. The novel side of Protocol 2 entails the use of a single-step multiplex F-PCR protocol including the DM mutation marker and the polymorphic marker D19S112. This protocol was able to detect the presence of contamination as well as the presence of ADO. ADO is the amplification of only one of the two parental alleles present in the single cell and can lead to misdiagnosis especially during PGD of a dominant genetic disease (Handyside *et al,* 1997; section 16.3.4.2). The D19S112 marker has a polymorphic nature therefore contamination can be detected in a sample specific fashion. An additional aspect of the D19S112 marker is that it is linked to the DM locus, and so can provide back-up diagnostic information for example when ADO has been detected for the DM locus. The use of linked markers for the adenomatous polyposis disease during clinical PGD has been reported previously (Ao *et al,* 1998). However, clinical application of a duplex F-PCR protocol for PGD of DM using the D19S112 linked marker was carried out only during this study. Unfortunately, no pregnancy was achieved in either of the PGD cycles performed.

Due to the extensive work in devising a patient-specific PGD protocol for the diagnosis of DM, the development of a universal-like protocol for the diagnosis of DM was tested initially in genomic DNA and subsequently in single cell DNA. The protocol was based on our previous experience with whole genome amplification procedures such as DOP-PCR and the use of fluorescent PCR markers. The protocol entailed amplification of DNA using WGA and then subsequent amplification of the DOP-PCR amplified DNA with the DM mutation marker (DM) as well as linked (D19S112) and unlinked polymorphic markers (D13S305, D18S535, D21S1414) for the DM disease. Initial testing a the genomic DNA level produced excellent results of amplification of the mutation and polymorphic markers (96-98%). Such amplification results prompted testing at the single cell DNA level. Twenty single cells from two heterozygote individuals were amplified using DOP-PCR and at least ten subsequent reactions (2 reactions per marker) were performed for each single cell. A decreased amplification rate and increased ADO rate was observed for the amplification (even during testing on various single cell DNA where results were not shown) of single

cells. The DM marker only showed acceptable amplification and an ADO rate of 85% and 10%. Respectively, CGH analysis was performed on the DOP-PCR amplified single cells and proved successful (85%). Hence, it can be concluded that the idea of a universal-like protocol for the PGD of DM is feasible however, requires refinement of the whole genome amplification protocol which may be achieved by changing the protocol to MDA (above) which offers a more reliable representation of genome. Further work should include purification of the whole genome amplified product which might enhance the amplification fidelity of the polymorphic markers. Overall, it was shown that this protocol can be used for low quantities of DNA, however, certain alterations and further optimisation should be carried out to improve the reliability in the minute quantities present in single cells.

6.2 Ethical Considerations towards PGD

The wide media coverage regarding advances in ART is the source of most of the ethical concerns raised today concerning what could become possible in the future. Cloning, prenatal diagnosis, and gene therapy (Fletcher, 1978; Fiddler and Pergament, 1995; Fiddler *et al,* 1999) are research fields mainly linked with fear of excessive embryo and fetal experimentation. Consequently most countries where this technology exists now have in place, or are in the process of defining, ethical guidelines or legislation to regulate human embryo research.

The philosophy of PGD is to provide couples at risk of transmitting an inherited disorder with the option and assurance of selectively having an unaffected child, in cases of an unacceptably high risk for a defective child. The great advantage from the ethical point of view that PGD provides is that it avoids implantation of defective embryos, and this process of selection eliminates the need for future TOP. Couples at high risk are offered the opportunity to overcome the worrisome burden of a possible abortion, as affected embryos are detected *in vitro* and only healthy embryos are implanted (Raeburn, 1995). Furthermore, PGD is able to prevent disabling inherited disorders prior to embryonic development (Viville *et al,* 1998). By applying PGD in countries where abortions are illegal, the number of terminations due to genetic factors can be reduced (Viville *et al*, 1998). PGD avoids the heated debate of selective abortion in society and in individual cases (Fasouliotis and Schenker, 1998). The goal

of avoiding the birth of offspring with severe genetic handicaps is part of the procreative liberty and parental decision (Kanavakis and Synodinos, 2002). However, the selection of embryos on genetic grounds is not yet ethically acceptable by all countries.

