Preimplantation Genetic Diagnosis - new methods for the detection of genetic abnormalities in human preimplantation embryos.

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Abbreviations

Below is a list of abbreviations – along with explanations - commonly used in this thesis. Other less commonly used abbreviations are explained within the text whenever appropriate.

aCGH	Array-CGH
ADO	Allele dropout
AE	Amplification efficiency
AMA	Advanced maternal age
allo-HSCT	Allogeneic haematopoietic stem cell transplantation
AOA	Artificial oocyte activation
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
Ca^{2+}	Calcium ions
CCS	Comprehensive chromosome screening
CGH	Comparative genomic hybridisation
COH	Change of homozygosity
CVS	Chorionic villus sampling
ddNTPs	Dideoxy nucleotides
DGW	Density gradient washing
dNTPs	Deoxyribonucleoside triphosphates
DOP-PCR	Degenerate oligonucleotide primed PCR
DTT	Dithiothreitol
EDTA	Ethylene-di-amine-tetra-acetic-acid
ESHRE	European Society of Human Reproduction and Embryology
Exo I	Exonuclease I

F-PCR	Fluorescent PCR
FAM	6-carboxyfluorescein
FISH	Fluorescent *in situ* hybridisation
GOH	Gain of heterozygosity
H233L	Histidine to Leucine point mutation at position 233 of PLCζ amino acid sequence
H398P	Histidine to Proline point mutation at position 398 of PLCζ amino acid sequence
HEX	4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein
HLA	Human leukocyte antigen
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilisation
kb	Kilobase
LA-PCR	Linker adapter - PCR
Mb	Megabase
MDA	Multiple displacement amplification
MHC	Major histocompatibility complex
mtDNA	Mitochondrial DNA
NWB	Non-stick washing buffer
OAD	Oocyte activation deficiency
OGT	Oxford Gene Technology
OXPHOS	Oxidative phosphorylation
PB	Polar body
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
PLCζ	Phospholipase C zeta (ζ)

PND	Prenatal diagnosis
PK	Proteinase K
qPCR	Quantitative real time PCR
RCT	Randomized control trial
RIF	Repeated implantation failure
ROS	Reactive oxygen species
ROX	6-carboxy-X-rhodamine
SAP	Shrimp alkaline phosphatase
SMN	Survival motor neuron
SNP	Single nucleotide polymorphism
STR	Short tandem repeat
SART	Society of Assisted Reproductive Technologies
TAF	Total amplification failure
TAMRA	N,N,N',N'-tetramethyl-6-carboxyrhodamine
Taq	*Thermophilus aquaticus*
TBE	Tris/Borate/EDTA
TERT	Telomerase reverse transcriptase
UPD	Uniparental disomy
WGA	Whole genome amplification

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Abstract

Title: Preimplantation Genetic Diagnosis – new methods for the detection of genetic abnormalities in human preimplantation embryos.

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Preimplantation genetic diagnosis (PGD) refers to the testing of embryos produced through *in vitro* fertilization (IVF) in order to identify those unaffected by a specific genetic disorder or chromosomal abnormality. In this study, different methodologies were examined and developed for performance of PGD. Investigation of various whole genome amplification (WGA) methods identified multiple displacement amplification as a reliable method for genotyping single cells. Furthermore, this technology was shown to be compatible with subsequent analysis using single nucleotide polymorphism (SNP) microarrays. Compared to conventional methods used in this study to perform single cell diagnosis (e.g. multiplex PCR), WGA techniques were found to be advantageous since they streamline the development of PGD protocols for couples at high risk of transmitting an inherited disorder and simultaneously offer the possibility of comprehensive chromosome screening (CCS). This study also aimed to develop a widely applicable protocol for accurate typing of the human leukocyte antigen (HLA) region with the purpose of identifying embryos that will be HLA-identical to an existing sibling affected by a disorder that requires haematopoietic stem cell transplantation. Additionally, a novel microarray platform was developed that, apart from accurate CCS, was capable of reliably determining the relative quantity of mitochondrial DNA in polar bodies removed from oocytes and single cells biopsied from embryos. Mitochondria are known to play an important role in oogenesis and preimplantation embryogenesis and their measurement may therefore be of clinical relevance. Moreover, real-time PCR was used for development of protocols for CCS, DNA fingerprinting of sperm samples and embryos and the relative quantitation of telomere length in embryos (since shortened telomeres might be associated with reduced viability). As well as considering the role of genetics in terms of oocyte and embryo viability assessment and the diagnosis of inherited genetic disorders, attention was given to a specific gene (Phospholipase C zeta) of relevance to male infertility. A novel mutation affecting the function of the resulting protein was discovered highlighting the growing importance of DNA sequence variants in the diagnosis and treatment of infertility.

1) Introduction

Genetic disorders including single gene defects, sex-linked conditions and chromosomal rearrangements, have always been one of the major issues that medicine has had to face. It is estimated that over 10,000 disorders are caused due to single gene defects alone. When taken together, the prevalence of all monogenic disorders (disorders that occur from modification of a single gene) at birth is calculated to be approximately 1/100 (World Health Organization 2012). Importantly, the incidence of chromosome abnormalities is estimated to be at similar levels (1/200 newborns) (Luthardt and Keitges 2001). These figures indicate how common genetic disorders and chromosomal anomalies are in the human species.

Couples at risk of transmitting a genetic disease are often advised to undergo prenatal diagnosis (PND). Fertile or infertile couples – through the means of assisted reproductive technology (ART) - can attempt to establish a pregnancy and then opt for amniocentesis or chorionic villus sampling (CVS). However, these methods, carried out after a gestation has been established, necessitate the parents being prepared for the possibility of pregnancy termination following the detection of an affected pregnancy. Whether or not to terminate an affected pregnancy can be an extremely difficult decision with extensive psychological and physical consequences (Braude *et al.* 2002; Grace *et al.* 2004). Amniocentesis and CVS are invasive techniques that can cause complications to both the mother and the foetus. A woman has a 0.5% chance of miscarrying after amniocentesis while after CVS this percentage reaches 1-2%. CVS has also been linked to an increase in the occurrence of congenital malformations (Wells and Delhanty 2001). Additionally, termination of pregnancy is not considered an option for some couples because of religious or personal objections. Other options for couples at risk of transmitting a genetic disorder include using gametes from a donor not carrying the mutation - although this might not always be

acceptable because of moral or religious reasons – adoption or remaining childless (Braude *et al.* 2002; Grace *et al.* 2004).

Preimplantation genetic diagnosis (PGD) was developed so that these problems can be circumvented. In fact, the development of PGD was indirectly initiated by the pressure of couples who were in the risk of passing on a single gene disorder to their offspring (Delhanty and Harper 2000).

1.1 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis is considered to be an alternative to PND with the main difference being that the diagnosis of a genetic disorder happens before a clinical pregnancy has been established (Delhanty and Wells 2002). It involves the biopsy and testing of one or more cells from embryos (or in some cases polar bodies from oocytes) generated using *in vitro* fertilisation (IVF) techniques. The aim of this approach is to determine which of the embryos are free of the genetic disorder carried by the parent(s). Only unaffected or karyotypically normal embryos are transferred to the uterus of the woman and consequently the risks of an abnormal pregnancy are dramatically reduced (Katz *et al.* 2002; Wells and Delhanty 2001).

1.1.1 Historical aspect of PGD

Although the first steps towards PGD took place in 1968 with the transfer of sex-selected embryos into recipient rabbit females (Gardner and Edwards 1968), it was not until 1989 that the first clinical application of PGD was carried out. The

introduction of human *in vitro* fertilisation (Steptoe and Edwards 1978) and the development of diagnostic techniques applicable to the single cell level, such as fluorescence *in situ* hybridisation (FISH) and polymerase chain reaction (PCR), were essential requirements for the first clinical application of PGD to take place (Geraedts and De Wert 2009). The first clinical PGD cycles were carried out by Professor Alan Handyside and colleagues, focusing on the avoidance of recessive X-linked disorders in five couples. The strategy involved the biopsy of a single cell from cleavage stage embryos followed by sex determination (via PCR for Y-chromosome-specific DNA sequences) and transfer of female embryos (Handyside *et al.* 1990). However, the adoption of a strategy where diagnosis of an unaffected embryo is based upon a negative result (i.e. non-amplification of Y-chromosome-specific DNA sequences being indicative of an unaffected/carrier female), carried the risk of misdiagnosis due to amplification failure. Ultimately a misdiagnosis did occur, in which an established pregnancy was found to be an affected male. The pregnancy was terminated (Griffin *et al.* 1992). This incident, and a growing appreciation of the deficiencies of PCR-based approaches, led to the rapid replacement of PCR with FISH for PGD cycles, which provided more accurate and robust gender determination of embryos (Griffin *et al.* 1992, 1994). Before long, PGD was applied for the first time for diagnosis of an autosomal recessive disorder (cystic fibrosis) (Handyside *et al.* 1992; Verlinsky *et al.* 1990), while soon after, the feasibility of aneuploidy detection in human embryos was demonstrated (Munne *et al.* 1993), setting the stage for PGD of chromosome rearrangements and for what is known today as preimplantation genetic screening (PGS). It is thought that more than 50,000 cycles have been performed to date and that more than 10,000 babies have been born through PGD (Simpson 2010).

1.1.2 Indications of PGD

Since its first clinical application in 1989 PGD has expanded significantly to include a wide range of indications. Nowadays PGD is applied for detection of monogenic disorders (including late-onset diseases and genetic defects with incomplete penetrance), mitochondrial DNA disorders and chromosomal rearrangements such as reciprocal and Robertsonian translocations. Furthermore, the technology developed for PGD is being used in certain countries for social sexing (i.e. no medical indication; PGD is performed specifically for selection of sex). PGD has also been employed for human leukocyte antigen (HLA) typing, with or without a monogenic disease diagnosis, with the aim of retrieving stem cells from cord blood at the time of birth in order to use them for treating an existing affected sibling. Finally, PGD is being used extensively for screening oocytes and embryos for spontaneously arising (i.e. non-familial) aneuploidy, with the aim of enhancing IVF success rates in specific groups of patients - preimplantation genetic screening. As shown in Table 1.1, although PGD has expanded to a number of different applications, PGS remains the most common indication.

Table 1.1: Data collected from European Society of Human Reproduction and Embryology (ESHRE) and from Society of Assisted Reproductive Technologies (SART) (USA) regarding the different indications of PGD.

	ESHRE PGD Consortium 1997-2007[a]			**SART 2007-2008[b]**		
	Cycles performed	*No. embryos biopsied*	*No. embryos transferred / cycle*	*Cycles performed*	*No. embryos biopsied*	*No. embryos transferred / cycle*
Monogenic Disorders*	5 900 (21.4%)	35 297	1.8-1.9	1 246 (20.1%)	NA**	2.0
Social Sexing	671 (2.4%)	4 285	2.0	1 388 (22.4%)	NA	1.9
Chromosomal Rearrangements	4 253 (15.4%)	27 068	1.7	483 (7.8%)	NA	1.8
Aneuploidy (PGS)	16 806 (60.8%)	90 404	1.8	3 082 (49.7%)	NA	2.0
Total	27 630	157 054	---	6 199	NA	---

* ESHRE PGD Consortium had data for monogenic disorders divided into two categories: 'Single genes' and 'Sexing X-linked'. These were combined in one category for the purposes of this Table.
** 'NA' stands for Not Available
[a] Harper *et al.* 2012
[b] Ginsburg *et al.* 2011

1.1.2.1 PGD for single gene disorders

Technically, PGD can be used to test for any disorder caused by a mutation in a recognized single gene (Spits and Sermon 2009). Hence, to date, PGD has been performed for a large number of single gene disorders including some very rare ones such as Crouzon Syndrome and Incontinentia pigmenti (Abou-Sleiman *et al.* 2002; Pettigrew *et al.* 2000). In fact, it has been reported that PGD has been used for more than 200 different single gene disorders (Rechitsky *et al.* 2009). While PGD is primarily applied to the same range of disorders that are routinely referred for prenatal testing, it is also often used for late-onset diseases (e.g. Huntington disease) and

genetic defects with incomplete penetrance (e.g. cancer predisposition syndromes); classes of disorder which are less often the subject of routine prenatal diagnosis.

However, some ethical questions do arise when PGD is used for such conditions. It is argued that screening for conditions with incomplete penetrance might be unnecessary (since these conditions are multifactorial). However, parents want their children to be healthy and would prefer to avoid them having to go through procedures linked to management of disease risk (e.g. continued monitoring, prophylactic surgery) that will cause stress and discomfort and will not be able to guarantee disease prevention (Robertson 2003). The same is true for late-onset disorders. In fact, the argument in favor of couples going through PGD for late-onset conditions is even stronger since most diseases in this case are not preventable and have limited treatment options. Nevertheless, some people argue that 30 or 40 years of normal functioning, before the onset of disease, is a significant number of years for a person to achieve much and have a life worth-living (Krahn 2009).

1.1.2.2 PGD for HLA typing

HLA-typing is one of the newest indications for PGD. It refers to the usage of PGD for selection of preimplantation embryos that are HLA-identical to an existing affected sibling suffering from a disorder requiring stem cell transplantation. At birth haemotopoietic stem cells can be retrieved from the newborn's umbilical cord blood and used to treat the affected sibling.

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is used to cure many malignant and non-malignant conditions including haemoglobinopathies,

immunodeficiencies, inborn errors of metabolism, leukaemia, lymphoma and bone marrow failure syndromes (Samuel *et al.* 2009). In fact, for some of these conditions (e.g. haemoglobinopathies) allo-HSCT is the only curative treatment available (Smiers *et al*. 2010). The success of the transplantation is controlled by the HLA system, also known as the human Major Histocompatibility Complex (MHC). MHC, which is more than 4 Mb (Megabases) in length, is located within the 6p21.3 region of chromosome 6 and contains more than 220 genes (Robinson *et al.* 2011). Allo-HSCT requires the availability of an HLA-matched donor, ideally from within the patient's family (Samuel *et al.* 2009). Even after matching the major genes of the HLA system, the use of an unrelated donor is associated with an increased risk of morbidity and mortality (De Wert *et al.* 2007; Fiorentino *et al.* 2004; La Nasa *et al.* 2002; Szydlo *et al.* 1997). Transplants from HLA-identical siblings have higher success rates (Gaziev *et al.* 2000; La Nasa *et al.* 2002; Lucarelli *et al.* 2002; Orofino *et al.* 2003).

Although, PGD/HLA-typing has been used to date for a number of different conditions (e.g. *α*-thalassaemia, *β*-thalassaemia, sickle cell anaemia, leukaemia, Fanconi anaemia) (Fiorentino *et al*. 2004; Kahraman *et al.* 2011; Kuliev *et al.* 2005; Rechitsky *et al.* 2006; Verlinsky *et al.* 2004) and although the number of cycles performed for HLA-typing is growing annually (as shown by data collected by the ESHRE PGD Consortium) (Goossens *et al.* 2008b, 2009; Harper *et al.* 2010), some important ethical concerns regarding its usage exist. The major concern is 'instrumentalization' of the future child since it will be conceived and used to cure another child. People wrongly assume that the only reason for conceiving an additional child is to use it as a transplant donor (De Wert *et al.* 2007) and that its well-being will be ignored. However, it is argued that regardless of the circumstances

the birth of a child creates a powerful bond and its interests or wishes will not be ignored by the parents, nor it will be harmed. Indeed, conceiving another child to protect the first one shows that the parents really care about the well-being of their children and that the second child will be valued for its own sake (Robertson *et al.* 2002).

1.1.2.3 PGD for chromosomal rearrangements

Chromosomal rearrangements such as reciprocal/Robertsonian translocations and pericentric and paracentric inversions are well-recognised forms of genetic abnormality. In most cases carriers of such abnormalities are phenotypically normal and only find out about their condition when they attempt to have children. Subfertility, recurrent miscarriage and birth of children with congenital abnormalities and/or mental retardation are common outcomes for these patients. These problems are due to different modes of chromosome segregation during meiosis that result in the production of a large number of genetically unbalanced gametes (i.e. chromosomal material has been lost or gained). Given their effects on fertility and miscarriage, it is unsurprising that the occurrence of structural chromosome abnormalities is found at increased rates among patients undergoing IVF treatment with a history of recurrent miscarriages or repeated IVF failures. Specifically, the incidence in this group of patients is found to be ~25-fold higher (5%) than in the general population (Alfarawati *et al.* 2011a; Campana *et al.* 1986; Fryns and Buggenhout 1998; Stern *et al.* 1999).

Preimplantation genetic diagnosis can be used to detect chromosomal rearrangements in embryos produced from these patients with the aim of transferring only

normal/balanced ones. A large number of cases has been carried out since the first clinical application of PGD for chromosomal rearrangements in 1998 (Conn *et al.* 1998). Different studies reporting on the outcome of PGD cases for translocation carriers have demonstrated an increase in the chances of sustaining a pregnancy to full term: 85% of pregnancies on average were lost before PGD, while only 0-25% miscarried after PGD (reviewed in Munne *et al.* 2010). Furthermore, PGD reduces the time needed for carriers of structural chromosome abnormalities to achieve a sustained pregnancy. Studies have shown that patients undergoing PGD achieve pregnancy in an average of 1.2-1.4 IVF cycles (<4 months) while patients without PGD require a much longer interval (mean 4-6 years) (Fischer *et al.* 2010; Goddijn *et al.* 2004; Otani *et al.* 2006; Stephenson and Sierra 2006).

1.1.2.4 Preimplantation Genetic Screening

It has been documented that human oocytes and embryos generated for the purpose of IVF treatment are frequently chromosomally abnormal. The fraction of abnormal embryos is appreciable for women in their early thirties, affecting more than 50% (Munne *et al*., 2006a), but becomes much more of a problem with advancing age, with more than 80% of embryos found to be abnormal for women ≥40 years old (reviewed in Cohen *et al.* 2007). Most of the aneuploidies detected are derived from errors occurring during female meiosis (i.e. during oocyte formation). However, chromosome abnormalities can also occur in sperm or during the mitotic divisions after formation of the zygote (Delhanty *et al.* 1997; Katz-Jaffe *et al.* 2004; Munne *et al.* 2002). In the vast majority of cases embryonic aneuploidy is not compatible with successful development to term. This was evidenced by early studies showing that

>50% of early spontaneous abortions are chromosomally abnormal (Boue *et al.* 1975; Hassold *et al.* 1980), while more recent studies suggest that this percentage might actually exceed 65% (Fritz *et al.* 2001; Menasha *et al.* 2005).

The high frequency of aneuploidy in human oocytes and embryos and the knowledge that chromosomal imbalance is usually lethal to the embryo have let to the suggestion that screening of embryos from IVF cycles for aneuploidy, followed by the transfer of euploid embryos might improve the pregnancy rate, decrease the spontaneous miscarriage rate and reduce the risk of giving birth to babies with aneuploid syndromes such as Down (Munne *et al.* 1993; Verlinsky *et al*. 1995; Wells *et al.* 2008).

In IVF treatment, the most common strategy employed in order to identify the embryo(s) most likely to produce a viable pregnancy relies on the assessment of morphological and developmental criteria, such as cell number, existence of multinucleation in blastomeres, cleavage rate and pattern, compaction and degree of fragmentation (Cummins *et al.* 1986; Moayeri *et al.* 2008; Sakkas and Gardner 2005; Van Royen *et al.* 1999). However, although a correlation between morphology and implantation rates does exist (Borini *et al.* 2005; Hardarson *et al.* 2001; Nomura *et al.* 2007), it is universally acknowledged that the link is relatively weak. A significant number of embryos with normal morphology are found to be chromosomally abnormal and probably have little potential for producing a child (Alfarawati *et al.* 2011b; Baltaci *et al.* 2006, Munne *et al.* 2007).

Indications for usage of PGS, as recently outlined by the ESHRE PGD Consortium (Harton *et al.* 2011a), include:

- Advanced maternal age (AMA): Women at the age of 35 and older are generally considered to be of AMA, although this varies between different centres since some consider that women ≥36, ≥37 or even ≥40 should be included in this category. Studies carried out on large number of human oocytes have clearly demonstrated that advancing maternal age is linked with increasing aneuploidy rates (Fragouli *et al.* 2006, 2009, 2010b; Hassold *et al.* 2007; Kuliev *et al.* 2003; Pellestor *et al.* 2003; Sandalinas *et al.* 2002).
- Recurrent miscarriage: Recurrent miscarriage is usually defined as three or more pregnancy losses. A number of different studies have shown increased aneuploidy rates in embryos obtained from women falling in this category (Garrisi *et al.* 2009; Mantzouratou *et al.* 2007; Rubio *et al.* 2003).
- Repeated implantation failure (RIF): Usually RIF is considered for patients that have had 3 or more failed IVF attempts or have had failure of implantation after transfer of ≥10 embryos in multiple transfers. Studies carried out on embryos derived from RIF patients have revealed occurrence of multiple chromosome errors (Fragouli *et al.* 2010b; Mantzouratou *et al.* 2007; Voullaire *et al.* 2002, 2007).
- Severe male infertility: A number of studies carried out with patients affected by severe male infertility (e.g. azoospermia, oligospermia, teratozoospermia, oligoasthenoteratozoospermia, macrocephalic spermatozoa) have indicated increased rates of chromosomal abnormalities correlated with this group of patients (Calogero *et al.* 2001; Cinar *et al.* 2008; Kahraman *et al.* 2004; Levron *et al.* 2001; Platteau *et al.* 2004; Silber *et al.* 2003). Therefore, it is

suggested that performing PGS on embryos derived from such patients would be beneficial.

Although PGS has been practiced for more than 15 years and initially a number of studies had shown a positive impact of this method for IVF patients (Gianaroli *et al.* 1997; Munne *et al.* 1999, 2003, 2006b), several randomized control trials (RCTs) performed later had failed to find an improvement in IVF outcome and some even concluded that PGS decreases implantation and pregnancy rates (Debrock *et al.* 2007; Hardarson *et al.* 2008; Jansen *et al.* 2008; Mastenbroek *et al.* 2007; Mercereau *et al.* 2008; Meyer *et al.* 2009; Schoolcraft *et al.* 2009; Staessen *et al.* 2004, 2008; Stevens *et al.* 2004). It has been suggested that the unexpected results obtained from these RCTs maybe a result of technical problems such as damage to embryos during biopsy, removal of too many cells for testing, poor fixation of biopsied cells and inappropriate choice of chromosomes to be analysed (Munne *et al.* 2010). Also, almost all of these studies were carried out on day-3 embryos, a stage at which the diagnostically problematic phenomenon of chromosomal mosaicism is common (as explained in section 1.1.3.1). An additional technical problem and one of the major points of criticism for these studies is the usage of FISH to test the chromosomal status of the embryos. Fluorescence *in situ* hybridisation can only test a limited number of chromosomes for each embryo. In the RCTs that examined PGS the number of chromosomes tested ranged from 5 to 9. Given that any chromosome can potentially suffer aneuploidy during the cleavage stage of development, testing of 5-9 chromosomes will not reveal every abnormality. Indeed, only 31-72% of aneuploidies are expected to be detected (Lathi *et al.* 2008). As a result, some of the diagnosed euploid embryos transferred were inevitably aneuploid and this is likely to have

reduced the value of PGS in terms of implantation and pregnancy rates in studies using FISH.

In order to accurately assess the efficiency of PGS, techniques that will be able to test the full chromosomal status of embryos should be used to perform studies. Recently, a few studies were carried out using such techniques and yielded encouraging results, showing exceptionally high pregnancy rates (69.2-82.2%) (Fragouli *et al.* 2010b; Schoolcraft *et al.* 2010, 2011). Although these results look promising the studies were not randomised. However, results obtained very recently from a RCT performed on good prognosis patients (≤35 years old, no previous miscarriage) using such techniques, confirmed results received from the studies above (Yang *et al.* 2012). Patients whose embryos had undergone comprehensive chromosome screening (CCS) showed a considerably high clinical pregnancy rate (70.9%) which was found to be significantly higher than the clinical pregnancy rate calculated for patients whose embryos had not gone through CCS (45.8%).

1.1.3 Biopsy procedures

At the present, all embryos screened with PGD are obtained through ART. Patients go through controlled ovarian stimulation and all oocytes obtained are stripped from their surrounding cumulus cells, since these cells can potentially provide a source of non-embryonic DNA, causing contamination during PGD (Spits and Sermon 2009; Thornhill *et al.* 2005). In most of the cases, intracytoplasmic sperm injection (ICSI) is used to fertilize the oocytes in order to reduce the rate of fertilisation failures and also to prevent any contamination caused by DNA from residual sperm attached to the zona pellucida (Liebaers *et al.* 1998; Thornhill *et al.* 2005). After fertilisation the

embryos can be biopsied at different developmental stages and one or more cells can be retrieved for usage in PGD. Polar body biopsy can also be performed to obtain the 1st and 2nd polar bodies extruded from the oocyte.

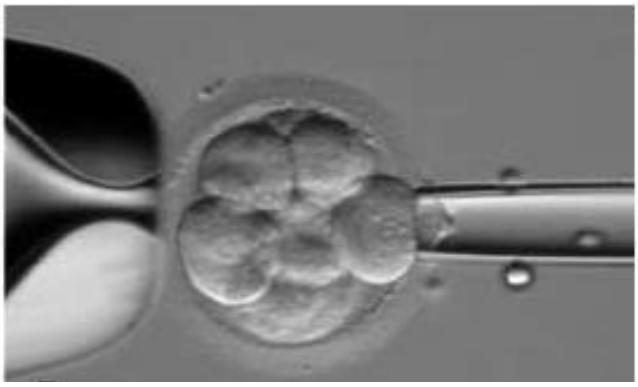
1.1.3.1 Polar body biopsy

Genetic material for PGD can be obtained pre-fertilisation by the removal of the 1st polar body from a mature (metaphase II) oocyte. The 1st polar body can be used to deduce the genotype of the oocyte before fertilisation, while upon fertilisation the 2nd polar body can also be obtained and tested (Braude *et al.* 2002). Polar body PGD was first applied by Verlinsky and colleagues (Verlinsky *et al.* 1990) and the accuracy of this method has been shown in a large number of PGD cases carried out for several genetic disorders (Rechitsky *et al.* 1999). Of note, for PGD of single gene disorders it is essential that both the first and second polar body are tested, since meiotic recombination can lead to unclear (heterozygous) results in the 1st polar body. Polar body biopsy is also widely used for aneuploidy screening of the oocytes. The advantage of this method is that it does not involve biopsy of the embryo and therefore avoids any potential harm (even if minimal). Furthermore, in some countries (e.g. Germany) where testing of embryos is forbidden or highly restricted, it is the only choice for obtaining material for PGD. However, it only provides information regarding the oocyte genotype and therefore it cannot be used for screening paternally derived genetic disorders (Ogilvie *et al.* 2005). Additionally, any chromosomal aneuploidies inherited from the sperm and any aneuploidies that arise because of mitotic errors after the zygote formation will not be detected (Delhanty *et al.* 1993; Vanneste *et al.* 2009a).

1.1.3.2 Cleavage stage biopsy

The most widely used method of obtaining genetic material for PGD is by performing biopsy on cleavage stage embryos on day-3 post-fertilisation (Figure 1.1) (Harper *et al.* 2012).

Figure 1.1: Biopsy of a cleavage stage embryo (day-3 post-fertilisation) to remove a single blastomere. Reproduced from Ogilvie *et al.* (2005) with permission.



Embryos grow in vitro until they reach day-3 and at this point the embryo biopsy takes place by breaching the zona pellucida (Sermon *et al.* 2004). At this stage the embryo typically contains 6-12 cells which are totipotent (Braude *et al.* 2002). One or two blastomeres are removed and used for PGD, although removal of two cells has decreased in popularity since studies linking this practice with decreased implantation potential and reduced rate of blastocyst formation have been published (Cohen *et al.* 2007; De Vos *et al.* 2009; Goossens *et al.* 2008a). Suitable embryos are transferred to the uterus of the woman on day-4 or day-5 post-fertilisation. Using blastomeres from day-3 embryos offers the potential of checking the embryo for genetic disorders inherited from both parents and also testing for any meiotic or mitotic chromosomal imbalances. However, a common problem regarding screening of cleavage stage

embryos for chromosomal anomalies is mosaicism. This phenomenon refers to the presence of two or more distinct cell lines with different chromosomal constitutions in the same embryo and occurs due to errors that happen during mitosis after zygote formation - especially during the first three mitotic divisions (Delhanty *et al.* 1997; Fragouli *et al.* 2011; Katz-Jaffe *et al.* 2004; Munne *et al.* 2002). These errors have been suggested to occur due to the absence of cell-cycle checkpoints during early cleavage divisions (Delhanty and Handyside 1995). The incidence of mosaicism in cleavage stage embryos, assessed through utilisation of CCS methods, has been reported to be ~66% (Voullaire *et al.* 2000; Wells and Delhanty 2000). Therefore, it can be concluded that a single blastomere biopsied from a day-3 embryo might not always be representative of the rest of the embryo. This can potentially lead to false positive and false negative results and consequently to the transfer of aneuploid embryos or the exclusion from transfer of euploid ones (Fishel *et al.* 2010; Li *et al.* 2005). Contrary to preimplantation cleavage stage embryos, a very low degree of mosaicism is detected in postimplantation embryos. Mosaicism in spontaneous miscarriage specimens is seen at a degree of <10% (Santos *et al.* 2010), while for first trimester ongoing pregnancies the incidence is even lower (1-2%) (Los *et al.* 2004). It therefore seems that mosaicism disappears prior to the period of first trimester through mechanisms that involve loss of mosaic embryos or shift of the mosaic embryos towards normality (i.e. selection against abnormal cells in the embryo) (Los *et al.* 2004; Santos *et al.* 2010).

1.1.3.3 Blastocyst biopsy

A final option for obtaining genetic material from the embryo is through blastocyst (day-5) biopsy. At this stage the inner cell mass, which will give rise to the foetus, has differentiated from the trophoectoderm, which will later on develop into the extraembryonic tissues (Braude *et al.* 2002). Biopsy at this stage involves the removal of a number of cells (3-10) from the trophoectoderm. This type of biopsy is advantageous in that no cells are extracted from the inner cell mass and also, in contrast to the other two biopsy strategies, this method obtains multiple cells for carrying out PGD, which theoretically leads to improved accuracy. Furthermore, blastocysts are more robust than earlier embryonic stages and tolerate biopsy exceptionally well. Two drawbacks of this method is that only about 50% of the embryos growing *in vitro* reach the blastocyst stage (Van Landuyt *et al.* 2005) and the time left for diagnosis after biopsy is limited since the embryos need to be transferred by day-6 (Spits and Sermon 2009). However, the introduction of vitrifcation as a method to cryopreserve blastocysts (Youssry *et al.* 2008) and the high survival rates obtained even after biopsy (Keskintepe *et al.* 2009; Liebermann 2009; Schoolcraft *et al.* 2010) make blastocyst biopsy increasingly attractive for PGD. Cryopreservation of biopsied blastocysts allows an unlimited amount of time for diagnosis to take place. As in cleavage stage embryos mosaicism exists in blastocysts (Bielanska *et al.* 2005; Coonen *et al*. 2004; Daphnis *et al.* 2005; Fragouli *et al.* 2011; Santos *et al.* 2010); the incidence however is found to be considerably lower (24-32.4%) (Fragouli *et al.* 2011; Northrop *et al.* 2010). Through data collected from different studies it is suggested that a proportion of mosaic embryos undergo developmental arrest before reaching the blastocyst stage (Evsikov and Verlinksy 1998; Sandalinas *et al.* 2001; Santos *et al.* 2010). This explains the lower rates of mosaicism at the blastocyst stage

when compared to day-3 embryos, while it also suggests that culture to the blastocyst stage might be advantageous since it would identify embryos of increased developmental competence. Furthermore, the possibility of misdiagnosis is believed to be considerably lower since only a small number of mosaic blastocysts are found to have a significant number of euploid cells (Fragouli *et al.* 2011) and since, in contrast to day-3 biopsy, 5-10 cells are obtained for analysis and thus diagnosis is not reliant on a single cell. In fact, it was determined by two different studies that array platforms commonly used for performance of CCS are able to detect a mosaicism rate of as low as 25% in a trophectoderm biopsy (e.g. when 1/4 cells of biopsy is abnormal) (Mamas *et al.* 2012; Northrop *et al.* 2010), preventing therefore in a large degree the transfer of mosaic embryos which would possibly have a negative effect on IVF outcome.

1.1.4 Techniques and methodologies used in PGD

Different techniques and methodologies have been used to date in preimplantation genetic diagnosis, all with different advantages and disadvantages.

1.1.4.1 PGD for Single-Gene Disorders

The small amount of DNA (5-10 pg) found in a single diploid cell makes development of PGD protocols very challenging and introduces several problems not usually seen in routine diagnostic laboratories (e.g. prenatal diagnosis laboratories). These problems include high risk of contamination, amplification failure, extreme preferential amplification of one allele and allele dropout (ADO) (Thornhill and Snow 2002). Allele dropout refers to the failure of one of the alleles of a heterozygous locus

to amplify. This makes a heterozygous cell appear homozygous for a certain locus and can lead to misdiagnosis (Spits and Sermon 2009; Wells and Delhanty 2001).

The polymerase chain reaction is the method used in the great majority of cases for performance of PGD for single gene disorders, although FISH has been used extensively for identification of embryo gender when PGD for X-linked disorders is carried out (Harper *et al.* 2012). In single cell PCR, amplification failure is a common phenomenon. A number of factors are involved in amplification failure. The cell might be lost during transfer to the microcentrifuge tube or lyse before it is placed in the tube during biopsy, washing or tubing. This leads to DNA degradation and therefore amplification failure. After the cell has been transferred the lysis protocol used to release the DNA also influences amplification efficiency (AE) (Kim *et al.* 2009; Sermon *et al.* 1995; Tsuchiya *et al.* 2005). Furthermore, the quality of the cell biopsied seems to affect the amplification success (e.g. apoptotic cells and cells from degraded embryos amplify less well) (Cui and Matthews 1996; Gutierrez-Mateo *et al.* 2009; Ray *et al.* 1998; Thornhill and Snow 2002; Wells and Sherlock 1998).

Another major challenge for single cell diagnostics is DNA contamination. The high number of PCR cycles necessary for amplification of a DNA fragment from a single genome makes PCR-based PGD protocols highly prone to contamination. Potential sources of contamination include any DNA from sperm or cumulus cells after biopsy of embryos derived from IVF and also skin cells from lab staff that accidentally enter the test tube. Contamination can also result from ‘carry over’ of amplified products generated from previous experiments. This is probably the most significant problem regarding contamination since PCR products accumulate in the work environment. In order to avoid contamination stringent experimental practices should be followed (Thornhill and Snow 2002; Wells and Delhanty 2001; Wells and Sherlock 1998).

Although contamination and amplification failure are serious problems in PGD, ADO is perhaps the most important obstacle for accurate diagnosis of single gene disorders (Wells and Sherlock 1998). ADO occurs at random, affects both alleles in a heterozygous cell with the same frequency and it is a particular problem for diagnosis of dominant disorders; if ADO of the affected allele occurs it could result in the transfer of an affected embryo. The occurrence of ADO varies between studies and has been reported to be as high as 25% (De Vos *et al.* 1998). However, experienced PGD laboratories should be able to achieve ADO rates of 5-15% only. There are several suggestions as to what might influence occurrence of ADO including cell lysis method used, length of amplified fragment, PCR denaturation temperature and time, freezing and thawing of cells and DNA damage (Kim *et al.* 2009; Piyamongkol *et al.* 2003; Ray and Handyside 1996; Thornhill *et al.* 2001).

1.1.4.1.1 Strategies for Diagnosis of Single-Gene Disorders

In order to reduce the incidence of the problems described above different PCR amplification methods are employed including nested PCR, multiplex PCR and fluorescent PCR. Nested PCR increases the specificity and sensitivity of PCR and also decreases the risk of 'carry-over' contamination (Braude *et al.* 2002; Wells and Sherlock 1998). This method uses two sequential rounds of PCR to amplify unique sequences from single cells. In the first amplification step a pair of primers amplifying the fragment of interest is used. A low number of PCR cycles are employed, which is insufficient for visualising the DNA fragment. Next, PCR products from the first reaction provide the DNA templates for a second 'inner' reaction. In the second reaction a set of primers situated inside the initial set of

primers is used, amplifying a smaller fragment containing the mutation to a detectable level. This way the sensitivity of the PCR is increased and also the fragments produced in the second step cannot be amplified by the initial outer set of primers. Therefore, these fragments create no contamination threat to any subsequent primary amplifications, which are the most critical in terms of contamination (Thornhill and Snow 2002; Wells and Sherlock 1998).

Another method widely used in PGD for single gene disorders, which has been considered the gold standard for several years now, is fluorescent PCR. Fluorescent PCR (F-PCR) involves the labelling of the 5´ end of one of the primers in a pair with a fluorochrome. Each different fluorescent molecule has its own distinct wavelength of emitted light. After PCR amplification an automated sequencer encompassing a laser detection system is used to visualise the amplified fragments (Spits and Sermon 2009; Thornhill and Snow 2002). Fluorescent PCR is found to be a thousandfold more sensitive than conventional non-radioactive methods (Harper and SenGupta 2012). Consequently, fewer cycles of PCR are needed, shortening the time required for diagnosis. Fluorescent PCR is found to be highly accurate permitting sizing of amplified DNA fragments to the single base pair level, while avoiding the use of hazardous radioisotopes or toxic DNA stains (Findlay *et al.* 1995a, 1996). Furthermore, it is found to reduce the ADO rates observed with conventional PCR product detection methods (e.g. ethidium bromide staining). This is due to the fact that cases of extreme preferential amplification appear as ADO with the less sensitive conventional methods (Sermon *et al.* 1998; Sherlock *et al.* 1998).

In almost all cases PGD is associated with a single minute specimen and as a result only a single PCR reaction can be carried out. In order to increase the amount of information that can be obtained, improving diagnostic accuracy, multiplex PCR is

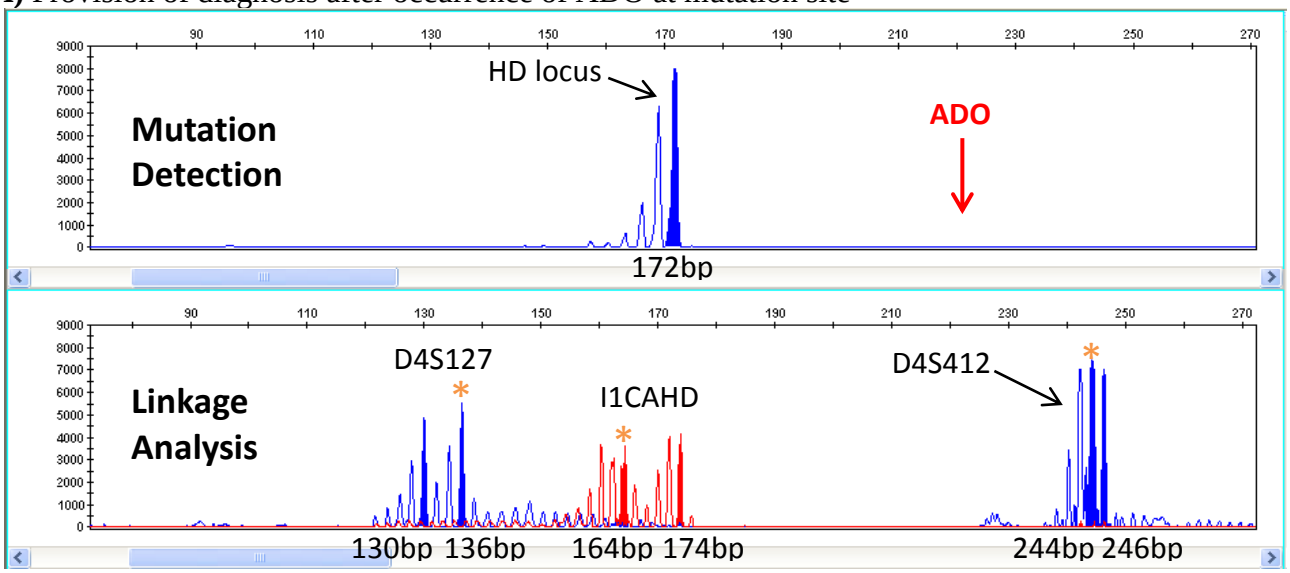
usually employed. Multiplex PCR is a method that allows analysis of several different loci from a single cell. It is achieved by carrying out DNA amplification in the presence of multiple distinct sets of primers, which produce simultaneous amplification of several loci. In most cases multiplex PCR strategies used for PGD involve amplification of the causative mutation site and one or more polymorphic markers that are closely linked to the gene of interest (linked markers) (Spits and Sermon 2009; Wells 2004). Linked markers are a very useful tool in PGD. By typing linked polymorphisms in the prospective parents (one or both of whom are mutation carriers) and genotyping the same loci in other first degree relatives (or more distant relatives if they are known to carry the mutation) it can be deduced which allele of each linked marker is found on the same chromosome as the mutation and therefore inherited along with it. Ideally, at least two informative linkage markers, flanking the gene, should be used so that if recombination happens between the mutation site and the marker it will not lead to misinterpretation of the genotype and misdiagnosis. In order for a linkage marker to be fully informative the parents should be both heterozygous for that marker and not share any of the alleles. In cases where ADO affects the mutation site, it is still possible to achieve a correct diagnosis by assessing the linked informative polymorphisms [Figure 1.2 (A)]. Although ADO can potentially affect the polymorphic loci as well as the mutation site, it has been shown to be independent for each amplified fragment in a multiplex reaction (Ao *et al.* 1998; Rechitsky *et al.* 1998). Since the probability that ADO will occur in two separate loci simultaneously is very low, the chances of misdiagnosis are greatly reduced in multiplex PCR incorporating linked markers (Wells and Sherlock 1998).

Not only does multiplex PCR allow the possibility of combined mutation detection and linkage analysis, but it can also assist in the detection of DNA contamination

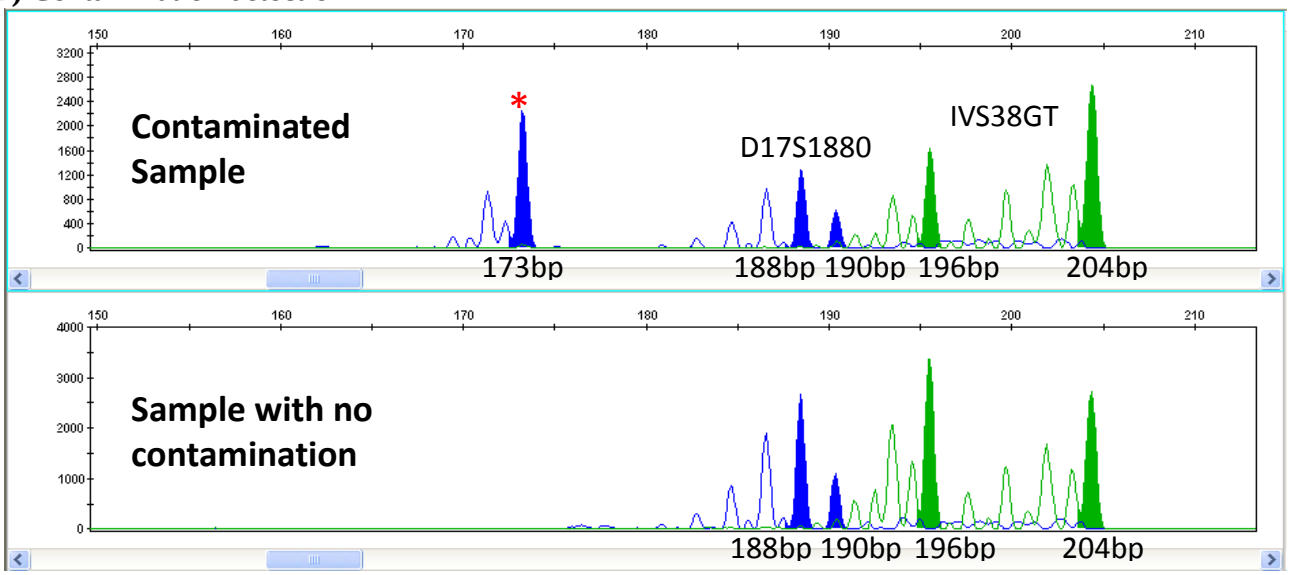
[Figure 1.2 (B)]. The polymorphisms amplified are of a hyper-variable nature, giving a basic form of DNA 'fingerprint' for the embryo. Since the embryo should have inherited one allele from each parent, the detection of any additional alleles can be taken as an indication of contamination with extraneous DNA (or in some cases, where excess parental alleles are present, aneuploidy) (Findlay *et al.* 1995b; Pickering *et al.* 1994). These advantages have made multiplex PCR the standard strategy for PGD of monogenic disorders (Dreesen *et al.* 2000; Kakourou *et al.* 2008; Pecina *et al.* 2010) and for HLA typing (Fiorentino *et al.* 2005; Van de Velde *et al.* 2004).

Figure 1.2: Usefulness of linked markers in PGD for single gene disorders. **A)** Provision of diagnosis after occurrence of ADO at mutation site. **B)** Contamination detection.

A) Provision of diagnosis after occurrence of ADO at mutation site



B) Contamination detection



Notes: **A)** Electropherograms show results for a sample tested for Huntington disease. ADO is identified at the mutation site (HD locus). Only one allele inherited from the unaffected father is detected. Diagnosis however, is still possible through usage of three linked markers included in the protocol which identify the specific embryo as affected. Orange asterisk shows alleles of polymorphic markers linked with the mutant allele of HD locus inherited from the affected mother. **B)** Electropherograms show results obtained from two different embryos having the same genotype for linked markers D17S1880 and IVS38GT (Neurofibromatosis type I). One of the samples is contaminated. Presence of contamination is indicated by one of the linked markers (D17S1880) which shows an extra allele at 173bp (shown with red asterisk) of non-parental origin.

1.1.4.1.2 Detection Methods

After DNA from a single cell is amplified different methods can be used to detect the PCR products and make a diagnosis. During the early years of PGD various different methods were used including heteroduplex analysis, single strand conformational polymorphism analysis, denaturing gradient gel electrophoresis, amplification refractory mutation system and sequencing (Bermudez *et al.* 2003; El-Hashemite *et al.* 1997; Gibbons *et al.* 1995; Ioulianos *et al.* 2000; Kanavakis *et al.* 1999; Moutou *et al.* 2003; Ray *et al.* 1998; Thornhill *et al.* 2000). However, the methods which are most commonly used nowadays include restriction endonuclease digestion, minisequencing and real time PCR.

Restriction endonuclease digestion is based on differences in DNA sequence such as those seen with single nucleotide polymorphisms (SNPs) and alterations caused by mutations. Different bacterial endonucleases recognize different DNA sequences and cleave the DNA fragments having these specific recognition sites, allowing alleles/mutations to be distiguished. Gel electrophoresis is used to separate the digested from the undigested products (Spits and Sermon 2009; Wells and Sherlock 1998).

Minisequencing is a newer method than the others mentioned above, applied to PGD for the first time by Fiorentino and colleagues in 2003 (Fiorentino *et al.* 2003). Minisequencing is a primer extension method which involves the usage of specially modified nucleotides, the dideoxy nucleotides (ddNTPs). Mutation detection involves the use of a primer designed so that its 3´ end anneals exactly one nucleotide upstream of a known mutation or polymorphic site. As well as the primer a DNA polymerase, ddNTPs and the DNA template to be tested (usually a PCR product) are added. After

the first ddNTP is added to the primer no further nucleotides can be added, due the absence of a 3'-OH group in ddNTPs. Each of the four nucleotides is labelled with a different fluorochrome, thus revealing the identity of the base added to the primer, which is complimentary to the base at the mutation/polymorphic site of the template strand (Bermudez *et al.* 2003; Fiorentino *et al*. 2003).

Real time PCR has also been used in a small number of studies for detection of mutations in single cells (Chen *et al.* 2011; Hung *et al.* 2010; Nakabayashi *et al.* 2007; Rice *et al.* 2002; Vrettou *et al.* 2004). This method employs simultaneous amplification and detection of the DNA sequences of interest. Fluorescently labelled probes or dyes are used that bind to amplicons produced during PCR and emit fluorescence. The fluorescence accumulates with each PCR cycle and is monitored throughout the amplification procedure (Traeger-Synodinos 2006).