Attitudes to embryo research vary widely from country to country, which has raised great debate towards the diagnosis of embryos at the preimplantation stage. Most concerns have been pointed to the fact that PGD can and will be used for eugenic purposes i.e. the possibility that embryos could be analysed for characteristics not related to health issues (Wells and Delhanty, 2001). PGD has been referred to as a vehicle of eugenics more powerful than any of its predecessors (Fasouliotis and Schenker, 1998). Therefore, in the UK centres that offer PGD are controlled by the Human Fertilisation and Embryology Authority (HFEA) (Harper and Delhanty, 2000). The Human Fertilisation and Embryology Act 1990 permits embryo research due to the following reasons: (1) promoting advances in the treatment of infertility, (2) Increasing knowledge about the causes of congenital disease, (3) Increasing knowledge about the causes of miscarriage, (4) Developing more effective techniques of contraception and (5) Developing methods for detecting the presence of gene or chromosome abnormalities in embryos before implantation. However, prohibited are reproductive cloning, inter-species fertilisation or transfer of human embryos into other species and gene therapy in the pre-embryo.

"Designer babies" and PGD raise real ethical dilemmas in certain unusual cases (Braude *et al,* 1998; Braude *et al,* 2001). A dilemma might occur with inherited deafness. In a recent case a non-hearing child was deliberately conceived to be deaf like its lesbian parents (Savulescu *et al,* 2002). Sex selection referred to as "family balancing" remains controversial (Gleincher and Karande, 2002) and many consider it not to be a legitimate use of PGD (Robertson, 2002). Finally, the attempt to save the life of a sibling by having another child provide a suitable tissue match can be rationalised and commended however, this process has met with great controversy (Boyle and Savulescu, 2002). In addition, further problems that PGD faces is the possibility of misdiagnosis (Harper *et al*, 1999; Harper and Delhanty, 2000; Wells, 2004).

CHAPTER 7

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Chapter 8 Appendix

Table 8.1. Analytical CGH and FISH results from each cell for chapter 4

Pt No. (age)	**EMB No.**	**Cell**	**CGH Result** (day 3)	**FISH Result** (day 5) **X/Y/18 (cell no)**	**Re-FISH Result** (day 5) **9c/16c/22LSI (cell no)**
1 (38)	**1.1**	**a**	No result	X, Y, 18,18 (1,5,6,7,8,9,10,11,13) X, Y, 18 (2,12) X, X, Y, Y, 18,18 (3,4)	9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,9,10,11, 12,13) 9, 9, 16, 16, 22, 22, 22 (7,8)
		b	rev ish XY		
	1.2	**a**	rev ish XY, dim(22)	X, 18 (1) X,X,X,X,X,X,X,18,18,18,18,18,18,18 (2)	9, 9, 22 (1) 9,9,9,9,9, 16,16,16,16,16, 22,22,22,22 (2)
		b	No result		
	1.3	**a**	rev ish XY	X, X, Y, Y, 18,18, 18, 18 (1) X, Y, 18,18 (2)	9, 9, 16, 16, 22, 22 (1,2)
2 (29)	**2.1**	**a**	rev ish XY	X, Y, 18,18 (1,2,3,4)	9, 9, 16, 16, 22, 22 (1,2,3,4)
		b	No result		

<table>
<tr><th>Pt No. (age)</th><th>EMB No.</th><th>Cell</th><th>CGH Result (day 3)</th><th>FISH Result (day 5)

X/Y/18 (cell no.)</th><th>Re-FISH Result (day 5)

9c/16c/22LSI (cell no.)</th></tr>
<tr><td rowspan="2">2 (29)</td><td rowspan="2">2.2</td><td>a</td><td>rev ish XY, dim(18)</td><td rowspan="2">Nullisomy for all chromosomes (1)
X, Y, 18 (2)
18 (3)</td><td rowspan="2">9, 22 (1,3)
9, 9, 16 (2</td></tr>
<tr><td>b</td><td>No result</td></tr>
<tr><td rowspan="2">3 (35)</td><td rowspan="2">3.1</td><td>a</td><td>rev ish XY, enh(5pter, 9qter, 17), dim(4,19)</td><td rowspan="2">Y,18,18,18 (1,2)</td><td rowspan="2">16, 22 (1)
9, 9, 16, 22 (2)</td></tr>
<tr><td>b</td><td>No result</td></tr>
<tr><td rowspan="3">4 (35)</td><td>4.1</td><td>a</td><td>rev ish XY</td><td>No cells to FISH</td><td>No cells to FISH</td></tr>
<tr><td rowspan="2">4.2</td><td>a</td><td>rev ish XX</td><td rowspan="2">X, X, 18, 18 (10,12,16,17,19,20,22, 26,32)
X, 18, 18 (4,9,13,23,24,27,30,31)
X, X, 18 (2,6,8,14,28)
X, 18 (11,15,18,29)
18, 18 (5,7,21)
X, X, 18, 18, 18 (25,33)
X, X, 18, 18, 18, 18 (1)
X, X, X, 18, 18, 18 (3)</td><td rowspan="2">9, 9, 16, 16, 22, 22 (2,4,6, 12,14,16,21, 22,23,24,25,26,27, 29,30,31,32,33)
9, 9, 16, 16, 22 (7,8,9, 15)
9, 9, 16, 22 (10,11,13)
9, 9, 16, 22, 22 (17, 18, 19)
9, 9, 16, 22 (3)
9, 9, 16 (1,5,28)</td></tr>
<tr><td>b</td><td>rev ish XX, enh(1, 22), dim(16pter, 18)</td></tr>
</table>