1.1.4.1.3 Limitations of the current PGD methods

Although the use of F-PCR and mutation detection techniques, such as minisequencing, has made the design of PCR protocols more straight-forward than in the early days of PGD, the development of reliable methods is still a difficult and a very demanding task. The inherent problems of single-cell PCR (e.g. contamination, amplification failure, ADO) have led to the wide usage of multiplex PCR. However, in order to produce a robust multiplex PGD protocol that will be reliable and accurate enough to be used for clinical cases it is necessary to spend long periods of time designing, optimising and validating the procedure. As a result, it is not unusual for couples requesting PGD to have to wait for several months before they can start their IVF cycle and have their embryos tested (Fiorentino *et al.* 2006). This problem can be

partially addressed by using linkage analysis, since such tests focus on polymorphisms present in all patients rather than mutations that might be specific to a single family (Dreesen *et al.* 2000). Protocols designed to assess multiple linked polymorphisms either side of the mutant gene have been developed for several common inherited disorders. This approach, in which a single linkage-based protocol applicable to multiple patients is produced, is sometimes referred to as preimplantation genetic haplotyping (PGH) (Renwick *et al.* 2006). However, some couples desiring PGD do not have access to DNA from other family members (e.g. individuals with *de novo* mutations) and therefore construction of haplotypes is impossible.

Another limitation of current single gene PGD techniques is that they are not compatible with PGS for full chromosomal screening. It is not uncommon for couple requesting PGD to be of advanced reproductive age (>35 years old). It is possible that the pregnancy chances for these patients could be increased and the risks of miscarriage reduced if their embryos were screened for aneuploidies. Some studies have used multiplex PCR protocols for single gene disorders that included microsatellite markers located in certain chromosomes in order to test for chromosomal aneuploidy (Kuliev *et al.* 2005). Although this method succeeds in combining PGD with PGS only a very limited number of chromosomes can be tested for aneuploidy. Because of the high degree of aneuploidy seen in human embryos (Cohen *et al.* 2007) many aneuploidies will inevitably not be detected.

1.1.4.2 Whole Genome Amplification

A technique that has been employed in PGD in order to avoid the limitations of single-cell multiplex PCR is whole genome amplification (WGA). Whole genome amplification aims to achieve non-specific amplification of the entire genome from a single cell or a number of cells (Wells and Sherlock 1998). In theory, after single cell WGA the amplified DNA produced can be treated in the same way as a larger DNA sample having many copies of the genome (e.g. a blood sample). Potentially, this makes the length of time needed to develop a PGD protocol shorter and the optimisation easier compared with conventional methods used in PGD (i.e. single cell multiplex PCR). Also, with WGA the amplified samples can be used for other applications as well such as aneuploidy screening via molecular cytogenetic approaches (e.g. comparative genomic hybridisation).

An ideal WGA amplification method should have a high efficiency, even when applied to a single cell, and provide a balanced and faithful representation of all chromosomal regions without any bias (Lovmar and Syvanen 2006; Peng *et al.* 2007).

1.1.4.2.1 Different types of WGA methods

Over the years different methods of WGA have been developed including interspersed repetitive sequence PCR, linker adapter PCR (LA-PCR), primer extension preamplification (PEP), degenerate oligonucleotide primed PCR (DOP-PCR), tagged random primer PCR and multiple displacement amplification (MDA) (Dean *et al.* 2002; Grothues *et al.* 1993; Lovmar and Syvanen 2006; Ludecke *et al.* 1989; Nelson *et al.* 1989; Tanabe *et al.* 2003; Telenius *et al.* 1992; Zhang *et al.* 1992).

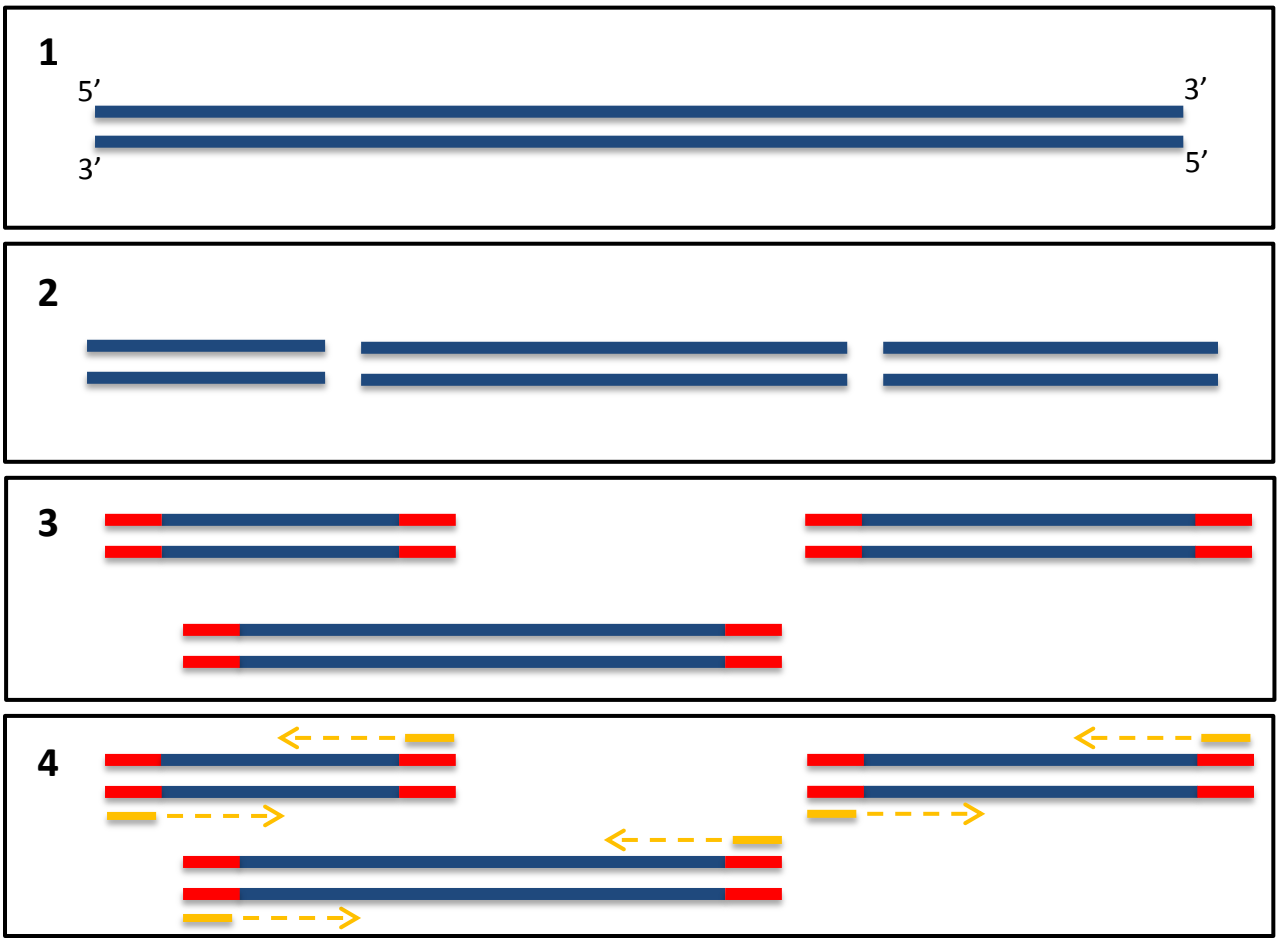
In PGD some of these methods (LA-PCR, PEP, DOP-PCR, MDA) have been used to develop protocols for single gene disorders or for aneuploidy screening (Ao *et al.* 1998; Fragouli *et al.* 2006; Hellani *et al.* 2005; Jiao *et al.* 2003; Lledo *et al.* 2006; Sermon *et al.* 1996; Treff *et al.* 2010b; Voullaire *et al.* 2000; Wells and Delhanty 2000).

A number of drawbacks observed with PEP and DOP-PCR however, have discouraged their use for PGD of monogenic disorders. Increased amplification bias including, incomplete coverage of the genome and also unequal amplification of alleles at heterozygous microsatellite and SNP loci have been observed at low DNA concentrations (Cheung and Nelson 1996; Dean *et al.* 2002; Dietmaier *et al.* 1999; Paunio *et al.* 1996; Zhang *et al.* 1992). Furthermore, amplification artefacts (deletions and insertions) are frequently observed for microsatellite loci (Wells *et al.* 1999).

LA-PCR on the other hand is expected to decrease the extent of amplification biases seen with methods such as DOP-PCR and PEP because it allows more uniform priming. This approach utilizes different methods (e.g. enzymatic digestion, random shearing) to fragment the DNA and through the usage of adapters it performs amplification of the whole genome (Lovmar and Syvanen 2006) (Figure 1.3). One WGA method that uses this approach is GenomePlex (developed by Rubicon Genomics, USA; available from Sigma-Aldrich). GenomePlex has been used in PGD for combined HLA typing and diagnosis of β-thalassaemia and it has also been employed in PGD in conjunction with DNA microarrays for aneuploidy screening and for DNA fingerprinting of the embryos (Chen *et al.* 2008; Fishel *et al.* 2010; Gutierrez-Mateo *et al.* 2011; Schoolcraft *et al.* 2011; Treff *et al.* 2010a, 2010b, 2010c, 2011a). Despite its successful application in most of these studies some disadvantages of this method have been observed including decreased genome coverage,

amplification artefacts and inconsistent results (Ballantyne *et al.* 2007; Fiegler *et al.* 2007; Treff *et al.* 2010c).

Figure 1.3: Schematic diagram showing principle of linker-adapter PCR. Figure created by MK.



Notes: 1) Intact, double-stranded DNA (blue line); 2) DNA is cleaved into fragments of a few hundred base pairs; 3) Universal adapters (red line) are allowed to bind to the 5' and 3' ends of each of the DNA fragments; 4) Adaptor-specific primers (yellow line) anneal to fragmented DNA and amplify the whole genome through PCR.

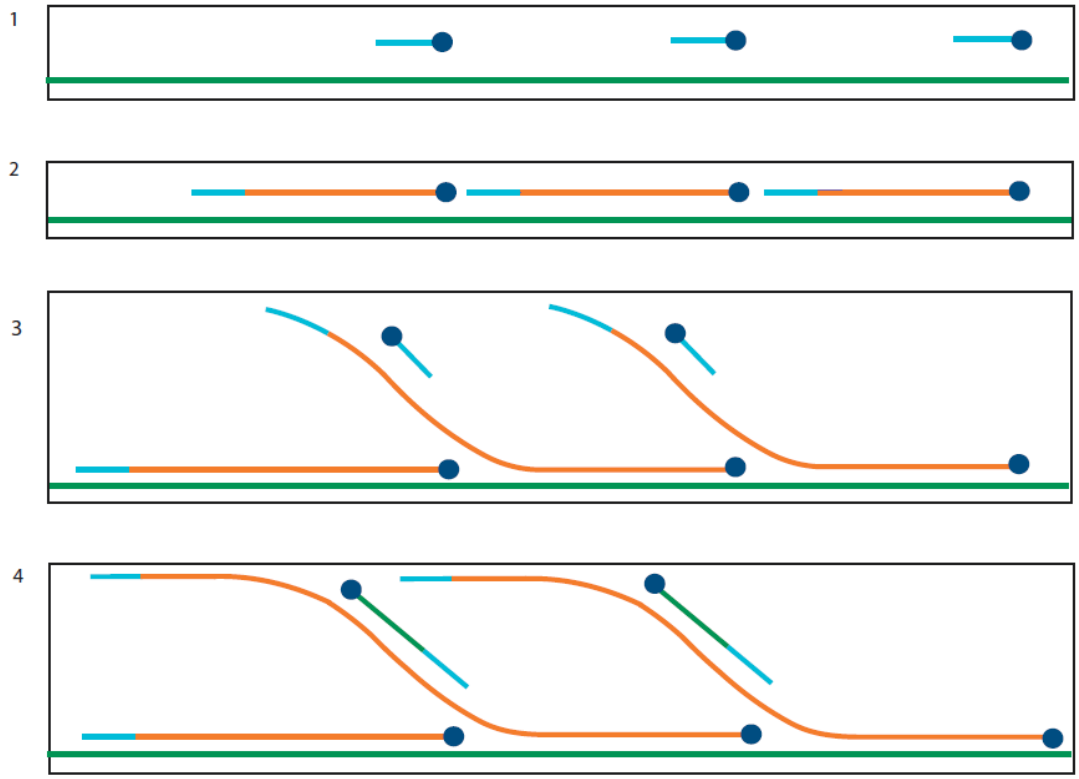
Recently, another WGA method based on LA-PCR has been developed namely SurePlex. SurePlex like GenomePlex has been developed by Rubicon Genomics. This method is now used widely as part of a commercial test employing WGA and microarrays (24sure, BlueGnome Ltd, UK) to assess human embryos and oocytes for

chromosomal abnormalities. Although several studies have been published using the 24sure microarrays (Alfarawati *et al.* 2011a; Fiorentino *et al.* 2011; Geraedts *et al.* 2011; Gutierrez-Mateo *et al.* 2011), there are as yet no studies that specifically assess the efficiency of SurePlex in amplifying single cells for other PGD applications.

Apart from the PCR-based WGA methods described above, MDA (a non-PCR-based WGA method) has also found application in PGD. MDA is an isothermal method carried out at 30°C (Dean *et al.* 2002). This method utilises the Φ29 bacteriophage DNA polymerase or the large fragment of the *Bacillus stearothermophilus* (*Bst*) DNA polymerase to carry out the amplification (Lage *et al.* 2003). Random hexamers or heptamers are used (Dean *et al.* 2002; Lage *et al.* 2003) which anneal at multiple sites along the human genome (Figure 1.4). Φ29 DNA polymerase is found to be highly accurate in replicating the DNA, having a low error rate ($< 3 \times 10^{-6}$) (Esteban *et al.* 1993; Nelson *et al.* 2002) and a high 3´ - 5´ proofreading capacity (Blanco and Salas 1985; Lovmar and Syvanen 2006). Additionally, MDA seems to offer excellent coverage of the genome (86.2 – 93%) when applied to single cells (Handyside *et al.* 2004; Jiang *et al.* 2005; Ling *et al.* 2009). However, MDA using Φ29 DNA polymerase has some limitations. It shows increased preferential amplification when applied to single cells (Glentis *et al.* 2009; Handyside *et al.* 2004; Hellani *et al.* 2004; Ling *et al.* 2009; Renwick *et al.* 2006; Spits *et al.* 2006a) and is also found to give under-representation of centromeric and telomeric regions (Ballantyne *et al.* 2007; Berthier-Schaad *et al.* 2007; Dean *et al.* 2002; Iwamoto *et al.* 2007; Lage *et al.* 2003). Most importantly, MDA shows high ADO rates calculated to be in the range of 10 – 38.9% (Burlet *et al.* 2006; Glentis *et al.* 2009; Handyside *et al.* 2004; Hellani *et al.* 2005; Iwamoto *et al.* 2007; Ling *et al.* 2009; Lledo *et al.* 2006; Ren *et al.* 2007; Renwick *et al.* 2007; Spits *et al.* 2006a). MDA has been more widely used for the

PGD of single-gene disorders than any other WGA method (Burlet *et al.* 2006; Chow *et al.* 2009; Fassihi *et al.* 2010; Hellani *et al.* 2005; Lledo *et al.* 2006, 2007; Ren *et al.* 2007, 2009). Furthermore, it has also been used for aneuploidy screening through the utilisation of microarrays (Brezina *et al.* 2011; Handyside *et al.* 2010; Hellani *et al.* 2004, 2008; Iwamoto *et al.* 2007; Le Caignec *et al.* 2006; Ling *et al.* 2009; Rabinowitz *et al.* 2012; van Uum *et al.* 2012; Vanneste *et al.* 2011).

Figure 1.4: Schematic diagram of multiple displacement amplification (MDA). Reproduced from Spits *et al.* (2006b) with permission.



Notes: 1) The random hexamers/heptamers (blue line) bind to the DNA (green line); 2) The DNA polymerase (blue circle) elongates each primer until it reaches newly-synthesized double stranded DNA (orange line); 3) The polymerase displaces the strand and continues elongation, while the displaced DNA strand serves as template for new priming events; 4) Elongation of the attached primers initiates on the displaced strands. As the reaction proceeds a hyperbranched network is formed generating multiple copies of the original DNA.

1.1.4.3 Diagnosing or testing for chromosomal abnormalities

Methods commonly used in PGD for detection of chromosomal imbalances include: FISH, CGH and methods utilizing DNA microarrays.

1.1.4.3.1 Fluorescent *in situ* hybridisation

Fluorescence *in situ* hybridisation was the first technique allowing the direct enumeration of chromosome copy number in single cells from human embryos (Griffin *et al.* 1992). The method has evolved greatly since its first application and has been widely used for the analysis of embryos and oocytes produced by carriers of structural chromosome abnormalities (Conn *et al.* 1998; Gianaroli *et al.* 1999; Kuliev *et al.* 2003; Munne *et al.* 2000, 2005, 2006a; Rubio *et al.* 2005; Verlinsky *et al.* 1995; Vialard *et al.* 2008). Fluorescence *in situ* hybridisation involves the fixation of the biopsied cell on a glass slide and subsequent hybridisation of fluorescently labelled DNA probes onto the spread chromosomes/nuclei, followed by analysis using a fluorescence microscope (Seli *et al.* 2010). The major limitation of FISH is the limited number of chromosomes that can be tested in single cells. PGD laboratories have tested a maximum of 15 chromosomes in a clinical context, although the vast majority of analyses have involved analysis of 5-12 chromosomes per sample (Munne *et al.* 2010). The restricted number of chromosomes that can be simultaneously tested is primarily related to the limited number of spectrally distinct fluorochromes (i.e. colours) available for probe labelling. Furthermore, FISH is not compatible with PCR and consequently this technique does not allow combination of PGD for monogenic disorders with PGS. These limitations of the FISH technique (especially the limited number of chromosomes assessed) and the development of new techniques able to

perform CCS (described below in sections 1.1.4.3.2 and 1.1.4.3.3) have caused a decline in the usage of FISH for PGS in the recent years. Nevertheless, FISH still finds wide application in PGD of chromosomal rearrangements such as reciprocal and Robertsonian translocations and also, in PGD of X-linked disorders through embryo sexing. PGD for these indications can be very efficiently carried out through utilisation of FISH since only limited number of probes is required to perform diagnosis.

1.1.4.3.2 Comparative genomic hybridisation

Another technique that has been used in PGD for detection of aneuploidy is comparative genomic hybridisation (Fragouli *et al.* 2008, 2010b; Schoolcraft *et al.* 2010; Voullaire *et al.* 2002, 2007; Wells *et al.* 2002). This technique was initially developed in 1992 (Kallioniemi *et al.* 1992) for detecting aneuploidies in tumours. The technique was optimised for application on quantities of DNA (0.2-1.0µg) far larger than the ones found in a single cell (5-10pg). As with most other techniques applied to single cells, application of CGH on single cells was very challenging and required extensive optimisation before it could be accurately used for CCS at this level. One of the key changes to the conventional CGH protocol was the inclusion of a WGA step (performed through DOP-PCR). Through this step sufficient quantities of DNA were obtained from single cells and utilisation of the technique for PGD purposes became feasible (Voullaire *et al.* 1999; Wells and Delhanty 2000; Wells *et al.* 1999).

Comparative genomic hybridisation involves the competitive hybridisation of sample and reference DNAs, labelled with different fluorochromes, to normal metaphase

chromosomes on a slide. DNA from the sample is traditionally labelled green and the chromosomally normal reference is labelled red. The radio of green:red fluorescence along each chromosome reveals the copy number of the sample in comparison with the reference DNA (i.e. an excess of green fluorescence on a specific chromosome is equivalent with gain while an excess of red indicates loss) (Wells *et al.* 2008). While CGH is more comprehensive than FISH, since it checks all of the chromosomes in biopsied cells, it is found to have some disadvantages that make its use in PGS difficult. It is a lengthy procedure taking 4-5 days to complete, making the cryopreservation of biopsied embryos a necessity. Although recent protocols have been developed for performance of the whole procedure in ~24 hours (Rius *et al.* 2010, 2011), CGH still remains a complex technique and a cumbersome procedure that requires expertise in cytogenetic and molecular genetic methods. Furthermore, usage of DOP-PCR for amplification of the samples renders this method incompatible with most PCR diagnoses for single gene disorders.

1.1.4.3.3 DNA microarrays

In recent years, there has been a rapid increase in the utilisation of DNA microarrays in PGD/PGS. DNA microarrays can be used in a simple and rapid way to provide full chromosomal screening at the single cell level. Different types of DNA microarrays have been used to date for embryo and oocyte testing including BAC (bacterial artificial chromosome) arrays, oligonucleotide arrays and SNP arrays.

BAC arrays consist of thousands of individual probes, produced inside bacteria. This type of array utilizes a variant of the CGH technique. The DNA fragments comprising the probes cover large parts of chromosomes [150-200kb (kilobases)] facilitating the

binding of multiple labelled DNA fragments produced using WGA. The hybridisation of so many fragments to each BAC probe, representing hundreds of distinct loci, dilutes the impact of technical artefacts such as ADO and preferential amplification (Wells *et al.* 2008). This type of array has found wide application for the purpose of PGS and for PGD of chromosome rearrangements and is the platform upon which the 24sure test is based (section 1.1.4.2.1) (Alfarawati *et al.* 2011a; Fiorentino *et al.* 2011; Fishel *et al.* 2010; Le Caignec *et al.* 2006; Vanneste *et al.* 2009b, 2011).

Oligonucleotide microarrays are another type of array that can be used for CGH. In contrast to BAC arrays, the probes (oligonucleotides) used are synthesised *in situ* and are much smaller in length (25-85 nucleotides in length). High-density arrays are produced consisting thousands of probes (Wells *et al.* 2008). This type of array has been successfully applied in some PGD studies although BAC microarrays remain the dominant platform for single cell analysis (Hellani *et al.* 2008; Traversa *et al.* 2011).

Another method for genetic diagnostics that involves oligonucleotide microarrays is the analysis of SNPs. Microarrays designed for the analysis of SNPs typically assess a large number (10,000 – 500,000) of polymorphic loci scattered throughout the human genome. In most cases the labelled test DNA and a reference DNA are hybridised to separate areas of the slide and the copy number of the chromosomes is determined by comparing fluorescence intensities obtained from these two samples. A significant advantage of this type of array is that analysis of SNPs, apart from providing information of the chromosomal status of an embryo, also provides genotyping information (Wells *et al.* 2008). The inheritance of specific genetic loci associated with disease can be determined by tracking the alleles of linked SNPs from one generation to the next. Analysis of several individuals from the same family can allow haplotypes (groups of linked SNPs) to be established across the entire genome.

Essentially this allows PGH to be conducted for any single gene disorder using a single protocol, an approach sometimes referred to as karyomapping (Handyside *et al*. 2010; Johnson *et al.* 2010). The combination of genotype and cytogenetic data provided by SNP microarrays makes them attractive for PGD/PGS and a few published studies have indicated that they are applicable in a clinical context, although the length of standard procedures for analysis using SNP microarrays is longer than ideal for embryo testing (Handyside *et al.* 2010; Iwamoto *et al.* 2007; Johnson *et al.* 2010; Ling *et al.* 2009; Schoolcraft *et al.* 2011; Treff *et al.* 2010a, 2010b, 2010c, 2011a, 2011d).

While usage of DNA microarrays has many advantages over competing technologies, some drawbacks do exist. The protocols used for some of the platforms can be laborious and long (i.e. SNP arrays) and the cost of the equipment and reagents used with DNA microarrays is relatively high.

1.2 Alternative methods for embryo screening to enhance IVF outcome

Apart from morphologic criteria and aneuploidy screening that have been used in the past, other methods have been investigated with the aim of selecting the IVF embryos with the greatest chance of producing a child. The development of an accurate and reliable test to assess oocyte and embryo viability, in the hope to reduce multiple gestations while maintaining or increasing overall pregnancy rates, is considered to be one of the most important aims in reproductive medicine (Seli *et al.* 2010). Multiple gestations are associated with increased health risks. Health consequences for the mother include an increase in pregnancy-associated hypertension and excessive bleeding during labor and delivery. Furthermore, an increased rate of preterm deliveries is observed along with a rise in infant deaths and an increase in the risk of disability for the newborn (e.g. cerebral palsy) (Keith *et al.* 2000; Luke and Brown 2007). The medical and financial complications caused by multiple pregnancies have led a number of countries to legally restrict the number of embryos permitted for transfer (Sakkas and Gardner 2005). However, adoption of a strategy which only allows 1 or 2 embryos to be transferred could result in decreased IVF success rates especially if it is considered that approximately 8 out of 10 embryos transferred fail to implant (Bromer and Seli 2008). Therefore, a number of studies have been conducted to investigate invasive and especially non-invasive methods using 'omics' (genomics, transcriptomics, metabolomics, proteomics) to supplement the methods currently used for embryo assessment (Assou *et al.* 2011; Dominguez *et al.* 2008; Fragouli *et al.* 2010a; Gebhardt *et al.* 2011; Jarkovska *et al.* 2010; Jones *et al.* 2008; Katz-Jaffe *et al.*

2006, 2009; Seli *et al.* 2007, 2010; Stokes *et al.* 2007; Sturmey *et al.* 2009; Wathlet *et al.* 2011).

Although some of these methods have shown positive results and were able to identify some potential biomarkers that might potentially be used for embryo selection, RCTs are lacking and most in the field do not consider these techniques to have yet reached the point where they can be used clinically to accurately identify the most viable embryo for transfer. Research is ongoing for identification of new embryo selection methods.

1.2.1 The role of mitochondria on embryo development

Mitochondria are maternally inherited organelles that contain their own specific genome of 16.6 kb which contains a set of 37 genes that control oxidative phosphorylation (OXPHOS). Mitochondria are responsible for the production of most of the energy needed by the cell for biosynthetic, metabolic and physiological processes. The energy they provide is in the form of adenosine triphosphate (ATP) and is produced though utilisation of the OXPHOS pathway (Anderson *et al.* 1981; Cummins 2004; Steuerwald *et al.* 2000).

Through a number of clinical and animal studies carried out it has been documented that mitochondria have an important role in oogenesis and preimplantation embryogenesis (Van Blerkom 2009). During early oogenesis and in the primordial germ cell there are only a very small number of mitochondria (probably fewer than 10) that have persisted through the 'mitochondrial bottleneck' (Cummins 2004). These mitochondria will later expand to give rise to the entire mitochondrial

constitution of an individual. This limited number of 'founding' mitochondria is replicated during the growth phase of oogenesis (Van Blerkom 2011) and results in the presence of thousands of copies in the mature, unfertilized oocyte; estimations for the number of oocyte mitochondria range from <50,000 to >1,000,000 (Van Blerkom 2009). The number of mitochondria in the embryo remains constant and is distributed amongst daughter cells during the preimplantation stages. Mitochondrial replication resumes after the blastocyst stage (Cummins 1998; Piko and Taylor 1987). Thus, during the early stages of embryogenesis each cell in the embryo has to rely on the activity of the mitochondria inherited from the oocyte to carry out relevant processes. This is of particular importance for the embryo since any mitochondrial dysfunction (e.g. caused by mutation of the mitochondrial genome or insufficient numbers of mitochondria) can negatively influence embryo development (Wang *et al.* 2009). It is important to note that inheritance of mitochondria in human is considered to be strictly maternal and active mechanisms exist that eliminate any paternal mitochondria that might enter the oocyte during fertilisation (Cummins 2004). Thus, the entire mitochondrial constitution of an embryo is expected to be derived solely from mitochondria contributed from the oocyte.

Decreased amounts of mtDNA and diminished bioenergetic capacity have been linked with fertilization failure, impaired oocyte quality and abnormal organization and function of meiotic spindle (May-Panloup *et al.* 2005; Reynier *et al.* 2001; Santos *et al.* 2006; Zeng *et al.* 2007). Furthermore, bioenergetic capacity has been correlated with embryo developmental potential and IVF outcome (Van Blerkom *et al.* 1995).

Apart from their function in energy production, mitochondria have roles in other essential cellular activities including regulation of apoptosis, control of protein glycosylation, calcium homeostasis, steroid hormone synthesis, Fe-S protein

synthesis, and pyrimidine and haem synthesis (Barritt *et al.* 2002; Delbart 2000 cited in May-Panloup *et al.* 2005, p.593).

Given the importance of mitochondrial activity for individual cells primarily and for the whole embryo ultimately, investigation of mitochondrial function in oocytes and embryos could provide key insights into embryo viability and development and thus help select the best embryo for transfer.

1.2.2 Telomere length and its role on mammalian reproduction

Telomeres are special functional complexes located at chromosome ends (Blackburn 2000). They are composed of repeated DNA sequences of TTAGGG and proteins that form a loop to cap chromosome ends and prevent end-to-end fusions (Keefe *et al.* 2007). In most cells telomeres shorten with each cell division due to DNA polymerase being unable to completely replicate the end of chromosomes (Watson 1972; Wright *et al.* 2001). Furthermore, telomeres lose length from exposure to reactive oxygen species (ROS) that cause their oxidation and eventually their shortening which is facilitated by a DNA damage response that removes the oxidised sequences (Keefe and Liu 2009; Passos and von Zglinicki 2005). Progressive shortening of telomeres by the different mechanisms eventually results in a critically short length which causes cell cycle arrest, apoptosis and genomic instability. However, not all cells are affected by telomere shortening. Some types of cells such as male germ cells, stem cells and cancer cells are able to avoid telomere shortening due to the function of telomerase, a reverse transcriptase that adds telomeric sequence (Blackburn 2005a, 2005b; Blasco *et al.* 1999; Keefe *et al.* 2007).

In regard to mammalian reproduction telomeres seem to have an important role in meiosis. Specifically, during meiosis I telomeres promote the alignment, pairing and synapsis of chromosomes and facilitate the formation of chiasmata (Bass *et al.* 1997; Keefe *et al.* 2007; Roig *et al.* 2004; Scherthan *et al.* 1996, 2006). It has been demonstrated from experiments carried out in mammals with shortened telomeres that reduced telomere length causes adverse outcomes in oocytes and embryos. Although oocytes do not divide, the female germ cells do divide mitotically before they enter meiosis. The process of replicative senescence - the inefficient replication of chromosome ends that causes telomere shortening - is expected to begin during division of germ cells. Telomere shortening in oocytes is also expected to occur from exposure to ROS during their prolonged stay in the ovaries (up to 45 years in some women) (Keefe and Liu 2009). Liu and colleagues (Liu *et al.* 2004) found that shortened telomeres cause a reduction in chromosome pairing and synapsis and disrupt recombination during meiosis I. Additionally, telomere deficiency negatively affects the function of meiotic spindles and causes misalignment of chromosomes (Liu *et al.* 2002a). Moreover, shortened telomeres can cause apoptosis eventually disrupting embryo viability (Keefe *et al.* 2005; Liu *et al.* 2002b).

It is clear that by facilitating cell cycle arrest and apoptosis, shortened telomeres have the potential to exert a negative effect on embryo development and viability. Therefore, determination of telomere length in oocytes and embryos could be beneficial for IVF procedures and could assist in selecting the most competent embryo for transfer. Of particular relevance is a study carried out on spare human oocytes from patients undergoing IVF which established that telomere lengths were longer in oocytes from patients that became pregnant than in oocytes from patients that failed to achieve a pregnancy (Keefe *et al.* 2003).

1.3 Phospholipase C zeta and infertility

Whether it is aneuploidy leading to implantation failure and miscarriage, reduction in the number of telomeric repeats causing cellular apoptosis and embryo arrest, or mutations of the mitochondrial DNA compromising vital energy requiring functions, genetics seems to be at the very heart of determining oocyte/embryo viability. However, viability is not the only important aspect of human fertility to be influenced by genetics. The process of spermatogenesis can be adversely affected or even completely prevented by mutation, especially by deletion of genes from the Y-chromosome. More recently, evidence that processes such as oocyte activation can be affected by genetic alterations has begun to emerge.

Intracytoplasmic sperm injection has evolved as a technique in ART to treat cases of male factor infertility which account for 35-40% of infertility in couples (Forti and Krausz 1998; Kumtepe *et al.* 2009). Although ICSI is a highly successful technique, achieving normal fertilisation in approximately 70% of cases (Nasr-Esfahani *et al.* 2010), complete or almost complete fertilization failure is still seen in 1-5% of ICSI cycles (Flaherty *et al.* 1998; Mahutte and Arici 2003; Yanagida *et al.* 2008). It has been concluded that the principal cause of fertilization failure, accounting for >80% of cases, is failure of oocyte activation (Flaherty *et al.* 1998). Oocyte activation refers to the process by which ovulated mature metaphase II oocytes are stimulated to resume meiosis. It involves a number of changes occurring in the cell including exocytosis of cellular granules, completion of meiosis and formation of pronuclei (Yanagida *et al.* 2008; Swann and Yu 2008). Oocyte activation is induced by a rise in intracellular calcium (Ca^{2+}) concentration which is followed by a series of calcium transients known as 'calcium oscillations' (Heytens *et al.* 2009).

After a number of theories formulated to explain Ca^{2+} oscillations and the general mechanism of oocyte activation, it is now generally accepted that Ca^{2+} signalling in mammalian fertilisation is initiated though the action of a sperm factor which is released into the oocyte after fusion of the gametes (Swann and Yu 2008). This factor has been identified as phospholipase C zeta (PLCζ) and is found to be exclusively expressed in testis (Saunders *et al.* 2002). Studies have shown that mouse oocytes injected with recombinant PLCζ RNA and protein, express calcium oscillations that resemble the ones seen at fertilization (Cox *et al.* 2002; Kouchi *et al.* 2005; Saunders *et al.* 2002). Furthermore, absence or reduced expression of PLCζ in sperm is found to suppress or prematurely stop Ca^{2+} release in the oocyte (Knott *et al.* 2005; Saunders *et al.* 2002; Yoon *et al.* 2008). More recently, a study carried out in nine patients whose sperm was unable to activate oocytes identified a novel mutation in the PLCζ gene for one of these patients (Heytens *et al.* 2009). The mutation (H398P) was found to cause an amino acid change (Histidine to Proline) at position 398 in the amino acid sequence of the PLCζ protein. After modeling the structure of the modified protein it was concluded that the amino acid change would disrupt the tertiary structure of the protein. Injection of cRNA carrying this mutation into mouse oocytes resulted in highly abnormal patterns of calcium oscillations that are inefficient for oocyte activation (Heytens *et al.* 2009) while no Ca^{2+} oscillations were observed after injection of recombinant $PLC\zeta^{H398P}$ into mouse oocytes (Kashir *et al.* 2011).

Given the established role of PLCζ in oocyte activation, any defects in the functional ability of the protein or its abnormal expression might explain some cases of male infertility where oocyte activation deficiency (OAD) is observed (Kashir *et al.* 2012). Similar to aneuploidy screening of human oocytes and embryos, screening the PLCζ gene for mutations in infertile men could help to guide treatment, as well as revealing

the basis of their infertility. Cases of OAD can potentially be treated through utilization of artificial oocyte activation (AOA) techniques which use a wide range of chemical, physical and mechanical stimuli to elicit Ca^{2+} release in the oocytes (Alberio *et al.* 2001).

1.4 Aims

1) Development of novel PGD protocols and their usage to carry out clinical cases for single gene disorders.

This project involved the utilization of a wide variety of conventional techniques employed in PGD (e.g. PCR, minisequencing) to develop a number of new PGD protocols that were subsequently used to carry out clinical cases. In total, 31 novel PGD protocols were developed. Development of the protocols involved the exploration and adaptation of different ways of performing PGD using established techniques. Through creation of these protocols it was possible to critically assess the conventional methodologies in PGD and compare them with new methodologies.

2) Development of a novel, universal HLA-typing protocol

The few PGD centres carrying out HLA-typing around the world employ a strategy whereby different markers and loci are used for each case (Kahraman *et al.* 2011; Rechitsky *et al.* 2004; Van de Velde *et al.* 2009). As a result a new protocol has to be developed for each couple. The aim of this project was the creation of an accurate, universal protocol that could be used for all cases for HLA-typing avoiding the need for development of a new protocol with each case. Given the extreme urgency of PGD-HLA typing, due to the deteriorating condition of the affected child, a protocol that could be applied without requiring a long design and work-up is highly desirable. A protocol encompassing 19 highly heterozygous short tandem repeat (STR) markers spread across the entire HLA region on chromosome 6 was developed, validated and applied in clinical cases.

3) Evaluation of different WGA methods for their applicability in PGD

This project aimed at the comprehensive evaluation of three WGA methods - GenomePlex, SurePlex and MDA - for their applicability in different indications of PGD. Ten clinically relevant STR markers were amplified from WGA products in order to assess characteristics such as amplification efficiency and ADO. Furthermore, WGA products were applied on two distinct types of array: an oligonucleotide array and a SNP array. This not only helped to assess the WGA methods but also helped to assess if the specific microarrays used have any applicability in PGD. Finally, clinical cases were carried out using some of the WGA methods.

4) Development of a customised array for usage in PGD

The aim of this project was the development of a customised oligonucleotide array that would be able to combine comprehensive aneuploidy screening with the measurement of mtDNA copy number and telomere length in the applied sample. Adding these extra features to aneuploidy screening could assist in selecting the best embryo for transfer and therefore increase IVF success rates, as well as yielding unique biological data concerning the association (or lack thereof) between aneuploidy, maternal age, mitochondria and telomeres. The possibility for usage of the array for embryo fingerprinting and contamination detection was also investigated. In order to validate the developed array, 116 clinical samples were applied to it. Furthermore, amplified and unamplified genomic DNA extracted from different cell lines was used to assess the array for its ability to accurately determine the relative quantities of mtDNA and telomere DNA in applied samples.

5) Measurement of telomere length in clinical samples

Apart from the development of a novel array for measurement of telomere DNA in clinical samples, the development of a quantitative real time PCR (qPCR) protocol was also attempted. The protocol was aimed for usage in parallel with the test used currently at Reprogenetics UK (UK) and also in many other laboratories worldwide for CCS of clinical samples (24sure test). Therefore, the protocol was optimised based on the usage of SurePlex WGA products. SurePlex amplified DNA (genomic or single cell) and unamplified genomic DNA extracted from different cell lines was used to assess the developed protocol.

6) Development of a protocol for rapid comprehensive aneuploidy screening

The methods used in PGD to perform comprehensive chromosome screening involve a lot of hands on work and require a considerable amount of time (>12 hours), which is problematic for application at the blastocyst stage. Additionally, the cost of reagents used in some of these methods is high (e.g. micoarrays). Therefore, this project aimed at the development of a relatively inexpensive method utilizing qPCR that would be able to provide accurate and rapid aneuploidy screening of the full chromosomal complement. The developed protocol was assessed through application on genomic DNA, single cells and clumps of cells (simulating the samples obtained from trophectoderm biopsies).

7) DNA fingerprinting of clinical samples

Incidences of sample mix-up have occurred before in ART (Bender 2006) and despite the adaptation of new technologies and utilization of strict guidelines issued by different authorities, errors continue to happen. This project involved the development of a rapid, low-cost protocol for fingerprinting of sperm samples collected in IVF clinics in order to ensure their match to the expected patients. Furthermore, a protocol was optimised for fingerprinting embryos in order to confirm they are a correct match to the parental DNA. The protocol has a further function in identifying DNA contamination during a PGD case. The optimised protocols were applied on 10 sperm samples derived from different individuals and also on single cells and WGA single cell products.

8) Explanation of male infertility through PLCζ gene

Further examination of DNA derived from patient^{H398P} mentioned in Heytens *et al.* (2009) revealed an additional mutation (H233L). As part of this project tests utilising real time PCR and minisequencing were constructed to detect these two mutations in DNA samples. Single sperm isolated from this patient were amplified and tested for these two mutations in order to confirm mode of inheritance. Furthermore, DNA from 100 individuals was tested for presence of these mutations in order to get an indication for prevalence in the general population.

2) Materials and Methods

2.1 General laboratory practice and methods used in multiple different projects

The laboratory where experiments were carried out consisted of a main lab room and a separate ‘single cell’ room which had no connection with the main lab. The ‘single cell’ room was kept under positive pressure at all times. The circulating air in the room was passed through a High-Efficiency Particulate Air (HEPA) filter and was frequently renewed, while the ventilation system of the room was not connected with other rooms of the building. These precautions were taken in order to prevent any DNA contaminants from entering the room. For this reason laboratory workers using the room wore overshoes (VWR, UK), lab coats and gloves at all times.

Single cell isolations were always carried out in this room using a binocular microscope (M8 type MDG17, WILD Heerbrugg, Switzerland). All reaction mixtures described in this thesis were prepared in PCR cooler 96-well racks (Eppendorf, UK) inside cabinets in the ‘single cell’ room and they always included positive and negative controls. All reagents, equipment and disposables used for DNA amplification were stored in the ‘single cell’ room and were never taken out of the ‘single cell’ room while reagents and apparatus from the main lab were never taken into the room.

Thermocyclers and most other items of equipment were located in the main laboratory. All PCRs described in this chapter were carried out using an Eppendorf Mastercycler ep gradient S (Eppendorf, UK) and an Eppendorf Mastercycler Pro (Eppendorf, UK). Work portion presented

It should be noted that although the great majority of research included in this study was carried out by the person presenting this thesis, some experimental work was performed by other scientists/collaborators. Whenever appropriate, detailed

information regarding research undertaken by other scientists are provided and the people involved are acknowledged.

2.1.1 Capillary electrophoresis

Capillary electrophoresis was carried out in order to analyze all fluorescent PCR products (including minisequencing products) with the use of a 3130 Genetic Analyzer (Applied Biosystems, USA). Specifically, 9.25µl of Hi-Di Formamide (Applied Biosystems, USA) were mixed with 0.25µl of size standard (GeneScan – 120 LIZ for minisequencing products; GeneScan – 500 ROX or 500 LIZ for the rest of the products) (Applied Biosystems, USA) and 0.5µl of product. These were loaded into MicroAmp – Optical 96 well reaction plates (Applied Biosystems, USA) and denatured at 95°C for 3 minutes before loading into the 3130 Genetic Analyzer. Capillary electrophoresis was carried out on the denatured samples using Performance Optimised Polymer – 7 (POP-7) (Applied Biosystems, USA). Capillary electrophoresis for minisequencing products was carried out as follows: E5 dye set, 12 second injection time, 8,500V, 60°C, 15-20 minutes. For all other products capillary electrophoresis was carried out using the following: G5 dye set, 16 second injection time, 15,000V, 60°C, 20-30 minutes.

The data received from capillary electrophoresis were analyzed using ‘GeneMapper software v4.0’ (Applied Biosystems, USA).

2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to analyse amplified products that were not fluorescently labeled and also restriction enzyme digestion products. UltraPure Agarose (Invitrogen, UK) was dissolved in 1x Tris/Borate/EDTA (TBE) buffer (Fisher Bioreagents, UK) to make 1% - 3% agarose gels (e.g. for 1% agarose gel 1g of agarose was dissolved in 100ml of 1x TBE buffer). The mixture was then heated in a microwave until the agarose was completely dissolved in TBE and was left for a few minutes at room temperature to cool. Subsequently, ethidium bromide (Invitrogen, UK) was added to the gel (0.9µg/ml in the agarose gel) and the gel was poured into a gel caster and was allowed to set at room temperature. 5µl of amplified product were mixed with 0.5-1µl of 10x BlueJuice gel loading buffer (Invitrogen, UK) and loaded into the gel. Electrophoresis was carried out at 90-100 volts until bands had migrated a sufficient distance through the gel for fragment size to be determined. Depending on the size of the products analyzed a 100bp (base pair) Low Scale DNA Ladder (Fisher Bioreagents, UK) or a 1kb Plus DNA ladder (Fisher Bioreagents, UK) was used to determine the size. The PhotoDoc-It Imaging System (UVP, UK) was used to visualize the DNA bands.

2.1.3 Ethics

Whenever considered necessary, ethical approval was obtained for usage of clinical samples in projects presented in this thesis.

Samples essential for the design, optimisation and validation of PGD protocols were obtained from patients who had requested preimplantation testing of their embryos

(sections 2.2, 2.3, 2.4.1.6). This formed part of the routine clinical PGD service and no ethical approval was necessary. Signed informed consent for PGD and the development of the necessary protocols was obtained from patients in all instances.

On two occasions, PGD was performed - on clinical samples - using a novel methodology (SNP arrays / karyomapping) while, in parallel the same clinical samples were diagnosed using standard clinical PGD methodology (section 2.4.1.7). Since the standard PGD testing was carried out in parallel to the new methodology in order to provide diagnosis of the biopsied embryos, and since the novel technology provided essentially the same information as the traditional clinical test, it was considered that ethical approval was unnecessary for this project. Importantly however, informed signed consent was obtained from the patients for both clinical cases carried out regarding usage of the clinical samples with the new methodology.

For the project presented in section 2.5, WGA products derived from embryos that had been previously assessed for aneuploidy using standard clinical diagnostic techniques, were reanalysed using a newly developed array platform (section 2.5.2.2.1). Under the scope of the same project, WGA samples derived from two clinical cases carried out for PGD of single gene disorders underwent SNP fingerprinting (section 2.5.2.3). Since analysis of these samples using the new array platform did not involve any new embryonic material, provided no additional data of clinical or biological relevance and was essentially confirmatory of tests already carried out, no additional ethical approvals were considered necessary. Usage of whole embryos for FISH analysis, donated from clinics in the United States and analysed in the Reprogenetics laboratory (section 2.5.2.2.1), was covered by approvals obtained from Western Institutional Review Board (Reprogenetics LLC, USA). Furthermore, mitochondrial analysis on samples applied on the developed

array was carried out with informed consent of the patients, under appropriate licencing from the regulator and with approval from the local ethics committee (RO149).

The sperm analysis work correlated to DNA fingerprinting described in this thesis (section 2.8) was approved under Research Ethics Committee (REC) reference number 12/SC/0468 [National Research Ethics Service (NRES) Committee South Central, UK].

Regarding usage of samples for investigation of the PLCζ gene (section 2.9), ethical approval was obtained from different ethics committees and boards. Utilisation of DNA samples and sperm obtained from patients undergoing fertility treatment at Ghent University Hospital (Belgium) was approved by the Ethical Committee of Ghent University Hospital. In addition, local ethical permission was granted by the Oxford Tropical Research Ethics Committee (OXTREC; 31/09). Screening of 100 fertile individuals was ethically approved by the Western Institutional Review Board.

2.2 PGD – Clinical cases

A total of 46 PGD clinical cases were carried out. All of the cases described in this section were performed using PCR to amplify the DNA found in each clinical sample in order to perform diagnosis of a known genetic disorder.

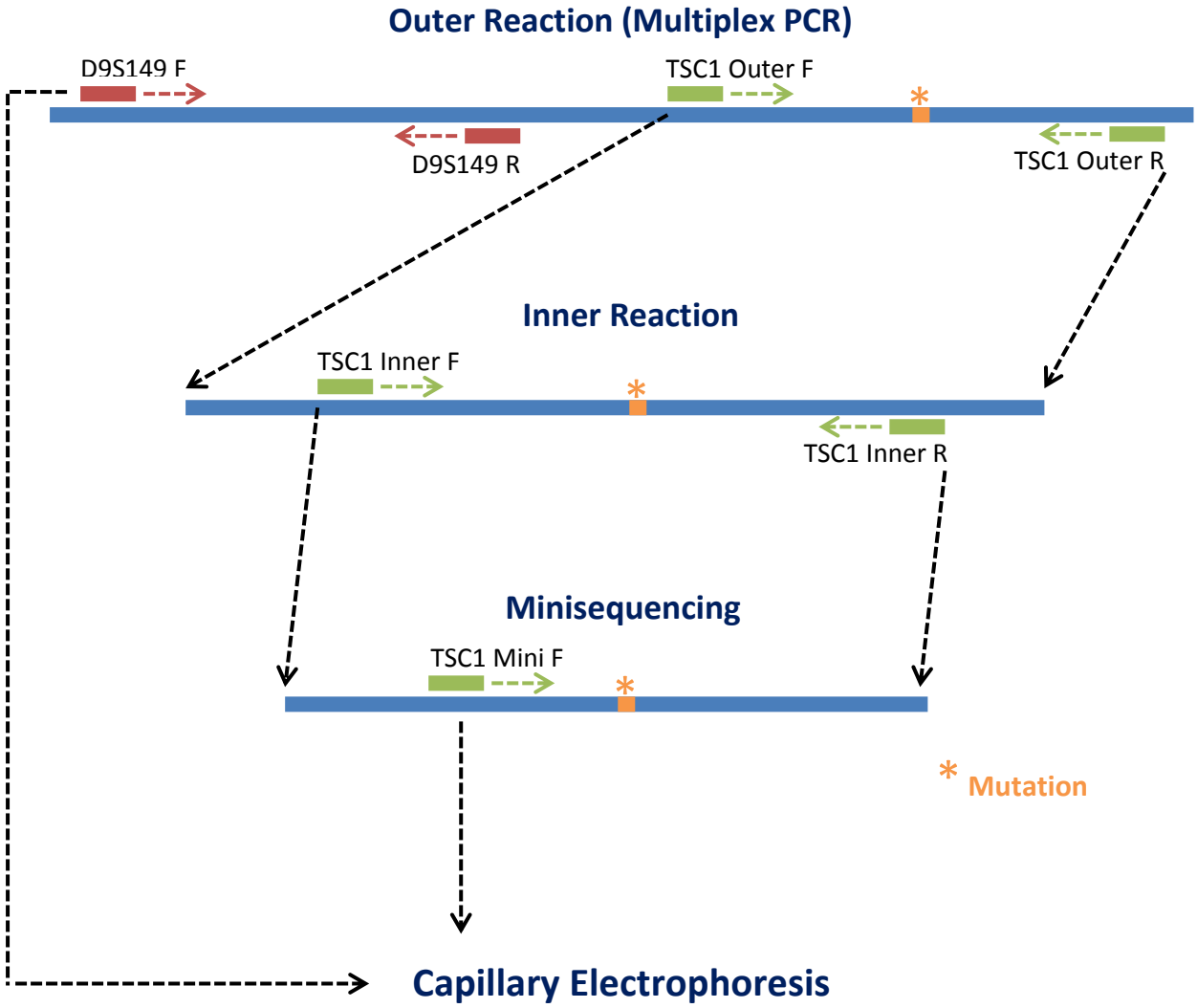
2.2.1 Amplification strategies used in PGD protocols

A number of different amplification strategies were employed in the PGD protocols developed. In most protocols a multiplex, nested (or heminested) PCR strategy was used for direct mutation detection and simultaneous amplification of linkage markers (Figure 2.1). This strategy involved an outer reaction which was essentially a multiplex reaction amplifying the mutation site and one or more linked markers. A relatively high number of PCR cycles (n=50-55) was used in the outer reaction in order to amplify the linkage markers to a level detectable. All linked markers used were microsatellites, amplified using fluorescent labeled primers and detected via capillary electrophoresis. Subsequently, an aliquot (0.5µl) from the outer reaction was used for the inner reaction (35 cycles). This was a singleplex reaction used to further amplify the mutation site so that the mutation would be detectable after carrying out minisequencing or restriction enzyme digestion on the amplified fragments. A variation of this strategy involved a multiplex with a low number of PCR cycles (n=20) for the outer reaction which was followed by separate inner reactions with a higher number of PCR cycles (n=45-55) in order to amplify to a detectable level the linkage markers and the mutation site.

Another method employed for PGD cases was linkage analysis in the absence of direct mutation detection (e.g. for families where the causative mutation was

unknown or refractory to PCR). Linkage analysis involved the amplification of a number of highly polymorphic microsatellite markers in a single multiplex reaction or in more reactions (i.e. one outer multiplex reaction with a low number of PCR cycles followed by different inner reactions with higher numbers of PCR cycles) in order to perform indirect detection of the mutant gene.

Figure 2.1: Schematic diagram showing the strategy followed for development of PGD test for tuberous sclerosis 1 (mutation: c.912T>G).



Notes: The first step was a multiplex PCR (55 cycles) using primers to amplify an informative STR marker and also using the 'outer' primers to amplify the mutation site in the gene of interest. The second step involved further amplification (35 cycles) of the mutation site using 'inner' primers which bind to the fragments produced by the 'outer' primers. The final step involved minisequencing using the inner reaction amplicons for direct mutation detection. Aliquots of samples produced from the 'outer' multiplex reaction and also from minisequencing were used to perform capillary electrophoresis in order to obtain and analyse results regarding the linked STR used and also detection of mutation.

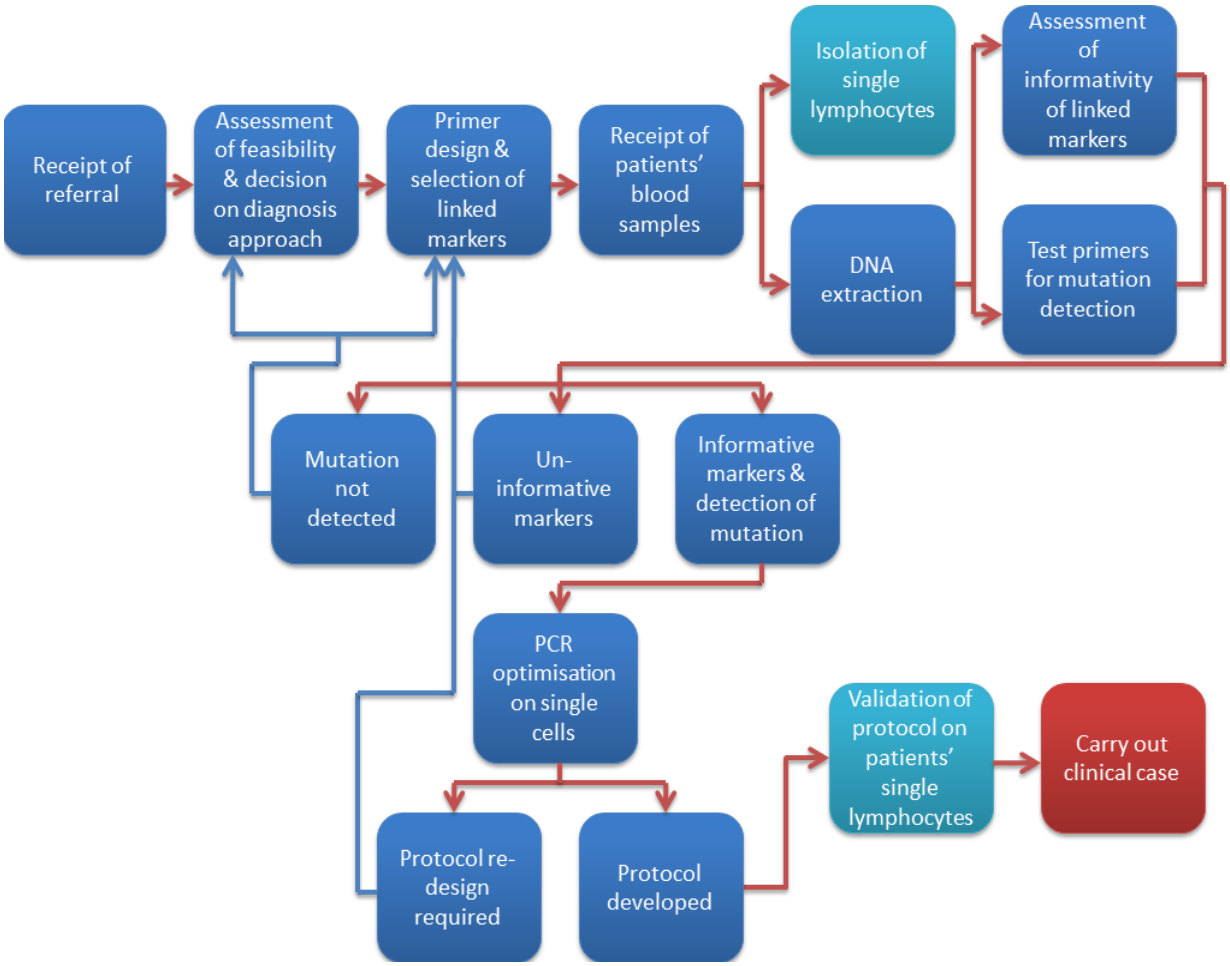
2.2.2 Pre-clinical workup

There are a lot of steps involved in the work up carried out before a clinical case can be performed (Figure 2.2). Development of a PGD protocol is a lengthy procedure involving a lot of primer design and troubleshooting. Numerous rounds of optimisation are required and extensive validation of each protocol is essential. Some protocols might take up to 6 months to be developed. In this study an average of 3 months was needed to develop each of the tests.

2.2.2.1 Locating mutations in the genes of interest

After receipt of an official genetic report describing the parental mutation responsible for the disorder being tested, the mutation was located by finding the correct, updated genomic DNA sequence of the gene of interest. The 'Entrez Gene' database (http://www.ncbi.nlm.nih.gov/gene) was used to find the genomic DNA, mRNA and protein sequences. Part of the genomic sequence that included the mutation was used for primer design.

Figure 2.2: Steps involved in the development of a PGD protocol



Notes: Firstly, assessment of the referral for a PGD case is carried out in order to determine if a PGD test can be developed for diagnosis. The approach that is going to be used is decided and if required, primers for direct mutation detection are designed. Hypervariable microsatellite markers (STRs) closely linked to the disease-causing gene are also included in the protocols developed in order to avoid misdiagnosis due to ADO of the mutation site. After patients' blood samples are received single lymphocytes are isolated via micromanipulation. DNA is also extracted from the blood samples. Subsequently, primers designed for the amplification of the mutation site are tested and linked markers are amplified and assessed for informativity (in order for a linked marker to be fully informative the parents should both be heterozygous for that marker and not share any of the alleles). If linked markers are informative and mutation detection primers are shown to work well then PCR optimisation on single cells is initiated. In cases where linked markers are uninformative then new linked markers have to be selected and tested. If the mutation is not detected there are two solutions: change of the diagnostic approach used or design of new primers. After many rounds of optimisation on DNA and then on single cells the PGD protocol is developed. Validation of the protocol is performed by testing 30 single lymphocytes isolated from the patients' blood sample. Finally, the clinical case is carried out. If after extensive optimisation the protocol is still not ready then primers have to be re-designed and optimisation of the protocol begun all over again.

2.2.2.2 Primers

2.2.2.2.1 Primer Design

Primers were designed using the 'Primer3' software (Rozen and Skaletsky, 2000). Selected primers were further tested by two more software packages, the 'Oligo Calc: Oligonucleotide Properties Calculator' (Kibbe 2007) and the 'SNPCheck' (National Genetics Reference Laboratory Manchester, 2005). These programs allowed primers to be assessed for potential hairpin formation, 3' complementarity and potential self-annealing sites. Furthermore, these software assessed the DNA sequence to which the primers annealed to determine if it includes any SNPs, while also a 'BLAST' (the online Basic Local Alignment Search Tool) was performed to determine the specificity of the designed primers.

Regarding minisequencing primers, these were designed without using the 'Primer3' software. The primers were designed manually so that their 3' end annealed exactly one nucleotide upstream (forward primer) or downstream (reverse primer) of a known mutation. The minisequencing primers designed were of 18-24 bases long. As with other primers, designed minisequencing primers were assessed using the 'Oligo Calc: Oligonucleotide Properties Calculator' and the 'SNPCheck'.

All primers designed and used in PGD cases are included in Appendix 1.

2.2.2.2.2 Selection of linkage markers and usage of loci for gender determination

At least one linkage marker was used in each protocol developed for PGD cases. Additionally, for some cases where gender determination was useful for diagnosis, loci for distinguishing male from female embryos (i.e. X- and Y-chromosome specific) were used.

The linkage markers were selected to be intragenic or less than 1Mb away from the gene of interest in order to reduce the possibilities of recombination [a marker that is 1Mb or cM (centimorgan) away from the gene of interest is expected to have about 1% chance of crossing over during meiosis] (Russel 2006; Sturtevant 1913). Furthermore, markers that were found to have high heterozygosity rates were preferred. For STRs, high heterozygosity rates are usually indicative of a marker with a large number of different alleles in the population. Such loci will have a high chance of being informative for the couple requesting PGD.

Linked markers close to the gene of interest were located through 'Ensembl' Genome database (http://www.ensembl.org/index.html). Heterozygosity rates of the linkage markers were found through genetic linkage maps created by the Mammalian Genotyping Service (http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp) (Broman *et al.* 1998).

All of the linkage markers used in the PGD cases described in this study are included in Appendix 1. The loci used for gender determination are included in Appendix 2.

2.2.2.3 Receipt and process of blood samples and buccal swabs

Blood samples from couples requesting PGD and other family members were collected in sodium EDTA tubes. The samples were stored at 4°C for a maximum of 48 hours before being processed or at -80°C for longer periods of time. The QIAamp DNA blood MiniKit (Qiagen, Germany) was used to extract DNA from the blood samples. The 'Blood and Body Fluid Spin Protocol' included in the kit was used according to manufacturer's instructions.

On some occasions buccal swabs were used to collect DNA. Buccal swabs (DNA Isolation Kit, Isohelix, Cell Projects, UK) were sent to the patients or family members and a sample was collected. After receipt the samples were stored at 4°C for a maximum of 48 hours before being processed. The buccal swabs were processed according to manufacturer's instructions.

DNA extracted from blood samples and buccal swabs was stored in PCR clean 1.5ml tubes (Eppendorf, UK) at 4°C.

2.2.2.3.1 Extraction and isolation of single lymphocytes

Single lymphocytes were isolated from patient's fresh (i.e. not frozen) blood samples in order to be used for validation of the developed PGD protocols.

2.2.2.3.1.1 Extraction of lymphocyte layer from blood samples

The lymphocyte layer was separated from other components of the blood through the usage of Ficoll-Paque PLUS (GE Healthcare, UK). In a 15ml sterile, centrifuge tube (Corning, USA) 2ml of Dulbecco's Phosphate-Buffered Saline (PBS, without

magnesium or calcium) (Invitrogen, UK) were mixed with 2ml of blood. Using a sterile Pasteur pipette (Fisherbrand, UK) the diluted blood sample was carefully layered on top of 3ml of Ficoll-Paque Plus located in another 15ml centrifuge tube. The tube was then centrifuged at 400 x g for 35 minutes. If layers were not separated after centrifugation, the tube was further centrifuged at 500 x g for 10 minutes. After centrifugation was completed, the upper layer (plasma) was carefully removed with a sterile Pasteur pipette and discarded. The next layer in the tube was the lymphocyte layer. This was transferred to a clean 15ml centrifuge tube using a Pasteur pipette. The tube containing the lymphocytes was transferred into the 'single cell' room and 6-7ml of NWB (non-stick washing buffer) [PBS, without magnesium or calcium + 0.1% polyvinyl alcohol (Sigma–Aldrich, Germany)] were added to the tube to dilute the densely packed lymphocytes. At this point the sample was ready for cell isolation.

2.2.2.3.1.2 Isolation of single lymphocytes

Before starting isolations, the NWB was sterile filtered into PCR clean 1.5ml tubes by using a 50ml syringe (Becton Dickinson, USA) and a 0.2µm filter disc Puradisc 25 AS (Whatman, UK). This was carried out in order to remove any debris or crystals that might had accumulated in the NWB as these could interfere with the lymphocyte isolation procedure. The procedure was carried out inside a laminar flow hood. After NWB was sterile filtered, 30 sterile 0.2ml PCR tubes (Molecular BioProducts, USA) were placed onto a PCR cooler 96-well rack inside a laminar flow hood. Subsequently, a number of 5µl microdrops of NWB were pipetted onto a sterile Petri dish (60mm x 15mm) (Thermo Scientific Nunc, USA) along with a 20µl drop from the lymphocyte suspension. Isolations were carried out via micromanipulation with

the use of a handheld micropipette (Drummond Microdispenser) (Drummond Scientific, USA) while observing cells using a binocular microscope. Single lymphocytes were picked up with the micropipette and each one was washed by passage through 3 clean microdrops before being transferred to sterile 0.2ml PCR tubes in 1µl of clean NWB. Thirty lymphocytes were isolated for each PGD case. After isolations were finished the PCR tubes were pulse centrifuged to collect the isolated lymphocytes at the bottom of each tube and placed onto 96 well PCR tube racks (Fisherbrand, UK). The lymphocytes were stored at -80°C until required.

2.2.2.4 Optimisation of PGD protocols

After primers were designed, case optimisation was initiated.

2.2.2.4.1 Gradient PCR

In order to determine the optimal annealing temperature (i.e. clear band or peak of expected size visualized after electrophoresis, with no secondary products detected) of each set of primers a gradient PCR was carried out. Gradient PCR involved the amplification of a primer set using different annealing temperatures that ranged from 53ºC to 62ºC (specifically: 53ºC, 54.5ºC, 56.7ºC, 59ºC, 60.9ºC and 61.8ºC). The HotMaster *Taq* DNA polymerase (5 Prime, Germany) was used. Reaction mixtures contained PCR grade water (Roche, Germany), 1x HotMaster *Taq* Buffer (with 25 mM Mg^{2+}), dNTPs (200µM each) (Thermo Scientific, USA), 0.8µM each primer, 0.6 units HotMaster *Taq* DNA polymerase and 0.5µl DNA for a final volume of 15µl. Thermal cycling consisted of an initial denaturation step of 96ºC for 1 min, followed

by 35 cycles of 94ºC for 15 s, 53ºC - 61.8ºC for 15 s, and 65ºC for 45 s, then a final extension step of 65ºC for 2 min.

Depending on whether or not labeled primers were used for PCR, analysis of amplified products was carried out through capillary electrophoresis and gel electrophoresis as described in sections 2.1.1 and 2.1.2.

2.2.2.4.2 Testing informativity of linkage markers and mutation detection

After optimal annealing temperatures were established for each set of primers, DNA from the couple requesting PGD and their family members was tested for informativity of the linkage markers selected. Furthermore, primers involved in mutation detection were also amplified and analysed through capillary electrophoresis (for labeled primers detecting deletion or repeat expansion mutations) or further processed with minisequencing or restriction enzyme digestion.

Almost all linked markers and mutation sites were amplified with HotMaster *Taq* DNA polymerase as described in section 2.2.2.4.1 and by using the established optimal annealing temperature. The only exception was the amplification of the CAG repeat expansion (HD locus) for Huntington disease which was amplified with AmpliTaq Gold 360 DNA polymerase (Applied Biosystems, USA). The reaction mixture contained PCR grade water, 1x AmpliTaq Gold 360 buffer, 2mM $MgCl_2$, 3µl 360 GC Enhancer, dNTPs (200µM each), 0.8µM each primer, 1.5 units AmpliTaq Gold 360 DNA polymerase and 0.5µl DNA for a final volume of 15µl. Thermal cycling consisted of an initial denaturation step of 95ºC for 10 min, followed by 55

cycles of 96ºC for 10 s, 58ºC for 30 s, and 72ºC for 1 min, then a final extension step of 72ºC for 5 min.

Minisequencing and restriction enzyme digestion were carried out as described in sections 2.2.3.3.3 and 2.2.3.4.18, respectively.

Amplified products from labeled primers and minisequencing products were analysed through capillary electrophoresis as described in section 2.1.1. Restriction enzyme digestion products were analysed through gel electrophoresis as described in section 2.1.2.

As shown in Figure 2.2, if linkage markers were uninformative and/or the designed primers could not detect the mutation then new linkage markers were selected and new primers were designed and, if necessary, the protocol strategy was re-examined.

2.2.2.4.3 Protocol development

After optimal annealing temperatures were established and the primer sets that would be included in the protocol along with the amplification strategy followed were confirmed, development of the multiplex-PCR protocol was initiated. During development of PGD protocols several parameters were varied in order to achieve optimal results, including the DNA polymerase used, thermal cycling conditions, primer concentration, $MgCl_2$ concentration and usage (or not) of PCR additives such us glycerol, DMSO (dimethylsulphoxide) and Q solution (Qiagen, Germany). Different amplification kits used included HotMaster *Taq* DNA polymerase, AmpliTaq 360 DNA polymerase (Applied Biosystems, USA), AmpliTaq Gold 360 DNA polymerase, Expand Long Template PCR system (Roche, Germany), Expand

High Fidelity PCR System (Roche, Germany) and the Qiagen Multiplex PCR kit (Qiagen, Germany).

Development of the PGD protocols was carried out on single buccal cells donated from members of the lab and isolated as described before in section 2.2.2.3.1.2.

2.2.2.5 Protocol validation

At the point when protocols had been optimised at the single cell level, a final 'protocol validation' was carried out. Protocol validation involved the amplification of 30 single lymphocytes that were previously isolated from the couple. This was performed in order to obtain amplification efficiency rates, ADO rates and other amplification characteristics (e.g. the exact appearance of linked marker alleles after analysis using capillary electrophoresis) that would be helpful for making a diagnosis when the clinical case is carried out.

2.2.3 Final PGD protocols and performance of clinical cases

Ultimately, thirty-one novel PGD protocols were developed for 21 different single gene genetic disorders (Table 2.1).

Positive and negative controls were always used in every clinical case carried out. Genomic DNA extracted from family members involved in each case was used as positive control. Reactions including only the reagents used to amplify the samples (i.e. without DNA template) were used as negative controls. Furthermore, blanks were obtained and amplified for each sample tested (section 2.2.3.1).

Capillary electrophoresis was used as described before (section 2.1.1) to analyze fluorescent amplified STR products and minisequencing products.

Table 2.1: List of PGD clinical cases carried out.

Disease	Inheritance Pattern*	Gene	Mutation**	No. of Protocols Developed
Autosomal Dominant Hyper IgE Syndrome	AD	*STAT3*	c.1281+1G>A	1
Autosomal Dominant Polycystic Kidney Disease	AD	*PKD1*	Not Defined	1
Alpha-1-antitrypsin Deficiency	AC	*SERPINA1*	c.1096G>A	1
Branchio-oto-renal Syndrome	AD	*SIX1*	c.386A>G	1
Cystic Fibrosis	AR	*CFTR*	c.1521_1523delCTT / c.3659delC	1
Cystic Fibrosis	AR	*CFTR*	c.1521_1523delCTT	---
Cystic Fibrosis	AR	*CFTR*	c.1521_1523delCTT / c.350G>A	---
Dominant Dystrophic Epidermolysis Bullosa	AD	*COL7A1*	c.6501+1G>C	1
Duchenne Muscular Dystrophy	X	*DMD*	Not Defined	1
Ehlers Danlos Syndrome type IV	AD	*COL3A1*	c.2492 G>A	1
Hereditary Multiple Exostoses	AD	*EXT1*	c.811T>G	1
Huntington Disease	AD	*HTT*	CAG repeat expansion	2
Krabbe Disease	AR	*GALC*	c.1180delA	1
Marfan Syndrome	AD	*FBN1*	c.1904A>G	1
Marfan Syndrome	AD	*FBN1*	c.5721C>G	1
Marfan Syndrome	AD	*FBN1*	c.6569G>A	1
Marfan Syndrome	AD	*FBN1*	c.2097T>A	1
Mitochondrial DNA Depletion syndrome	AR	*POLG*	c.1399G>A / c.2542G>A	1
Neurofibromatosis type I	AD	*NF1*	c.5944-2A>G	1
Neurofibromatosis type I	AD	*NF1*	EX3_6del	1
Neurofibromatosis type I	AD	*NF1*	c.3916C>T	1

Table 2.1 (continued)

Disease	Inheritance Pattern*	Gene	Mutation**	No. of Protocols Developed
Neurofibromatosis type I	AD	*NF1*	c.3457_3460del CTCA	1
Otopalatodigital Syndrome	AD	*FLNA*	c.1664C>A	1
POMGNT1 related muscular dystrophy	AR	*POMGNT1*	c.1539+1G>A / c.1738C>T	1
Sickle Cell Anaemia	AR	*HBB*	c.20A>T	1
Spinal Muscular Atrophy (type 1)	AR	*SMN1*	[EX7del] + [EX8del]	1
Thalassaemia B	AR	*HBB*	c.142+5G>C	2
Tuberous Sclerosis 1	AD	*TSC1*	c.1525C>T	1
Tuberous Sclerosis 1	AD	*TSC1*	c.912T>G	1
X-linked Hydrocephalus	X	*L1CAM*	c.551G>A	2

* Inheritance pattern: AD (autosomal dominant); AC (autosomal codominant); AR (autosomal recessive); X (X-linked)

** Mutation nomenclature: e.g. c.912T>G → substitution of nucleotide T by nucleotide G at nucleotide position 912 of the coding DNA reference sequence.

2.2.3.1 IVF and embryo biopsy

All embryos generated for PGD purposes were produced through ICSI and all cumulus cells were carefully removed from the zona pellucida before biopsy. For most of the cases reported here, the embryos were biopsied on day 3 removing a single blastomere. For some cases however, polar body biopsy (1^{st} and 2^{nd} polar bodies obtained) and trophectoderm biopsy (3-10 cells retrieved) were carried out. After biopsy, isolations were carried out as described above (section 2.2.2.3.1.2). Negative controls (blanks) for each embryo were also prepared by aliquoting 1μL of NWB from the last microdrop in which each cell was washed before transfer to the PCR tube. In the cases where proteinase K (PK) lysis was used to release the DNA,

PCR-grade mineral oil (Sigma–Aldrich, Germany) was also used to overlay the samples.

2.2.3.2 Cell lysis

Cell lysis was carried out using either proteinase K lysis or alkaline lysis. Proteinase K lysis was carried out by adding 1µl of 1.75 mM sodium dodecyl sulphate (SDS) (Promega, USA) into 200 µg/ml proteinase K solution (Roche, Germany). The lysis solution was mixed thoroughly and 3µl were added to each sample. Cell lysis was performed at 37°C for 1 hour followed by enzyme inactivation at 95°C for 10 minutes.

Alkaline lysis was carried out by adding 0.45µl of PCR-grade water, 0.75µl of 0.1M DL-Dithiothreitol (DTT) (Sigma–Aldrich, Germany) and 0.3µl of 1M NaOH (Sigma–Aldrich, Germany) to each tube followed by incubation at 60°C for 10 minutes. Tricine (Sigma–Aldrich, Germany) was included in the PCR reaction mix whenever alkaline lysis was used in order to neutralise the pH of the PCR reaction.

2.2.3.3 Mutation detection – Inner reactions and minisequencing

Inner reactions amplifying the mutation in order to create products to be used for minisequencing were always the same (sections 2.2.3.3.1 and 2.2.3.3.2) and utilized one of two DNA polymerases; the HotMaster *Taq* DNA polymerase and the AmpliTaq 360 DNA polymerase. The minisequencing reaction that followed is described below (section 2.2.3.3.3).

2.2.3.3.1 Protocol for HotMaster *Taq* DNA Polymerase – Inner reaction

Reaction mixture and thermocycler conditions used are shown in Table 2.2.

Table 2.2: Protocol for HotMaster *Taq* DNA Polymerase

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	12.34
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
Amplified product from outer reaction	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	35
Annealing	Variable for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

2.2.3.3.2 Protocol for AmpliTaq 360 DNA Polymerase – Inner reaction

Reaction mixture and thermocycler conditions used are shown in Table 2.3.

Table 2.3: Protocol for AmpliTaq 360 DNA Polymerase

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	10.54
10x AmpliTaq 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A†	0.6
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq 360 DNA polymerase (5U/µl)	0.6 U/reaction	0.12
Amplified product from outer reaction	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 3min	1
Denaturation	95°C for 30s	35
Annealing	Variable for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

† N/A stands for non-applicable.

2.2.3.3.3 Minisequencing

Minisequencing was performed for direct mutation detection. The procedure for carrying out minisequencing involved two separate reactions. Initially, amplified products from the inner reaction were treated with Exonuclease I (Exo I) (USB, Affymetrix, USA) and Shrimp Alkaline Phosphatase (SAP) (USB, Affymetrix, USA) or ExoSAP-IT (USB, Affymetrix, USA). When Exo I and SAP were used the reaction was carried out as follows: 1.7µl of SAP (1 unit/µl) and 0.7µl of Exo I (10 units/µl) were added to 5µl of amplified product. The samples were then incubated at 37°C for 30 min and 75°C for 15 min (enzyme inactivation). This procedure was only followed

for a few cases. For the rest of the cases ExoSAP-IT was used according to manufacturer's instructions.

Treated products were then subjected to minisequencing using the SNaPshot Multiplex Kit (Applied Biosystems, USA). Specifically, the reaction mixture contained 2.5µl of SNaPshot Multiplex Ready Reaction Mix, 0.5µl of 2µM minisequencing primer, 0.5µl PCR grade water and 1.5µl of treated amplified product for a final volume of 5µl. Thermal cycling consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 30 s.

2.2.3.4 Protocols and actual clinical cases

Unless stated otherwise, alkaline lysis was used as described in section 2.2.3.2 to release the DNA from biopsied samples in cases carried out.

2.2.3.4.1 Autosomal dominant hyper IgE syndrome

Day-3 biopsy was carried out for this couple and 6 embryos were tested. Protocol used for outer reaction (Table 2.4) utilized the Qiagen Multiplex PCR Master Mix which contains HotStarTaq DNA Polymerase, Multiplex PCR buffer (with 6mM $MgCl_2$) and dNTP mix.

Table 2.4: Autosomal dominant hyper IgE syndrome – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.66
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1281+1G>A outer F+R (10µM)	0.2µM	0.3
D17S1299 F+R (10µM)	0.16µM	0.24
D17S1861 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Amplified products from the outer reaction were used to carry out an inner reaction. The inner reaction was carried out as described in Table 2.2 (annealing temperature: 59°C) using the c.1281+1G>A Outer F - Inner R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1281+1G>A Mini R primer (Appendix 1).

2.2.3.4.2 Autosomal dominant polycystic kidney disease

Two PGD cycles were carried out for this couple. Day-3 biopsy was carried out on both occasions for PGD while for the 2nd PGD cycle the couple also wanted to test for chromosome aneuploidy and for this reason 1st polar bodies were biopsied and tested with 24sure test (BlueGnome Ltd, UK) as described in Appendix 3. Overall, 7 embryos were tested. Protocols used for outer and inner reactions are shown below (Table 2.5).

Table 2.5: Autosomal dominant polycystic kidney disease – PGD protocol. **A)** Outer reaction. **Bi)** and **Bii)** Inner reactions I and II.

A) Outer reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.3
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D16S3082 F+R (10µM)	0.2µM	0.3
D16S291 F+R (10µM)	0.2µM	0.3
D16S3399 F+R (10µM)	0.2µM	0.3
16PTEL06 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	20
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Bi) Inner reaction I

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	5.9
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D16S3082 F+R (10µM)	0.2µM	0.3
D16S291 F+R (10µM)	0.2µM	0.3
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	45
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Bii) Inner reaction II

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	5.9
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D16S3399 F+R (10µM)	0.2µM	0.3
16PTEL06 F+R (10µM)	0.2µM	0.3
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	45
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.3 Alpha-1-antitrypsin deficiency

Day-3 biopsy was carried out for this case and 6 embryos were tested. The protocol used is shown below (Table 2.6).

Table 2.6: Alpha-1-antitrypsin deficiency – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1096G>A outer F+R (10µM)	0.2µM	0.3
D14S553 F+R (10µM)	0.2µM	0.3
D14S62 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	58°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: An inner reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.1096G>A inner F and outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1096GA Mini F primer (Appendix 1).

2.2.3.4.4 Branchio-oto-renal syndrome

Two PGD cycles were carried out for this couple. Day-3 biopsy was carried out in both occasions. Overall, 8 embryos were tested. The protocol used is shown below (Table 2.7).

Table 2.7: Branchio-oto-renal syndrome – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.386A>G outer F+R (10µM)	0.2µM	0.3
D14S1038 F+R (10µM)	0.2µM	0.3
D14S997 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	57°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: An inner reaction was carried out as described in Table 2.2 (annealing temperature: 54.5°C) using the c.386A>G inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.386AG Mini F primer (Appendix 1).

2.2.3.4.5 Cystic fibrosis

All in all, 5 PGD cycles were carried out for Cystic Fibrosis (one of the couples went through 2 PGD cycles). Day-3 biopsy was carried out for all 5 cycles and 24 embryos were tested overall. The same protocol (Table 2.8) was used to process all cycles.

Table 2.8: Cystic fibrosis – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.48
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1521_1523delCTT F+R (10µM)	0.1µM	0.15
D7S486 F+R (10µM)	0.3µM	0.45
D7S2847 F+R (10µM)	0.28µM	0.42
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.6 Dominant dystrophic epidermolysis bullosa

Two PGD cycles were carried out for this couple. Day-3 biopsy was carried out on both occasions and a total of 14 embryos were tested. However, the results received from the 1st PGD cycle indicated that 4 of the biopsied cells (or embryos) might be haploid or monosomic for chromosome 3. Therefore, day-5 biopsy was carried out on these embryos and the cells were amplified and processed using the 24sure test (Appendix 3) to test for aneuploidy. Additionally, SurePlex WGA products were used as described in section 2.4.1.6.1 to repeat the PGD test on these 4 embryos.

The protocol used to process samples obtained from day-3 biopsy is shown below (Table 2.9).

Table 2.9: Dominant dystrophic epidermolysis bullosa – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.72
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.6501+1G>C outer F+R (10µM)	0.2µM	0.3
D3S2409 F+R (10µM)	0.12µM	0.18
D3S1581 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: An inner reaction was carried out as described in Table 2.2 (annealing temperature: 61°C) using the c.6501+1G>C inner F and outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.6501+1G>C Mini F primer (Appendix 1).

2.2.3.4.7 Duchenne muscular dystrophy

Embryos tested for this case were biopsied on day-5. Ten embryos were biopsied in total. The protocol used for the case is shown below (Table 2.10). In the protocol developed, apart from the linkage markers included for diagnosis of the disease, loci for gender determination (AMEL, SRY) were also incorporated in order to be able to distinguish female carriers from affected males (since Duchenne muscular dystrophy is an X-linked disorder).

Table 2.10: Duchenne muscular dystrophy – PGD protocol

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.09
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
DXS1214 F+R (10µM)	0.18µM	0.27
DXS1236 F+R (10µM)	0.2µM	0.3
DXS997 F+R (10µM)	0.18µM	0.27
SRY F+R (10µM)	0.18µM	0.27
AMEL F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	58°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.8 Ehlers Danlos syndrome type IV

Two PGD cycles were carried out for this couple and 2 embryos were tested. Day-3 biopsy was carried out in both occasions. The protocol used is shown below (Table 2.11).

Table 2.11: Ehlers Danlos syndrome type IV – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.9
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.2492G>A outer F+R (10µM)	0.2µM	0.3
D2S389 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: An inner reaction was carried out as described in Table 2.2 (annealing temperature: 61°C) using the c.2492G>A inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.2492G>A Mini R primer (Appendix 1).

2.2.3.4.9 Hereditary multiple exostoses

Three PGD cycles were carried out for this couple and 6 embryos were tested. Day-3 biopsy was performed in all occasions. The protocol used is shown below (Table 2.12).

Table 2.12: Hereditary multiple exostoses – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	6.68
Tricine (0.4M)	40mM	1.5
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
EXT-1 outer F (100µM)	0.8µM	0.12
EXT-1 outer F (100µM)	0.8µM	0.12
D8S592 F (100µM)	1.2µM	0.18
D8S592 R (100µM)	1.2µM	0.18
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	0.6 U/reaction	0.12
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	59°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Notes: A inner reaction was carried out as described in Table 2.3 (annealing temperature: 60.9°C) using the c.811 T>G inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (first 2 cycles Exo I and SAP were used; 3rd cycle ExoSAP-IT was used) using the c.811 T>G Mini F primer (Appendix 1).

2.2.3.4.10 Huntington disease

Two PGD cycles were performed for Huntington disease for 2 different families. Day-3 biopsy was carried out for both cycles. Nine embryos were tested, overall. The PGD protocols used for the two cases are described in Tables 2.13 and 2.14.

Table 2.13: Huntington disease – PGD protocol A. **A)** Outer reaction. **Bi)** and **Bii)** Inner reactions I and II.

A) Outer reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	1.19
Tricine (0.4M)	40mM	1.5
Q Solution	N/A	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D4S127 F+R (10µM)	0.08µM	0.12
HD F+R (10µM)	0.22µM	0.33
I1CAHD F+R (10µM)	0.1µM	0.15
D4S412 F+R (10µM)	0.14µM	0.21
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 45s	20
Annealing	62°C for 90s	
Extension	72°C for 90s	
Final Extension	72°C for 10min	1
Hold	4°C for ∞	1

Bi) Inner reaction I

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	7.46
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	3
HD F (100µM)	0.8µM	0.12
HD R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	1.5 U/reaction	0.3
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	96°C for 10s	55
Annealing	59°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 5min	1
Hold	4°C for ∞	1

Bii) Inner reaction II

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	5.99
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D4S127 F+R (10µM)	0.1µM	0.15
I1CAHD F+R (10µM)	0.1µM	0.15
D4S412 F+R (10µM)	0.14µM	0.21
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	50
Annealing	62°C for 90s	
Extension	72°C for 90s	
Final Extension	72°C for 10min	1
Hold	4°C for ∞	1

Table 2.14: Huntington disease – PGD protocol B. **A)** Outer reaction. **Bi), Bii)** and **Biii)** Inner reactions I, II and III.

A) Outer reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	1.01
Tricine (0.4M)	40mM	1.5
Q Solution	N/A	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D4S127 F+R (10µM)	0.1µM	0.15
HD F+R (10µM)	0.22µM	0.33
I1CAHD F+R (10µM)	0.1µM	0.15
D4S412 F+R (10µM)	0.14µM	0.21
D4S136 F+R (10µM)	0.08µM	0.12
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	20
Annealing	60°C for 90s	
Extension	72°C for 90s	
Final Extension	72°C for 10min	1
Hold	4°C for ∞	1

Bi) Inner reaction I

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	7.46
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	3
HD F (100µM)	0.8µM	0.12
HD R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	1.5 U/reaction	0.3
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	59°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Bii) Inner reaction II

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	10.04
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
D4S136 F (100µM)	0.8µM	0.12
D4S136 R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	0.6 U/reaction	0.12
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	59°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Biii) Inner reaction III

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	5.99
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
D4S127 F+R (10µM)	0.1µM	0.15
I1CAHD F+R (10µM)	0.1µM	0.15
D4S412 F+R (10µM)	0.14µM	0.21
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	50
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.11 Krabbe Disease

Two PGD cycles were carried out for this couple. For the 1st cycle, 6 day-3 embryos were biopsied and tested. However, the embryos produced were of very poor quality and did not produce a pregnancy. Therefore, for the 2nd cycle it was decided to carry out PGD on the 1st and 2nd polar bodies with the aim of detecting only the allele (mutant or normal) inherited from the mother. This way the embryos would not have to go through the stressful procedure of biopsy and would be available for transfer at an earlier stage, potentially preserving their viability. First and second polar bodies were obtained from 6 oocytes. The protocol used for both PGD cycles is shown below (Table 2.15).

Table 2.15: Krabbe disease – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1180delA outer F+R (10µM)	0.2µM	0.3
D14S67 F+R (10µM)	0.2µM	0.3
D14S68 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	59°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: An inner reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.1180delA outer F and inner R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1180delA Mini R primer (Appendix 1).

2.2.3.4.12 Marfan syndrome

Four PGD cycles for 4 different couples (each one involving a different mutation) were carried out for this disorder. Day-3 biopsy was carried out for all four cycles. Sixteen embryos were biopsied. Proteinase K lysis was used for one of the cycles (c.1904A>G) while for the rest of the cycles alkaline lysis was used to lyse the blastomeres obtained (section 2.2.3.2). The protocols used to carry out the cases are described in Tables 2.16 - 2.19.

For one of the cases (mutation: c.6569G>A), a sperm sample was collected from the affected male on the day of the biopsy and single sperm was isolated as described before (section 2.2.2.3.1.2). The sample was processed by embryologists with density gradient washing (DGW) before isolation of individual spermatozoa was attempted.

Single sperm were used to help obtain the haplotype involving the two linkage markers and mutation site used in the PGD protocol developed, since no DNA was available from other family members. In order to achieve this single sperm was lysed using alkaline lysis. Alkaline lysis was used as described before (section 2.2.3.2) with a few changes (0.75µL of 0.3M DTT were used instead of 0.1M; incubation was carried out at 60°C for 20 minutes instead of 10 minutes). Single sperm was amplified along with biopsied cells as described in Table 2.18.

Table 2.16: Marfan syndrome – PGD protocol A (c.1904A>G)

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	6.8
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
c.1904A>G outer F (100µM)	0.8µM	0.12
c.1904A>G outer R (100µM)	0.8µM	0.12
D15S143 F (100µM)	0.8µM	0.12
D15S143 R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	0.6 U/reaction	0.12
Lysis buffer + released DNA	Variable	4

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	50
Annealing	56.7°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.2 (annealing temperature: 56.7°C) using the c.1904A>G inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (Exo I and SAP were used) using the c.1904A>G Mini F primer (Appendix 1).

Table 2.17: Marfan syndrome – PGD protocol B (c.5721C>G)

	Concentration	*Volume for 15µl reaction (µl)*	*Thermocycler Conditions*		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.6	**Initial Denaturation**	95°C for 15min	1
Tricine (0.4M)	40mM	1.5	**Denaturation**	94°C for 30s	
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5	**Annealing**	59°C for 90s	55
c.5721C>G F+R (10µM)	0.2µM	0.3	**Extension**	72°C for 60s	
D15S196 F+R (10µM)	0.3µM	0.45	**Final Extension**	60°C for 10min	1
D15S659 F+R (10µM)	0.1µM	0.15	**Hold**	4°C for ∞	1
Lysis buffer + released DNA	Variable	2.5			

Notes: Inner Reaction was carried out as described in Table 2.3 (annealing temperature: 60.9°C) using the c.5721C>G inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.5721C>G Mini F primer (Appendix 1).

Table 2.18: Marfan syndrome – PGD protocol C (c.6569G>A)

	Concentration	*Volume for 15µl reaction (µl)*	*Thermocycler Conditions*		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.6	**Initial Denaturation**	95°C for 15min	1
Tricine (0.4M)	40mM	1.5	**Denaturation**	94°C for 30s	
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5	**Annealing**	59°C for 90s	55
c.6569G>A F+R (10µM)	0.2µM	0.3	**Extension**	72°C for 60s	
D15S196 F+R (10µM)	0.3µM	0.45	**Final Extension**	60°C for 10min	1
D15S659 F+R (10µM)	0.1µM	0.15	**Hold**	4°C for ∞	1
Lysis buffer + released DNA	Variable	2.5			

Notes: Inner Reaction was carried out as described in Table 2.3 (annealing temperature: 60.9°C) using the c.6569G>A inner F and outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.6569G>A Mini F primer (Appendix 1).

Table 2.19: Marfan syndrome – PGD protocol D (c.2097T>A)

	Concentration	*Volume for 15µl reaction (µl)*		*Thermocycler Conditions*		
				Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.6		**Initial Denaturation**	95°C for 15min	1
Tricine (0.4M)	40mM	1.5		**Denaturation**	94°C for 30s	55
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5		**Annealing**	58°C for 90s	
c.2097T>A F+R (10µM)	0.2µM	0.3		**Extension**	72°C for 60s	
D15S196 F+R (10µM)	0.3µM	0.45		**Final Extension**	60°C for 10min	1
D15S659 F+R (10µM)	0.1µM	0.15		**Hold**	4°C for ∞	1
Lysis buffer + released DNA	Variable	2.5				

Notes: Inner Reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.2097T>A inner F and outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.2097T>A Mini F primer (Appendix 1).

2.2.3.4.13. Mitochondrial DNA depletion syndrome

Day-3 biopsy was carried out for this case and 5 embryos were tested. Protocol used is shown below (Table 2.20).

Table 2.20: Mitochondrial DNA depletion syndrome – PGD protocol

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.63
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1399G>A outer F+R (10µM)	0.14µM	0.21
c.2542G>A outer F+R (10µM)	0.14µM	0.21
D15S979 F+R (10µM)	0.14µM	0.21
D15S116 F+R (10µM)	0.16µM	0.24
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	58°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Two separate inner reactions were carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.1399G>A inner F - outer R primers and the c.2542G>A inner F - outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) in two separate reactions using the c.1399G>A Mini F primer and the c.2542G>A Mini F primer (Appendix 1).

2.2.3.4.14 Neurofibromatosis type I

Four PGD cycles for 4 different couples (each one involving a different mutation) were carried out for this disorder. Day-3 biopsy was carried out for all four cases. Twenty-one embryos were biopsied. The protocols used to carry out the cases are described in Tables 2.21 - 2.24.

For one of the cases, which involved a *de novo* mutation (EX3_6del) carried by the male, it was decided to use linkage analysis alone to make the diagnosis. In order to achieve this, a genetic haplotype including linked STRs had to be obtained. Since the couple had no children and a *de novo* mutation was involved (therefore no family

members could be used to construct the haplotype), it was decided to use single sperm. Sperm samples were collected from the affected male a few weeks before the case and on the day of the case. The samples were processed by embryologists with DGW and analysed. Single sperm was isolated as explained previously (2.2.2.3.1.2). Alkaline lysis was used to release the DNA from single sperm as described before (section 2.2.3.2) with a few changes (0.75µL of 0.5M DTT were used instead of 0.1M; incubation was carried out at 60°C for 20 minutes instead of 10 minutes). Released DNA was amplified using the protocol described in Table 2.22.

Table 2.21: Neurofibromatosis type I – PGD protocol A (c.5944-2A>G)

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.5499-2A>G outer F+R (10µM)	0.2µM	0.3
D17S1800 F+R (10µM)	0.2µM	0.3
IVS38GT F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.5499-2A>G inner F - outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.5499-2A>G Mini F primer (Appendix 1).

Table 2.22: Neurofibromatosis type I – PGD protocol B (EX3_6del). **A)** Outer reaction. **B)** Inner reaction

A) Outer reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.3
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
Exon 4 F+R (10µM)	0.2µM	0.3
D17S1880 F+R (10µM)	0.2µM	0.3
IVS38GT F+R (10µM)	0.2µM	0.3
D17S783 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	20
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

B) Inner reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	12.34
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
Amplified product from outer reaction	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	55
Annealing	60°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: Each of the STR markers included in the outer reaction were amplified in singleplex inner reactions for both single sperm and single blastomere samples. 'Exon 4' primer set was only used to amplify the single sperm samples in order to construct the haplotype. Gel electrophoresis (section 2.1.2) was carried out to obtain results from 'Exon 4' amplification. No amplification of 'Exon 4' indicated that the sample (single sperm) had inherited the mutant allele which involves a large deletion from exon 3 to exon 6. Therefore, the alleles of the STR markers obtained from that sample were linked to the mutant allele. This way the STR markers' haplotype was determined and was used to make diagnosis.

Table 2.23: Neurofibromatosis type I – PGD protocol C (c.3916C>T)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.36
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.3916C>T outer F+R (10µM)	0.2µM	0.3
D17S1824 F+R (10µM)	0.18µM	0.27
IVS38GT F+R (10µM)	0.2µM	0.3
D17S1294 F+R (10µM)	0.18µM	0.27
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner reaction was carried out as described in Table 2.2 (annealing temperature: 60°C) using the c.3916C>T inner F - outer R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.3916C>T Mini R primer (Appendix 1).

Table 2.24: Neurofibromatosis type I – PGD protocol D (c.3457_3460del CTCA)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.06
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.3457-3460delCTCA F+R (10µM)	0.18µM	0.27
D17S1800 F+R (10µM)	0.2µM	0.3
IVS38GT F+R (10µM)	0.2µM	0.3
IVS2728 F+R (10µM)	0.2µM	0.3
D17S1294 F+R (10µM)	0.18µM	0.27
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	57°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.15 Otopalatodigital syndrome

Day-3 biopsy was carried out for this couple and 3 embryos were tested. The protocol used is shown below (Table 2.25).

Table 2.25: Otopalatodigital syndrome – PGD protocol

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1664C>A F+R (10µM)	0.2µM	0.3
DXS1073 F+R (10µM)	0.2µM	0.3
DXS8061 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner reaction was carried out as described in Table 2.2 (annealing temperature: 60°C) using the c.1664C>A outer F - inner R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1664C>A Mini R primer (Appendix 1).

2.2.3.4.16 POMGNT1 related muscular dystrophy

Day-3 biopsy was carried out for this couple and 11 embryos were tested. The protocol used is shown below (Table 2.26).

Table 2.26: POMGNT1 related muscular dystrophy – PGD protocol. **A)** Outer reaction. **B)** Inner reaction

A) Outer reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.3
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1539+1G>A F+R (10µM)	0.2µM	0.3
c.1738C>A F+R (10µM)	0.2µM	0.3
D1S2677 F+R (10µM)	0.2µM	0.3
D1S2797 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	20
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

B) Inner reaction

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	11.84
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
Amplified product from outer reaction	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	50
Annealing	61°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: Each of the STR markers included in the outer reaction were further amplified in singleplex reactions using this protocol. Inner reactions were also carried out for the two mutation sites (in singleplexes) using the c.1539+1G>A F+R primers and the c.1738C>T F+R primers (Appendix 1). Minisequencing then followed and was carried out in two separated reactions as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1539+1G>A Mini R and the c.1738C>T Mini R primers (Appendix 1).