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result (day 5) 9c/16c/22LSI (cell no.)
4 (35)	4.3	a	rev ish XY	No cells to FISH	No cells to FISH
	4.4	a	rev ish XY, enh(X)	No cells to FISH	No cells to FISH
6 (30)	6.1	a	No result	No cells to FISH	No cells to FISH
	6.2	a	rev ish XY	X, X, 18, 18 (1,2,3,4,5)	9, 9, 16, 16, 22, 22 (1,3,4) 9, 9, 16, 16, 22 (2,5)
		b	rev ish XY		

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result (day 5) 9c/16c/22LSI (cell no.)
6 (30)	6.3	a	rev ish XY, enh(6p25-p21.1)*	X, X, Y, 18, 18 (1,2) X, 18, 18 (3,4,7,8) X, Y, 18, 18 (5,6)	9, 9, 16, 16, 22, 22 (1,2,5,6,7) 9, 9, 16, 22, 22 (3) 9, 9, 16, 16, 22, 22, 22 (4) 9, 9, 16, 16, 22 (8)
		b	rev ish XY, enh(Y)		
7 (39)	7.1	a	rev ish XX	X, X, 18, 18 (3,6,7,8) X, X, 18 (1,2,4) X, X, 18, 18, 18 (5)	9, 9, 16, 16, 22, 22 (1,2,3,5,7,8) 9, 9, 16, 22, 22 (4) 9, 9, 16, 16, 22 (6)
	7.2	a	rev ish XYY, enh(1p36.2-36.1, 2q31-p25, 5, 7, 8, 18, 19, 20, 21 and Y), dim(1p31-q44, 2q32-q37, 3, 6, 11, 13, 14, 15)	X, X, X, 18, 18, 18 (1,3) X, X, X, X, X, Y, Y, 18,18, 18 (2)	<u>**3c/11c/13LSI**</u> 3, 13, 13 (1) 3 (2) 3, 3, 3, 13 (3)
		b	No result		
	7.3	a	rev ish XY, dim(22q11.1-q13)	X, X, 18, 18 (3,5,6,7,9,10,11,12, 13, 14,15,17,18,19, 20,21,22,23,24,25, 26,27,28,29,30) X, 18, 18 (4,8) X, X, X, X, 18, 18 (1,2) X, X, X, X, 18, 18, 18, 18 (16) X, X, 18 (24)	9, 9, 16, 16, 22, 22 (6,13,15,23,24) 9, 9, 16, 16,22,22,22 (7,14,18,19,26) 9, 9, 16, 22 (12,22) 9, 9 (17,20) 22 (3,8) 9, 9, 22 (4,11) 9, 16, 16, 22 (1) 9, 9, 9, 16, 22 (2) 9, 16, 16, 22, 22 (5) 9,9,9,9, 16,16,16,16, 22,22,22,22 (16)
		b	rev ish XY		

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result(day 5) 9c/16c/22LSI (cell no.)
8 (31)	8.1	a	rev ish XX	X, X, 18, 18 (1,2,3) X, X, 18, 18, 18, 18 (4)	**<u>10c / 14q</u>** 10, 14q, 14q (1,2) 10, 14q, 14q, 14q (3,4)
		b	rev ish XX, enh(4, 12p11.2-q24.3, 14q21-q32) dim(2q31-q37, 10)		
	8.2	a	rev ish XX, enh(9 and 16)	X, X,18,18 (1,2,3,4,5,6,7,8,9,10)	9, 9, 16, 16, 22, 22 (2,3,4,6,7,10) 22, 22 (1,8,9) 9, 22, 22 (5)
9 (33)	9.1	a	rev ish XY, enh(Xp11.2-q22)*	X, Y, 18, 18 (1,2,3,4,5,6,7)	9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,7)
	9.2	a	rev ish XY, dim(Yq12)*	X, Y, 18, 18 (2,3,4,5,7,8,9,10,11, 12,1516,17,18,19,20 21,22,23,24,2526,27 28,29,30,31) X, Y, 18 (1,13,14) Y, 18, 18 (6)	9, 9, 16, 16, 22, 22 (2,3,4,5,6,7,8,9, 10,11,12,14,15, 16,17,18,19,20, 21,22,23,24,25, 26,27,29,31) 9, 9, 16, 22, 22, 22 (1,30) 9, 9, 9, 16, 22, 22, 22 (13) 9, 9, 16, 16, 22, 22, 22, 22 (28)