2.2.3.4.17 Sickle cell anaemia

Five PGD cycles for 5 different couples were carried out for this disorder and a total of 29 embryos were tested. All embryos were biopsied on day-3 and single blastomeres were obtained. Protocol used is shown below (Table 2.27).

Table 2.27: Sickle cell anaemia – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*	*Thermocycler Conditions*		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.51	**Initial Denaturation**	95°C for 15min	1
Tricine (0.4M)	40mM	1.5	**Denaturation**	94°C for 30s	
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5	**Annealing**	61°C for 90s	55
c.20A>T outer F+R (10µM)	0.2µM	0.3	**Extension**	72°C for 60s	
D11S4181 F+R (10µM)	0.26µM	0.39	**Final Extension**	60°C for 10min	1
D11S1760 F+R (10µM)	0.16µM	0.24	**Hold**	4°C for ∞	1
D11S2362 F+R (10µM)	0.04µM	0.06			
Lysis buffer + released DNA	Variable	2.5			

Notes: Inner reaction was carried out as described in Table 2.2 (annealing temperature: 61°C) using the c.20A>T outer F - inner R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.20A>T Mini R primer (Appendix 1).

2.2.3.4.18 Spinal muscular atrophy type I

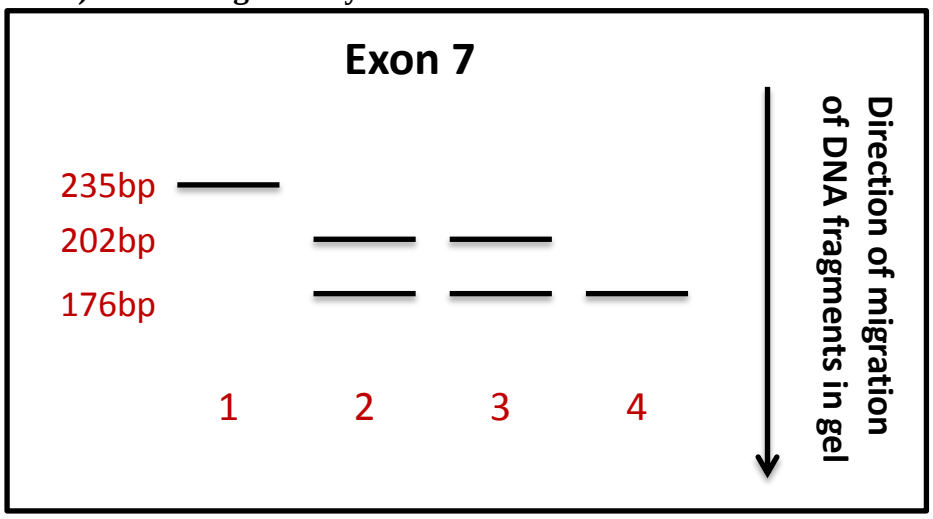
The protocol developed for this case, utilized primers and restriction endonucleases described by Girardet *et al.* (2008). The gene that causes spinal muscular atrophy (SMA), the *survival motor neuron (SMN)*, has two almost identical copies (*SMN1* and *SMN2*). However, only deletions and mutations in the *SMN1* copy are responsible for

SMA (Wirth 2000). The two copies of the gene differ only by 5 nucleotides. Two of these nucleotides are located in exons 7 and 8 (Burglen *et al*., 1996; Lefebvre *et al*., 1995). These differences are used to distinguish *SMN1* from *SMN2*. In this case, two restriction endonucleases (DdeI and DraI) (New England BioLabs, USA) were used to distinguish the two copies and make diagnosis of the disorder possible. DdeI is used to digest exon 8 of *SMN2* leaving *SMN1* undigested. DraI digests exon 7 of *SMN1* once and exon 7 of *SMN2* twice (Figure 2.3).

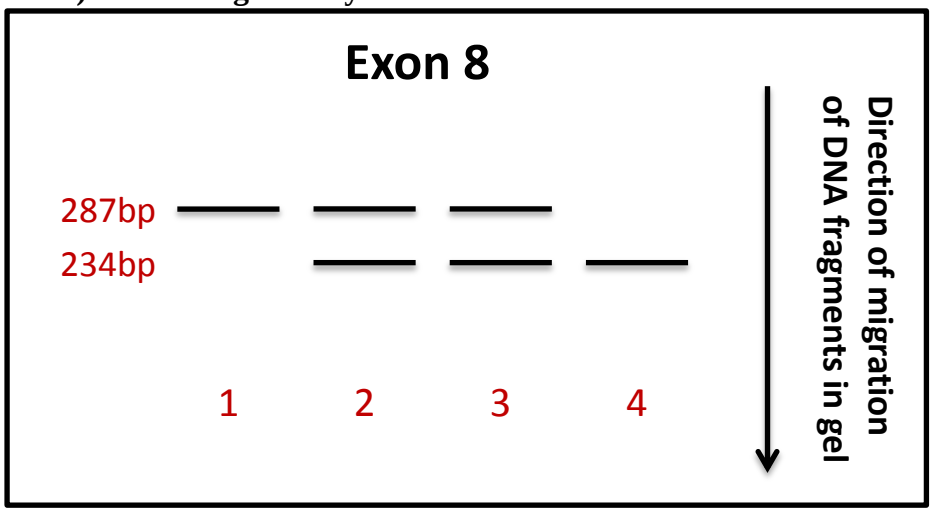
Day-3 biopsy was carried out for this case and single blastomeres were retrieved from 9 embryos. The protocol used to carry out the outer multiplex reaction is shown below (Table 2.28). Subsequently, two separate inner reactions were carried out as described in Table 2.2 (annealing temperature: 61°C) using the Exon 7 outer F – inner R primers and the Exon 8 outer F – inner R primers (Appendix 1). Afterwards, amplified products from Exon 7 inner reaction were digested using DraI while amplified products from Exon 8 inner reaction were digested using DdeI. The reaction used to digest the products with DraI was as follows: 11.5µl of PCR grade water, 2µl of 10x NE buffer 4 (New England BioLabs, USA) and 1.5µl of DraI (20U/µl) were added to 5µl of amplified product for a final volume of 15µl. For DdeI reaction, 11.5µl of PCR grade water, 2µl of 10x NE buffer 3 (New England BioLabs, USA) and 1.5µl of DdeI (10U/µl) were added to 5µl of amplified product for a final volume of 15µl. Samples were incubated at 37°C overnight. Gel electrophoresis (3% agarose gel used) was carried out as described in section 2.1.2 to collect the results.

Figure 2.3: Schematic diagram of two agarose gels showing the migration of exon 7 and 8 (*SMN* gene) DNA fragments after digestion with DraI and DdeI. **A)** Exon 7 digested by DraI. **B)** Exon 8 digested by DdeI.

A) Exon 7 digested by DraI



B) Exon 8 digested by DdeI



Notes: For both diagrams (A and B) lane 1 corresponds to an undigested product, lane 2 is a digested product from an unaffected individual, lane 3 is a digested product from a carrier of the mutation and lane 4 is a digested product from an affected individual. **A)** This diagram shows digested and undigested products after amplified with Exon 7 outer F – inner R primers. At 202bp is digested *SMN1* amplified product and at 176bp is digested *SMN2* product. As can be seen from the diagram there is no product at 202bp for lane 4 indicating that this DNA sample must be carrying the mutation in both *SMN1* alleles and therefore it is affected with SMA. The DNA sample in lane 3 even though is a carrier of the disorder still gives a product for *SMN1* exon 7. The product comes from the amplification of the normal allele. As a result, in order to distinguish carriers from unaffected, linkage analysis is used. **B)** This diagram shows digested and undigested products after amplified with Exon 8 outer F – inner R primers. At 287bp is undigested *SMN1* amplified product and at 234pb is digested *SMN2* product. As with exon 7, at lane 4 there is no product present for *SMN1* indicating that for this DNA sample there is deletion of exon 8 of *SMN1* gene in both alleles and is therefore affected.

Table 2.28: Spinal muscular atrophy type I – PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.09
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
Exon 7 outer F+R (10µM)	0.2µM	0.3
Exon 8 outer F+R (10µM)	0.2µM	0.3
D5S610 F+R (10µM)	0.14µM	0.21
D5S629 F+R (10µM)	0.18µM	0.27
D5S637 F+R (10µM)	0.22µM	0.33
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	61°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

2.2.3.4.19 Thalassaemia B

Two PGD cycles for 2 different couples were carried out for this disorder. Day-3 biopsy was performed for one of the cycles and day-5 biopsy for the other. Fifteen embryos were biopsied. The protocols used to carry out the cases are described in Tables 2.29 and 2.30.

Table 2.29: Thalassaemia B – PGD protocol A (c.142+5G>C)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	6.56
Tricine (0.4M)	40mM	1.5
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
c.142+5G>C outer F (100µM)	2µM	0.3
c.142+5G>C outer R (100µM)	2µM	0.3
D11S4181 F (100µM)	0.4µM	0.06
D11S4181 R (100µM)	0.4µM	0.06
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	0.6 U/reaction	0.12
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	54.5°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.3 (annealing temperature: 60.9°C) using the c.142+5G>C inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (Exo I and SAP were used) using the c.142+5G>C Mini R primer (Appendix 1).

Table 2.30: Thalassaemia B – PGD protocol B (c.142+5G>C)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.9
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.142+5G>C outer F+R (10µM)	0.2µM	0.3
D11S2362 F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	54.5°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.3 (annealing temperature: 61°C) using the c.142+5G>C inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.142+5G>C Mini R primer (Appendix 1).

2.2.3.4.20 Tuberous sclerosis 1

Two PGD cycles for 2 different couples were carried out for this disorder. Day-3 biopsy was performed for both cycles. Nine embryos were biopsied. PK lysis was used for one of the cases (c.912T>G) to lyse the cells and alkaline lysis was used for the other (c.1525C>T) as described in section 2.2.3.2. The protocols used to carry out the cases are described in Tables 2.31 and 2.32.

Table 2.31: Tuberous sclerosis 1 – PGD protocol A (c.912T>G)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	6.8
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
c.912T>G outer F (100µM)	0.8µM	0.12
c.912T>G outer R (100µM)	0.8µM	0.12
D9S149 F (100µM)	0.8µM	0.12
D9S149 R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5u/µl)	0.6 U/reaction	0.12
Lysis buffer + released DNA	Variable	4

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	60.9°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.2 (annealing temperature: 53°C) using the c.912T>G inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (Exo I and SAP were used) using the c.912T>G Mini F primer (Appendix 1).

Table 2.32: Tuberous sclerosis 1 – PGD protocol B (c.1525C>T)

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.69
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.1525C>T outer F+R (10µM)	0.2µM	0.3
D9S149 F+R (10µM)	0.2µM	0.3
PM4 F+R (10µM)	0.14µM	0.21
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	59°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.1525C>T inner primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.1525C>T Mini F primer (Appendix 1).

2.2.3.4.21 X-linked hydrocephalus

Two PGD cycles were carried out for this couple and 11 embryos were tested. Day-3 biopsy was carried out for all embryos. Although the 2 PGD cycles were carried out for the same couple, different PGD protocols were used in each cycle. The first time the couple requested PGD, the disease-causing mutation had not been identified as yet, while no DNA from family members was available to construct a haplotype. For this reason a protocol was developed for sex determination of embryos with the aim of identifying female embryos for transfer. Before the 2nd PGD cycle was initiated the disease-causing mutation had been identified by a specialised genetics laboratory. Therefore, for performance of the 2nd PGD cycle a protocol was developed that was superior from the 1st one and involved sex determination coupled with mutation detection (Table 2.34).

Table 2.33: X-linked hydrocephalus – PGD protocol A

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.6
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
AMEL F+R (10µM)	0.2µM	0.3
X22 F+R (10µM)	0.2µM	0.3
SRY F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	
Annealing	58°C for 90s	55
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Table 2.34: X-linked hydrocephalus – PGD protocol B

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	2.39
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
c.551G>A outer F+R (10µM)	0.18µM	0.27
DXS1227 F+R (10µM)	0.18µM	0.27
AMEL F+R (10µM)	0.2µM	0.3
X22 F+R (10µM)	0.18µM	0.27
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	58°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Inner Reaction was carried out as described in Table 2.2 (annealing temperature: 58°C) using the c.551G>A outer F – inner R primers (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.551G>A Mini F primer (Appendix 1).

2.3 HLA typing

A universal HLA-typing protocol was developed and validated. Three PGD clinical cases for three different families were carried out with the protocol developed. It should be noted that the three clinical cases were carried out by collaborators at Reprogenetics LLC; further details regarding this matter are given in section 2.3.3.

2.3.1 Protocol optimisation

To begin with, 24 STR markers were selected from a panel of 50 described in Handyside *et al.* (2005). Selection was based on the position of the STR markers in the HLA region (Figure 2.4) and the fragment size produced by the primers amplifying each STR marker.

Initially, three different methods were examined for amplification of the STR markers: a large multiplex-PCR, MDA and SurePlex. Human fibroblast cells from a normal female cell line (46,XX) (coded 'Pink') were obtained from the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. Single fibroblasts were isolated as described before (section 2.2.2.3.1.2) and DNA was extracted using the QIAamp DNA blood MiniKit ('Blood and Body Fluid Spin Protocol' was used according to manufacturer's instructions). Three single fibroblast cells were amplified with each method. Positive (genomic DNA) and negative (water) controls were used.

For direct PCR, single cells were lysed using alkaline lysis (section 2.2.3.2) and amplified using the Qiagen Multiplex PCR kit. Specifically, the reaction included PCR grade water, 0.04M Tricine, 1x Qiagen Master Mix, 0.08μM of each primer and

the lysed cell suspension (2.5µl) for a total volume of 30µl. The cells were amplified using the conditions described in Table 2.35 (20 cycles, annealing temperature: 55ºC).

Regarding the WGA methods, the single cells were lysed and amplified as described in section 2.4.1.2 for MDA and SurePlex.

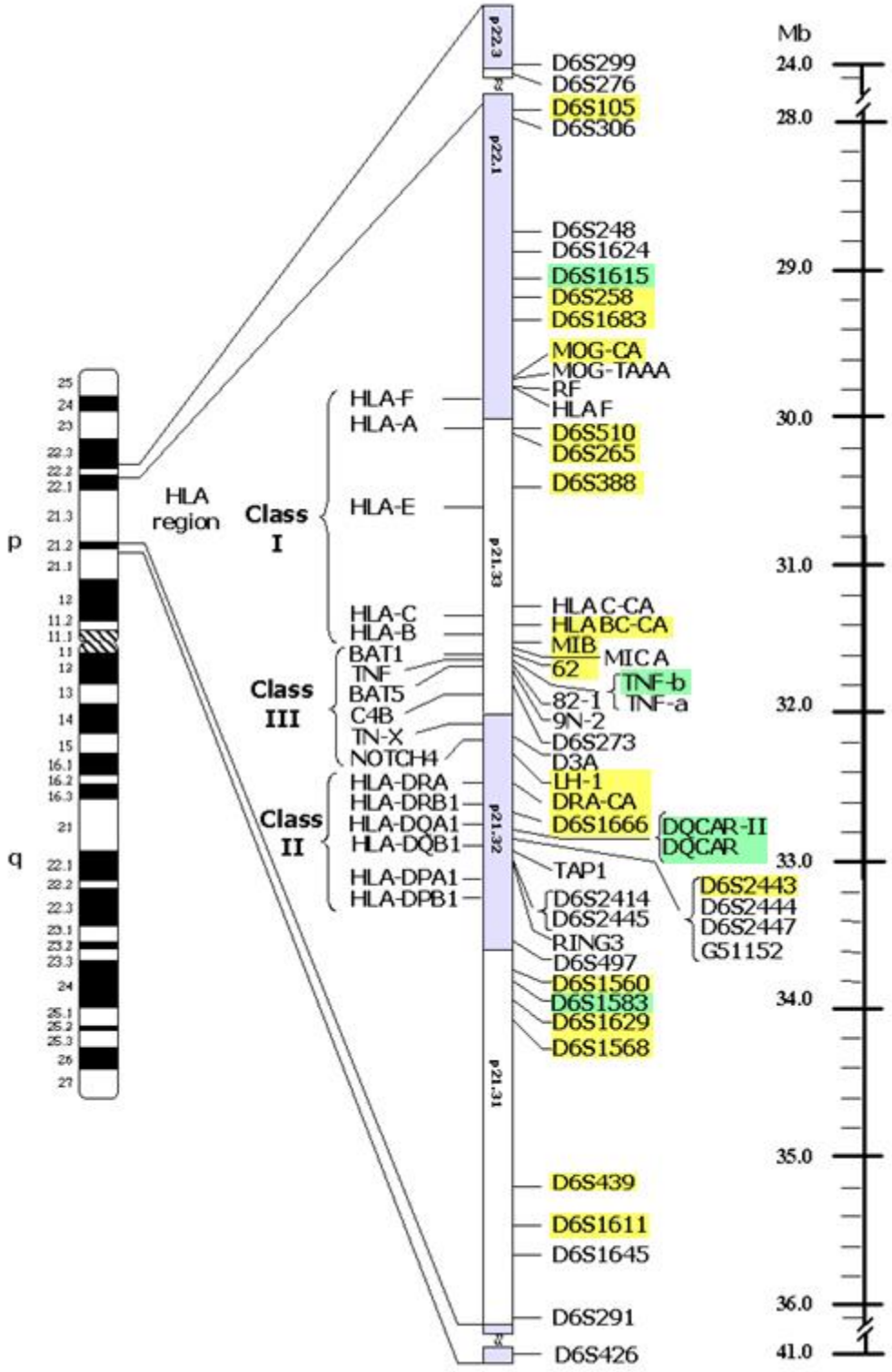
Table 2.35: Thermocycler conditions for Qiagen Multiplex PCR kit

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	
Annealing	variable for 90s	Variable
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

After direct multiplex-PCR, MDA or SurePlex had been carried out on single cells, amplified products were used to perform singleplex reactions for each STR marker and final products underwent capillary electrophoresis (section 2.1.1) in order to collect the results. The purpose of the singleplex reactions was to establish which loci had been successfully amplified by the different WGA and PCR methods. Singleplex reactions were carried out using HotMaster *Taq* DNA polymerase as described before (Table 2.2) for 50 cycles (annealing temperature: 55ºC). 1µl of direct PCR product, 1µl of MDA product and 2µl of SurePlex product were used for each singleplex reaction.

After carrying out these initial tests, it was decided to use the direct PCR method to develop the HLA typing protocol. Some of the STRs were excluded from the protocol based on criteria related to amplification characteristics.

Figure 2.4: Position of the 24 STR markers included in protocol optimisation in the HLA region, on chromosome 6. Image adapted from Handyside *et al.* (2005).



Notes: Highlighted in yellow are the 19 STRs included in the final protocol (Table 2.36). Highlighted in green are the STRs that were initially included in protocol optimisation but eventually excluded from the final protocol. At the top of the image are the STR markers closer to the telomere of chromosome 6p and at the bottom are those closer to the centromere.

2.3.2 Final Protocol and Validation

The final protocol developed consisted of 19 STR markers amplified simultaneously in an outer multiplex reaction. The amplified product from the outer reaction was used to further amplify each marker using three separate multiplex-PCRs, each one amplifying a different set of STR markers. Alkaline lysis was used in the protocol to lyse the single cells (section 2.2.3.2).

The outer reaction mixture contained PCR grade water, 0.04M Tricine, 1x Qiagen Master Mix, primers for 19 markers at final concentrations described in Table 2.36 and the lysed cell suspension (2.5µl) for a total volume of 30µl. Amplification was carried out as described in Table 2.35 (20 cycles, annealing temperature: 61°C). Subsequently, the inner multiplex reactions were carried out by using amplified product from the outer reaction. The reaction mixtures contained: PCR grade water, 1x Qiagen Master Mix, primers at final concentrations described in Table 2.36 and 1µl of amplified product for a final volume of 15µl. Amplification of each of the inner multiplexes occurred as described in Table 2.35 (45 cycles, annealing temperature: 61°C).

The primer sequences used are shown in Appendix 4.

The final protocol was validated by amplifying 36 lymphocytes extracted and isolated from two individuals as described before (section 2.2.2.3.1).

Table 2.36: HLA-typing protocol - Primer concentrations

Panel*	STR Marker	Outer Reaction - Primer Concentration (µM)	Inner Reaction - Primer Concentration (µM)
1	D6S105	0.03	0.06
	D6S2443	0.03	0.06
	D6S1629	0.1	0.2
	LH-1	0.07	0.14
	MOG-CA	0.13	0.26
	D6S1560	0.2	0.4
2	D6S265	0.02	0.04
	62	0.05	0.1
	D6S439	0.025	0.05
	DRA-CA	0.06	0.12
	MIB	0.11	0.22
	D6S388	0.035	0.07
3	D6S1666	0.015	0.03
	D6S1611	0.04	0.08
	D6S510	0.04	0.08
	HLABC-CA	0.05	0.1
	D6S1683	0.2	0.4
	D6S258	0.2	0.4
	D6S1568	0.2	0.4

* After outer reaction was carried out, the STR markers were separated into 3 panels and the markers found in each panel were amplified together in a multiplex inner reaction.

2.3.3 Clinical cases

Three PGD cycles (each one for a different couple) were carried out using the developed protocol. Preparation of cases was carried out in the laboratory in Oxford by M.K and the final protocol, along with primers, were sent to Reprogenetics LLC in the USA to carry out the clinical cases. The cases were performed by Ms. Renata Prates, Mr. Jorge Sanchez and Ms. N-neka Goodall (Reprogenetics LLC). For the preparation of the cases, genomic DNA extracted from the couples and family members was used (sent from Reprogenetics LLC).

2.3.3.1 HLA typing

Two of the three clinical cases were carried out for HLA typing alone. In total, 11 embryos were tested. Day-3 biopsy was carried out initially for both cases and a single blastomere was removed from each embryo. For one of the cases, 2 of the embryos were re-biopsied on day-5 and trophectoderm cells were obtained and re-analyzed. The re-biopsy was performed because the cells obtained from these two embryos on day-3 failed to give amplification for any of the loci assessed and therefore no HLA typing could be carried out.

The protocol for HLA-typing was carried out as described above (section 2.3.2) with one difference; PK lysis was used instead of alkaline lysis. This was due to the fact that the laboratory at Reprogenetics LLC was more familiar with PK lysis than alkaline lysis. PK lysis was carried out as described before (section 2.2.3.2). The outer reaction mixture contained PCR grade water, 1x Qiagen Master Mix, primers for 19 markers at final concentrations described in Table 2.36 and the lysed cell suspension

(4µl) for a total volume of 30µl. Amplification was carried out as described in Table 2.35 (20 cycles, annealing temperature: 61°C). Subsequently, the inner multiplex reactions were carried out as described in section 2.3.2.

2.3.3.2 HLA typing, Mucopolysaccharidosis type 1 and Gender selection

The other clinical case combined HLA typing with the amplification of other loci in order to carry out PGD for an autosomal recessive disorder (Mucopolysaccharidosis type 1) and also to identify the gender of each biopsied embryo. An additional STR marker (DQCAR) was included for HLA typing. This marker was part of a protocol developed which preceded the final protocol decided for HLA-typing (section 2.3.2). This case was processed before the final protocol had been developed and the protocol available at the time was one including the specific STR along with the 19 STRs included in the final protocol.

Two embryos were biopsied on day-5 and trophectoderm cells were obtained for this case. Alkaline lysis was used to lyse the biopsied cells as described before (section 2.2.3.2). The outer reaction mixture contained PCR grade water, 0.04M Tricine, 1x Qiagen Master Mix, primers amplifying 26 different loci at final concentrations described in Table 2.37 and the lysed cell suspension (2.5µl) for a total volume of 30µl. Amplification was carried out as described in Table 2.35 (20 cycles, annealing temperature: 61°C). Subsequently, the inner reactions were carried out by using amplified product from the outer reaction. The reaction mixtures contained: PCR grade water, 1x Qiagen Master Mix, primers at final concentrations described in Table 2.37 and 1µl of amplified product for a final volume of 15µl. Amplification of each of

the inner reactions occurred as described in Table 2.35 (45 cycles, annealing temperature: 61°C).

Table 2.37: HLA typing, Mucopolysaccharidosis type 1 and gender selection protocol - Primer concentrations

Panel	STR Marker	Outer Reaction - Primer Concentration (μM)	Inner Reaction - Primer Concentration (μM)
1	D6S105	0.03	0.06
	D6S2443	0.03	0.06
	D6S1629	0.1	0.2
	LH-1	0.07	0.14
	MOG-CA	0.13	0.26
	D6S1560	0.2	0.4
2	D6S265	0.02	0.04
	62	0.05	0.1
	D6S439	0.025	0.05
	DQCAR	0.08	0.16
	DRA-CA	0.06	0.12
	MIB	0.11	0.22
	D6S388	0.035	0.07
3	D6S1611	0.04	0.08
	D6S510	0.04	0.08
	HLABC-CA	0.05	0.1
	D6S1683	0.2	0.4
	D6S258	0.2	0.4
	D6S1568	0.2	0.4
4	D6S1666	0.015	0.03
5	D4S3038	0.05	0.1
	D4S3360	0.05	0.1
	HPRT	0.09	0.18
6	D4S2936	0.05	0.1
	AMEL	0.07	0.14
	SRY	0.08	0.16

Notes: After outer reaction was carried out, the primers amplifying the different loci were separated into 6 panels and the loci found in each panel were amplified in multiplex or singleplex reactions. Unlike the final HLA-typing protocol (section 2.3.2), the 6-FAM-labeled D6S1666 marker was not amplified together with the other STR markers in Panel 3 during the inner reactions. This was due to the fact that one of the alleles carried by the parents for this marker had the same size with an allele of another 6-FAM-labeled marker in Panel 3 (D6S510). The primers used for this case are included in Appendices 1, 2 and 4.

2.4 Whole Genome Amplification

Three WGA methods were studied for their applicability in PGD using a variety of different methodologies. Eventually, PGD clinical cases were carried out using the WGA methods found to perform the best.

Some parts of this project were carried out by collaborators. Specifically, application of WGA samples on two different types of array (section 2.4.1.5) and some analysis of the results obtained were performed by scientists at The Wellcome Trust Centre for Human Genetics (University of Oxford, UK). Furthermore, application of MDA products (obtained from two PGD clinical cases) on SNP arrays and analysis of results through karyomapping (section 2.4.1.7) were carried out by scientists at Univesrity of Cambridge, UK and at BlueGnome Ltd, UK. More details regarding these collaborations are given in sections 2.4.1.5 and 2.4.1.7.

2.4.1 Investigation of whole genome amplification methods

In order to study the different WGA methods (MDA, GenomePlex, SurePlex), aneuploid cell lines were cultured and single cells were isolated. The isolated cells underwent WGA and amplified products were used in different applications.

The following cell lines were used: 46,XX (‘Pink’); 47,XY,+13; 47,XY,+15; 47,XY,+18; 47,XY,+21; 47,XX,+22; 45,X0 and 48,XXY,+18. All of these cell lines were fibroblast cell lines apart from cell line 47,XX,+22 which was a chorionic-villus derived cell line. All cell lines were obtained from Coriell Cell Repositories, USA apart from cell lines 47,XY,+15 and 48,XXY,+18, that were kindly provided by Prof J. Navarro (Universitat Autonoma de Barcelona, Spain) and ‘Pink’ cell line (section

2.3.1). It should be noted that cell line 47,XX,+22 was only used for application on the NimbleGen microarray platform (section 2.4.1.5).

2.4.1.1 Cell Culture, Single Fibroblast Isolation and DNA Extraction

The following aneuploid fibroblast cell lines were cultured: 47,XY,+13; 47,XY,+18; 47,XY,+21; 45,X0. The rest of the cell lines were not cultured; provided cell suspensions were used to carry out DNA extraction and single cell isolations as described in this section.

Fibroblast cells were cultured under standard conditions using Chang medium C (Irvine Scientific, USA) supplemented with L-Glutamine (Invitrogen, UK) and penicillin-streptomycin (Invitrogen, UK). The cells were incubated at 37°C / 5%CO_2. Trypsin-EDTA (1x) (Sigma Adlrich, USA) was used whenever appropriate. Cultured cells were used to carry out DNA extraction for each cell line and also single cell isolations. The QIAamp DNA blood MiniKit ('Blood and Body Fluid Spin Protocol' used) was used for DNA extractions according to manufacturer's instructions. Single cell isolations were performed as described before (section 2.2.2.3.1.2). The single fibroblasts isolated were stored at -80°C until required.

2.4.1.2 WGA Methods and Processing

SurePlex and GenomePlex (Sigma–Aldrich, Germany) were carried out according to the manufacturer's instructions to amplify 100 and 57 single cells, respectively. Regarding MDA the Repli-g Midi kit (Qiagen, Germany) was used with modifications from the original protocol to amplify 72 single cells. Specifically, to

each sample 1.5µL of PCR-grade water were added in order to get an overall volume of 2.5µL before cell lysis. Alkaline lysis was then carried out by adding 0.75µL of PCR-grade water, 1.25µL of 0.1M DTT and 0.5µL of 1M NaOH to each tube followed by incubation at 60°C for 10 minutes. The reaction mixture was then added to the lysed cell. The mixture contained 5µl of PCR grade water, 10µl of 0.1M Tricine and 29µl Repli-g Midi Reaction buffer for a total volume of 44µl. Following, 1µl of Repli-g Midi DNA polymerase was added to each sample individually. The final reaction volume was 50µl (including reaction mixture and lysed cell). The samples were then incubated in a thermocycler at 30°C for 2 hours followed by enzyme inactivation at 65°C for 5 minutes.

After WGA, some of the samples were left unprocessed while others were purified by one of three methods.

Some of the samples were column purified using the GenElute PCR Clean-Up Kit (Sigma–Aldrich, Germany). Specifically, 40µl of MDA product and 65µl of SurePlex and GenomePlex products were used to perform the purification following instructions from the manufacturer. The only modification to the manufacturer's protocol was that at the end of the procedure the purified products were collected in 60µl of Elution Solution (GenElute PCR Clean-Up Kit) instead of 50µl.

Other samples were column purified using the 'Blood and Body Fluid Spin Protocol' of the QIAamp DNA blood MiniKit. Before the procedure was carried out, 45µl of MDA product were mixed with 155µl of nuclease free water (Promega, USA) for each sample, while for SurePlex and GenomePlex 65µl of amplified product were mixed with 135µl of nuclease free water. The 200µl of sample prepared were used to perform the procedure according to the recommendations of the manufacturer. The

only change at the procedure was introduced at the end where the purified products were collected in 60µl of AE Buffer (QIAamp DNA blood MiniKit) instead of 200µl.

Finally, samples were simply precipitated. 40µl of MDA sample and 65µl of SurePlex and GenomePlex sample were used for the precipitation procedure. Briefly, sodium acetate solution (Sigma–Aldrich, Germany) was added to the sample at a volume of 1/10 of the final volume of the WGA product (e.g. 6.5µL of sodium acetate were added to 65µL of WGA product). Then ethanol 100% (molecular grade) (Sigma–Aldrich, Germany) was added to each tube at x2.5 the volume of sodium acetate + WGA product. Tubes were then placed at -80°C for 1 hour. Afterwards, tubes were centrifuged for 30 minutes at full speed [i.e. 16.1 rcf (relative centrifugal force)]. Supernatant was removed from each tube and the DNA pellets were re-suspended in 60µL of nuclease-free water.

The WGA products created (processed and unprocessed) were used for gel electrophoresis and DNA concentration measurement (section 2.4.1.3), for PCR amplification (section 2.4.1.4) and for application on DNA microarray platforms (section 2.4.1.5).

2.4.1.3 Concentration Measurement and Gel electrophoresis

Concentration measurements of the different WGA products (purified and unpurified) were taken using a Nanodrop ND-1000 spectrometer (Nanodrop Technologies, USA).

Gel electrophoresis was carried out as explained before (section 2.1.2) to determine the size of the WGA products (1.5% agarose gels used).

2.4.1.4 PCR amplification of WGA products

Aliquots from WGA products were used to amplify 10 STR markers found in various positions in the human genome. The STR markers chosen are clinically relevant, since they are found in close proximity to genes associated with serious inherited disorders. Three multiplex and two singleplex protocols were used in total. The protocols included the following: Myotonic dystrophy - multiplex (DMPK locus, APOC2, D19S112), Huntington disease - multiplex (HD locus, D4S136, D4S127), Sandhoff disease - multiplex (D5S1988, D5S2003), Huntington disease - singleplex (D4S412) and Hereditary multiple exostoses - singleplex (D8S592). The reaction protocols used are shown in Tables 2.38-2.41. Primer sequences used to amplify each of these loci, apart from the loci amplified in the Myotonic dystrophy multiplex, are included in Appendix 1. The protocol and the primer sequences used for the Myotonic dystrophy multiplex were obtained from Kakourou *et al.* (2007). In some occasions, some of the loci (HD locus, D4S127, D5S2003) included in the multiplexes did not show any amplification. When this occurred, amplification of these loci was attempted a second time, in a singleplex reaction. The singleplex reaction for HD locus was carried out using AmpliTaq Gold 360 DNA polymerase as described before (section 2.2.2.4.2). Singleplex reactions for D4S127 and D5S2003 were carried out using HotMaster *Taq* DNA Polymerase as explained in Table 2.2 for 55 cycles (annealing temperature used for D4S127: 58°C, annealing temperature used for D5S2003: 56.7°C).

Table 2.38: Myotonic dystrophy multiplex

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	11.24
10x Expand High Fidelity Buffer (15mM $MgCl_2$)	1 x	1.5
DMPK F+R (10µM)	0.2µM	0.3
APOC2 F+R (10µM)	0.3µM	0.45
D19S112 F+R (10µM)	0.3µM	0.45
dNTPs (10mM each)	0.2mM	0.3
Expand High Fidelity Enzyme Mix (3.5U/µl)	0.91 U/reaction	0.26
WGA Product	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 2min	1
Denaturation	†94°C for 15s	40
Annealing	58°C for 45s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

† For the first 10 cycles of the PCR program the denaturation temperature was 96°C.

Table 2.39: Huntington disease multiplex

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	7.63
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	3
HD 3 F (100µM)	0.8µM	0.12
HD 4 R (100µM)	0.8µM	0.12
D4S136 F (100µM)	0.6µM	0.09
D4S136 R (100µM)	0.6µM	0.09
D4S127 F+R (10µM)	0.1µM	0.15
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5U/µl)	1.5 U/reaction	0.3
WGA Product	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	96°C for 10s	55
Annealing	58°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 5min	1
Hold	4°C for ∞	1

Table 2.40: Sandhoff disease multiplex

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	10.18
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
D5S1988 F (100µM)	1.2µM	0.18
D5S1988 R (100µM)	1.2µM	0.18
D5S2003 F (100µM)	0.8µM	0.12
D5S2003 R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5U/µl)	0.6 U/reaction	0.12
WGA Product	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	55
Annealing	56.7°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Table 2.41: Singleplex protocol for amplification of D4S412 and D8S592

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	12.34
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
WGA Product	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	55
Annealing	† Variable for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

† For D4S412 annealing temperature used was 58°C. For D8S592 annealing temperature used was 61°C.

2.4.1.5 DNA microarrays

Products from the 3 different WGA methods created as described before (section 2.4.1.2) were utilized for application on two microarray platforms; the NimbleGen 12x135K array (135,000 probes tested for each sample) (Roche, Germany) and the Illumina HumanCytoSNP-12 BeadChip (~300,000 SNPs tested for each sample) (Illumina Inc., USA). The NimbleGen array was used for aneuploidy screening, while the Illumina SNP array was used for aneuploidy screening and also genotyping of applied samples. WGA products of single cells derived from the following cell lines were used: 47,XY,+13; 47,XY,+15; 47,XY,+18; 47,XY,+21; 47,XX,+22; 45,X0 (section 2.4.1). In total, 82 WGA products were applied on Illumina SNP array, while 72 WGA products were applied on NimbleGen array. For the NimbleGen microarray platform control male DNA samples were amplified as well (using the same WGA method as the method used for the samples tested in each batch). The control samples were used as hybridisation controls and they were prepared by amplifying 1µl of male genomic DNA (1ng/µl). WGA products were used column purified (utilizing the GenElute PCR Clean-Up Kit), precipitated and unprocessed. The concentration of the products was determined using a Nanodrop spectrometer (section 2.4.1.3) before they were applied on the microarray platforms.

For the NimbleGen array platform 1µg of WGA DNA was used for each sample and control. Procedures were carried out following manufacturer's instructions (60-72 hours of hybridisation were used). Eventually, it was decided to test a few SurePlex column purified samples using reduced hybridisation time in an attempt to make the procedure more applicable to PGD and be able to provide a diagnosis on the following day of the biopsy. Specifically, 16h of hybridisation were tested.

For the Illumina array platform 200ng of WGA DNA were used for each sample. Additionally, 200ng of unamplified genomic DNA extracted from the cell lines was tested. Results obtained from single-cell WGA DNA were compared to results from the extracted unamplified cell line DNA. Procedures for application of samples on the Illumina array were carried out following the 'Infinium HD Assay Ultra Manual' (Illumina Inc., USA) according to manufacturer's instructions.

Experiments for application of the samples on the two array platforms and initial data assessment were carried out by Dr. Elham Sadighi Akha (The Wellcome Trust Centre for Human Genetics, University of Oxford, UK). Further assessment and analysis of data using 'Nexus Copy Number' software (BioDiscovery Inc., USA) was carried out by Dr. Samantha Knight (The Wellcome Trust Centre for Human Genetics, University of Oxford, UK). Bioinformatics analysis of data obtained from experiments carried out with the Illumina platform was performed by Ms. Natasha Saghal (The Wellcome Trust Centre for Human Genetics, University of Oxford, UK). Production of WGA samples applied on the arrays and further analysis of results obtained were carried out by M.K

2.4.1.6 PGD clinical cases

WGA methods were used to carry out five clinical cases (Table 2.42). Five PGD protocols were developed for 5 different single gene disorders. IVF and embryo biopsy were carried out as described before (section 2.2.3.1).

Table 2.42: List of PGD clinical cases carried out using WGA methods.

Disease	Inheritance Pattern	Gene	Mutation	No. of Protocols Developed
Dominant Dystrophic Epidermolysis Bullosa	AD	*COL7A1*	c.6501+1G>C	1
Marfan Syndrome	AD	*FBN1*	c.235C>T	1
Phenylketonuria	AR	*PAH*	c.194T>C / c.1241A>G	1
Sandhoff Disease	AR	*HEXB*	c.115delG	1
Smith-Lemli-Opitz Syndrome	AR	*DHCR7*	Not Defined	1

2.4.1.6.1 Dominant dystrophic epidermolysis bullosa

As explained in section 2.2.3.4.6, 4 embryos were re-biopsied on day-5 for the 1st PGD cycle of this case and re-tested. SurePlex WGA was used according to manufacturer's instructions to amplify the 4 samples. The WGA products were then utilized in singleplex PCR reactions (Table 2.43) in order to amplify the loci relevant to the disorder. Additionally, the 24sure test was carried out for all samples as described in Appendix 3.

Table 2.43: Dominant dystrophic epidermolysis bullosa – WGA PGD protocol

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	12.37
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.45 U/reaction	0.09
SurePlex WGA Product	Variable	0.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	35
Annealing	61°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: The STR markers (D3S2409, D3S1581) and the mutation site were amplified in separate singleplex reactions using this protocol. For amplification of the mutation site the c.6501+1G>C inner F and outer R primers were used (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.6501+1G>C Mini F primer (Appendix 1).

2.4.1.6.2 Marfan syndrome

Day-3 biopsy was carried out for this case and 6 embryos were tested. For two of the embryos 2 cells were obtained instead of the usual one. The cells were lysed using alkaline lysis and amplified using MDA as described before (section 2.4.1.2). The MDA products were then used for amplification of linked markers and also amplification of the mutation site (Table 2.44). Additionally, aliquots of the MDA products were sent to BlueGnome Ltd in Cambridge to apply on SNP arrays (section 2.4.1.7).

Table 2.44: Marfan syndrome - WGA PGD protocol

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	11.84
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
MDA WGA Product	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	50
Annealing	60°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: The STR markers (D15S143, D15S196. D15S659) and the mutation site were amplified in separate singleplex reactions using this protocol. For amplification of the mutation site the c.235C>T inner F+R primers were used (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (ExoSAP-IT was used) using the c.235C>T Mini F primer (Appendix 1).

2.4.1.6.3 Phenylketonuria

Day-3 biopsy was carried out for this case and 12 embryos were tested. For one of the embryos 2 cells were obtained instead of one. Lysis and amplification of the samples was carried out using the SurePlex DNA amplification system according to manufacturer's instructions. The WGA products were then used for amplification of linked markers and also amplification of the two mutation sites (Table 2.45).

Table 2.45: Phenylketonuria - WGA PGD protocol. **A)** Protocol A - amplification of linkage markers. **B)** Protocol B - amplification of mutation sites.

A) Protocol A - amplification of linkage markers

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	11.075
10x Expand Long Template buffer 3 (27.5mM $MgCl_2$)	1 x	1.5
Primer F (100µM)	2µM	0.3
Primer R (100µM)	2µM	0.3
dNTPs (10mM each)	0.35mM	0.525
Expand Long Template Enzyme Mix (5u/µl)	1.5 U/reaction	0.3
SurePlex WGA Product	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 2min	1
Denaturation	† 94°C for 40s	45
Annealing	54.5°C for 1min	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

† For the first 10 cycles of the PCR program the denaturation temperature was 96°C.

B) Protocol B - amplification of mutation sites

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	11.84
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
SurePlex WGA Product	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	45
Annealing	54.5°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: The STR markers (STR-3, VNTR-13) were amplified in separate singleplex reactions using Protocol A. The mutation sites were amplified in separate singleplex reactions using Protocol B. For amplification of the mutation sites the c.194T>C inner F+R primers and the c.1241A>G inner F+R primers were used (Appendix 1). Minisequencing was carried out as described in section 2.2.3.3.3 (Exo I and SAP were used) using the c.194T>C Mini F and the c.1241A>G Mini F primers (Appendix 1).

2.4.1.6.4 Sandhoff disease

Six embryos were biopsied on day-3 for this case and single blastomeres were obtained. Due to the fact that the mother was of advanced maternal age (39 years old) it was decided to screen the embryos for aneuploidy as well as testing them for Sandhoff disease.

Lysis and amplification of the samples was carried out using the SurePlex DNA amplification system according to manufacturer's instructions. The WGA products were then used for amplification of linked markers and also amplification of the mutation site (Table 2.46). For this case, two different sets of primers were optimised and used to detect the same mutation.

Aliquots from the WGA products were also used to carry out the aneuploidy screening of the embryos using the 24sure test as described in Appendix 3.

Table 2.46: Sandhoff disease - WGA PGD protocol. **A)** Protocol A - amplification of linkage markers. **B)** Protocol B - amplification of mutation site.

A) Protocol A - amplification of linkage markers

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	10.84
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
SurePlex WGA Product	Variable	2

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	50
Annealing	59°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

B) Protocol B - amplification of mutation sites

	Concentration	*Volume for 15µl reaction (µl)*
PCR grade H_2O	---	9.04
10x AmpliTaq Gold 360 Buffer	1 x	1.5
$MgCl_2$ (25mM)	2mM	1.2
360 GC Enhancer	N/A	0.6
Primer F (100µM)	0.8µM	0.12
Primer R (100µM)	0.8µM	0.12
dNTPs (10mM each)	0.2mM	0.3
AmpliTaq Gold 360 DNA polymerase (5U/µl)	0.6 U/reaction	0.12
SurePlex WGA Product	Variable	2

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 10min	1
Denaturation	95°C for 30s	50
Annealing	59°C for 30s	
Extension	72°C for 1min	
Final Extension	72°C for 7min	1
Hold	4°C for ∞	1

Notes: The STR markers (D5S1988, D5S2003) were amplified in separate singleplex reactions using Protocol A. The mutation site was amplified in separate singleplex reactions using Protocol B. For amplification of the mutation site two sets of primers were used; the c.115delG F1+R1 primers and the c.115delG F1 + R2 primers (Appendix 1).

2.4.1.6.5 Smith-Lemli-Opitz syndrome

1st and 2nd polar body biopsy was carried out for this case for 6 oocytes. The biopsied polar bodies were screened for aneuploidy using the 24sure test (Appendix 3). Following, Day-3 biopsy was carried out for 5 embryos and single blastomeres were removed. For two of the embryos 2 cells were obtained instead of one. The single blastomeres were used to carry out PGD for diagnosis of the single gene disorder. They were lysed using alkaline lysis and amplified using MDA as described before (section 2.4.1.2). The MDA products were then used for amplification of linked markers (Table 2.47). Diagnosis was carried out through linkage analysis.

Aliquots of the MDA products were sent to Bluegnome Ltd in Cambridge to apply on SNP arrays (section 2.4.1.7).

Table 2.47: Smith-Lemli-Opitz syndrome - WGA PGD protocol

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	10.88
10x HotMaster *Taq* buffer with 25mM Mg^{2+}	1 x	1.5
Primer F+R (10µM)	0.8µM	1.2
dNTPs (10mM each)	0.2mM	0.3
HotMaster *Taq* DNA polymerase (5U/µl)	0.6 U/reaction	0.12
MDA WGA Product	Variable	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	96°C for 1min	1
Denaturation	94°C for 15s	55
Annealing	58°C for 15s	
Extension	65°C for 45s	
Final Extension	65°C for 2min	1
Hold	4°C for ∞	1

Notes: The STR markers (D11S4139, D11S4143. D11S4207) were amplified in separate singleplex reactions using this protocol. The primers used to amplify the STR markers were provided by Genoma - Molecular Genetics Laboratory, Italy.

2.4.1.7 SNP arrays - Karyomapping

Aliquots from MDA amplified products generated from the processing of clinical samples from two clinical cases (sections 2.4.1.6.2 and 2.4.1.6.5) were applied on SNP arrays. Genomic DNA derived from parents and a child for each case, was also applied on SNP arrays. Experiments and karyomapping analysis were carried out as described before (Handyside *et al.* 2010) with some modifications performed in order to reduce the length of the procedure to approximately 36 hours. The aim of this experiment was to investigate if the optimised methodology used to apply the

products on the SNP arrays and also the analysis method used (karyomapping) provide accurate diagnosis and can be used clinically to perform PGD. Application of samples on SNP arrays was performed by Ms. Kerry Cliffe and Mr. Christopher Reitter (Department of Pathology, University of Cambridge, UK). Karyomapping analysis was carried out by Professor Alan Handyside (BlueGnome Ltd, UK). Results from this experiment were compared with results obtained using PCR amplification of samples (for single gene diagnosis) and 24sure test (for aneuploidy detection).

2.5 Development of a customised array

A customised oligonucleotide array utilizing CGH technology was developed in collaboration with Oxford Gene Technology (OGT), UK. The idea for development of the specific array was conceived by Dr. Dagan Wells (Nuffield Department of Obstetrics and Gynaecology, University of Oxford, UK) at a time when no other microarray platforms for the purpose of PGD/PGS were commercially available. The developed array included oligonucleotide probes representing all human chromosomes for accurate detection of whole chromosome aneuploidies. Additionally, the developed array contained oligonucleotide probes covering the mitochondrial genome and telomeric regions in order to provide a relative measurement of the mitochondrial DNA quantity and the telomere length. Furthermore, the option of adding oligonucleotide SNP probes on the array for embryo fingerprinting was investigated.

WGA and preparation of samples for application on arrays was carried out in the laboratory at Reprogenetics UK by M.K (unless otherwise stated) and then samples were sent to OGT. Manufacture of the array and of specialized analysis software, application of samples on the array (including labelling of amplified products, hybridisation, washes and scanning of the arrays) and initial interpretation of results were performed by scientists at OGT (Dr. Douglas Hurd, Dr. Marta Paolucci, Mr. John Shovelton). Further interpretation and analysis of results was carried out by M.K.

Details for any parts of this project that were carried out by scientists other than the ones mentioned in the above paragraph are given in sections 2.5.2.2.1 and 2.5.2.2.2.

2.5.1 Optimisation

Amongst other procedures specific to the array platform, optimisation involved the testing of different WGA methods. SurePlex, GenomePlex and MDA were tested for their applicability in combination with the customised array. The WGA methods were performed as described before (section 2.4.1.2) on aneuploid single cells. WGA products were processed using one of two methods (column purification by using the QIAamp DNA blood MiniKit and precipitation as described in section 2.4.1.2) or unprocessed.

2.5.2 Optimised array platform and its validation

The optimised array contained 14,334 unique probes for aneuploidy detection, 261 unique probes for mitochondrial DNA measurement and 10 probes (2 probes included 5 times each) for telomere length measurement. The probes included on the final array were selected through the utilization of OGT proprietary bioinformatics algorithms.