<table>
<tr><th>Pt No. (age)</th><th>EMB No.</th><th>Cell</th><th>CGH Result (day 3)</th><th>FISH Result (day 5)
X/Y/18 (cell no.)</th><th>Re-FISH Result (day 5)
9c/16c/22LSI (cell no.)</th></tr>
<tr><td rowspan="5">9 (33)</td><td>9.3</td><td>a</td><td>rev ish XY, dim(15q15-q26, 16)</td><td>X, Y, 18, 18 (1,2,3,4)</td><td>9, 9, 16, 16, 22, 22 (1,4)
9, 9, 16, 22, 22, 22 (2,3)</td></tr>
<tr><td>9.4</td><td>a</td><td>rev ish XX, dim(11q23-q25)*</td><td>X, X, 18, 18 (1,3,5)
X, X, X, X, 18, 18, 18, 18 (2,6)
X, X (4)</td><td>9, 9, 16, 16, 22, 22 (1,3,4,5)
9,9,9,9, 16,16,16,16, 22,22,22,22 (2,6)</td></tr>
<tr><td>9.5</td><td>a</td><td>rev ish XY</td><td>X, Y, 18, 18 (1,3,4,5,7,8,9)
Y, 18, 18 (2,6,10)
18 (11)</td><td>9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,7, 8,9,10)
9, 16, 22, 22 (11)</td></tr>
<tr><td rowspan="2">9.6</td><td>a</td><td>rev ish XX,
enh(2q21-q33, 3q11.1-q25), dim(1p36.1-p33, 16, 19 and 22)</td><td rowspan="2">X, X, 18, 18 (1,2,3,4,5,6,7,8,9,12,13 14,15,16,18,19,20,22)
X, X, 18 (10)
X, X, X, X, 18, 18, 18, 18 (11,17)
X, X, X, 18, 18 (22)</td><td rowspan="2">9, 9, 16, 16, 22, 22 (2,3,4,5,7,8, 9,10,13,16, 17,19,20,21 22,23)
9, 9, 16, 22 (6,14,15)
9,9,9,9, 16,16,16,16, 22,22,22,22 (11,17)
9, 9, 16, 16, 22 (12,18)
9, 9, 16, 22, 22, 22, 22 (1)</td></tr>
<tr><td>b</td><td>rev ish XX</td></tr>
</table>

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result (day 5) 9c/16c/22LSI (cell no.)
9 (33)	9.7	a	rev ish XY, enh(1p36.3-q21), dim(1q31-q44)	X, Y, 18, 18 (3,5) X, X, Y, 18, 18 (1) X, Y,Y, 18, 18 (2) Y, 18, 18 (4)	**1p / 1q / 16cep** 1p, 1q, 16, 16 (1) 1p, 1q, 1q, 1q, 16, 16 (2,5) 1p, 1p, 1p, 16, 16 (3) 1p, 1p, 1q, 1q, 16 (4)
		b	rev ish XY, dim(1)		
	9.8	a	rev ish XY, enh(17p13-q11, 18p11.3-q11.1), dim(3p26-p14)	X, Y, 18, 18 (1,2,3,4,5,6,7,8,9,10,12, 13,15,16,17) X, Y,Y Y, 18, 18 (11) Y, Y, 18, 18 (14)	**3cep/6cep/18cep** 3, 3, 6, 6, 18, 18 (1,2,3,5,6,7,8,12, 13,16,17) 3, 3, 6, 6, 6, 18, 18 (4,9,10) 3, 3, 6, 18, 18 (15) 3, 3, 6, 6, 18 (11)
		b	rev ish XY, enh(6)		
10 (32)	10.1	a	rev ish XX, enh(20)	X, X, 18, 18 (4,8,12,13,16,17,18) X, X, X, X, 18, 18 (1,2,3,5,6,11,14) X, X, X, 18, 18 (7,15,19) X, X, X, 18, 18, 18 (9) X, 18 (10) X, X, X, X, X, X, 18, 18, 18, 18 (17)	9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,7, 8,10,11,12,13, 15,16,17,18,19 9, 9, 16, 16, 16, 22, 22, 22 (9) 9, 9, 16, 16, 22 (14) 9, 9, 9, 9, 16, 16, 16, 16, 22, 22, 22, 22 (17)
11 (35)	11.1	a	rev ish XY, enh(Yp11.3-11.2)*	X, Y, 18, 18 (1,2,3,4,5,6,7,8,9,10)	9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,7, 8,9,10)