2.5.2.1 Final array protocol

The final protocol involved the amplification of the samples and control male DNA samples through the usage of SurePlex DNA amplification system. Control samples were used as hybridisation controls and they were prepared by amplifying 1µl of male genomic DNA (1ng/µl). Amplified DNA (8µl) was labelled using the CytoSure labelling kit which incorporates Cy-dCTP using exonuclease-free Klenow enzyme following standard protocols (OGT, UK). After the labelling, the DNA was purified using either CytoSure purification columns (OGT, UK) or CytoSure purification

plates (OGT, UK). The sample and reference DNA were pooled together, dried and then resuspended in hybridisation mix (Agilent, USA). The mixed DNA sample was applied on the microarray slide (8x15k format) and was left to hybridise overnight. Post-hybridisation washes were carried out using Agilent Oligo aCGH Wash Buffer 1 for 5 mins at room temperature and Agilent Oligo aCGH Wash Buffer 2 for 1 minute at 37 degrees. The microarray slide was then scanned at 5µm resolution on Agilent's DNA microarray scanner with Surescan High-Resolution Technology. Feature extraction was carried out using Agilent feature extraction software (v10.7.3.1) and data analysis carried out using a special version of 'CytoSure Interpret Software' (OGT, UK).

2.5.2.2 Validation of developed array

2.5.2.2.1 Validation of array for aneuploidy screening

Validation of the developed array was carried out through the utilization of SurePlex amplified clinical samples that had been screened previously for aneuploidies by using the 24sure test (Appendix 3). Preparation of these samples and performance of the clinical cases through usage of 24sure test were carried out at Reprogenetics UK by Dr. Elpida Fragouli (Reprogenetics UK), Mr. Samer Alfarawati (Nuffield Department of Obstetrics and Gynaecology, University of Oxford, UK) and M.K. Results obtained from the developed array were compared with results obtained from the 24sure test; a validated test for aneuploidy screening used in several PGD laboratories worldwide. Additionally, some of the embryos that were not transferred were donated from the patients for research. These embryos were biopsied and the remaining cells were used for FISH analysis. FISH was carried out in three rounds of hybridisation by scientists at Reprogenetics LLC as described previously (Colls *et al.*

2009; Munne *et al.* 1998) to test chromosomes X, Y, 8, 13, 14, 15, 16, 17, 18, 20, 21, and 22. An additional round of hybridisation was performed to test any chromosomes that were found to be abnormal by 24sure test and were not included in the first 3 rounds of hybridisations. SurePlex amplification and performance of 24sure test on clinical samples corresponding to the embryos tested by FISH were carried out by scientists at Reprogenetics LLC.

Overall, 97 clinical samples were tested (27 PBs, 50 blastomeres, 20 trophectoderm samples) from which 20 (19 blastomeres, 1 trophectoderm sample) were assessed by FISH as well.

2.5.2.2.1.1 Selection of new probes for chromosome 19

After validation of the array for aneuploidy screening was completed and results were obtained, it was decided to further optimise the array for chromosome 19. New probes for chromosome 19 were selected and added on the array. Probes were selected on the basis of signal intensity and also ratio change for chromosome 19 aneuploidies.

The new array was tested against 5 samples (single blastomeres) that were aneuploid for chromosome 19.

2.5.2.2.2 Validation of array for mitochondrial DNA quantity measurement

In order to assess if the developed array was accurate for measuring the relative quantity of mitochondrial DNA in a sample, genomic DNA extracted from 4 cell lines (A549, Red, B2342B, 206F) was used.

SurePlex amplified genomic DNA and unamplified genomic DNA were applied on the developed array as described above (section 2.5.2.1). Additionally, aliquots from these DNA samples were used to carry out relative quantitation of mitochondrial

DNA through the utilization of qPCR in order to check if the results obtained from the array were accurate. Preparation of samples for application on the array and also relative quantitation through qPCR were carried out by Ms. Lorna Macleod using standard techniques (Nuffield Department of Obstetrics and Gynaecology, University of Oxford, UK).

2.5.2.2.3 Validation of array for telomere length measurement

The samples used to test the mitochondrial probes included in the array (section 2.5.2.2.2) were also used to test the telomere probes. The eight samples (4 unamplified genomic DNA samples and 4 SurePlex amplified genomic DNA samples) were applied on the array (as described in section 2.5.2.1) and they were also used to carry out relative quantitation through usage of qPCR.

Relative quantitation was carried out by using a StepOne Real-Time PCR System (Applied Biosystems, USA). The 8 samples, along with the SurePlex amplified reference DNA sample that was used as control sample when samples were applied on the array, were used to carry out the experiment. All samples were diluted 1:10 with PCR grade water. Two sets of primers were used to amplify the samples. The primers ‘tel 1’ and ‘tel 2’ were used to amplify the telomeres and the primers ‘36B4u’ and ‘36B4d’ were used to amplify the *36B4* single copy gene (Cawthon 2002). The protocols used to amplify these targets (Tables 2.48 and 2.49) were modified from Cawthon (2002) to meet the requirements of the experiment. The *36B4* gene was amplified and used as an endogenous control in order to normalise the measurements taken for each sample regarding telomere amplification.

Each sample was amplified in triplicate for each target. The Power SYBR Green PCR Master Mix (Applied Biosystems, USA) was used to carry out the amplifications. Results were collected and analysed using the 'StepOne Software v2.2.2'. The comparative C_T method (Schmittgen and Livak 2008) was used to determine relative quantitation of telomere DNA.

Table 2.48: Amplification of telomere DNA - Protocol A

	Concentration	***Volume for 10µL reaction (µl)***	***Thermocycler Conditions***		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.883	**Initial Denaturation**	95°C for 10min	1
Power SYBR Green PCR Master Mix (2x)	1 x	5	**Denaturation**	95°C for 15s	40
tel 1 (100µM)	0.27µM	0.027	**Annealing / Extension**	54°C for 2min	
tel 2 (100µM)	0.9µM	0.09			
Amplified or unamplified sample	Variable	2			

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Comparative C_T ($\Delta\Delta C_T$)' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle. A melt curve was carried out at the end of the experiment using standard conditions provided by the software (temperature used as starting point was the same as the annealing/extension temperature used in the experiment).

Table 2.49: Amplification of *36B4* gene - Protocol A

	Concentration	*Volume for 10µL reaction (µl)*	*Thermocycler Conditions*		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.92	**Initial Denaturation**	95°C for 10min	1
Power SYBR Green PCR Master Mix (2x)	1 x	5	**Denaturation**	95°C for 15s	40
36B4u (100µM)	0.3µM	0.03	**Annealing / Extension**	54°C for 2min	
36B4d (100µM)	0.5µM	0.05			
Amplified or unamplified sample	Variable	2			

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Comparative C_T ($\Delta\Delta C_T$)' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle. A melt curve was carried out at the end of the experiment using standard conditions provided by the software (temperature used as starting point was the same as the annealing/extension temperature used in the experiment).

2.5.2.3 Assessment of SNP probes for inclusion on the developed array

A number of SNP probes were assessed for inclusion on the developed array. The reason for wanting to include SNP probes was to be able to perform embryo fingerprinting.

SNP probes were selected from the commercially available ISCA (International Standard Cytogenomic Array) UPD (uniparental disomy) array v1.0 (OGT, UK) on the basis of a preliminary experiment during which SurePlex amplified products were applied to the microarray. It could be concluded that probes displaying fluorescence were in regions of the genome successfully amplified by SurePlex and therefore suitable for use in conjunction with this WGA method. Selected SNP probes were then placed on a new customised 8x15k array (different from the one developed) to

create a specialist SNP array. In total 1,282 different SNPs were selected from an initial pool of 6,186. The SNP probes were placed on the array in triplicates.

In order to test the new SNP array, aliquots from leftover SurePlex products, amplified from 14 single blastomeres from two previous clinical cases (sections 2.4.1.6.3 and 2.4.1.6.4), were used. The amplified samples were applied on the SNP array along with genomic DNA extracted from the parents. Application of the samples on the array was carried out as described before (2.5.2.1). The data collected were analysed using 'CytoSure Interpret Software' (OGT, UK). The aim of this experiment was to test if the SNP array could be used to confirm parental origin of embryos and also detect non-matching embryos.

2.6 Development of a protocol for measurement of telomere length

2.6.1 Selection of primers and optimisation of PCR conditions

The development of a protocol that will be able to provide a relative quantitation of telomere length in clinical samples was attempted. It was intended that the test could be used in parallel with the aneuploidy screening procedures [based upon array-CGH (aCGH)]. Therefore, the test had to be developed based on the usage of SurePlex amplified products.

For the development of the test various primer sets amplifying different loci in the human genome were tested. Specifically, for telomere amplification the 'tel 1' and 'tel 2' primer set was used (Cawthon 2002) and also the 'telg' and 'telc' primer set was used (Cawthon 2009).

For endogenous control the *Alu* sequence was used which is found in multiple copies on all human chromosomes. A multicopy sequence was used for normalization to avoid any potential issues that could arise when using a single copy gene such as: ADO and aneuploidy. Normalization given by a multicopy gene would not be affected by ADO at a single locus while aneuploidy is not an issue since any change to chromosome copy number and therefore telomere quantity will be reflected by the number of *Alu* sequences as well. Primers ('Alu F', 'Alu R') described by Nicklas and Buel (2006) were used to amplify the multicopy *Alu* sequence.

The amplification protocols developed were modified from the published studies describing each set of primers and were optimised to be applicable on SurePlex WGA products.

2.6.2 Assessment of optimised protocols

In order to assess if the different protocols optimised would be able to provide correctly a relative quantitation of the telomeres in SurePlex WGA samples, an experiment was carried out. The experiment involved the amplification of the two telomere primer sets ('tel 1 - tel 2', 'telg - telc'), the Alu primer set and the 36B4 primer set (section 2.5.2.2.3). Although, the usage of a single copy gene for endogenous control when amplifying single cells has disadvantages as explained before, the 36B4 gene was used in this experiment as endogenous control when amplifying genomic DNAs. This was performed in order to assess results obtained from utilization of *Alu* sequence as an endogenous control (*Alu* being a multicopy sequence was expected to be more challenging to optimise). Results from protocols developed using the two endogenous controls would be expected to agree between them regarding relative quantitation of telomere DNA when genomic DNA is assessed. If disagreement was observed, it would be indicated that developed protocols are not accurate and further optimisation is needed. In that instance, obtained results would help identify the factors responsible for the inaccuracy seen and guide optimisation to the appropriate path.

Genomic DNA and single cells obtained from 5 fibroblast cell lines ('Pink'; 47,XY,+13; 47,XY,+15; 47,XY,+18; 45,X0) were used to carry out this experiment. These fibroblast cell lines were the same as described previously (sections 2.3.1 and 2.4.1). The genomic DNA was used unamplified while a single fibroblast cell from each cell line was amplified with SurePlex according to manufacturer's instructions and used. Protocols used to amplify each set of primers are shown in Tables 2.50-

2.52. Amplification of 'tel1 - tel2' primer set was carried out as described before (Table 2.48).

Results were collected and analysed using 'StepOne Software v2.2.2'. The comparative C_T method (Schmittgen and Livak 2008) was used to determine relative quantitation of telomere DNA. An additional SurePlex WGA sample was used as a reference to calculate fold changes in telomere quantity.

Table 2.50: Amplification of telomere DNA - Protocol B ('telg - telc' primer set)

	Concentration	***Volume for 10µl reaction (µl)***	***Thermocycler Conditions***		
			Stage	*Conditions*	*Cycle No.*
			Initial Denaturation	95°C for 10min	1
PCR grade H_2O	---	2.82	**Denaturation**	94°C for 15s	
Power SYBR Green PCR Master Mix (2x)	1 x	5	**Annealing / Extension**	49°C for 1min	2
telg (100µM)	0.9µM	0.09	**Denaturation**	94°C for 15s	
telc (100µM)	0.9µM	0.09	**Annealing**	62°C for 15s	38
			Extension	74°C for 1min	
Amplified or unamplified sample	Variable	2			

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Comparative C_T ($\Delta\Delta C_T$)' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle starting from the 3rd cycle. A melt curve was carried out at the end of the experiment using standard conditions provided by the software (temperature used as starting point was 62°C).

Table 2.51: Amplification of *Alu* sequence

	Concentration	***Volume for 10µl reaction (µl)***	***Thermocycler Conditions***		
			Stage	*Conditions*	*Cycle No.*
PCR grade H_2O	---	2.82	**Initial Denaturation**	95°C for 10min	1
Power SYBR Green PCR Master Mix (2x)	1 x	5	**Denaturation**	95°C for 15s	30
Alu F (100µM)	0.9µM	0.09	**Annealing / Extension**	60°C for 1min	
Alu R (100µM)	0.9µM	0.09			
Amplified or unamplified sample	Variable	2			

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Comparative C_T ($\Delta\Delta C_T$)' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle. A melt curve was carried out at the end of the experiment using standard conditions provided by the software (temperature used as starting point was the same as the annealing/extension temperature used in the experiment).

Table 2.52: Amplification of *36B4* gene - Protocol B

	Concentration	***Volume for 10µl reaction (µl)***	***Thermocycler Conditions***		
			Stage	*Conditions*	*Cycle No.*
			Initial Denaturation	95°C for 10min	1
PCR grade H_2O	---	2.82	**Denaturation**	94°C for 15s	2
Power SYBR Green PCR Master Mix (2x)	1 x	5	**Annealing / Extension**	49°C for 1min	
36B4u (100µM)	0.9µM	0.09	**Denaturation**	94°C for 15s	38
36B4d (100µM)	0.9µM	0.09	**Annealing**	62°C for 15s	
			Extension	72°C for 1min	
Amplified or unamplified sample	Variable	2			

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Comparative C_T ($\Delta\Delta C_T$)' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle starting from the 3rd cycle. A melt curve was carried out at the end of the experiment using standard conditions provided by the software (temperature used as starting point was 62°C).

2.7 Development of a Real-Time PCR protocol for comprehensive chromosome screening of human embryos

The development of a fast and reliable protocol utilizing Real-Time PCR technology for detection of aneuploidies in human embryos was attempted. For the development of the protocol TaqMan copy number assays (Applied Biosystems, USA) were used. In total, 96 assays were used (Appendix 5) along with a TaqMan copy number reference assay. The copy number assays selected amplified small parts of different genes spread across all human chromosomes. Specifically, 4 TaqMan copy number assays were used per chromosome; 2 assays per chromosome arm, excluding acrocentric chromosomes (13, 14, 15, 21, 22) for which all assays were amplifying sequences located on the same arm. The reference assay used targeted the telomerase reverse transcriptase (TERT) gene located on chromosome 5 (Applied Biosystems, USA). This sequence, like the sequences amplified by the selected copy number assays, is known to exist in two copies in a diploid human genome. The reference assay is required for the relative quantitation of copy number targets since it is used to normalize the results obtained from the copy number assays. Therefore, the reference assay was always amplified together with the copy number assays in a multiplex reaction initially and in duplex reactions eventually. The reference assay was equipped with a different reporter dye from the copy number assays in order to allow the Real-time PCR system used to detect and quantify it separately (Appendix 6).

2.7.1 Testing of protocol on genomic DNA

A protocol was developed and was tested on genomic DNA before tested on single cells. The protocol involved pre-amplification of the samples using TaqMan PreAmp Master Mix (Applied Biosystems, USA) and further amplification using TaqMan Genotyping Master Mix (Applied Biosystems, USA). Specifically, DNA extracted from the following cell lines was used: 47,XY,+13; 47,XY,+18 and 'Pink' (46,XX) (sections 2.4.1 and 2.3.1). For the pre-amplification of samples an assay mix was prepared by combining equal volumes of each copy number assay and the reference assay and diluting them with PCR grade water. The final concentration of each assay in the mix was 0.2x. The pre-amplification reaction was carried out as described in Table 2.53.

Table 2.53: Pre-amplification of genomic DNA with TaqMan copy number and reference assays.

	Concentration	***Volume for 50µl reaction (µl)***
PCR grade H_2O	---	10.5
TaqMan PreAmp Master Mix (2x)	1x	25
Assay mix (0.2x)	0.05x	12.5
DNA (1ng/µl)	---	2

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Enzyme Activation	95°C for 10min	1
Denaturation	95°C for 15s	15
Annealing / Extension	60°C for 4min	
Enzyme Inactivation	99°C for 10min	1

After pre-amplification, a spectrophotometer (Eppendorf Biophotometer Plus v1.04) (Eppendorf, UK) was used to get an estimation of the amplified DNA concentration in each sample. Samples were then diluted in PCR grade water to ~10ng/µl and used to carry out qPCR. Aliquots from the diluted pre-amplified samples were used to amplify each copy number assay in duplex reactions along with TERT reference assay (Table 2.54). Amplification of each assay was carried out in quadruplicates for each sample. Pre-amplified samples from DNA extracted from 47,XY,+13 and 47,XY,+18 cell lines was used to amplify all assays while samples from 'Pink' cell line were only used to amplify assays targeting the X and Y chromosomes. Amplification and data collection was performed through usage of the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Analysis of results was carried out using the 'StepOne Software v2.2.2' initially and eventually the 'CopyCaller Software v1.0' (Applied Biosystems, USA).

Table 2.54: Protocol used to amplify each copy number assay

	Concentration	***Volume for 10µl reaction (µl)***
PCR grade H_2O	---	2
TaqMan Genotyping Master Mix (2x)	1x	5
TaqMan Copy number assay (20x)	1x	0.5
TERT reference assay (20x)	1x	0.5
Pre-amplified DNA	---	2

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Enzyme Activation	95°C for 10min	1
Denaturation	95°C for 15s	40
Annealing / Extension	60°C for 1min	

Notes: For setup of the experiment on the StepOne software, the 'Quantitation - Standard Curve' was used as experiment type (as instructed by Applied Biosystems) and the ramp speed of the instrument was set as 'Standard'. Data collection by the software was taking place at the end of each amplification cycle.

2.7.2 Testing of protocol on single cells

Two single cells representing the type of material obtained from day-3 biopsies and polar body biopsies (i.e. single blastomeres and polar bodies) were tested. The single cells used were derived from the fibroblast cell lines 47,XY,+15 and 45,XO (section 2.4.1). The single cells were lysed using alkaline lysis as described before (section 2.2.3.2). The released DNA was pre-amplified in each sample as described in Table 2.55. The assay mix used in the reaction was prepared as described earlier (section 2.7.1).

Table 2.55: Pre-amplification of released single cell DNA with TaqMan copy number and reference assays.

	Concentration	***Volume for 50µl reaction (µl)***
PCR grade H_2O	---	5
Tricine (0.4M)	40mM	5
TaqMan PreAmp Master Mix (2x)	1x	25
Assay mix (0.2x)	0.05x	12.5
Lysis buffer + released DNA	---	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Enzyme Activation	95°C for 10min	1
Denaturation	95°C for 15s	16
Annealing / Extension	60°C for 4min	
Enzyme Inactivation	99°C for 10min	1

Pre-amplified products were diluted 1:50 in PCR grade water and used to carry out qPCR as shown in Table 2.54. Although all 96 copy number assays were used for pre-amplification, only 48 were processed with qPCR. Specifically, the 48 assays that were found to perform the best on genomic DNA were used to amplify single cell pre-amplified products. Two assays were selected per chromosome located one on each chromosome arm or both on the same arm for acrocentric chromosomes.

Amplification of each assay was carried out in triplicates for each sample. Data collection and analysis of results was carried out as described in section 2.7.1.

2.7.3 Testing of protocol on clumps of cells

In addition to the experiments described above, more experiments were carried out using several isolated cells (simulating the amount of material typically obtained in a trophectoderm biopsy). Lymphocytes were donated for this purpose from a male and a female lab member and were isolated as described before (section 2.2.2.3.1); the only difference being that instead of single lymphocytes groups of 3 lymphocytes were isolated. Lysis and pre-amplification of the lymphocytes was carried out as described in section 2.7.2 (16 cycles were used for pre-amplification). Pre-amplified products were diluted 1:15 in PCR grade water and used to carry out qPCR for each copy number assay as described in Table 2.54. All 96 assays were tested. Each assay was amplified in triplicates for each sample. Six samples were used in total (3 female, 3 male).

2.8 Protocols developed for DNA fingerprinting of clinical samples

Protocols were developed utilizing 12 TaqMan SNP genotyping assays (Applied Biosystems, USA) (Appendix 7) for DNA fingerprinting of sperm samples and embryos with the aim of avoiding sample mix-ups and efficiently detecting contamination. The SNPs detected by the assays were very carefully selected in order to be highly informative (i.e. highly heterozygous) across different human populations and therefore be widely applicable. Specifically, allele frequency data were considered for Caucasian, African, African American, Chinese and Japanese populations and the mean minor allele frequency of all the SNPs selected was >46% (with a maximum of 50%) (Appendix 7). The 12 assays selected were amplifying SNPs located on 12 different chromosomes (autosomes) in order to avoid any linkage possibilities and make sure each SNP would behave as a separate entity when inherited.

All experiments were carried out using a StepOne Real-Time PCR System. Analysis of results was carried out using the 'StepOne Software v2.2.2' and the 'TaqMan Genotyper Software (v1.0.1)' (Applied Biosystems, USA).

2.8.1 Application of SNP assays on sperm samples

In order to be able to fingerprint sperm samples, first DNA had to be extracted. A fast, efficient and inexpensive method of DNA extraction was sought for this purpose.

2.8.1.1 Selection of the best DNA extraction method

Three different DNA extraction methods were optimised and tested on a sperm sample. The sample was stored at -80°C until required. The alkaline lysis extraction method was tested and also the TaqMan Sample-to-SNP kit (Applied biosystems, USA) and QIAamp DNA blood Mini kit were used for DNA extraction.

Alkaline lysis was carried out by adding 3.7µl of PCR-grade water, 4.5µl of 0.5M DTT and 1.8µl of 1M NaOH to 5µl of neat sperm sample for a final volume of 15µl. The sample was then incubated at 60°C for 20 minutes. After incubation, 4µl of 1.6M tricine and 1µl of PCR grade water were added to the sample.

Regarding DNA extraction using TaqMan Sample-to-SNP kit, 50µl of lysis solution were added to 5µl of neat sperm sample and incubation was carried out at 95°C for 3 minutes. The sample was then allowed to equilibrate at room temperature for 30 seconds and 50µl of stabilizing solution were added to it.

The 'Blood and Body Fluid Spin Protocol' was used according to manufacturer's instructions to extract DNA with the QIAamp DNA blood Mini kit. 200µl of neat sperm sample were used.

DNA extracted with the three methods was used to carry out SNP genotyping as described in Table 2.56. DNA extracted with alkaline lysis was diluted 1:50 in PCR

grade water before used. DNA extracted with QIAamp DNA Mini kit was diluted 1:10 while DNA extracted with TaqMan Sample-to-SNP kit was used undiluted.

Table 2.56: Protocol for amplification of TaqMan SNP genotyping assays

	Concentration	*Volume for 10µl reaction (µl)*
PCR grade H_2O	---	2.75
TaqMan Genotyping Master Mix (2x)	1x	5
TaqMan SNP genotyping assay (40x)	1x	0.25
DNA	---	2

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Pre-PCR Read	60°C for 30s	1
Enzyme Activation	95°C for 10min	1
Denaturation	95°C for 15s	variable
Annealing / Extension	60°C for 1min	
Post-PCR Read	60°C for 30s	1

Notes: Each TaqMan SNP genotyping assay was amplified in singleplex reactions using the protocol described in this Table. 50 cycles of amplification were used for all reactions carried out apart for the reactions carried out for DNA extracted using the TaqMan Sample-to-SNP kit. For this, 60 cycles of amplification were used. For setup of the experiment on the StepOne software, 'Genotyping' was used as experiment type and the ramp speed of the instrument was set as 'Standard'. Data collection by the software took place during the pre-PCR read, the post-PCR read and at the end of each amplification cycle.

2.8.1.2 Validation of the developed protocol

Ten sperm samples were used to validate the developed protocol. The concentration of sperm samples used ranged from low to high levels (8.6-168 million/ml). This helped assess the protocol in the most critical way and under conditions that would be met in a real clinical setting.

DNA from each sperm sample was extracted using alkaline lysis and the QIAamp DNA blood Mini kit as described in section 2.8.1.1. DNA extracted using the

QIAamp DNA blood Mini kit was used as the positive control to which results obtained from alkaline lysis extraction would be compared. Amplification of each SNP assay was carried out as described in section 2.8.1.1.

2.8.2 Application of SNP assays on single cells

The amplification of the 12 SNP assays was tested on 20 single cells derived from the following fibroblast cell lines: 47,XY,+13; 47,XY,+18; 47,XY,+21; 45,X0 (section 2.4.1). This was performed in order to assess if the protocol can be applied on clinical samples (e.g. single blastomeres) commonly obtained for PGD of single gene disorders. Genomic DNA extracted from these cell lines was used as positive control.

Alkaline lysis was used to extract the DNA from these cells as described before (section 2.2.3.2). Following the extraction, DNA was pre-amplified in a multiplex PCR including all the assays (Table 2.57). For this reaction an assay mix was prepared by combining equal volumes of each SNP genotyping assay and diluting them with PCR grade water. The final concentration of each assay in the mix was 0.2x.

Table 2.57: Pre-amplification of single cells with TaqMan SNP genotyping assays

	Concentration	*Volume for 25µl reaction (µl)*
PCR grade H_2O	---	1.25
Tricine (0.4M)	40mM	2.5
2x QIAGEN Multiplex PCR Master Mix	1x	12.5
Assay Mix (0.2x)	0.05x	6.25
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	18
Annealing	60°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Pre-amplified products were diluted 1:50 in PCR grade water and were used to carry out real-time PCR for amplification of the SNP genotyping assays as described in Table 2.56 (50 cycles of amplification used).

2.8.3 Application of SNP assays on WGA products

MDA and SurePlex WGA single cell products were used to test amplification of the 12 SNP assays, providing an assessment of whether the DNA fingerprinting protocol could be applied to WGA products (such as those that might be produced during routine aneuploidy screening procedures). MDA and SurePlex were carried out as explained before (section 2.4.1.2) on single fibroblast cells derived from the following cell lines: 47,XY,+13; 47,XY,+15; 47,XY,+18; 47,XY,+21; 45,X0 and 'Pink' (sections 2.3.1 and 2.4.1). Genomic DNA extracted from these cell lines was used as positive control.

In total, 11 MDA products and 13 SurePlex products were assessed. Amplification of the SNP assays was carried out as described in Table 2.56 (50 cycles of amplification used). MDA products were diluted 1:50 and SurePlex products 1:20 in PCR grade water before used.

2.9 Using PLCζ gene to explain infertility problems in males involved in IVF treatment

A previously identified PLCζ mutation (H398P) (Heytens *et al.* 2009) and a newly identified PLCζ mutation (H233L) were investigated in this study. The new mutation was identified in genomic DNA provided by patient^{H398P} (Heytens *et al.* 2009).

Some experiments included in this project were performed by a collaborator. Specifically, all procedures described in section 2.9.1 and DGW of sperm sample described in section 2.9.3 were performed by Dr. Junaid Kashir (Nuffield Department of Obstetrics and Gynaecology, University of Oxford, UK). The rest of the experiments described in this project were carried out by M.K.

2.9.1 Sequencing PLCζ gene in infertile males

To assess whether the PLCζ gene mutations described in patient^{H398P} are responsible for deficiencies of oocyte activation in other infertile men, the nucleotide sequence of each exon of the PLCζ gene was determined in 9 infertile males (including patient^{H398P}) whose sperm was found to lack oocyte activation ability. All of these patients were undergoing fertility treatment at the Department of Reproductive Medicine, Ghent University Hospital, Belgium.

Buccal samples were obtained from the patients and DNA extraction was carried out by using the Oragene DNA Self-Collection Kit (DNAgenotek, USA) following the manufacturer's instructions. PCR amplification of the PLCζ gene was carried out on the extracted DNA samples as described previously (Heytens *et al.* 2009) and the

sequence of the amplified nucleotides was determined (Geneservice, UK). Results were compared to the published wild type PLCζ open reading frame sequence (accession number NM_033123). Procedures were performed by Dr. J Kashir.

2.9.2 Design of primers and SNP genotyping assays

Primers were designed as described before (section 2.2.2.2.1) in order to be used for the detection of the H398P and H233L mutations in the PLCζ gene (Appendix 8). Additionally, two custom TaqMan SNP genotyping assays were developed using the 'Custom TaqMan assay design tool' (Applied Biosystems, USA) for detection of the two mutations.

2.9.3 Mutation detection in single sperm and genomic DNA

To confirm whether the two mutations detected in patient patient^{H398P} are situated on the same parental chromosome or different chromosomes, and to gain an insight into the mode of inheritance, single sperm were tested for the presence of the two mutations. A sperm sample was obtained from patient^{H398P} and was used for isolation of single sperm. The sample was subjected to DGW through the usage of PureSperm 40/80 (Nidacon International AB, Sweeden); procedure was carried out by Dr. J Kashir. Subsequently, single spermatozoa were isolated as described before (section 2.2.2.3.1.2). Isolated sperm along with extracted DNA from patient^{H398P} and 4 members of his family (mother, father, half-brother, and daughter – conceived by ICSI and using an assisted oocyte activation methodology), were used to determine the inheritance pattern of the two mutations (i.e. if the two mutations are located on

the same PLCζ allele or on two different alleles of patient^{H398P}). Mutation detection was carried through minisequencing. Additionally, DNA extracted from the four family members was used for sequencing as described in section 2.9.1.

Single sperm was lysed using alkaline lysis as described before (section 2.2.3.2) with a few changes (0.75µL of 0.5M DTT were used instead of 0.1M; incubation was carried out at 60°C for 20 minutes instead of 10 minutes). Samples were processed as described in Table 2.58.

Table 2.58: Protocol used for detection of H398P and H233L mutations

	Concentration	***Volume for 15µl reaction (µl)***
PCR grade H_2O	---	2.9
Tricine (0.4M)	40mM	1.5
2x QIAGEN Multiplex PCR Master Mix	1 x	7.5
H398P outer F+R (10µM)	0.2µM	0.3
H233L outer F+R (10µM)	0.2µM	0.3
Lysis buffer + released DNA	Variable	2.5

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Initial Denaturation	95°C for 15min	1
Denaturation	94°C for 30s	55
Annealing	58°C for 90s	
Extension	72°C for 60s	
Final Extension	60°C for 10min	1
Hold	4°C for ∞	1

Notes: Two separate inner reactions were carried out as described in Table 2.2 (annealing temperature: 58°C, 40 cycles) using 1µl of amplified outer reaction product. The H398P outer F - inner R primers and the H233L inner F - outer R primers (Appendix 8) were used to carry out the inner reactions. Minisequencing was performed as described in section 2.2.3.3.3 (ExoSAP-IT was used) in two separate reactions using the H398P Mini F primer and the H233L Mini F primer (Appendix 8).

2.9.4 Screening of 100 individuals for H398P and H233L mutations

In order to determine whether the variants identified in patient^{H398P} are polymorphisms present in the general population or should be considered to be mutations, 100 individuals were anonymously screened for carriage of the H398P and H233L mutations. Blood samples were obtained and DNA was extracted as described before (section 2.2.2.3). Extracted DNA was used to carry out SNP genotyping reactions for the two mutations as described in Tables 2.59-2.60. DNA samples derived from the patient and his family members (section 2.9.3) were used as positive control in the reactions carried out.

Table 2.59: Protocol used for H398P TaqMan SNP genotyping assay

	Concentration	***Volume for 5µl reaction (µl)***
PCR grade H_2O	---	1.375
TaqMan GTXpress Master Mix (2x)	1x	2.5
H398P TaqMan SNP genotyping assay (40x)	1x	0.125
DNA	---	1

Thermocycler Conditions		
Stage	*Conditions*	*Cycle No.*
Pre-PCR Read	25°C for 30s	1
Enzyme Activation	95°C for 20s	1
Denaturation	92°C for 3s	35
Annealing / Extension	62°C for 20s	
Post-PCR Read	25°C for 30s	1

Notes: For setup of the experiment on the StepOne software, 'Genotyping' was used as experiment type and the ramp speed of the instrument was set as 'Fast'. Data collection by the software took place during the pre-PCR read, the post-PCR read and at the end of each amplification cycle.

Table 2.60: Protocol used for H233L TaqMan SNP genotyping assay

	Concentration	***Volume for 5µl reaction (µl)***	***Thermocycler Conditions***		
			Stage	*Conditions*	*Cycle No.*
			Pre-PCR Read	25°C for 30s	1
PCR grade H_2O	---	1.375	**Enzyme Activation**	95°C for 20s	1
TaqMan GTXpress Master Mix (2x)	1x	2.5	**Denaturation**	95°C for 3s	40
H233L TaqMan SNP genotyping assay (40x)	1x	0.125	**Annealing / Extension**	60°C for 20s	
			Post-PCR Read	25°C for 30s	1
DNA	---	1			

Notes: For setup of the experiment on the StepOne software, 'Genotyping' was used as experiment type and the ramp speed of the instrument was set as 'Fast'. Data collection by the software took place during the pre-PCR read, the post-PCR read and at the end of each amplification cycle.

2.10 Statistical analysis

Whenever appropriate, statistical analysis was carried out for samples included in the different projects described in this thesis. Depending on the nature of the data being analysed, different statistical tests were used including: Fisher's Exact Test (GraphPad, USA), Chi-Square Test, Mann-Whitney U Test and Independent-samples T Test (SPSS v19.0.0, USA).

3) Results

3.1 PGD - Clinical cases

3.1.1 Development of PGD protocols

Development of the PGD protocols presented in this thesis involved primer design, literature review and search of online databases for the selection of informative linkage markers, optimisation of the PGD method, validation of the developed protocol and finally, clinical application. As an example, results from the development of the PGD protocol for the Tuberous Sclerosis 1 case (c.1525C>T) are presented (Appendix 9). In brief, initially primers amplifying the DNA sequence encompassing the mutation were designed. After linkage markers were selected as well, primers were ordered. When primers were received, a gradient PCR was carried out for each primer set to determine the optimal annealing temperature for the primers in terms of amplification efficiency and specificity. Eventually this allowed determination of the most appropriate annealing temperature for the multiplex PCR. The linkage markers were then tested for informativity on DNA obtained from family members. Additionally, the amplified DNA fragment encompassing the mutation site was tested to verify that the mutation could be clearly detected. Following, development of the multiplex PCR was undertaken on single cells. Finally, amplification was attempted using single lymphocytes from the patients, verifying the performance of the protocol on cells carrying the specific familial mutation(s).In general, development of a multiplex PCR involved the modification of multiple parameters such as primer concentration, annealing temperature and the types of polymerase and associated buffers used. An average of 3 months was required for the development of a novel multiplex PGD protocol.

3.1.2 Performance of clinical cases

Forty-six PGD cycles were carried out over a period of 32 months. In total, 37 couples underwent PGD. Most of the couples (29) went through one cycle of PGD. Some couples however went through 2 cycles of PGD (7) and one couple underwent 3 cycles. Maternal age ranged from 27 to 41 years (average 33.3 ± 0.5 years). For most of the PGD cycles (40) women were ≤37 years old but for some cycles (6) women were of AMA (>37 years).

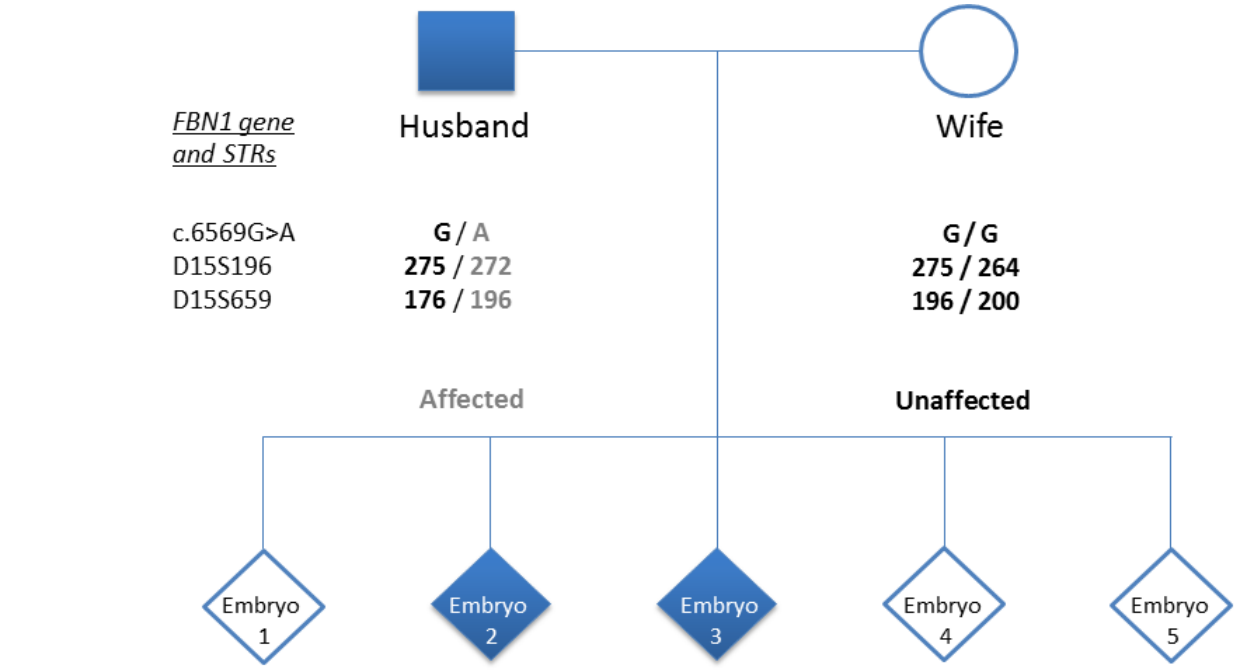
Most of the cycles carried out (35/46) were of the 'transport PGD' category. This involves the biopsy of the embryos in IVF laboratories distant from the PGD lab and the shipping of the biopsied cells to the PGD lab to perform the test. The transportation time ranged from 1.5-3 hours with an average of 2 hours. PGD cases were sent to the laboratory from 6 different IVF centres. Diagnosis was completed 22-24 hours after receipt of samples.

3.1.2.1 Protocols developed

A large portion of the PGD protocols developed involved direct mutation detection (27/31) (Figure 3.1). Only a small number of protocols utilized linkage analysis alone to make diagnosis (3/31) (Appendix 10), while one of the protocols developed utilized embryo sexing alone to perform PGD for an X-linked disorder. In one occasion, PGD for a single gene disorder was combined with comprehensive aneuploidy screening (Appendix 10). In two occasions where DNA from family members was not available and linkage analysis was desired, single sperm from the affected husband was isolated and amplified in order to construct a haplotype (Figure 3.1).

Figure 3.1: PGD carried out for Marfan Syndrome (c.6569G>A). **A)** Pedigree analysis. **B)** Results from linkage markers used in protocol. **C)** Minisequencing results.

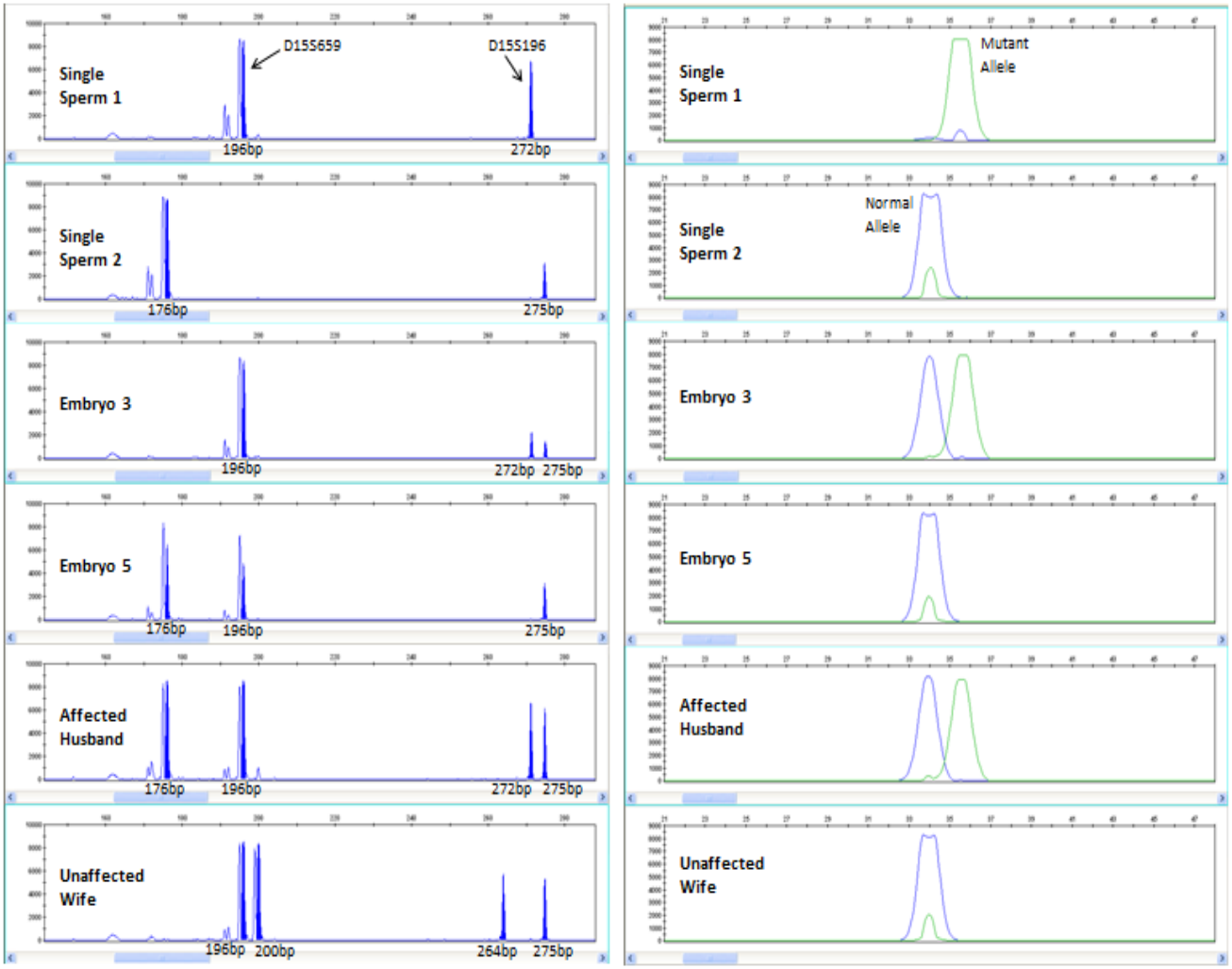
A) Pedigree analysis



FBN1 gene and STRs

	Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
c.6569G>A	G / G	G / A	G / A	G / G	G / G
D15S196	275 / 264	275 / 272	275 / 272	275 / 275	275 / 275
D15S659	176 / 200	196 / 196	196 / 196	176 / 196	176 / 196
	Unaffected	Affected	Affected	Unaffected	Unaffected

B) Results from linkage markers used in protocol. **C)** Minisequencing results.



Notes: Single spermatozoa isolated from the affected husband were used in this case to determine which alleles of the two linkage markers were inherited along with the mutant allele of *FBN1* gene. As seen above, 196bp and 272bp alleles for STRs D15S659 and D15S196 respectively, are inherited along with the mutant allele (single sperm 1). The remaining STR alleles are inherited along with the normal *FBN1* allele carried by the husband (single sperm 2). Based on these results embryo 3 was found to be affected and embryo 5 unaffected.

3.1.2.2 Number of embryos tested, contamination and aneuploidy

Overall, 233 embryos were tested (mean: 5.1 ± 0.5 embryos/cycle; range: 1-15 embryos/cycle) either directly (embryo biopsy) or indirectly (PB testing) (Table 3.1). Diagnosis was achieved for 86.7% (202/233) of the embryos. The main reason for no diagnosis was total amplification failure (TAF) of the biopsied cell whereby no

amplification took place for any of the loci included in the PGD protocol. TAF was responsible for 67.7% (21/31) of the embryos with no diagnosis. For the rest of the undiagnosed embryos, contamination was the cause for 6, while for 4 of them inconclusive results were obtained (i.e. results of linkage markers did not agree with results from mutation detection - possible ADO and/or recombination).

In total, contamination was detected in 9 of the 233 embryos tested (3.9%). In three embryos contamination was detected through the observation of an unexpected STR allele in the biopsied cell. For the rest of the embryos contamination was detected in the cell-specific negative control.

Suspected aneuploidy (i.e. monosomy or trisomy of the tested chromosome) was detected in 15 of the 202 diagnosed embryos (7.4%). Aneuploid embryos were considered separately for women of younger reproductive age (≤37 years) and women of AMA (>37 years). It was concluded that the incidence of aneuploidy was higher in women of AMA (2/22, 9.1%) compared to women of younger reproductive age (13/180, 7.2%), although no significant difference could be calculated ($P>0.05$). It is important to note that there is the possibility that some of the trisomy incidences observed might be due to contamination of samples with parental DNA and not due to aneuploidy; this resulting in a lower aneuploidy rate than the one reported.

Table 3.1: PGD cases – Overall data and clinical outcome

Disease*	**No. of embryos analysed**	**No. of biopsy specimens analysed**	**Amplification Efficiency**			**Embryos Diagnosed**	**No. of transfers / No. of embryos****	**Clinical Outcome / embryo transfer****				
			Loci Tested	*Loci amplified*	*Samples with TAF**			*Biochemical Pregnancy*	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
A/D Hyper IgE Syndrome	6	6	18	15	1	5	N / A*	---	---	---	---	---
A/D PKD	7	7	28	28	0	7	0 / 7	---	---	---	---	---
Alpha-1-antitrypsin Deficiency	6	6	18	9	2	3	2 / 6	0	0	0	0	0
BOR Syndrome	8	8	24	18	2	6	3 / 8	2	1	2	0	0
Cystic Fibrosis	24	24	72	62	3	19	2 / 6	1	1	0	0	1
DDEB	14	14	42	42	0	14	2 / 12	0	0	0	0	0
DMD	10	10	50	50	0	10	N / A	---	---	---	---	---

Table 3.1 (continued)

Disease*	No. of embryos analysed	No. of biopsy specimens analysed	Amplification Efficiency			Embryos Diagnosed	No. of transfers / No. of embryos**	Clinical Outcome / embryo transfer**				
			Loci Tested	*Loci amplified*	*Samples with TAF**			*Biochemical Pregnancy*	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
EDS 4	2	2	4	4	0	2	2 / 2	0	0	0	0	0
HME	6	6	12	10	1	4	2 / 6	2	2	1	0	1
Huntington Disease	9	9	42	37	0	9	2 / 6	0	0	0	0	0
Krabbe Disease	12	18	54	48	2	10	0 / 6	---	---	---	---	---
Marfan Syndrome	16	17	48	45	1	15	3 / 8	1	1	0	0	1
MDS	5	5	20	20	0	5	2 / 5	0	0	0	0	0
NF1	21	21	72	69	1	19	4 / 20	2	1	1	0	1
OPD Syndrome	3	3	9	9	0	3	N / A	---	---	---	---	---

Table 3.1 (continued)

Disease*	No. of embryos analysed	No. of biopsy specimens analysed	Amplification Efficiency			Embryos Diagnosed	No. of transfers / No. of embryos**	Clinical Outcome / embryo transfer**				
			Loci Tested	*Loci amplified*	*Samples with TAF**			*Biochemical Pregnancy*	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
POMGNT1 - muscular dystrophy	11	11	44	44	0	10	1 / 11	0	0	0	0	0
Sickle Cell Anaemia	29	29	116	112	1	26	2 / 14	1	1	0	1	0
SMA type I	9	9	45	25	4	5	1 / 9	1	1	1	0	0
Thal – β	15	15	30	23	3	12	3 / 15	1	1	0	0	1
Tuberous Sclerosis 1	9	9	25	25	0	8	4 / 9	1	1	0	0	1
X-linked Hydro-cephalus	11	11	34	34	0	10	1 / 5	0	0	0	0	0

Table 3.1 (continued)

Disease*	No. of embryos analysed	No. of biopsy specimens Analysed	Amplification Efficiency			Embryos Diagnosed	No. of transfers / No. of embryos**	Clinical Outcome / embryo transfer**				
			Loci Tested	*Loci amplified*	*Samples with TAF**			*Biochemical Pregnancy*	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
Total	**233**	**240**	**807**	**729 (90.3%)**	**21**	**202 (86.7%)**	**36 / 155**	**12 (33.3%)**	**10 (27.8%)**	**5**	**1**	**6**

* TAF: total amplification failure; N/A: not available; A/D PKD: autosomal dominant polycystic kidney disease; BOR: branchio-oto-renal; DDEB: dominant dystrophic epidermolysis bullosa; DMD: Duchenne muscular dystrophy; EDS: Ehlers-Danlos Syndrome; HME: hereditary multiple exostoses; MDS: mitochondrial DNA depletion syndrome; NF1: neurofibromatosis type I; OPD: Oto-Palatal-Digital; SMA: spinal muscular atrophy; Thal - β: thalassaemia beta.

** IVF centres provided results regarding clinical outcome only for some of the cases. Therefore results included in these columns are not for the overall but for a subset of cases.

3.1.2.3 Diagnostic accuracy, amplification efficiency and ADO

Diagnostic accuracy for vast majority of the diagnosed embryos was estimated to range from 95% to >99% based upon the number of loci (e.g. linked polymorphisms) simultaneously tested and the ADO rates determined during work-up. In a limited number of embryos (3) however, accuracy of diagnosis was lower ranging from 80% to 90%. Reduced accuracy was usually due to failure of some of the diagnostic loci (mutation sites or linked polymorphisms) to amplify. No misdiagnosis has been reported to date, although only 6 children have been born and tested so far.

In total, 807 loci found interspersed across 16 chromosomes of the human genome were tested. Overall amplification efficiency (AE) was 90.3% (729/807). Excluding samples with TAF, which is usually due to poor quality DNA or cell loss during transfer to the PCR tube, the AE was 98.5%.

Apart from AE the ADO rate was also calculated from the different cases carried out. The loci for which ADO could be assessed were determined to be 385. The overall ADO rate was calculated to be 5.2% (20/385).

3.1.2.3.1 Amplification efficiency and ADO rates in relation to fragment length

In order to investigate possible causes for reduced AE and increased ADO rates in single cells, the AE and ADO rates of a number of fragments with differing lengths amplified in single cells were determined. Specifically, AE and ADO rates of different loci amplified during validation of the developed PGD protocols on single lymphocytes were used to make this analysis. Thirty and 28 different loci located in 8 human chromosomes were used to make correlations between AE and fragment

length and between ADO and fragment length, respectively. In total, 1,013 loci were assessed for AE and 856 loci were assessed for ADO (Table 3.2). The fragments tested ranged from 94bp to 319bp in length.

Table 3.2: Fragment length of amplified products in relation to amplification efficiency and ADO.

Fragment Length (bp)	Amplification Efficiency		ADO	
	No. of loci tested	*Rate (%)**	*No. of loci tested*	*Rate (%)**
≤100	29	100^{A}	29	0^{a}
101 – 200	526	99^{A}	429	4.9^{a}
201 – 300	397	97.7^{A}	339	6.2^{a}
>300	61	96.7^{A}	59	15.3^{b}
Total	1013	---	856	---

* Differing superscripts (**e.g. a**,**b**) in each column indicate significant differences ($P<0.05$) in the values obtained for AE and ADO between different ranges of fragment length. No significant difference was observed regarding AE. Contrary, significant difference was observed regarding ADO rates.

It was concluded from results obtained that ADO rates were highly correlated with fragment size. Specifically, ADO rates in single cells were directly proportional to fragment length (i.e. ADO rates increase with increasing fragment length) (Figure 3.2). ADO rates were found to be significantly higher in fragments of >300bp than fragments of ≤300bp (Table 3.2). In contrast, AE was not found to be highly correlated to fragment length. Although there was a steady trend towards decreased AE rates with increasing fragment length, the change was too subtle to be considered statistically significant given the number of loci tested in this study (Figure 3.3, Table 3.2).

Figure 3.2: Relationship of fragment length with ADO rate.

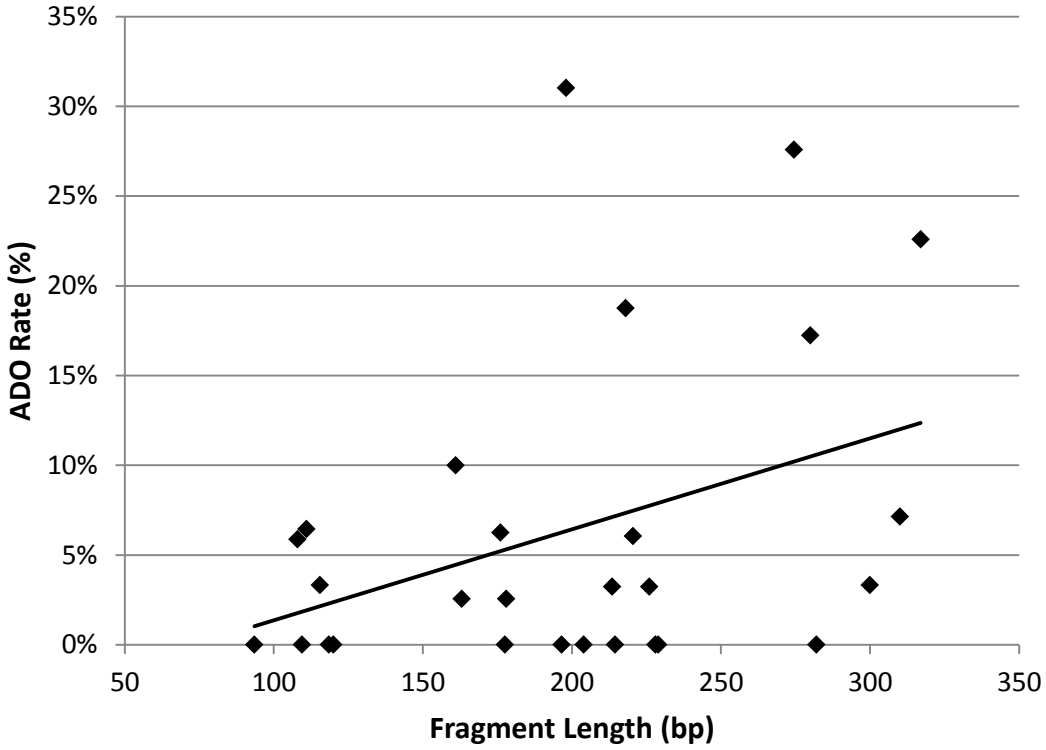
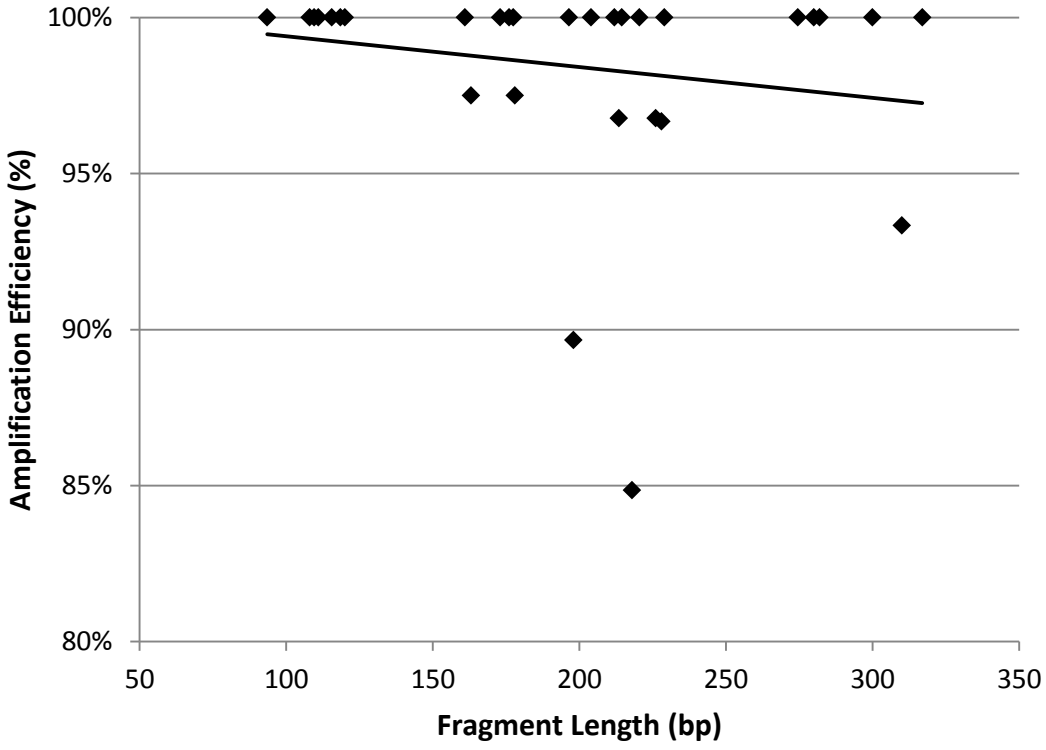


Figure 3.3: Relationship of fragment length with amplification efficiency.



3.1.2.3.2 Comparison of amplification characteristics between blastomeres and lymphocytes

Amplification efficiency and ADO rates for 27 loci for which data were available from both, single blastomeres (through cases) and single lymphocytes (through protocol validations), were calculated. The AE of single blastomeres for these loci was determined to be 98.6% (281/285) and the ADO rate was 4.4% (8/182). For single lymphocytes AE was 98% (902/920) while ADO rate was 5.9% (49/825). The AE and ADO rates between the two types of cells were found to be very similar and in fact no significant difference was calculated ($P>0.05$).

3.1.2.4 Clinical outcome

Data regarding clinical outcome were provided by IVF centres and were available only for a subset of cases. Specifically, results were available for 155 embryos out of the 233 tested (Table 3.1). A total of 36 embryos were transferred. Of the 36 embryos transferred 12 (33.3%) implanted giving positive human chorionic gonadotropin (hCG) levels approximately two weeks after the transfer took place. Ten of these embryos continued to form clinical pregnancies confirmed with foetal sacs and heart beat (27.8%) while two failed early after implantation. Three more pregnancies miscarried later into pregnancy. The reason for one of the miscarriages seen was confirmed to be aneuploidy. Specifically, the tissue from the foetus was found to be trisomic for chromosome 22. Six out of the seven ongoing pregnancies resulted in live birth, while one is in the third trimester, but yet to deliver.

3.2 HLA typing

3.2.1 Protocol optimisation

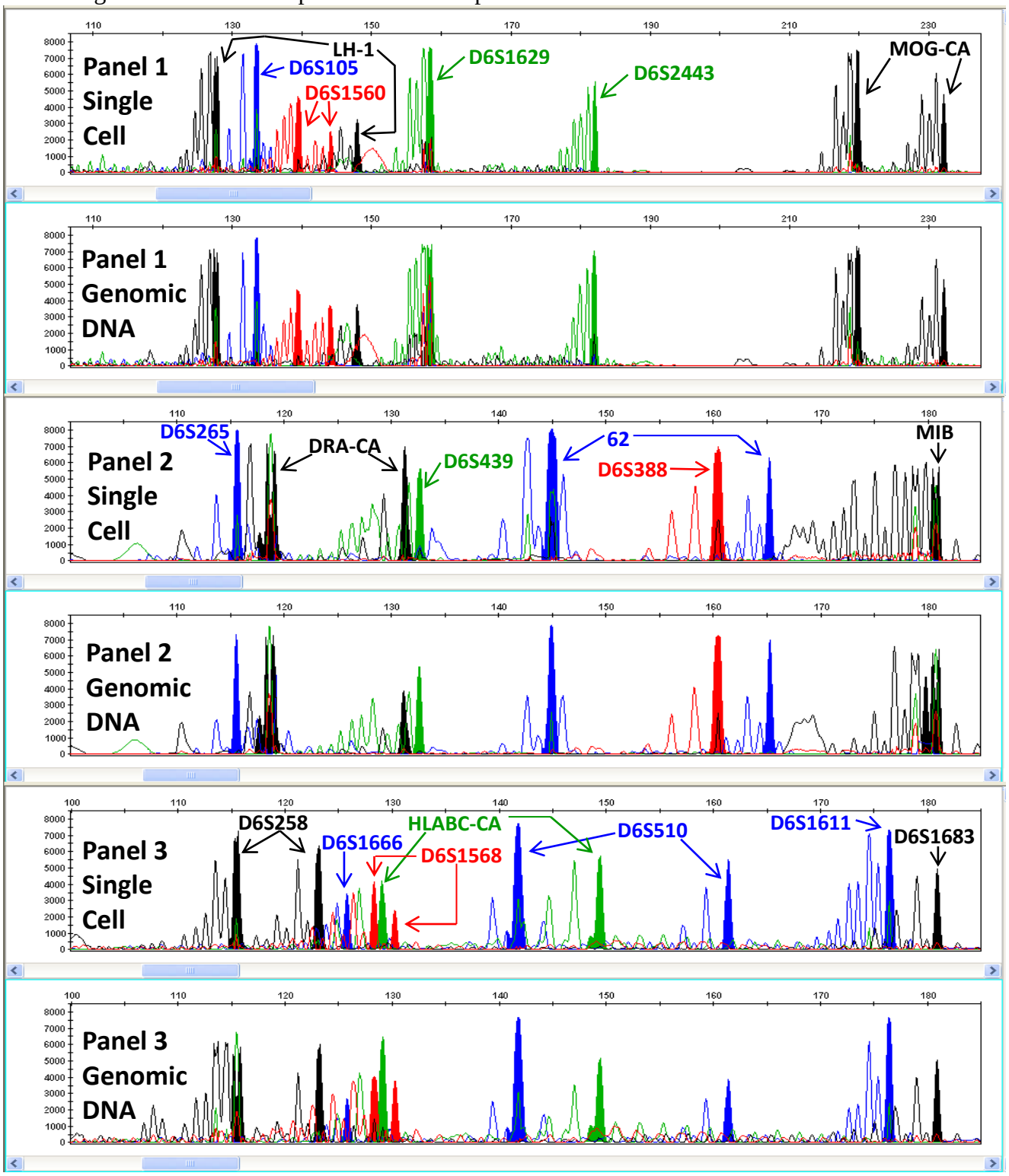
Initially, three different amplification strategies (direct PCR and two whole genome amplification methods: MDA and SurePlex) were tested for amplification of 24 STR markers located in the HLA region. Three single fibroblast cells were tested for each method. After initial multiplex PCR (for the direct PCR method) and WGA were carried out, the amplified product was used to carry out singleplex reactions for each marker. Amplification characteristics for each marker were investigated and it was eventually decided to remove three of the STR markers (D6S1615, D6S1583 and TNF-β) from protocol optimisation due to problematic amplification and difficulty in interpreting results obtained from their amplification.

Amplification efficiency and ADO rates were determined for the remaining 21 STR markers for all three amplification methods. In terms of amplification efficiency the best method was MDA (100%), followed by direct PCR (98%) and SurePlex (78%). Regarding ADO, direct PCR had the lowest rate (0%), followed by MDA (25%) and then SurePlex (43%). Taking into consideration these results it was decided to use the direct PCR method for development of the HLA-typing protocol.

Optimisation of the protocol was a lengthy and challenging procedure that involved many modifications of different variables such as annealing temperature, primer concentrations and PCR cycle number. During development of the protocol it was decided to remove two more STR markers (DQCAR, DQCARII) from the protocol because they produced inconsistent results. Therefore the final protocol contained 19 STR markers simultaneously amplified from a single cell (Figure 3.4). The included

STR markers are interspersed throughout the entire HLA region on chromosome 6 (Figure 2.4), providing an excellent opportunity to detect any recombination. The high number of markers means that the protocol should be informative for virtually all families seeking HLA matching.

Figure 3.4: Electropherograms showing the final protocol developed for HLA- typing containing 19 STR markers separated into three panels.

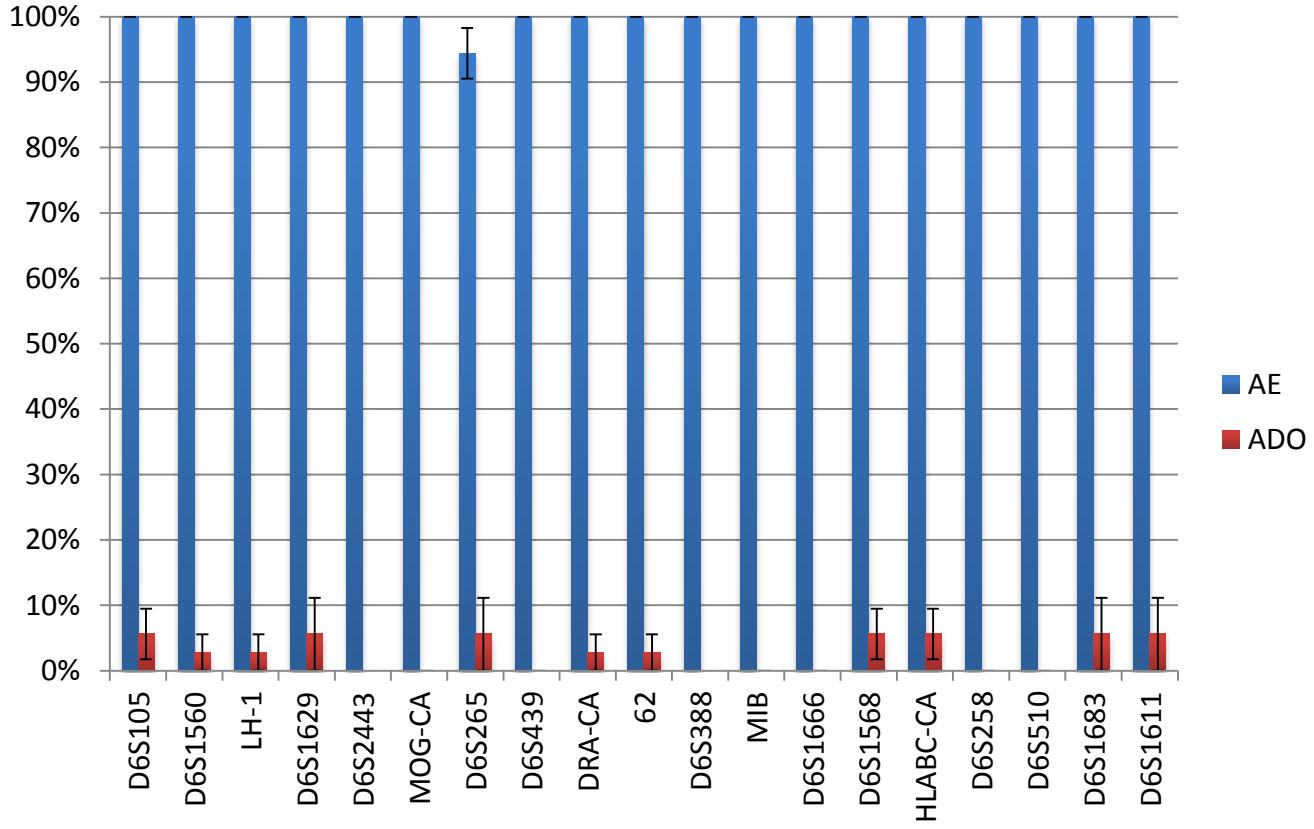


Notes: The different STRs are initially amplified altogether in a multiplex outer reaction. Following, the STRs are amplified to detectable levels through performance of three separate inner reaction multiplexes, each multiplex reaction amplifying a different set of STRs (section 2.3.2). As seen from these electropherograms, amplification of the STRs is very similar between single cells and genomic DNA.

3.2.2 Validation of final protocol

The final protocol was validated on 36 single lymphocytes isolated from two individuals (18 from each). The overall AE was calculated to be 99.7% (682/684 loci) while the ADO rate was determined to be 3.1% (14/450 heterozygous loci). The AE and ADO rates were also calculated for each marker individually in order to determine the performance of the multiplex PCR for each locus (Figure 3.5). ADO rates were not available for 2/19 markers (D6S2443, D6S388) because the 2 individuals from whom the single cells were derived from were homozygous for these loci. As seen in Figure 3.5 all STR markers included in the developed protocol performed equally well giving high amplification efficiency and low ADO rates.

Figure 3.5: Amplification efficiency and ADO rates of each STR marker included in the final HLA-typing protocol.



3.2.3 Clinical cases

Three PGD/HLA-typing clinical cases were carried out in the USA using the newly developed HLA typing protocol. Two of the cases were carried out for HLA typing alone while the third case was combined with PGD for an autosomal recessive disease (Mucopolysaccharidosis type 1) and also gender determination. During test development for the latest it was determined that the fragment sizes of two of the STRs (D6S1666, D6S510) included in Panel 3 of the protocol were overlapping (Tables 2.36, 2.37). Therefore, one of the STRs was removed from the panel and was amplified in a singleplex reaction. In future cases, this can be avoided by simply changing the fluorescent dye used to label the primers selected for amplification of the D6S510 STR from '6-FAM' to 'NED'.

In total, 13 embryos were tested 10 of which were given a diagnosis (Table 3.3). The reason for no diagnosis for the three embryos was TAF. From the 10 embryos given a diagnosis two were found to be chromosomally abnormal since they were found to have inherited only one copy of chromosome 6 (paternal or maternal). From the remaining eight embryos, seven were for HLA-typing alone. From these, only one embryo was found to be compatible with an existing sibling that was affected with acute myelogenous leukaemia and was in need of transplantation (Figure 3.6). The remaining diagnosed embryo was for the case for which HLA typing was combined with PGD for a single gene disorder and gender determination. The specific embryo was found to be non-compatible with the existing affected sibling and also affected with the disorder and was therefore unavailable for transfer.

Table 3.3: Overall data from PGD/HLA-typing cases carried out

	No. of embryos analysed	**No. of biopsy specimens analysed**	**Amplification Efficiency**			**Embryos Diagnosed**
			Loci Tested	*Loci amplified*	*Samples with TAF*	
HLA typing	11	11	150	108	3	8
HLA typing – Rebiopsy	2[†]	2	28	14	1	1
HLA typing + MPS1* + Gender determination	2	2	42	20	1	1
Total	**13**[†]	**15**	**220**	**142 (64.5%)**	**5**	**10 (76.9%)**

* MPS1: Mucopolysaccharidosis type 1

[†] These two embryos were not included in the total number of embryos analysed since these were re-biopsied and they were no new embryos.

3.2.3.1 Amplification efficiency and ADO rates

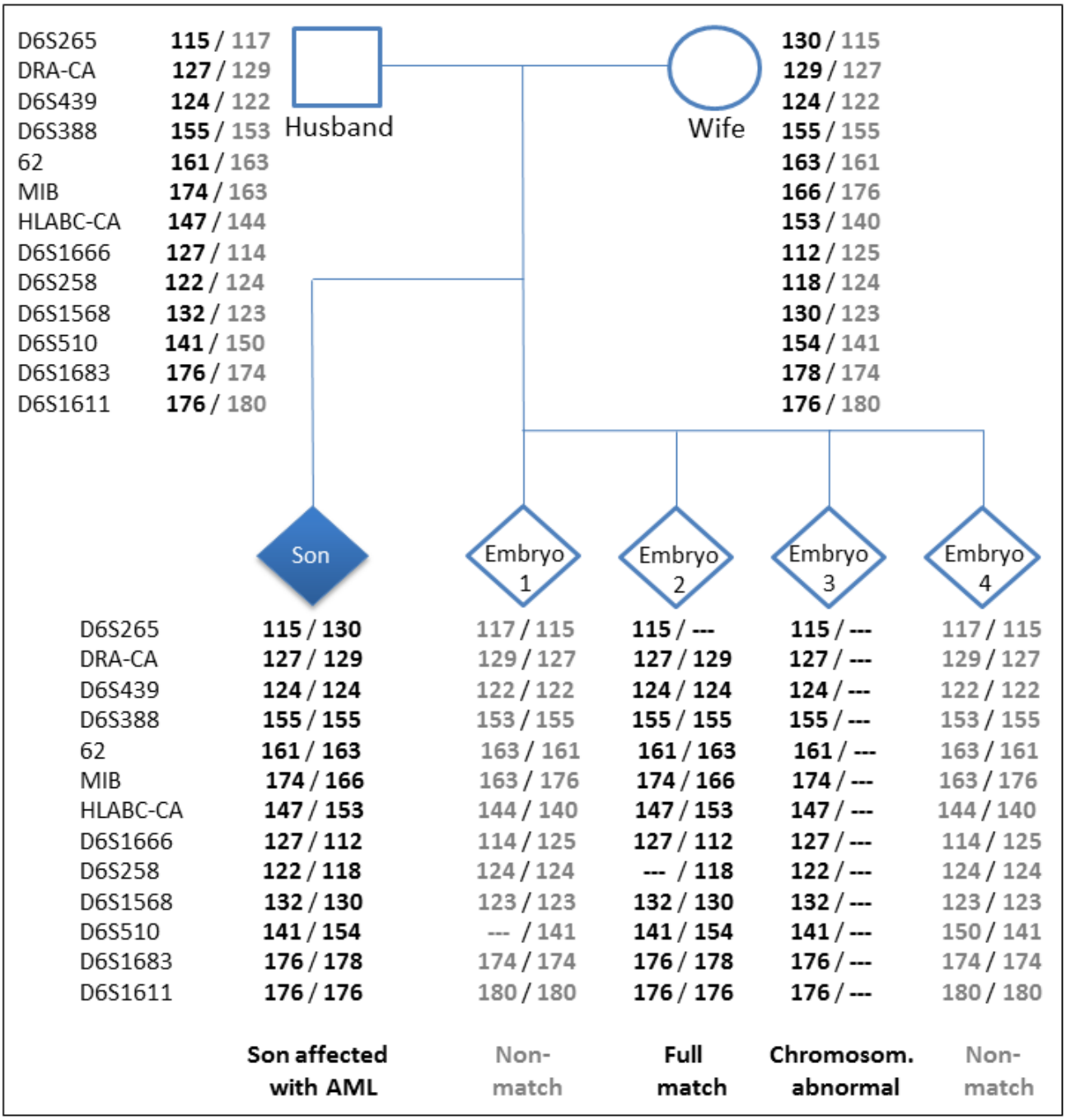
In total, 220 loci were tested (Table 3.3). From these, 142 amplified (64.5%). However, when the samples that gave TAF were excluded from calculations the AE was calculated to be 99.3% (142/143). It should be noted that the collaborating laboratory (Reprogenetics LLC) that carried out the clinical cases only scored results from a subset of markers included in the developed protocol. They only scored results from markers that they considered useful and informative enough for each case. Therefore, results presented here are based only on the markers scored by Reprogenetics LLC.

When only single blastomeres were considered, AE was determined to be 100% (108/108) - excluding cells that gave TAF. Amplification efficiency from loci amplified from trophectoderm cells was found to be 97.1% (34/35) - excluding samples with TAF.

Regarding overall ADO rate, this was calculated to be 7.1% (7/99). When separated into single blastomeres only, the ADO rate was 7.7% (5/65). The ADO rate for trophectoderm samples was 5.9% (2/34).

The AE and ADO rates calculated from the clinical cases for single blastomeres, were found to be similar to the rates obtained from validation of the developed protocol on single lymphocytes ($P>0.05$).

Figure 3.6: Results from an HLA typing case



Notes: In total, 13 STRs were used to make diagnosis for this case. Four embryos were tested from which two were found to be non-compatible with the existing son, one was chromosomally abnormal (haploid or monosomic for chromosome 6) and one embryo was found to be compatible.

3.3 Whole genome amplification

3.3.1 Concentration measurement and fragment length of WGA products

Concentration measurements were taken for WGA products before processing and after processing. As described in section 2.4.1.2, products were processed with one of three methods - column purification using the QIAamp DNA blood MiniKit (described as 'Qiagen column purification' from this point), column purification using the GeneElute PCR Clean-Up Kit (described as 'Sigma column purification' from this point) and precipitation.

As shown in Table 3.4, generally, DNA concentration was significantly higher ($P<0.05$) for non-processed products than for processed products for all 3 WGA methods. Furthermore, samples processed with precipitation were found to have significantly higher ($P<0.05$) DNA concentration than samples processed with column purification (Qiagen or Sigma).

DNA concentration measurements were also taken for non-processed WGA negative controls. The measurements received were very similar to the measurements received for WGA non-processed single-cell samples, suggestive of contamination. However, PCR amplification of these negative controls showed no amplification for any of the loci tested in any of the samples. Therefore, it was concluded that the negative controls were actually free of any contaminating human DNA.

In addition to concentration measurements, measurements of the 260/280 ratio and the 260/230 ratio were taken using Nanodrop in order to assess the purity of the amplified samples (Table 3.4) before application to microarrays. In general it is

indicated by different microarray manufacturers that the 260/280 ratio should be ≥1.8 while the 260/230 ratio should be ≥1.9 in order for the DNA to be of sufficient purity for microarray analysis (i.e. free of contaminating proteins and organic compounds) (Agilent Technologies 2007; Roche NimbleGen 2009). From the measurements obtained it is concluded that Sigma column purification provided the best purification for all 3 WGA methods although precipitation was also close to fulfilling the criteria mentioned above. Interestingly, MDA unprocessed products were also found to have appropriate 260/230 and 260/280 ratios for DNA microarray application; this indicating that MDA products might not need any processing/purification before applied on a microarray platform.

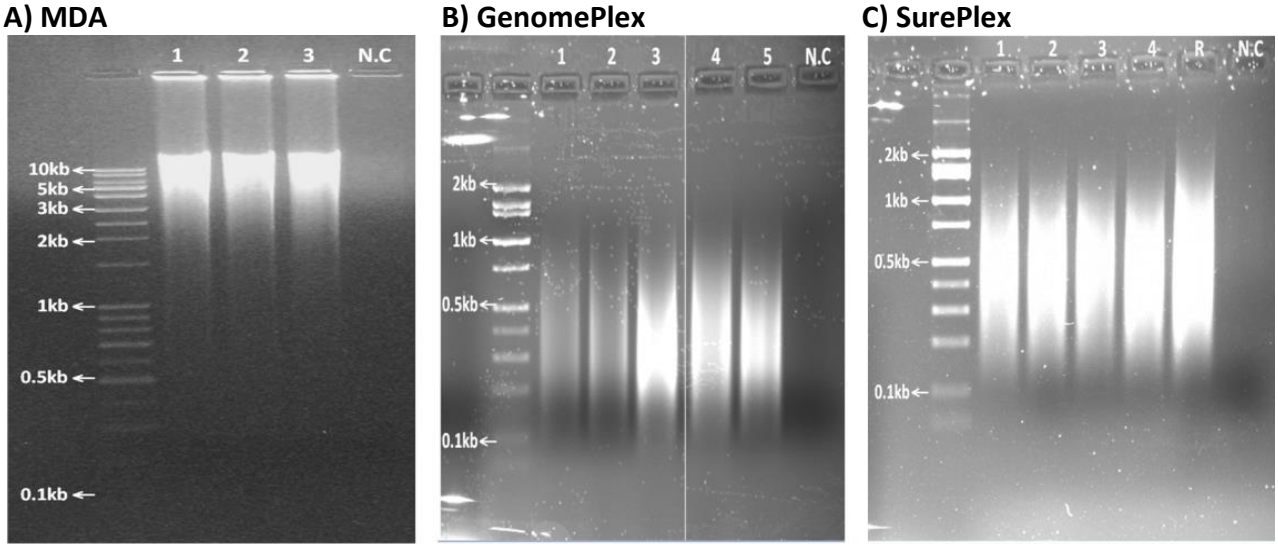
Table 3.4: Concentration and purity of processed and non-processed WGA DNA derived from single cells.

WGA Method	Processing Method*	Concentration (ng/µl)**	260/280 ratio	260/230 ratio
MDA	*Qiagen*	22.05[a]	---[†]	---[†]
	Sigma	126.87[b]	1.88	2.09
	Precipitation	301.42[c]	1.79	2.35
	Non-processed	2115.87[d]	1.77	2.18
GenomePlex	*Qiagen*	55.03[e]	1.89	1.64
	Sigma	40.03[e]	1.95	2.12
	Precipitation	451.00[c]	1.73	2.25
	Non-processed	895.07[f]	1.68	1.51
SurePlex	*Qiagen*	65.57[e]	1.91	1.58
	Sigma	62.41[e]	1.91	2.08
	Precipitation	470.12[c]	1.78	2.37
	Non-processed	819.20[c]	1.80	0.78

Note: In total, 259 samples were assessed to obtain these values.
***** 'Qiagen': Qiagen column purification; 'Sigma': Sigma column purification
****** Values with different superscripts ([a,b,c,d,e,f]) are significantly different (P<0.05).
[†] It was not possible to obtain any measurements for MDA samples processed with Qiagen column purification.

Gel electrophoresis was carried out to determine the size of the amplified products obtained from application of each WGA method on single cells. As seen in Figure 3.7, the length of the amplified products differed for each method. MDA produced the largest products ranging from 1kb to >10kb (most intense smear between 4kb and >10kb, mean fragment size: ~10kb). The product length obtained from the two PCR-based WGA methods was similar, both yielding much smaller fragments than MDA. Specifically, the size of GenomePlex products ranged from 0.1kb to 1kb (most intense smear between 0.2kb to ~0.6kb, mean fragment size: ~0.3kb) and the size of SurePlex products ranged from 0.1kb to 2kb (most intense smear between 0.2kb and 1kb, mean fragment size: ~0.4kb).

Figure 3.7: Gel electrophoresis of WGA products. **A)** MDA. **B)** GenomePlex. **C)** SurePlex.



Notes: N.C stands for 'Negative Control; R stands for 'Reference sample' (i.e. WGA genomic DNA).

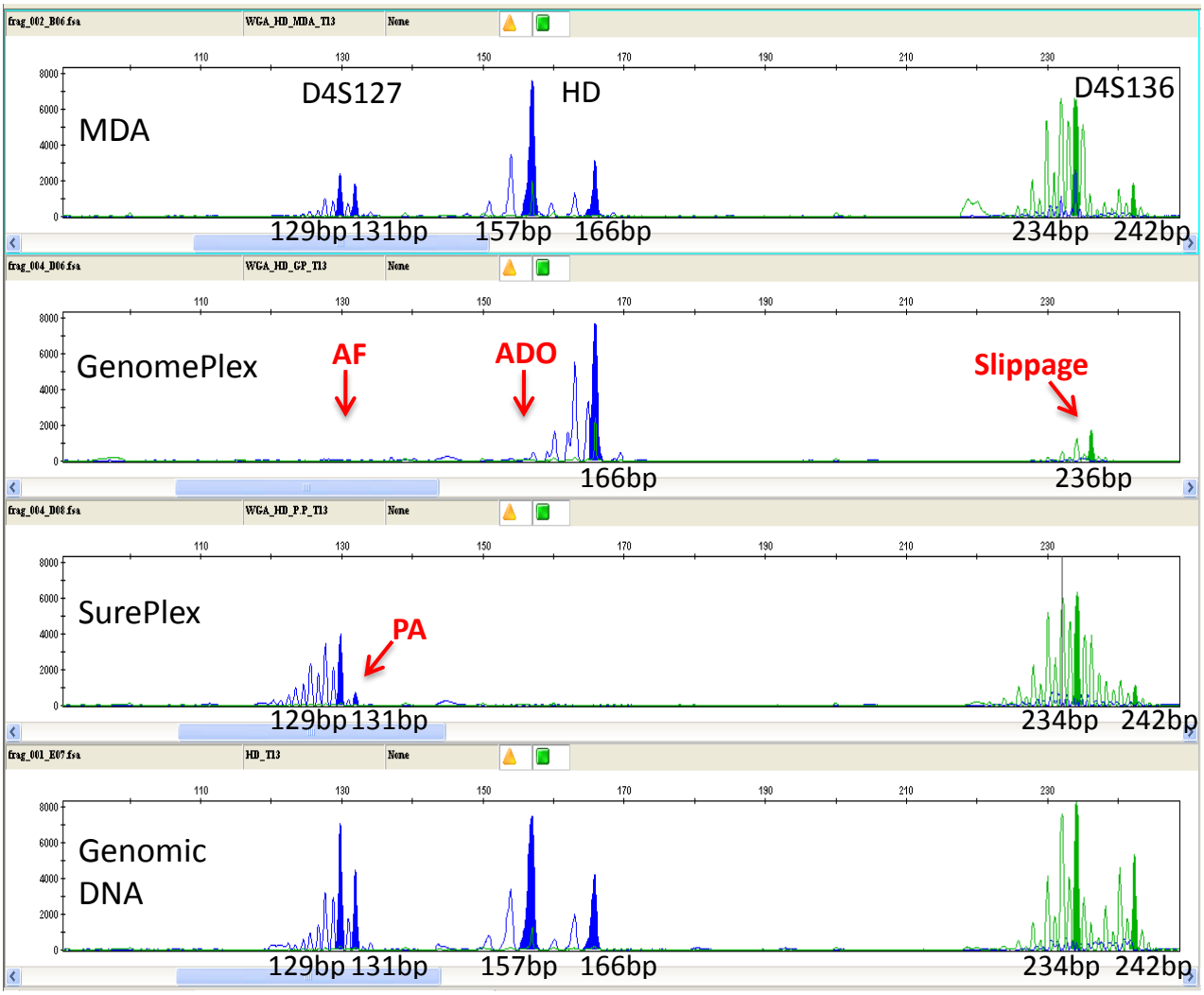
3.3.2 PCR amplification of WGA products

WGA products were used to amplify ten clinically relevant loci in order to assess the amplification characteristics of the 3 WGA methods. Different phenomena were observed from the results obtained after amplification of the WGA products. Apart from ADO, events such as preferential amplification and DNA polymerase slippage were observed (Figures 3.8 and 3.9). Such phenomena had also been observed in loci amplified directly from single cells without prior WGA (sections 3.1 and 3.2). Preferential amplification was considered to have occurred when the ratio of one allele to the other (determined by the amount of fluorescence detected) was >3:1 and it was seen in all three WGA methods tested.

DNA polymerase slippage was considered to have occurred when one allele of a specific locus was found to be one or more repeats shorter or longer than its actual length (as determined by direct analysis of genomic DNA without prior WGA) or when an additional, unexpected allele was observed (i.e. 3 alleles instead of 2 for a certain locus). It should be emphasized, that the DNA polymerase slippage rates reported here refer to the production of new, unexpected alleles; they do not refer to the 'stutters' commonly seen after amplification of repetitive DNA sequences that also occur through DNA polymerase slippage. Polymerase slippage occurred in 0.9% (5/554) of the MDA products tested, in 5.1% (4/78) of the GenomePlex products and in 6.1% (30/495) of the SurePlex products (Table 3.5). MDA samples were determined to have a significantly lower ($P<0.05$) polymerase slippage rate than the two other WGA method. No significant difference ($P>0.05$) was observed between the two PCR-based WGA methods (GenomePlex, SurePlex).

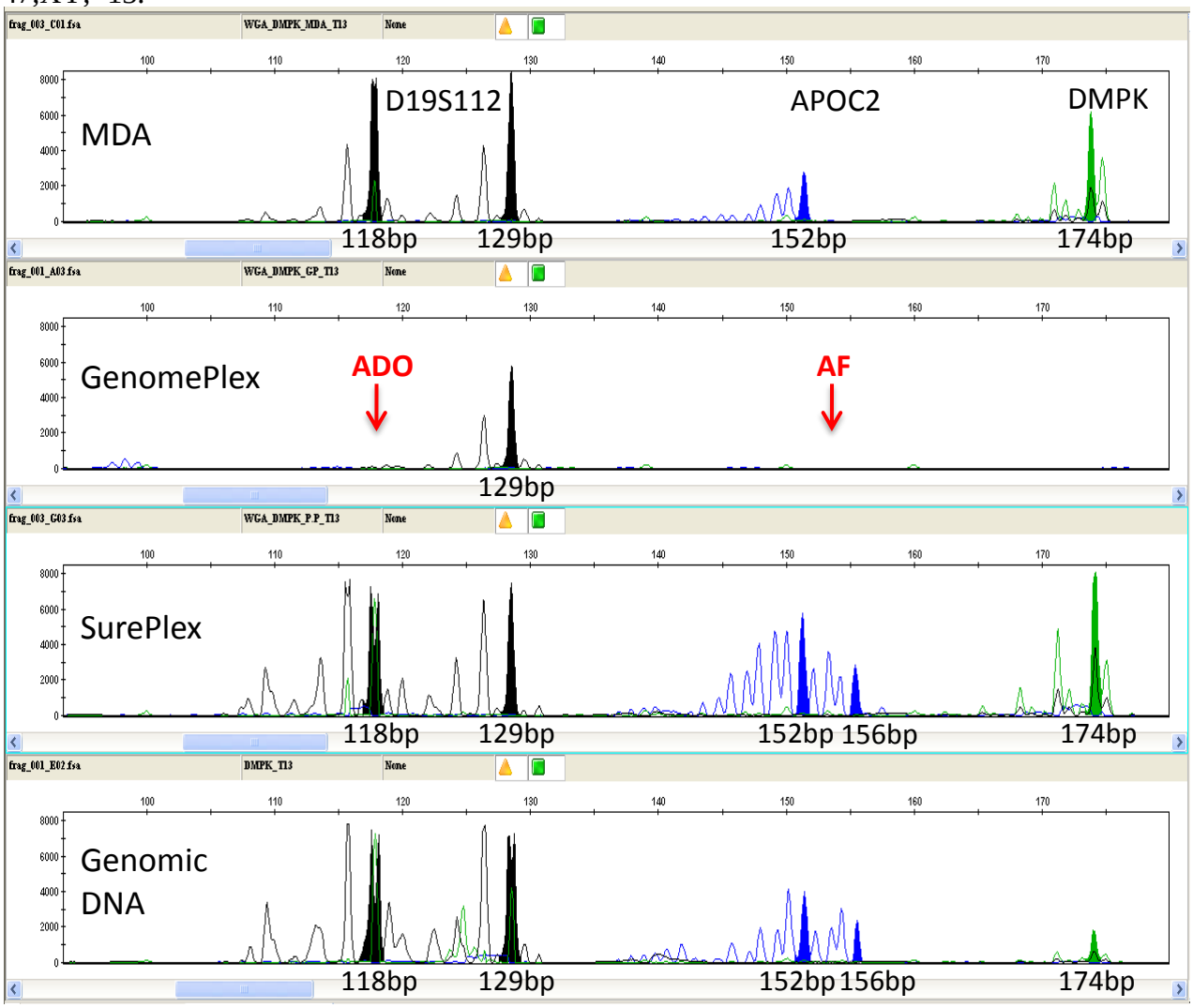
Overall, 177 whole genome amplified single cells went through PCR amplification and 1666 loci were tested (Table 3.5). MDA was found to have a significantly higher ($P<0.001$) AE than the other two methods. Furthermore, SurePlex had a significantly higher AE ($P<0.001$) than GenomePlex. In terms of ADO, SurePlex had the lowest rate (20.5%) and this was found to be significantly lower than GenomePlex's ADO rate (53.3%; $P<0.001$). When compared to the ADO rate of MDA (26.8%), the rate observed for SurePlex was not found to be significantly different although it was approaching statistical significance ($P=0.0534$). MDA was also determined to have significantly lower ADO rate than GenomePlex ($P<0.001$).

Figure 3.8: Electropherograms showing amplification of the Huntington multiplex using products from the 3 WGA methods and genomic DNA derived from cell line 47,XY,+13.



Notes: In this example, amplification failure (AF) is seen for D4S127 and the HD locus on GenomePlex and SurePlex products respectively. ADO is seen for the HD locus and D4S136 for GenomePlex products. Preferential amplification (PA) is seen for D4S127 on SurePlex products and for D4S136 on MDA and SurePlex products. Furthermore, slippage of DNA polymerase is noted for one of the alleles of D4S136 on GenomePlex products. In this occasion the specific allele should had been 234bp long according to the genomic DNA, but, instead the allele is 236bp.

Figure 3.9: Electropherograms showing amplification of the DMPK multiplex using products from the 3 WGA methods and genomic DNA derived from cell line 47,XY,+13.



Notes: In this example, amplification failure (AF) is seen for APOC2 and DMPK locus on GenomePlex products. ADO is observed for D9S112 and APOC2 on GenomePlex and MDA products respectively.

Table 3.5: Overall results from PCR amplification of WGA products derived from single cells.

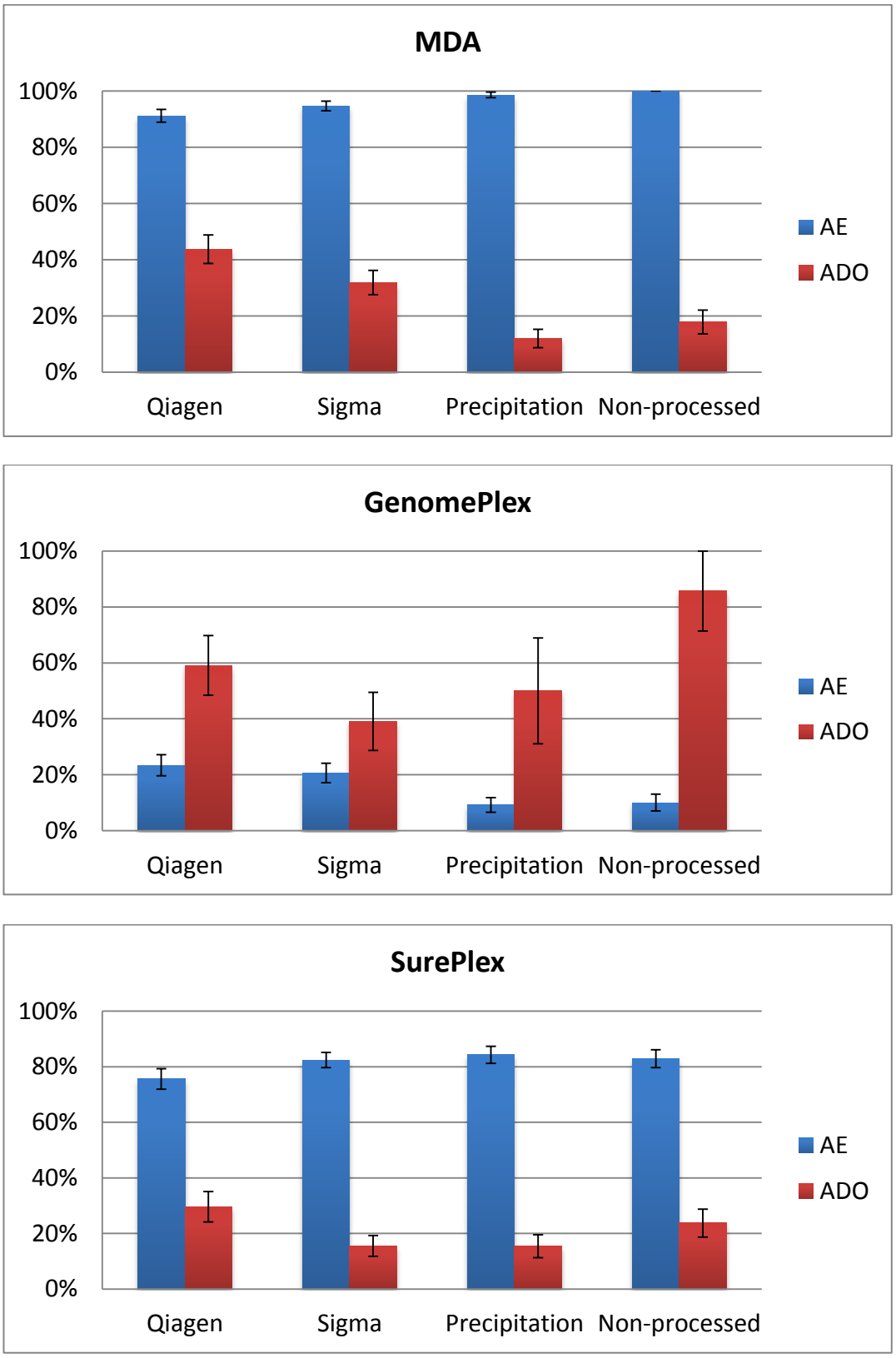
WGA Method	No. of cells	No. of Loci Tested	AE (%)*	ADO (%)*	DNA Polymerase Slippage (%)*
MDA	62	578	95.8^{A}	26.8^{a}	$0.9^{\#}$
GenomePlex	51	480	16.3^{B}	53.3^{b}	$5.1^{\#\#}$
SurePlex	64	608	81.4^{C}	20.5^{a}	$6.1^{\#\#}$
Total	177	1666	---	---	---

* Different superscripts ($^{A,B, \text{ or } a,b \text{ or } \#,\#\#}$) in each column show significant differences ($P<0.05$) in the values obtained between the three WGA methods.