Pt No. (age)	**EMB No.**	**Cell**	**CGH Result (day 3)**	**FISH Result** (day 5) **X/Y/18 (cell no.)**	**Re-FISH Result** (day 5) **9c/16c/22LSI (cell no.)**
12 (30)	**12.1**	**a**	rev ish XX	X, X, 18, 18 (1,3) X, X, 18 (2)	9, 9, 16, 16, 22, 22 (1) 9, 9, 16, 22, 22, 22, 22 (2) 9, 22, 22 (3)
	12.2	**a**	rev ish XY, enh(Y), dim(4pter)	X, Y, 18, 18 (1,2,3,4,5,11) X, Y, 18 (8,10) X, 18, 18 (6,7) 18 (9) Y, Y, 18, 18 (12)	**4cep/Ycep** 4, 4, Y (1,2,3,4,5,8,10,11) 4, 4 (6,7) Nullisomy 4 and Y (9) 4, Y, Y (12)
	12.3	**a**	rev ish XY, enh(Yq11.1-q12), dim(16q21-q24)	X, Y, 18 (1) X, Y, 18, 18 (2)	**Xc/Yc/16q** X, Y, 16q (1,2)
13 (36)	**13.1**	**a**	rev ish XY, enh(5)	No cells to FISH	No cells to FISH

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result (day 5) 9c/16c/22LSI (cell no.)
13 (36)	13.2	a	rev ish XX, dim(19p13.3-p13.2)*	X, X, 18, 18 (1,2,3,4,7,9,12,14) 18, 18 (5) X, X, X, X, 18, 18 (6) X, X, X, 18, 18, 18 (8) X, X, X, 18 (10) X, 18, 18, 18 (11) X, X, 18 (13) X, X, X, X, 18, 18, 18, 18 (15)	9, 9, 16, 16, 22, 22 (1,2,3,7,9,12,14) 9, 16, 16, 22, 22, 22 (4) 16, 22, 22, 22, 22 (5) 22, 22 (6) 9, 9, 9, 16, 16, 16, 22, 22, 22 (8) 9, 9, 16, 16, 22, 22, 22 (10) 9, 9, 16, 16, 22 (11) 9, 9, 9, 16, 16, 22, 22, 22 (13) 9, 9, 9, 9, 16, 16, 16, 16, 22, 22, 22, 22 (15)
		b	rev ish XX		
	13.3	a	rev ish XX	X, X, 18, 18 (1,2,3,4,5,6,8,9,10,12,15,16 17,20,22,23,24,25,26,27, 28,29,30,31,32,33,34,35) X, X, X, 18, 18, 18 (13,14) X, X, X, 18 (11,21) X, X, X, X, X, X, 18, 18, 18 18, 18 (7) X, X, 18, 18, 18, 18, 18 (18) X, X, 18, 18, 18 (19)	9, 9, 16, 16, 22, 22 (1,2,3,4,5,6,8,9,10,11, 12,15,16,17,18,20,21, 22,23,24,25,26,27,28, 29,30,31,32,33,34,35) 9, 9, 9, 16, 16, 16, 22, 22, 22 (13,14) 9, 9, 9, 16, 16, 22, 22, 22, 22 (7) 9, 16, 22, 22 (19)
		b	rev ish XX		
14 (28)	14.1	a	rev ish XX	No cells to FISH	No cells to FISH
		b	rev ish XX, enh(2,4, 9qter), dim(1, 16, 21)		

Pt No. (age)	EMB No.	Cell	CGH Result (day 3)	FISH Result (day 5) X/Y/18 (cell no.)	Re-FISH Result (day 5) 9c/16c/22LSI (cell no.)
14 (28)	14.2	a	rev ish XX, enh(1, 10, 16qter), dim(8, 13, 21, 22)	X, X, 18, 18 (1,2,5,6) X, X (3) X, 18 (4)	9, 9, 16, 16, 22, 22 (1) 9, 9, 16, 22, 22 (2) 9, 9, 16, 22 (3,5) 9, 9, 16, 16, 16, 22, 22, 22 (4,6)
		b	rev ish XX, enh(2,6), dim(9qter,13, 15, 16qter, 17)		
	14.3	a	rev ish XX	No cells to FISH	No cells to FISH