3.3.2.1 Processing methods

Amplification efficiency and ADO were scored for each WGA method according to the method of processing. As seen in Figure 3.10, non-processed MDA products had the highest amplification efficiency (100%) and this was found to be significantly higher ($P<0.05$) than the AE obtained from MDA products treated with Qiagen and Sigma column purification. The AE of precipitated MDA products (98.6%) was found to be similar to non-processed products ($P>0.05$). The lowest AE was observed for Qiagen column purified products (91.2%). When considering ADO, MDA precipitated products were determined to have a significantly lower rate ($P<0.001$) than Qiagen and Sigma column purification but a similar rate to unprocessed products ($P>0.05$). Nevertheless, MDA precipitated amplified DNA had the lowest ADO rate (12%). On the other hand, MDA products processed with Qiagen column purification had the highest ADO rate (43.75%).

Figure 3.10: Amplification efficiency and ADO rates of the 3 different processing methods for the 3 WGA methods



Notes: 'Qiagen' refers to Qiagen column purification. 'Sigma' refers to Sigma column purification.

Regarding GenomePlex, AE was relatively low while ADO rates were considerably high for all treatment methods (Figure 3.10). The highest AE was seen for Qiagen column purification (23.4%) and the lowest for precipitation (9.2%). Amplification efficiency of Qiagen column purification was significantly higher ($P<0.05$) than precipitation and unprocessed but similar to Sigma column purification ($P>0.05$). GenomePlex products processed with Sigma column purification had the lowest ADO rate (39.1%), while GenomePlex unprocessed products had the highest ADO rate (85.7%). No significant differences ($P>0.05$) were seen regarding ADO rates between the different processing methods.

Amplification efficiencies of the different processing methods for SurePlex WGA were all similar between them ($P>0.05$) (Figure 3.10). Precipitated products were found to have the highest AE (84.3%) and Qiagen column purified products the lowest (75.6%). Regarding ADO, the rates were found to be statistically similar ($P>0.05$) between Sigma column purification, precipitation and non-processed products. Precipitated products were found to have the lowest ADO rate (15.4%). This ADO rate was very similar to the ADO rate of Sigma column purified products (15.5%). Qiagen column purified products on the other hand, were found to have the highest ADO rate which was significantly higher ($P<0.05$) than Sigma column purified products and precipitated products.

All in all, the highest AE amongst all 3 WGA methods for all processing methods used was achieved with MDA unprocessed products (100%), firstly and then with MDA precipitated products, secondly (98.6%). These AE rates were found to be significantly higher than any other method ($P<0.001$). MDA precipitated products were also determined to have the lowest ADO rate (12%) followed by SurePlex

precipitated (15.4%) and SurePlex Sigma column purified products (15.5%). No significant differences were observed between these 3 methods (P>0.05).

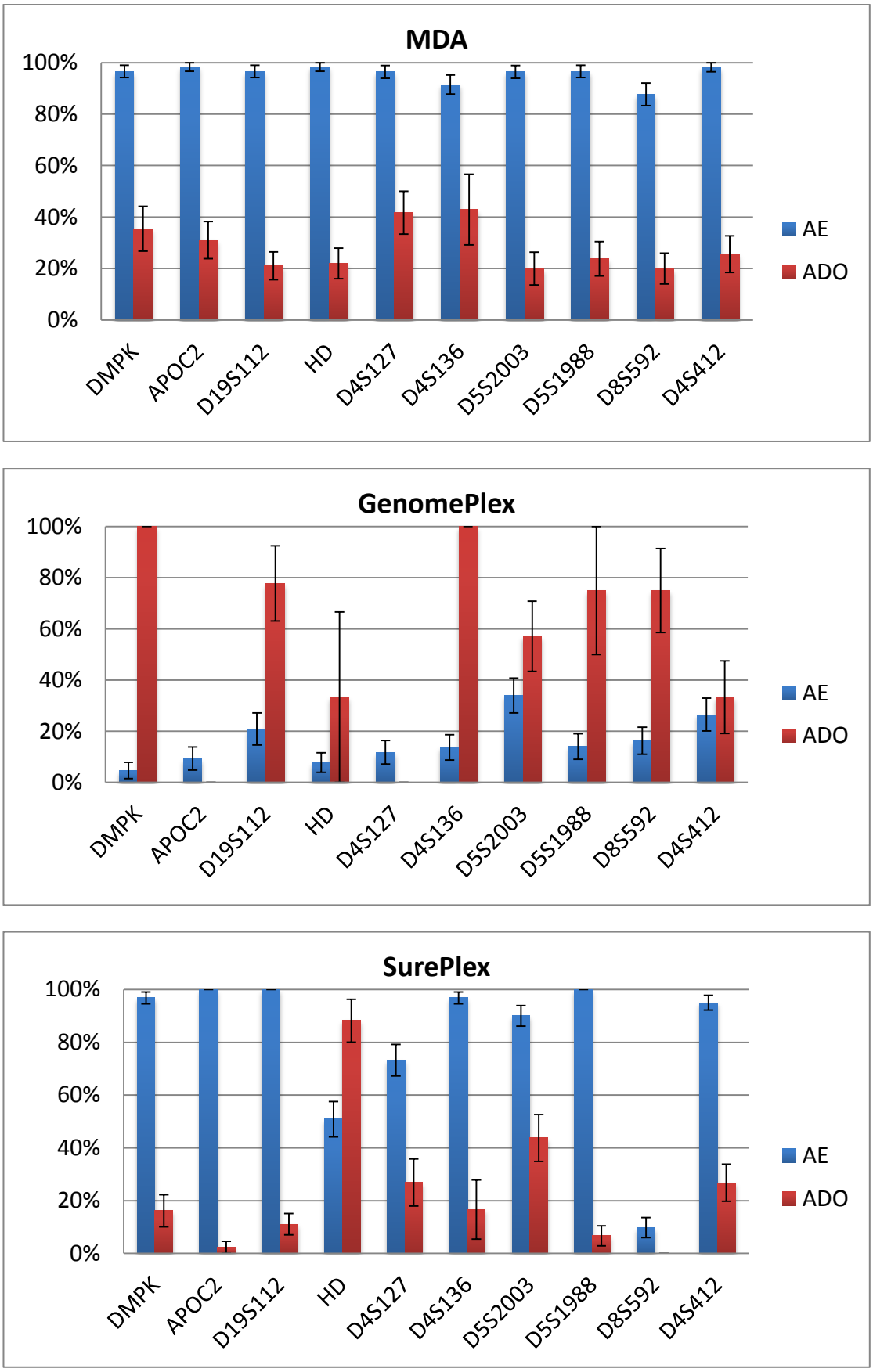
3.3.2.2 Amplification efficiency and ADO of the different loci amplified

Amplification efficiency and ADO rates were calculated for each locus individually for each WGA method. When MDA was considered, AE was uniformly high for all loci tested, ranging from 87.7% to 98.3% (Figure 3.11). Contrary, there was more variability between the 10 loci tested regarding ADO, with rates ranging from 20% to 42.9%.

Differently from MDA, GenomePlex had uniformly low AE rates for all loci tested (4.7% - 34%) (Figure 3.11). Regarding ADO, rates between the different loci were found to be greatly variable ranging from 0% to 100%. A possible explanation for this excessive variability is the low number of loci available for calculation of ADO rates since only a small number of loci amplified.

Contrary to the other two WGA methods, SurePlex was seen to amplify loci with differing efficiencies (Figure 3.11). Most of the loci tested showed a very high AE (90% - 100%). Other loci were amplified with lower efficiency (50.9% - 73.2%), while one locus (D8S592) was found to be amplified with very low efficiency (9.8%). When ADO was considered, rates were relatively low for most of the loci tested (0% - 26.9%). However, for 2 of the loci tested ADO rates were considerably higher (43.75% and 88.2%).

Figure 3.11: Amplification efficiency and ADO rates of the 10 STR markers tested for the 3 WGA methods.



3.3.3 DNA microarrays

Sigma column purified, precipitated and non-processed products from the 3 WGA methods were tested using two types of arrays - an oligonucleotide array and a SNP array. This was carried out in order to further assess the WGA methods and also determine if the two array platforms can be used in PGD. The single cells amplified using the different WGA methods were from well characterised cell lines containing known cytogenetic abnormalities.

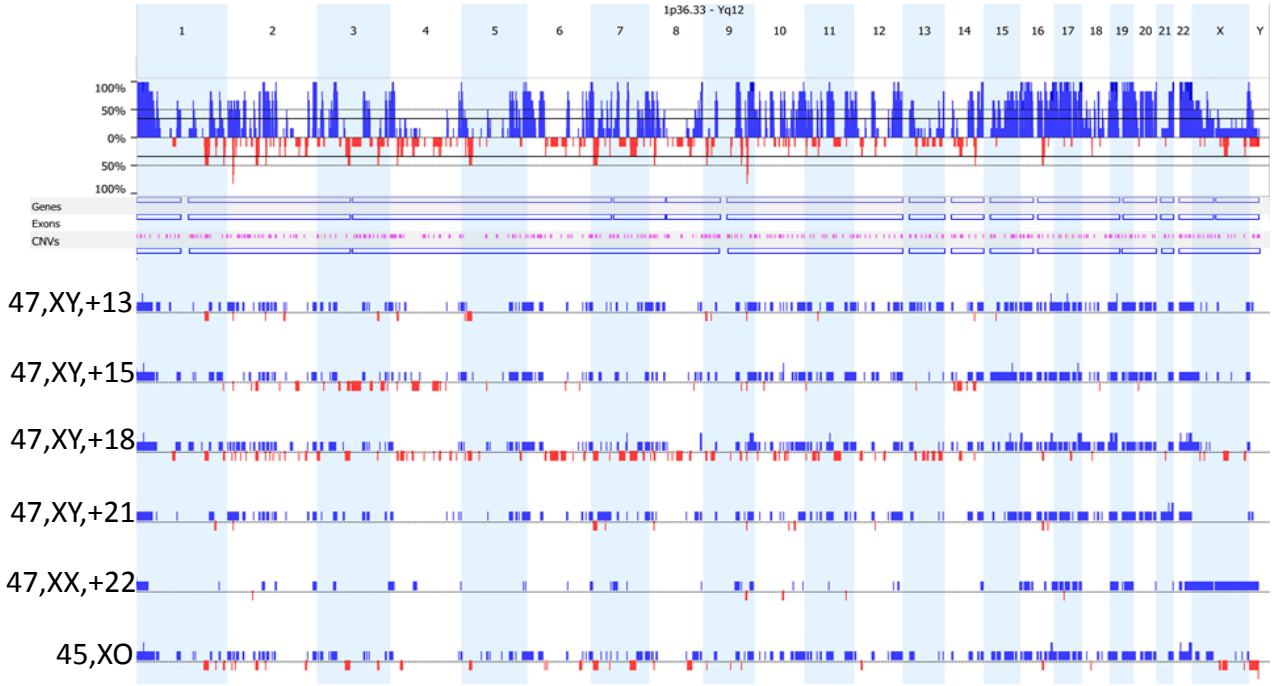
3.3.3.1 NimbleGen oligonucleotide array

In total, 12 non-processed samples were applied to microarrays from each WGA method.

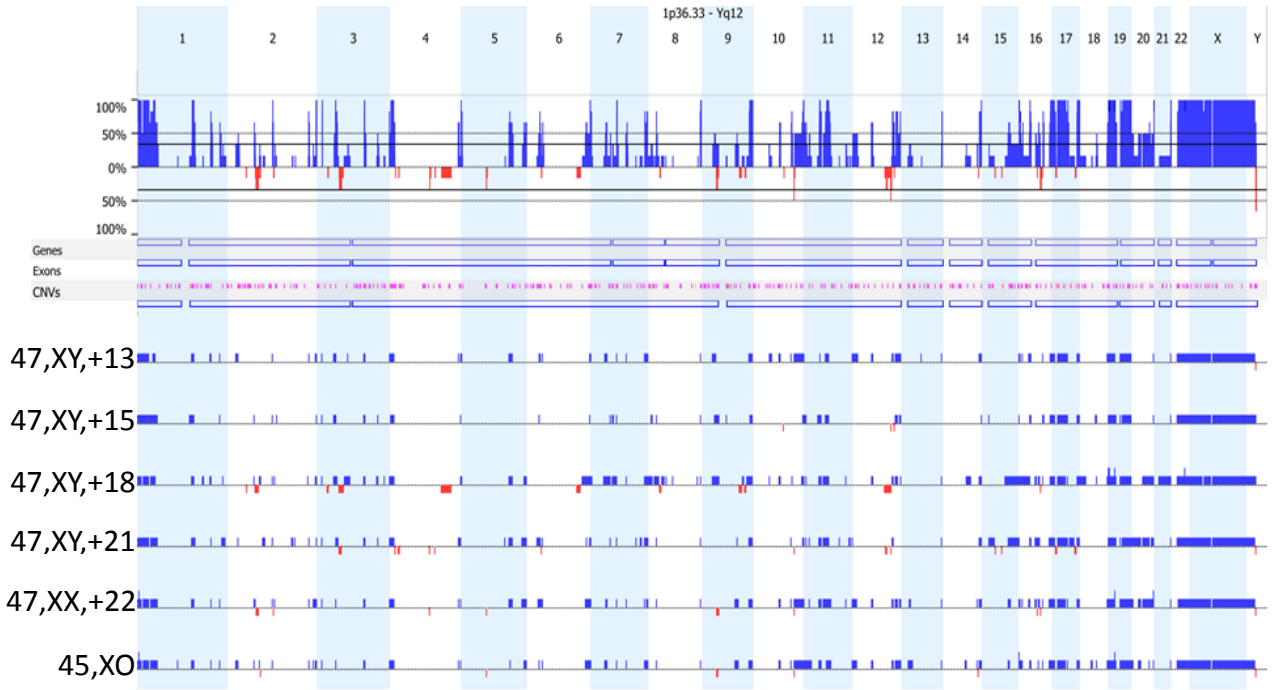
MDA and GenomePlex samples gave a large number of artefactual losses and gains of chromosomes in all the samples tested making the detection of the original aneuploidies impossible (Figure 3.12). SurePlex WGA samples on the other hand, showed a very low degree of artefactual losses and gains of chromosomes. As a result, the original aneuploidies were detected in a high percentage of the samples used. Consequently, SurePlex was determined to be the method of choice for usage on this type of array in order to perform aneuploidy screening.

Figure 3.12: Analysis of results through utilisation of the 'Nexus Copy Number' software after application of single cell WGA products on NimbleGen array. **A)** MDA samples. **B)** GenomePlex samples. **C)** SurePlex samples. Images provided courtesy of Dr Samantha Knight.

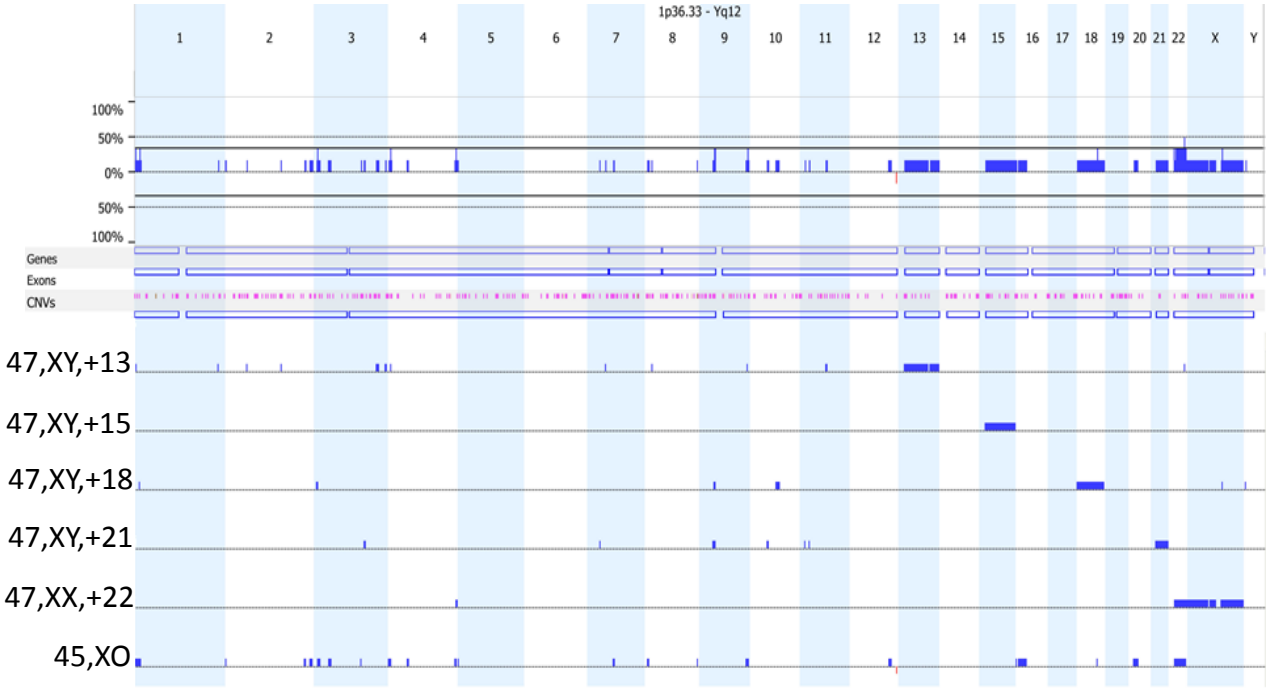
A) MDA samples



B) GenomePlex samples



C) SurePlex samples



Notes: Blue colour represents gain of chromosomal material while red colour shows loss of chromosomal material. All samples were tested against control male (46,XY) DNA samples.

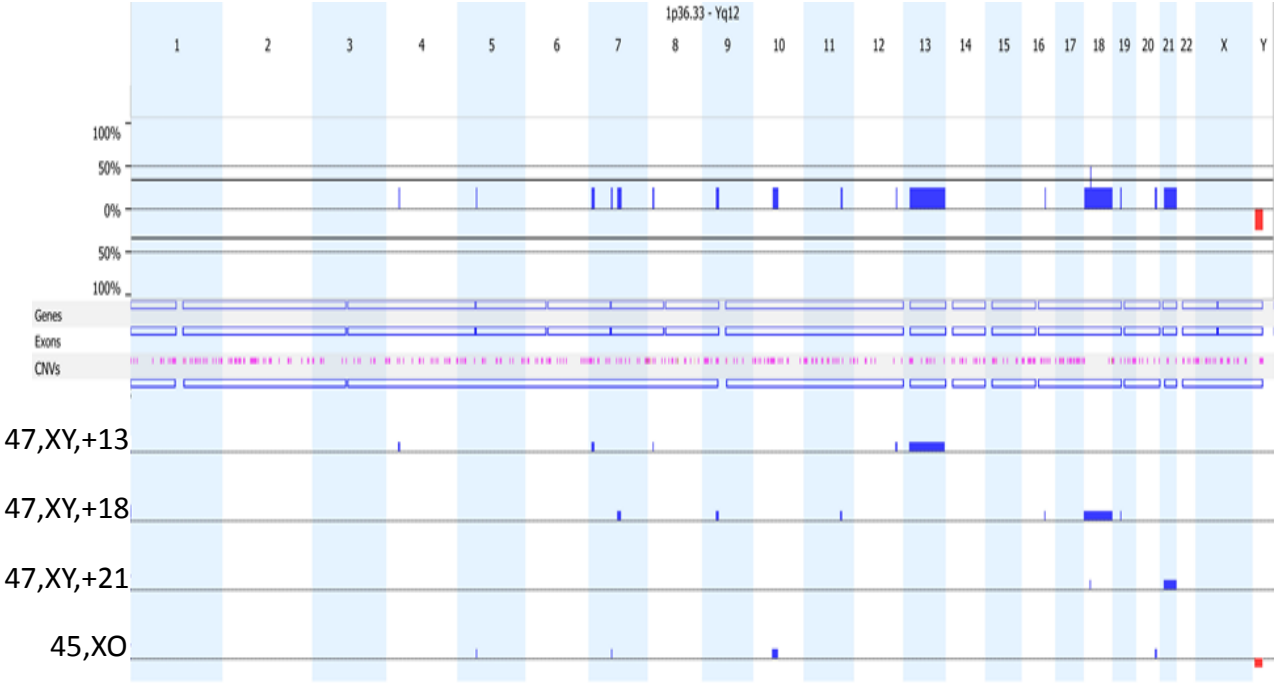
3.3.3.1.1 Further assessment of SurePlex

After SurePlex WGA was determined to be the best method from the three for usage on NimbleGen arrays, it was further assessed. Sigma column purified and precipitated SurePlex products were used to define if processing products improves results and if it does which processing method is the best. It was concluded that similar results regarding determination of chromosomal status are obtained when processing SurePlex products (through column purification or precipitation) and when using products unprocessed. However, usage of column purified products was found to require a lower number of labelling reactions in order to get to the optimal amount of labelling product needed for application on the array platform. This offered a great

reduction in the expense of the procedure compared to precipitated and unprocessed products and it was therefore selected as the method of choice.

Subsequently, a reduction in hybridisation time of applied samples was tested. This was performed in order to assess if utilization of NimbleGen arrays can be made more applicable for PGD without affecting their ability to determine the chromosomal status of samples. Specifically, the recommended hybridisation time (40-72h) was reduced to 16h. Sigma column purified products were used to assess the shortened protocol. Results obtained indicated that reducing the hybridisation time does not affect performance of the array. Aneuploidies were correctly identified in samples tested, while no increase in background 'noise' was observed (Figure 3.13).

Figure 3.13: Results from application of SurePlex-Sigma column purified samples for 16h of hybridisation on the NimbleGen array. Images provided courtesy of Dr Samantha Knight.



Notes: All samples were tested against control male (46,XY) DNA samples.

3.3.3.2 Illumina SNP array

In total, 82 single cells derived from 4 fibroblast cell lines (47,XY,+13; 47,XY,+18; 47,XY,+21; 45,X0) were used for application on Illumina SNP arrays. These cells were amplified using one of the 3 WGA methods and products were either left unprocessed or were processed (Sigma column purification, precipitation) before application to the array. During initial assessment 4 cells (1 from each cell line) were tested using each method. Following this initial evaluation, more cells were tested using the methods found to perform the best (Table 3.6). Results obtained from each WGA sample were compared to results received from genomic DNA of the corresponding cell line which was also applied on the SNP array.

As shown in Table 3.6, MDA was the WGA method with the greatest genome coverage since it provided the highest call rates for individual SNPs (i.e. yielded genotype results for the largest number of loci) ranging from 78.7% to 81.2%, depending on the method of processing. GenomePlex on the other hand had the lowest genome coverage with only 41.7% to 42.7% of loci producing data. SurePlex had slightly higher genome coverage than GenomePlex (44.1% - 51.5%).

Regarding MDA, when considering only the SNPs that provided a result, the proportion of calls that were identical to the control genomic DNA of each cell line ranged from 90.1% to 91.2%, with MDA non-processed samples having the highest rate. This was found to be significantly higher ($P<0.001$) than the other two MDA processing methods. The main reason for the false calling of the remaining SNPs was ADO. Allele dropout for MDA samples was high ranging from 36.5% to 50.6%, with MDA non-processed products showing a significantly lower rate than the other two methods ($P<0.001$). In addition to ADO, gain of heterozygosity (GOH) and change of

homozygosity (COH) contributed to the false calls, although these artefacts were relatively rare. Gain of heterezygosity was the situation where a homozygous locus (AA or BB) was called heterozygous (AB), while COH refers to a change of homozygosity such as a SNP being AA but called BB or vice versa (Table 3.6).

Compared to MDA, SurePlex had a significantly lower ($P<0.001$) percentage of identical calls to genomic DNA (83.5% - 85.5%). However, it also showed a lower degree of ADO (31.1% - 41.3%). Gain of heterozygosity was considerably higher than was observed for MDA ($P<0.001$), ranging from 8% to 10.9%, while COH was at low levels. Sigma column purification was the best method for SurePlex WGA having significantly higher genome coverage than the other two methods ($P<0.001$), significantly higher percentage of identical calls ($P<0.001$) and significantly lower GOH and COH rates ($P<0.001$).

Contrary to the other two WGA methods, results obtained from GenomePlex were found to be highly variable and inconsistent amongst the different samples. When the average values were considered, GenomePlex was the worst WGA method in terms of number of identical calls provided (35.4% - 49.7%). Also, ADO was substantially higher regardless of the method used for purification. Change of homozygosity was elevated compared with MDA and SurePlex ($P<0.001$). GOH was also found to be significantly higher in all GenomePlex methods than MDA methods ($P<0.001$). As with SurePlex, Sigma column purification was the best method for GenomePlex having the best rates for all categories apart from ADO which was found to be higher than the other two processing methods.

In conclusion, MDA unprocessed samples provided the best results in terms of SNP calling on the specific Illumina array. This method had the highest genome coverage, the highest rate of identical calls and the lowest GOH and COH rates. It also had one of the lowest ADO rates (36.5%).

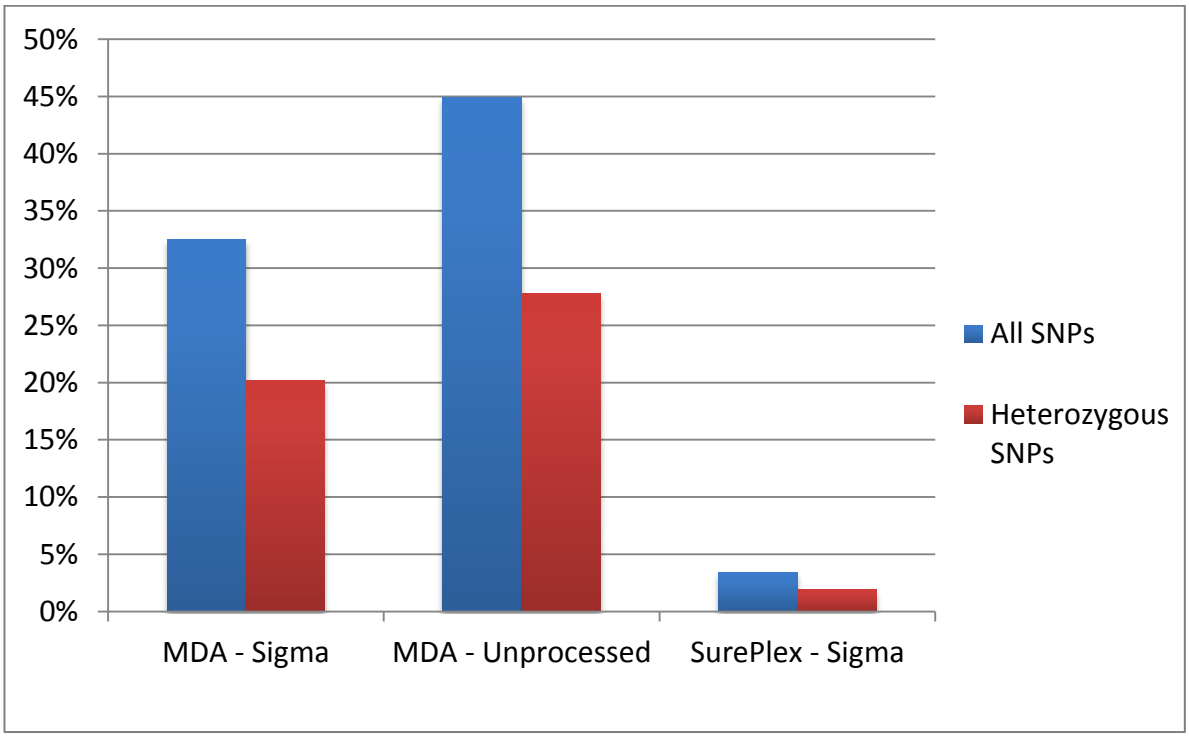
Table 3.6: Overall data from application of WGA products on Illumina SNP array

	MDA (%)			**GenomePlex (%)**			**SurePlex (%)**		
	*Sigma (15)**	*Precipitation (3)**	*Non-processed (16)**	*Sigma (4)**	*Precipitation (4)**	*Non-processed (4)**	*Sigma (16)**	*Precipitation (4)**	*Non-processed (16)**
Call Rate	80.4	78.7	81.2	42.7	41.7	42.6	51.8	44.6	44.1
Identical Calls	90.1	88.2	91.8	49.7	49.5	35.4	85.5	84.2	83.5
GOH	0.9	0.9	0.6	4.5	6.7	12.8	8	10.9	8.4
ADO	42.1	50.6	36.5	93.9	90.4	86	35.7	31.1	41.3
COH	0.01	0.01	0.01	28.5	28.6	43.4	0.2	0.3	0.7

* Numbers in brackets indicate the number of single cells tested for each method.

In addition to the above assessment of the different methods, the number of consistent-correct SNP calls was calculated for the three best performing methods: MDA-Sigma column purification, MDA-unprocessed and SurePlex-Sigma column purification (Figure 3.14). This was calculated by identifying the SNPs that were called correctly every time in samples tested, across all cell lines, for each of the methods. The twelve (3 from each cell line) best performing samples (in terms of characteristics described in Table 3.6) from each method were used to make the calculations. MDA-unprocessed was the method with the highest overall number of consistent-correct SNP calls; this number was 134,689 and corresponds to 44.9% of the total number of SNPs present on the specific array. Considering the heterozygous SNPs only, the percentage of consistent-correct calls for the same method was 27.8%. The second best method was MDA-Sigma column purification. SurePlex-Sigma column purification was the worst method amongst the three; only 3.4% of the SNPs present on the array provided consistent-correct calls.

Figure 3.14: Amount of consistent-correct SNP calls for three of the best performing methods on Illumina SNP array.



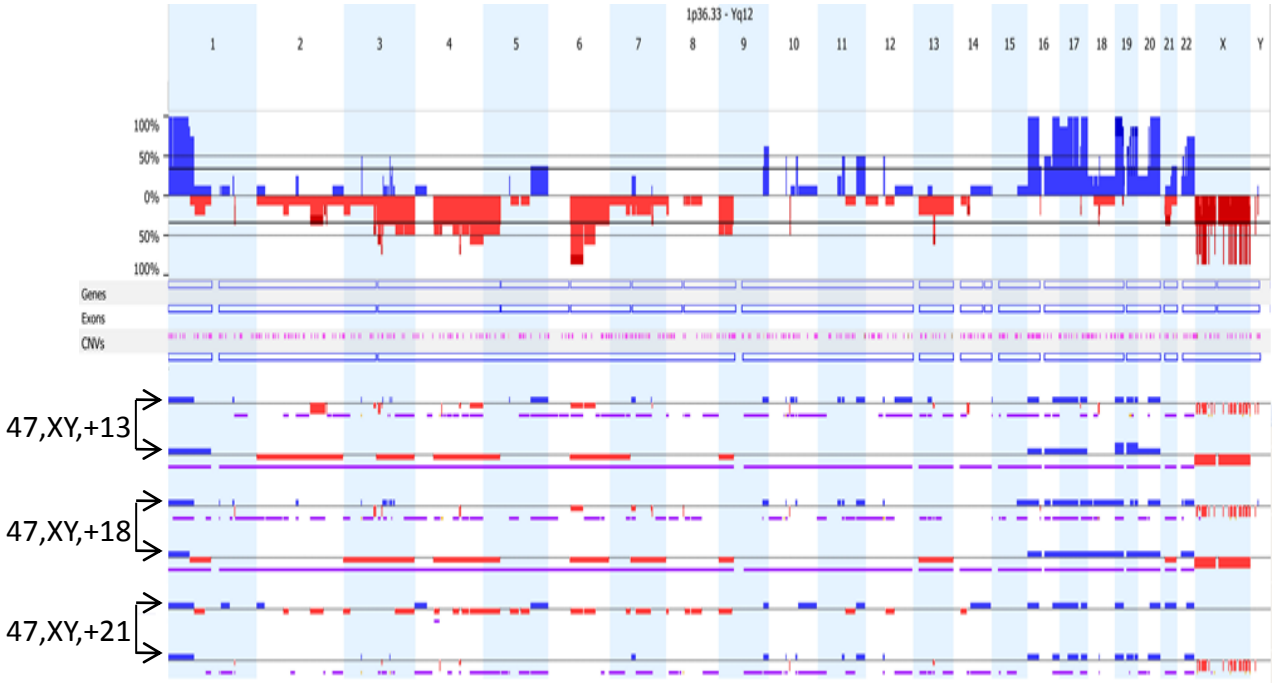
Notes: 'Sigma' refers to Sigma column purification.

3.3.3.2.1 Aneuploidy screening

As well as evaluating genotyping accuracy, the two best performing methods (MDA-unprocessed, MDA-Sigma column purified) were also assessed for aneuploidy detection using the Illumina SNP array. In order to assess aneuploidy detection, two types of reference DNA were used. The first involved combined data from 270 unamplified genomic DNA samples from male and female individuals derived from the International HapMap project. Analysis of results using this reference type could not identify the chromosomal imbalances of the samples used, while a large amount of false gain/loss of chromosomal material was observed (Figure 3.15). The second type of reference involved data obtained from MDA WGA of single cells. Results obtained from utilization of this type of reference were considerably better than the

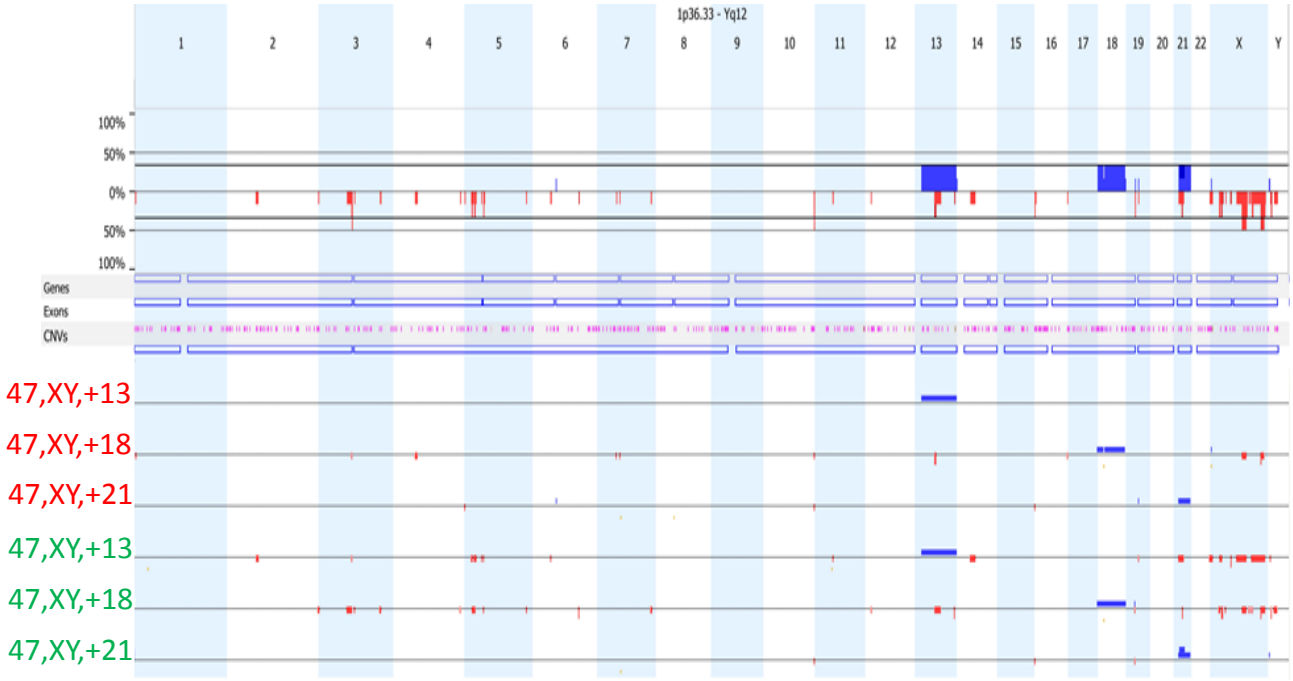
first type of reference. Aneuploidies were correctly identified, while background noise was minimal (Figure 3.16). Results were similar between the two MDA methods although Sigma column purified products were found to produce slightly less background noise. Samples were further assessed using the second type of reference with only the SNPs that provided consistent/correct calls. The usage of only consistent/correct calls was not found to provide any improvement in results compared to usage of all SNP calls from single cell MDA products.

Figure 3.15: Aneuploidy detection results from application of MDA-Sigma column purified samples on Illumina array and usage of unamplified genomic DNA samples for reference. Image provided courtesy of Dr Samantha Knight.



Notes: Blue colour represents gain of chromosomal material while red colour shows loss of chromosomal material. Purple colour indicates 'allelic imbalance'.

Figure 3.16: Aneuploidy detection results from application of MDA-Sigma column purified samples and MDA-unprocessed samples on Illumina array and usage of single cell MDA samples for reference. Image provided courtesy of Dr Samantha Knight.



Note: Sample names written in red denote MDA-Sigma column purified samples. Sample names written in green show MDA-unprocessed samples.

3.3.4 Clinical cases

In total, 5 clinical cases were carried out using WGA methods to process the biopsied cells (Table 3.7). Three cases were carried out using SurePlex WGA and 2 cases were performed using MDA WGA. WGA products from all cases were used without additional purification, providing templates for PCR amplification of the relevant loci. Contrary to when conventional methods were used to develop protocols (section 3.1.1), development of PGD tests for single gene disorders using WGA took only 15 days to 1 month to be completed. This was due to the fact that WGA products could be treated as genomic DNA avoiding the need for utilisation of specialized single-cell

multiplex protocols which require lengthy periods of time to be developed. For three of the cases aneuploidy screening was also carried out in addition to single gene testing.

Overall, single gene disorder testing was carried out for 33 embryos, while aneuploidy screening was performed for 16 embryos (Table 3.7). Diagnosis of the single gene disorder was achieved for 27 of the embryos (81.8%) and for aneuploidy screening for 13 of the embryos (81.25%). The main reason for no diagnosis of single gene disorder was TAF (4/6 embryos). One of the embryos could not be diagnosed due to inconclusive results (i.e. only one locus from the three assessed amplified successfully and in addition, ADO was detected) and one embryo was not diagnosed due to contamination, most likely maternal, detected for one of the STR loci. Lack of diagnosis regarding aneuploidy screening was due to TAF.

Aneuploidy screening was proven to be very useful for identifying the best embryo for transfer. Out of the 13 embryos that had results for aneuploidy screening, 8 were found to be unaffected or carriers of the single gene disorder. From these 8 embryos, 5 were determined to be aneuploid. Thus, 62.5% of the unaffected/carrier embryos, which would have been otherwise considered for transfer (i.e. if aneuploidy screening was not available), were aneuploid.

3.3.4.1 Amplification efficiency and clinical outcome

In total, 82 loci were tested from SurePlex WGA products during clinical cycles and 53 loci for MDA. Amplification efficiency for SurePlex was 72% (59/82) and for MDA was 90.6% (48/53). Excluding cells with TAF, SurePlex AE was 88.1% (59/67)

and MDA AE was 98% (48/49). When considering only single blastomeres, SurePlex AE was 86.2% (50/58) (excluding cells with TAF). ADO rate for SurePlex WGA samples was 20.6% (7/34) and for MDA WGA samples was 10.3% (3/29). When only single blastomere samples were considered for SurePlex, ADO rate was calculated to be 22.6% (7/31).

Clinical outcome data were available for 4/5 PGD cycles performed. Specifically, 5 embryos were transferred and 3 pregnancies occurred, one of which was a twin pregnancy (Marfan Syndrome) (Table 3.7). To date, 3/4 embryos that formed pregnancies have been delivered and one pregnancy is still ongoing. It is important to note that the twin pregnancy was delivered prematurely and one of the babies died soon after delivery due to infection. The initial genetic diagnosis has been confirmed for the two surviving babies (Phenylketonuria and Marfan Syndrome cases).

Table 3.7: Single gene testing, aneuploidy screening and clinical outcome data of PGD cases carried out through WGA.

Disease	Embryo	Single gene testing	Aneuploidy screening	Embryos transferred*	Clinical Outcome*				
					Biochemical Pregnancy	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
Dominant Dystrophic Epidermolysis Bullosa	1	Unaffected	Aneuploid	N / A[††]	---	---	---	---	---
	2	No Diagnosis	No Diagnosis		---	---	---	---	---
	3	Unaffected	Euploid		---	---	---	---	---
	10	Chromosomally abnormal[†]	Aneuploid		---	---	---	---	---
Marfan Syndrome	1	Unaffected	---	---	---	---	---	---	---
	2	Affected	---	---	---	---	---	---	---
	3	Chromosomally abnormal[†]	---	---	---	---	---	---	---
	4	Unaffected	---	✓	1	1	0	0	1
	5	Unaffected	---	✓	1	1	0	0	1
	6	Chromosomally abnormal[†]	---	---	---	---	---	---	---
Phenylketonuria	1	Affected	---	---	---	---	---	---	---
	2	Carrier	---	---	---	---	---	---	---
	3	No Diagnosis	---	---	---	---	---	---	---

Table 3.7 (continued)

Disease	Embryo	Single gene testing	Aneuploidy screening	Embryos transferred*	Clinical Outcome*				
					Biochemical Pregnancy	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
Phenylketonuria	4	Affected	---	---	---	---	---	---	---
	5	Affected	---	---	---	---	---	---	---
	6	Affected or carrier	---	---	---	---	---	---	---
	7	Carrier	---	---	---	---	---	---	---
	8	Unaffected	---	✓	1	1	0	0	1
	9	Unaffected	---	---	---	---	---	---	---
	10	Unaffected	---	---	---	---	---	---	---
	11	Carrier	---	---	---	---	---	---	---
	12	Carrier	---	---	---	---	---	---	---
Sandhoff Syndrome	1	Carrier	Euploid	✓	0	0	0	0	0
	2	Unaffected	Aneuploid	---	---	---	---	---	---
	3	No Diagnosis	No Diagnosis	---	---	---	---	---	---

Table 3.7 (continued)

Disease	Embryo	Single gene testing	Aneuploidy screening	Embryos transferred*	Clinical Outcome*				
					Biochemical Pregnancy	*Clinical Pregnancy*	*Miscarried*	*Ongoing*	*Born*
Sandhoff Syndrome	4	No Diagnosis	Aneuploid	---	---	---	---	---	---
	5	No Diagnosis	Aneuploid	---	---	---	---	---	---
	6	No Diagnosis	No Diagnosis	---	---	---	---	---	---
Smith-Lemli-Opitz Syndrome	1	Unaffected	Euploid	✓	1	1	0	1	0
	2	Carrier	Aneuploid	---	---	---	---	---	---
	3	Unaffected	Aneuploid	---	---	---	---	---	---
	4	Affected	Euploid	---	---	---	---	---	---
	5	Unaffected	Aneuploid	---	---	---	---	---	---
	6	Not Tested[†††]	Aneuploid	---	---	---	---	---	---

* IVF centres provided results regarding clinical outcome only for some of the cases.

[†] For the specific embryos, the characterization 'chromosomally abnormal' refers to the tested cell being haploid or monosomic for the chromosome at which the gene of interest is located.

[††] N/A stands for 'Not Available'.

[†††] This embryo was arrested and therefore it could not be tested for inheritance of the single gene disorder. Aneuploidy screening was carried out through testing the 1st and 2nd PBs corresponding to the embryo

3.3.5 Karyomapping

Aliquots of products from WGA of single blastomeres from two PGD cases (Marfan Syndrome, Smith-Lemli-Opitz Syndrome) were used for application on SNP arrays and subsequent analysis of results with karyomapping (genome wide linkage analysis). Based on results obtained from assessment of WGA products (sections 3.3.2 and 3.3.3) it was decided to use MDA non-processed products to perform the two cases. DNA samples from the parents and existing children were also tested, but without any prior MDA. The procedures were carried out after PGD using conventional methods had already been undertaken (section 3.3.4) (i.e. this should be considered a retrospective proof-of-principle study).

3.3.5.1 Marfan syndrome

Results obtained from karyomapping analysis regarding single gene testing were in complete agreement with results obtained from conventional PCR-based analysis (Table 3.8). Embryos 1, 4 and 5 were diagnosed as unaffected by both methods while embryo 2 was diagnosed as affected. Regarding embryo 3, it was identified as potentially haploid or monosomic for chromosome 15 after PCR amplification since only maternal alleles were detected. After testing of the sample using SNP array and following karyomapping analysis, the biopsied cell was confirmed to be haploid, having only maternal alleles across the entire genome. Embryo 6 was also concluded to be haploid or monosomic for chromosome 15 after PCR amplification, since again only maternal alleles were detected. Karyomapping analysis determined this embryo to be monosomic for chromosome 15 - missing the paternal copy. Embryos 4 and 5, which were found to be unaffected, were transferred and a pregnancy was achieved leading to the delivery of healthy twins. The transferred embryos were determined to

be euploid by karyomapping analysis. The rest of the embryos were found to be aneuploid (Table 3.8).

3.3.5.2 Smith-Lemli-Opitz syndrome

Single gene testing with standard methods was in complete agreement with single gene testing after karyomapping (Table 3.9). Embryo 2 was identified as carrier, embryos 3 and 5 were identified as unaffected and embryo 4 was diagnosed as affected by both methods. Embryo 1 was concluded to be unaffected and euploid and was transferred. This embryo was not tested by karyomapping. Also, embryo 6 was arrested and therefore it was not biopsied. As a result, it could not be tested for inheritance of the disorder and also karyomapping was not carried out.

Results obtained for aneuploidy screening were in agreement between aCGH and karyomapping for all embryos tested (Table 3.9). Embryos 2 and 4 were correctly identified as normal by karyomapping. Oocytes 3 and 5 were determined by aCGH test to be missing chromosomes 15 and 22, respectively. These results were confirmed after karyomapping since embryos 3 and 5 were found to be monosomic - having only one paternal copy for chromosomes 15 and 22, respectively.

The embryo transferred (embryo 1 - unaffected and derived from a euploid oocyte) resulted in the initiation of a pregnancy which is ongoing at this time.

Table 3.8: Results obtained from processing Marfan syndrome case with conventional methods and karyomapping

	Standard diagnostic methods		**Karyomapping**			
	Single gene testing (PCR amplification)		**Aneuploidy screening**		**Single gene testing**	
	Single blastomere 1	*Single blastomere 2*	*Single blastomere 1*	*Single blastomere 2*	*Single blastomere 1*	*Single blastomere 2*
Embryo 1	Unaffected	Unaffected	Monosomy 21 (maternal)	Normal	Unaffected	Unaffected
Embryo 2	Affected	---	Trisomy 1 (maternal)	---	Affected	---
Embryo 3	Haploid or monosomic for chromosome 15	---	Haploid (maternal genome only), gain of 10 & loss of 19 (maternal)	---	Haploid, maternal genome only	---
Embryo 4	Unaffected	---	Normal	---	Unaffected	---
Embryo 5	Unaffected	No amplification	Normal	No Amplification	Unaffected	No amplification
Embryo 6	Haploid or monosomic for chromosome 15	---	Monosomy 15 & loss of 20qter (paternal), trisomy 6 & 8 (maternal),	---	Monosomy 15 (loss of paternal chromosome)	---

Table 3.9: Results obtained from processing Smith-Lemli-Opitz syndrome case with conventional methods and karyomapping

	Methods currently used					**Karyomapping**			
	Aneuploidy screening (24sure Test)			**Single gene testing (PCR amplification)**		**Aneuploidy screening**		**Single gene testing**	
	1st PB	*2nd PB*	*Oocyte karyotype*	*Single blastomere 1*	*Single blastomere 2*	*Single blastomere 1*	*Single blastomere 2*	*Single blastomere 1*	*Single blastomere 2*
Embryo 1	Normal	Normal	23,X	Unaffected	Inconclusive	---	---	---	---
Embryo 2	Gain chromatid 22	Loss 22	23,X	Carrier	Carrier	Normal	Normal	Carrier	Carrier
Embryo 3	Normal	Gain 15	22,X,-15	Unaffected	---	Monosomy 15 (maternal), Monosomy 7 (paternal)	---	Unaffected	---
Embryo 4	Normal	Normal	23,X	Affected	---	Normal	---	Affected	---
Embryo 5	Normal	Gain 22	22,X,-22	Unaffected	---	Monosomy 22 (maternal), Deletion 15q (paternal)	---	Unaffected	---
Embryo 6	Normal	Gain 12, 14	21,X,-12,-14	---	---	---	---	---	---

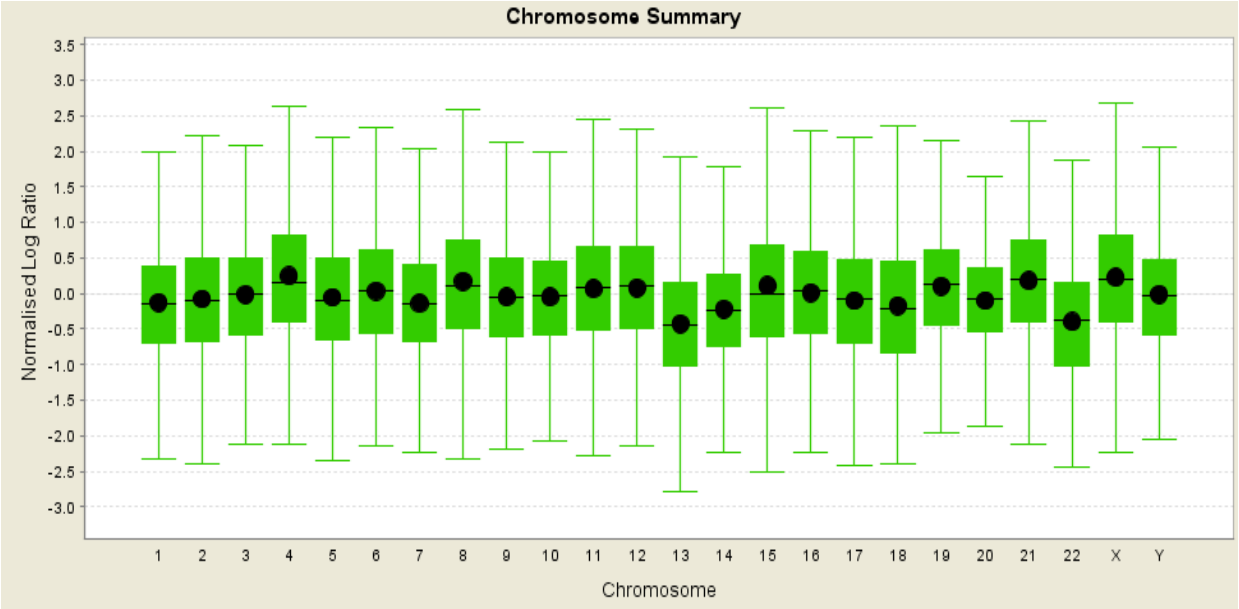
3.4 Development of a customised microarray

3.4.1 Optimisation

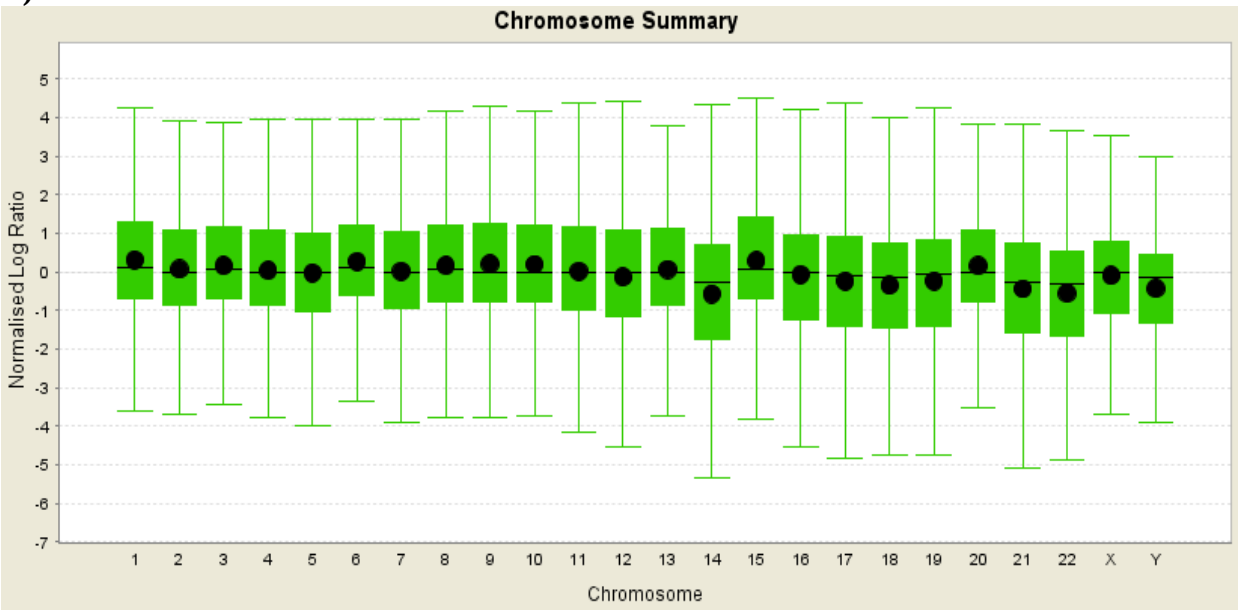
Fourty-eight single cell WGA products produced through utilization of three different WGA methods (MDA, GenomePlex, SurePlex) were tested to determine which is the best method for usage in the development of the array. WGA products were firstly used column purified (Qiagen) or precipitated. Precipitated products were found to provide better results. Regarding the best WGA method, this was determined to be SurePlex (Figure 3.17). The aneuploidies were successfully detected in all the SurePlex samples tested. In contrast, when MDA products were used aneuploidies were only detected in some of the samples while, none of the aneuploidies were detected when GenomePlex products were used. In addition, artefactual losses and gains of chromosomes were observed for MDA and GenomePlex samples. After SurePlex was determined to be the best method for usage on the array, non-processed SurePlex products were assessed. These were found to perform equally well as precipitated products and were therefore selected for application on the array since removing precipitation step would reduce the cost and the length of the procedure.

Figure 3.17: WGA products applied on customised microarray. Single cells from aneuploid cell line 47,XY,+13 were used. **A)** MDA. **B)** GenomePlex. **C)** SurePlex. Images provided courtesy of Dr Douglas Hurd.

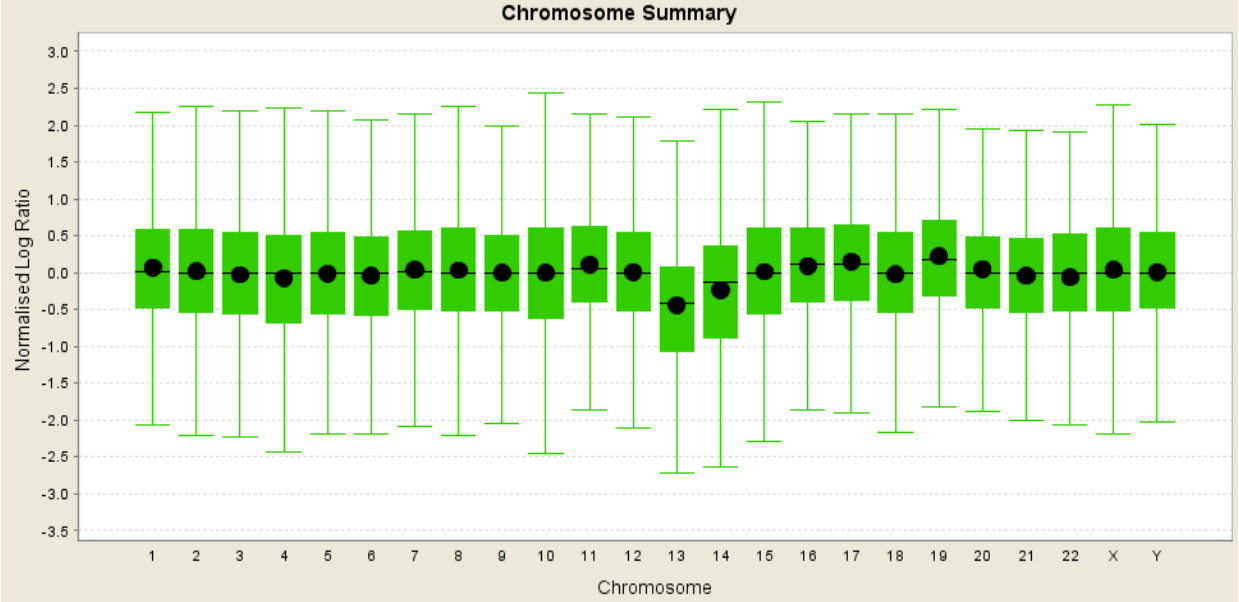
A) MDA



B) GenomePlex



C) SurePlex



Notes: The error bars represent the minimum and maximum values obtained from the fluorescence intensity of the probes attached on the microarray after applying the WGA samples and the reference sample and indicate that fluorescence intensities for individual probes varied greatly (i.e. the microarray results were very noisy). The black circles in the box plots represent the median values and the black lines indicate the mean values, showing that despite the noisy results obtained a useful diagnosis could be achieved by averaging together the results of multiple probes. In this example, the aneuploidy was correctly identified for a cell amplified using SurePlex but no conclusion could be drawn for the GenomePlex sample. The aneuploidy could also be seen in cells amplified using MDA, but other imbalances appeared to be present too. No shift was observed in the X and Y chromosomes for any of the samples indicating that this cell line comes from a male individual (since male DNA was used as reference).

3.4.2 Validation of developed microarray

In order to validate the developed array, 97 SurePlex amplified clinical samples that had been previously processed using 24sure test were applied on the array. Specifically, 27 PBs, 50 blastomeres and 20 trophectoderm samples were assessed. Assessment and scoring of samples was carried out by specialized OGT personnel in a blinded way (i.e. no information was given to personnel regarding 24sure test analysis results).

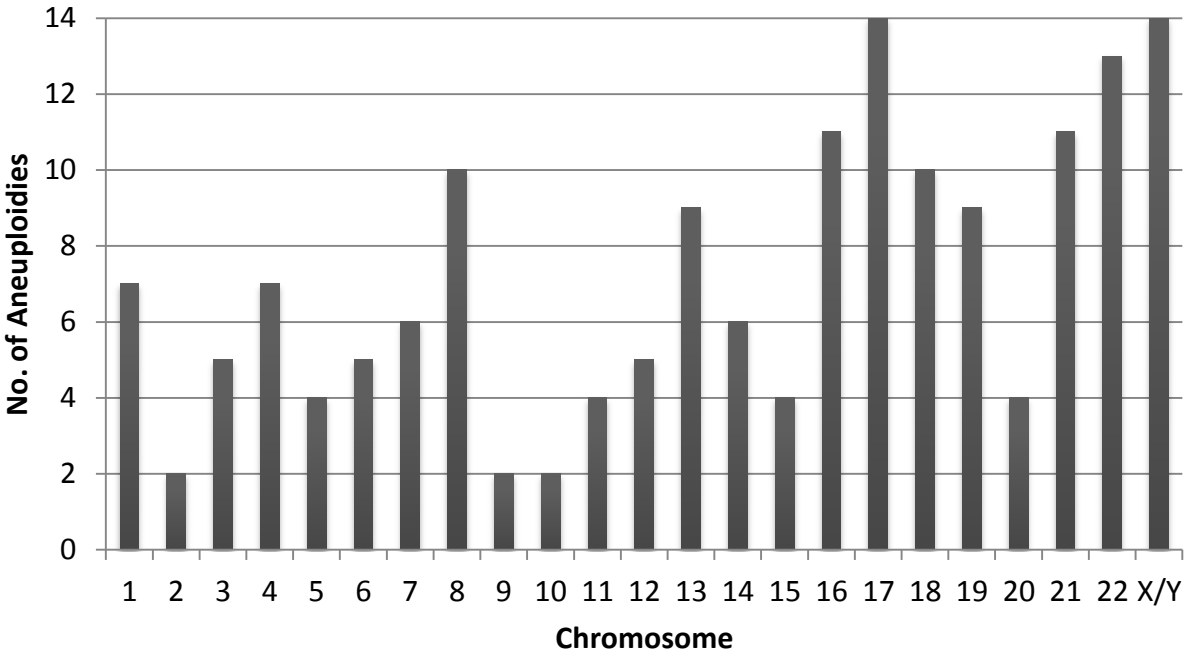
3.4.2.1 Validation of microarray for aneuploidy screening

In total, 164 aneuploidies affecting the entire chromosomal complement were assessed (Figure 3.18) - 30 aneuploidies for PBs, 118 for blastomeres and 16 for trophectoderm samples. The developed array successfully detected 156/164 aneuploidies (95.1% detection rate) while, it also detected 3 extra aneuploidies not detected by aCGH using the 24sure microarrays. Specifically, the array detected 28/30 aneuploidies for PB samples (93.3%), 114/118 aneuploidies for blastomere samples (96.6%) and 14/16 aneuploidies for trophectoderm samples (87.5%). No significant difference was calculated regarding the accuracy of the array between the three different sample types. Interestingly, 4/8 aneuploidies not detected and 2/3 extra aneuploidies detected involved chromosome 19, suggesting a potential problem in the determination of chromosome 19 copy number. Although the array was developed for detection of whole chromosome aneuploidies, 14 of the 164 aneuploidies tested were segmental abnormalities; size of deleted/duplicated segments ranged from 19.3Mb to 133.5Mb. The array successfully, detected 12/14 of these aneuploidies. The two segmental abnormalities not detected were correlated to chromosome 19 (size of segments not detected: 21.6Mb and 23.3Mb).

In terms of diagnosis (normal or abnormal at the level of the sample, rather than at the level of the chromosome), the developed microarray was concordant with aCGH using the 24sure microarray in 94/97 samples (96.9%). The array correctly identified 33 samples as normal and 61 samples as abnormal. Disagreement was observed for 3 samples that were found to be abnormal by the 24sure microarray but were determined normal by the developed microarray. Interestingly, the cause of disagreement for 2/3 samples was no detection of chromosome 19 aneuploidy by the developed array.

Furthermore, results obtained from the new array were assessed for correct identification of the gender of the processed samples. From the 86 samples that were found to be euploid for X and Y chromosomes, the new array agreed with 24sure test in 84 (84/86). Both discrepancies seen were regarding single blastomere samples.

Figure 3.18: Number of aneuploidies tested for each chromosome during validation of developed array.



3.4.2.1.1 Assessment of a subset of samples with FISH

The embryos associated with 20 of the samples tested using the customised microarray and 24sure (19 blastomeres and 1 trophectoderm sample) were disaggregated and then reanalyzed using FISH as described previously (Colls *et al.* 2009; Munne *et al.* 1998). The embryos corresponding to these samples were donated

to research by patients. Included in this cohort, were the 3 embryos for which the new array detected three extra aneuploidies not detected using the 24sure microarray.

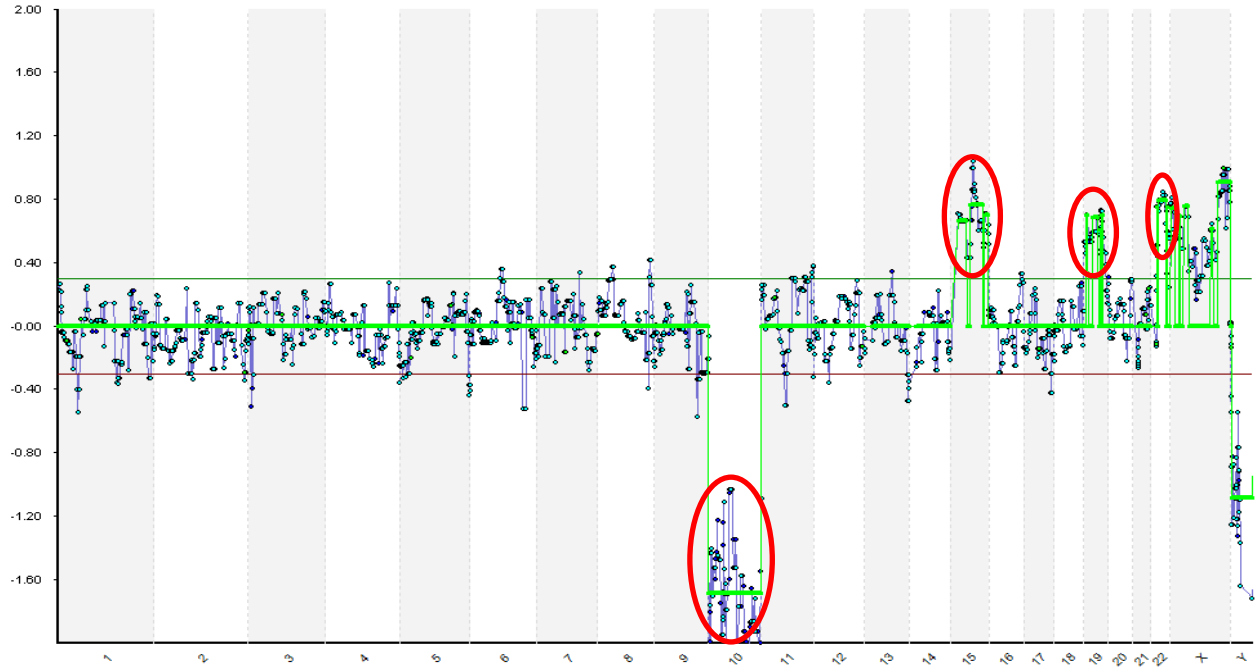
Overall, 26 aneuploidies were detected by FISH. The 24sure microarray platform succeeded in detecting all of these aneuploidies, while it also detected one extra aneuploidy. The new microarray detected 25 of the aneuploidies detected by FISH and 4 extra aneuploidies. One of the extra aneuploidies was the one also detected by 24sure microarray. The rest of the extra aneuploidies detected by the novel microarray were not detected by FISH or 24sure microarray, suggesting that they were artefacts.

3.4.2.1.2 Selection of new probes for chromosome 19

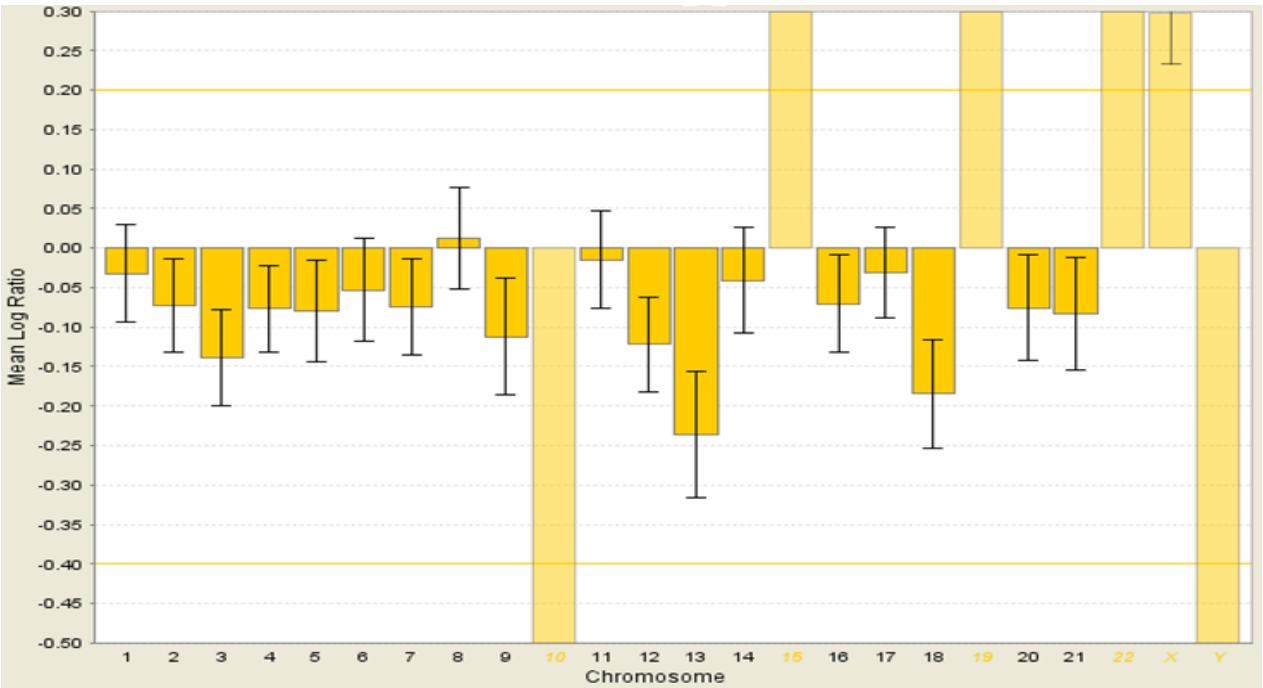
After initial validation of the new microarray was completed, it was concluded that chromosome 19 was responsible for about half (6/14) of the discrepancies seen between the array and the well-established 24sure test / FISH. Therefore, it was decided to select new probes to be included in the array for detection of chromosome 19 copy number. The newest version of the array was tested against 5 samples for which chromosome 19 aneuploidies had been detected after assessment with 24sure microarrays. The new array was able to correctly identify all of the chromosome 19 aneuploidies present in the samples (Figure 3.19). Taking into consideration the results obtained from validation of the array and also correction of chromosome 19 detection, it can be estimated that the detection rate of the latest version of the array could be as high as 97.6% (160/164 aneuploidies), while accuracy of diagnosis at the level of the sample could increase to 99% (96/97 samples).

Figure 3.19: Chromosome profile of an aneuploid blastomere (48,XX,-10,+15,+19,+22) processed with 24sure array and the developed array. **A)** 24sure array. **B)** Developed array (image provided courtesy of Dr Douglas Hurd).

A) 24sure array



B) Developed array

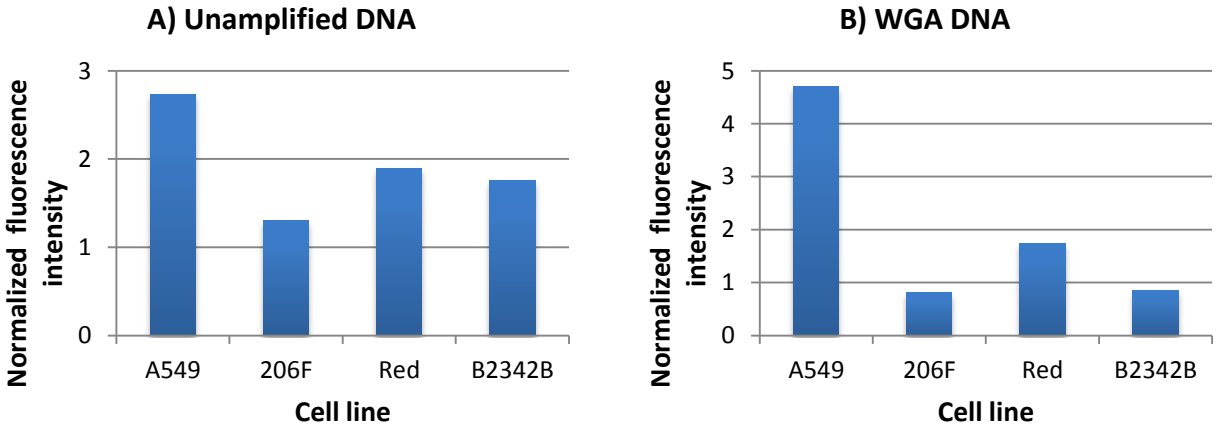


Notes: The developed array correctly identified loss of chromosome 10 and gain of chromosomes 15, 19 and 22. Furthermore, the developed array correctly determined this sample to be female.

3.4.2.2 Validation of array for mitochondrial DNA quantity measurement

The developed array was assessed for its ability to correctly determine the relative mitochondrial DNA (mtDNA) quantity of applied samples. Unamplified genomic DNA and SurePlex amplified DNA from 4 cell lines was applied to the array. Results for unamplified DNA agreed with results received for WGA DNA (Figure 3.20). Specifically, the array determined mtDNA quantity in unamplified DNA samples to be in the following order: A549 > Red > B2342B > 206F. The same exact order was also determined for WGA DNA. These results were independently verified by another scientist (Ms Lorna Macleod) through the method of quantitative real time PCR. Collectively, these results indicate that the mtDNA specific probes included on the array can accurately determine the relative mtDNA quantity of WGA samples and that WGA using the SurePlex method does not introduce significant distortions.

Figure 3.20: Relative quantitation of mtDNA in unamplified and WGA DNA derived from 4 different cell lines after application on the developed array. **A)** Unamplified DNA. **B)** WGA DNA.



3.4.2.2.1 Analysis of mtDNA quantity measurements obtained from samples used for validation of the developed array

After confirmation that relative mtDNA quantity can be determined using the microarray approach, results obtained from the samples used for aneuploidy screening validation (section 3.4.2.1) were analysed. Measurements were available for 89 samples (27 PBs, 42 blastomeres, 20 trophectoderm samples).

Analysis of results revealed that single blastomeres had significantly higher ($P<0.001$) quantities of mtDNA than PBs and trophectoderm samples (Table 3.10). Furthermore, trophectoderm samples were determined to have significantly higher ($P<0.001$) quantities of mtDNA than PBs. When chromosomal status was considered for each type of sample, abnormal samples showed a trend towards lower quantities of mtDNA compared with normal samples. This was true for all 3 sample types. However, none of the differences observed was found to be statistically significant ($P>0.05$). Results were also analyzed according to maternal age. Two groups were formed for each sample type; samples derived from younger patients (30-37 years old) and samples from patients of AMA (38-45 years old). In PB samples mtDNA quantity in the AMA group was found to be significantly lower than the younger age group ($P<0.05$). On the contrary, single blastomere and trophectoderm samples of the AMA group were determined to have larger quantities of mtDNA than the samples from younger patients. However, these differences were not statistically significant ($P>0.05$).

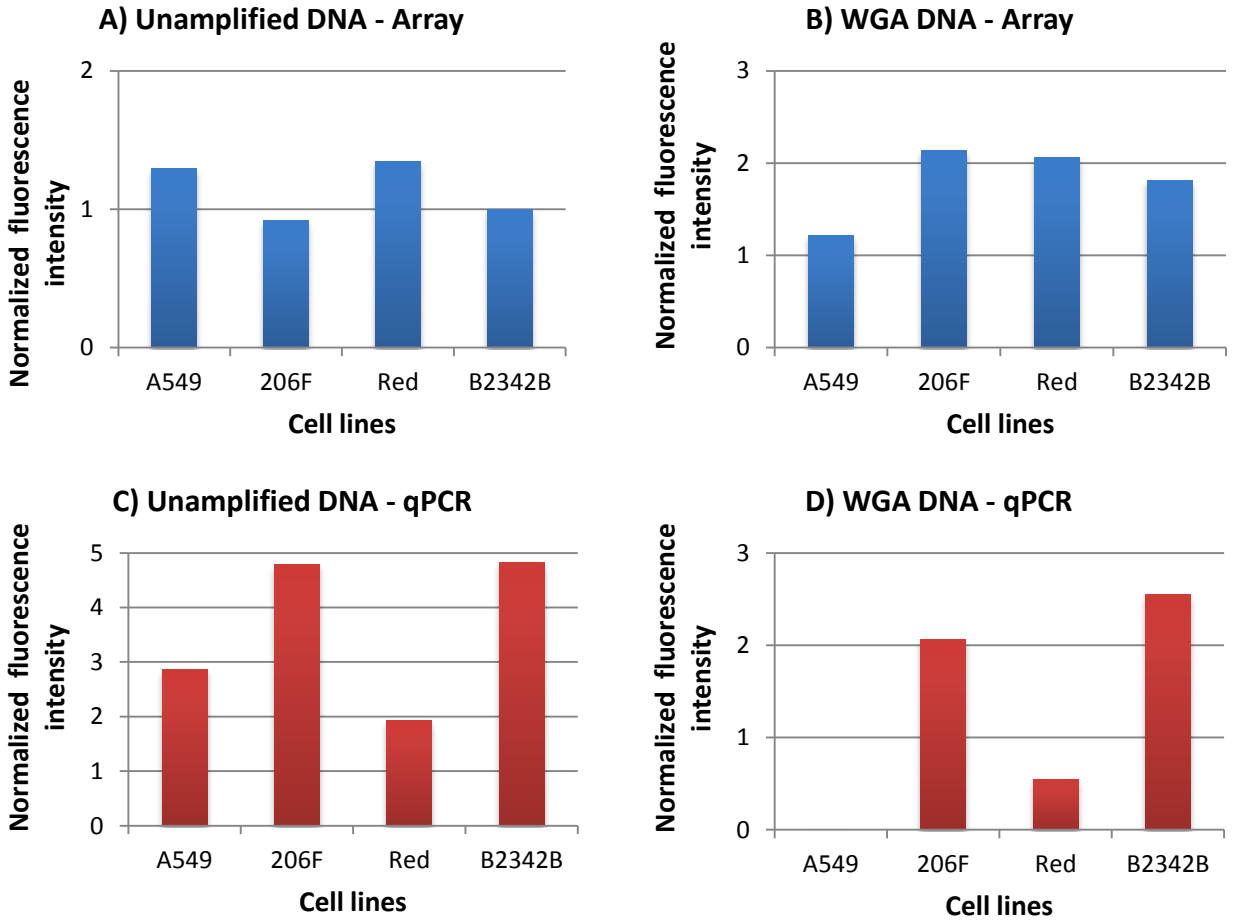
Table 3.10: Normalized fluorescence intensity values obtained for mtDNA from analysis of polar bodies, blastomeres and trophectoderm samples.

	Normalized Fluorescence Intensity				
	Overall	Chromosomal Status		Age	
		Normal	*Abnormal*	*30-37*	*38-45*
Polar Bodies	1.5	1.6	1.4	1.8	1.1
Blastomeres	20.7	21.4	20.4	18.9	21.8
Trophectoderm samples	5.5	5.9	5.2	4.4	6.7

3.4.2.3 Validation of microarray for telomere length measurement

As with mtDNA probes (section 3.4.2.2), telomere probes included on the array were assessed using unamplified genomic DNA and SurePlex amplified DNA from the 4 cell lines. As seen in Figure 3.21, results obtained from unamplified DNA did not agree with results received from WGA DNA when samples were tested using the microarray. Interestingly, it was observed that telomere probes on the array become saturated, even in samples with less telomeric DNA, preventing accurate quantification. Results obtained using a qPCR method for measuring telomere DNA did not agree with results obtained using the microarray for any of the sample types (unamplified or WGA) (Figure 3.21). Altogether, these results indicated that the usage of SurePlex WGA in combination with the developed array is not accurate in providing relative quantitation of telomere DNA in samples. Therefore it was decided not to use the developed array to collect results regarding telomere length from applied samples.

Figure 3.21: Relative quantitation of telomere length in unamplified and WGA DNA derived from 4 different cell lines. **A)** Unamplified DNA - Array. **B)** WGA DNA - Array. **C)** Unamplified DNA – qPCR. **D)** WGA DNA – qPCR.



3.4.3 Assessment of SNP probes for inclusion on the developed array

A specialist SNP array containing 6,186 selected SNP probes was produced. In order to test the efficiency and accuracy of the probes, aliquots from 14 single blastomere SurePlex samples from Sandhoff syndrome and Phenylketonuria cases (sections 2.4.1.6.3 and 2.4.1.6.4) were used. Genomic DNA from the parents in each case was also applied on the arrays.

Figure 3.22 (A) shows a comparison of the SNP calls obtained from an embryo and its parents. The 'CytoSure Interpret Software' uses dots of differing colours to show results obtained from informative SNPs. The informative SNPs giving a correct call (i.e. one that would match parental genotype) were found to be 40.1%. The SNPs giving Mendelian inconsistencies (i.e. SNPs showing genotypes incompatible with inheritance from the specific parents) constituted 13.6% of the overall informative SNPs. The rest of the informative SNPs displayed UPD. In summary, although this embryo was truly derived from the parents tested, a small number of SNPs were inconsistent with parental DNA (Mendelian inconsistencies). This was not unexpected since it had been previously determined that SurePlex amplification might provide erroneous SNP calls (section 3.3.3.2).

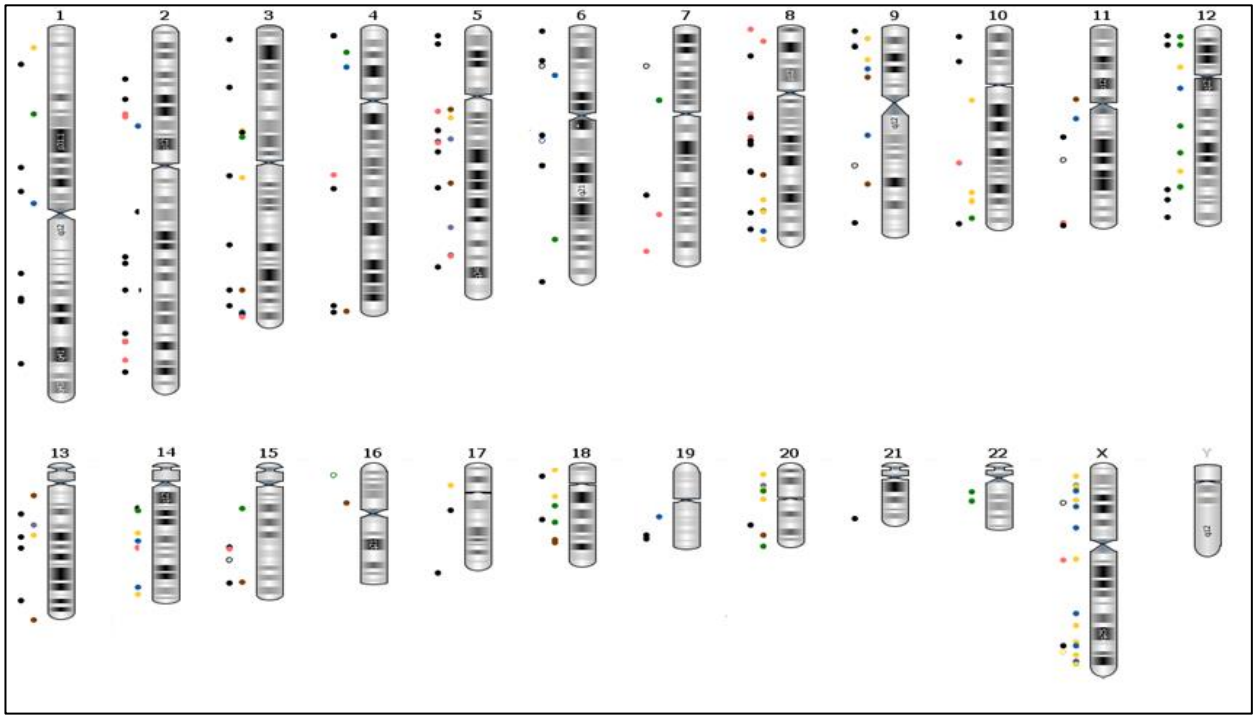
In order to determine if the SNPs included on the array would be able to provide embryo fingerprinting, results obtained from embryos were ran against results obtained from non-matching parents. Figure 3.22 (B) shows results from when SNP calls of an embryo were compared to non-matching parents. The SNP calls giving a correct result (i.e. a result matching parental genotype) were 9.8% of the overall informative SNPs while, SNP calls showing Mendelian inconsistencies were 25.1%. Compared to results from the first example [Figure 3.22 (A)], correct SNP calls in this example are found to be significantly lower ($P<0.001$) and SNP calls showing Mendelian inconsistency are significantly higher ($P<0.01$).

Results from all 14 samples applied on the array were similar to results obtained in the examples of Figure 3.22. These results show that the specific SNP probes can be used to determine if tested embryos are derived from the correct parents (avoiding mismatch incidences). Also, these results indicate that the probes can be used in other

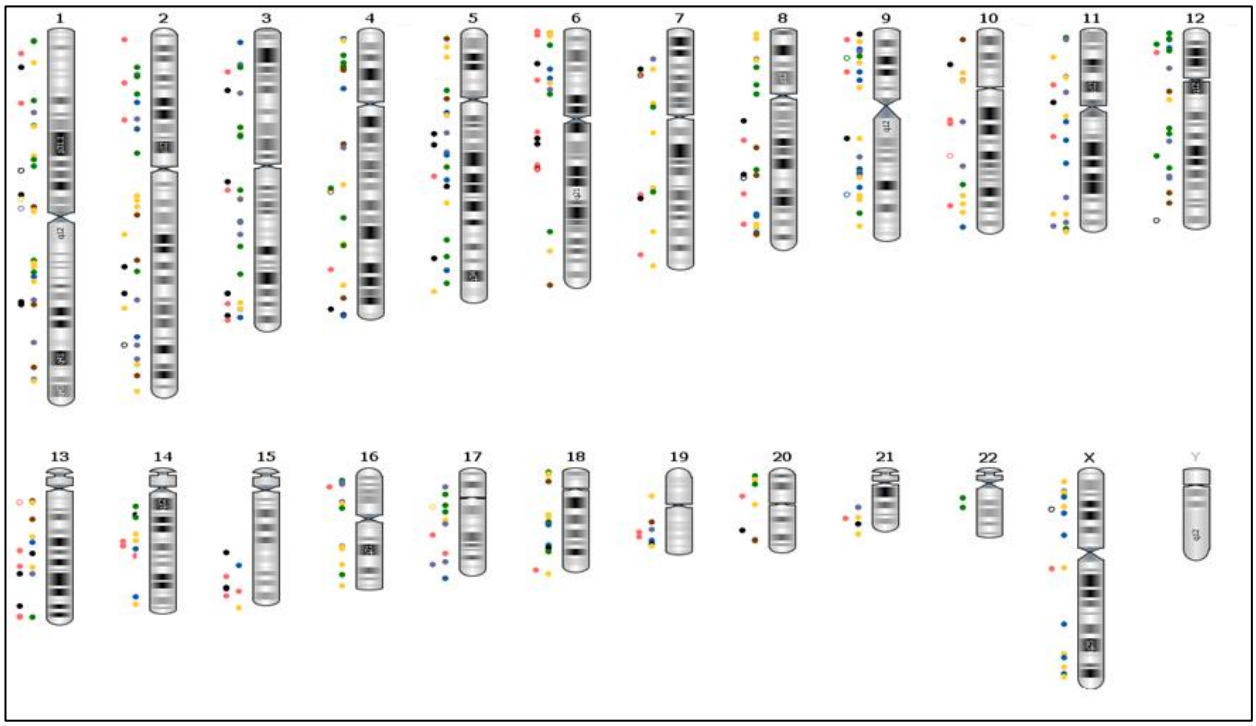
embryo fingerprinting applications, such as follow-up of embryos transferred and detection of DNA contaminants.

Figure 3.22: Results obtained for SNP probes through usage of 'CytoSure Interpret Software'. **A)** Embryo compared to matching parents. **B)** Embryo compared to non-matching parents. Images provided courtesy of Dr Douglas Hurd.

A) Embryo compared to matching parents.



B) Embryo compared to non-matching parents.

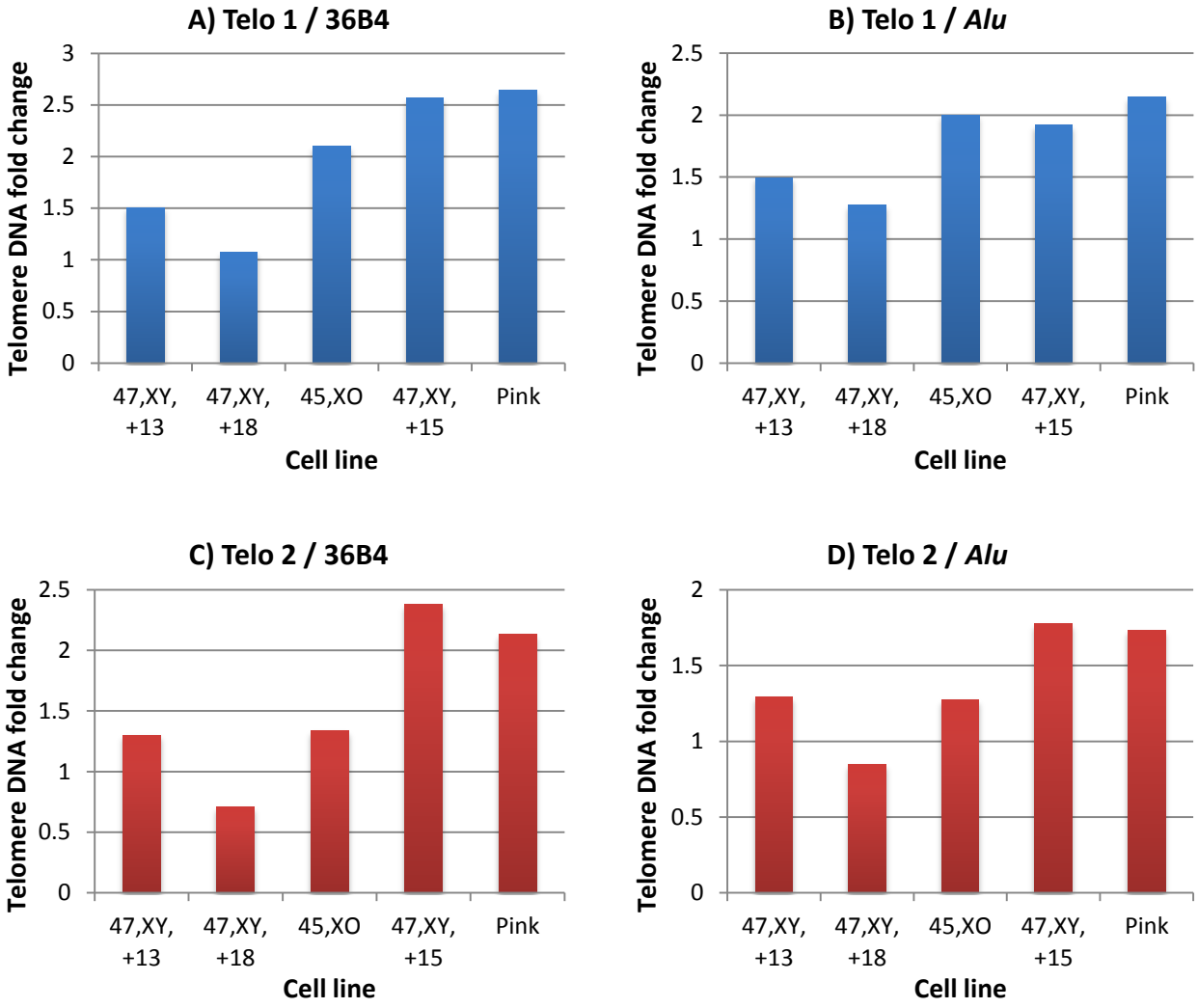


Notes: Both images show informative SNPs only. Each dot with different colour represents a different indication; black: heterozygous SNP - correct match to parental DNA, green: isodisomic UPD (paternal), brown: heterodisomic UPD (paternal), yellow: isodisomic UPD (maternal), blue: heterodisomic UPD (maternal), pink: Mendelian inconsistency (single), purple: Mendelian inconsistency (double).

3.5 Development of a protocol for measurement of telomere length

In order to test the optimised protocol, genomic DNA and SurePlex amplified single cells derived from 5 cell lines were used. As described in section 2.6.2, 2 sets of primers for telomere amplification were employed; 'tel1 - tel2' set (designated as 'telo 1' henceforth) and 'telg - telc' set (named 'telo 2' from this point). In addition, two targets were amplified and used as endogenous controls; the multicopy *Alu* sequence and a sequence from single gene 36B4. In order to assess if results obtained using the two endogenous controls were in agreement, genomic DNA from the 5 cell lines was amplified with all 4 primers sets and results were analysed. As seen in Figure 3.23, when the 'telo 1' primer set was used results from 36B4 and *Alu* controls did not agree. When considering the 'telo 2' primer set, results from the two endogenous controls looked very similar (Figure 3.23). However, when 36B4 was used as the endogenous control, 45,XO telomere DNA was found to be slightly more in quantity than 47,XY,+13 (1.37 compared to 1.30), while, when *Alu* was used as control relative quantitation of telomere DNA of 45,XO cell line is found to be slightly lower than 47,XY,+13 (1.27 compared to 1.29).

Figure 3.23: Relative quantitation of telomere DNA in samples of genomic DNA. **A)** 'Telo 1' primer set with 36B4 endogenous control. **B)** 'Telo 1' primer set with *Alu* endogenous control. **C)** 'Telo 2' primer set with 36B4 endogenous control. **D)** 'Telo 2' primer set with *Alu* endogenous control.

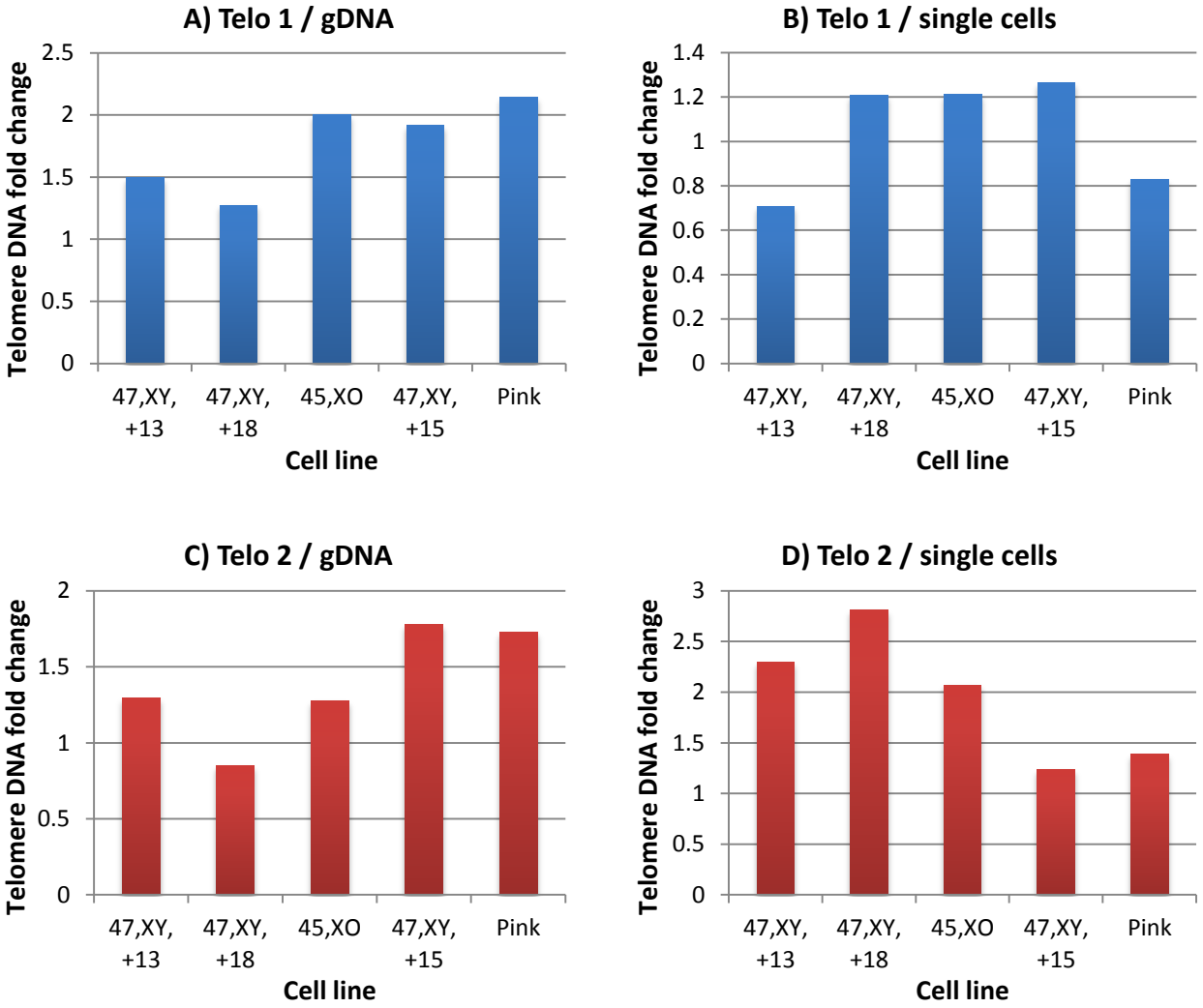


3.5.1 Testing of protocol with WGA single cells

The two telomere primer sets were further assessed through relative quantitation of SurePlex amplified single cells. *Alu* sequence was used as endogenous control for reasons explained before (section 2.6.1) and results were compared to results obtained from relative quantitation of corresponding genomic DNA.

Results obtained from SurePlex amplified single cells were very different from results obtained from genomic DNA (Figure 3.24). This was true for both primer sets used, ‘telo 1’ and ‘telo 2’. Furthermore, as observed with genomic DNA, results obtained regarding relative quantitation of telomere DNA in amplified single cells were different between the two primer sets amplifying the telomeres.

Figure 3.24: Relative quantitation of telomere DNA in samples of genomic DNA and SurePlex amplified single cells. **A)** 'Telo 1' primer set on genomic DNA. **B)** 'Telo 1' primer set on amplified single cells. **C)** 'Telo 2' primer set on genomic DNA. **D)** 'Telo 2' primer set on amplified single cells.



3.6 Development of a Real-Time PCR protocol for comprehensive chromosome screening of human embryos

The development of a protocol that would be able to test all 24 human chromosomes for aneuploidies in a fast and accurate way was undertaken. The protocol aimed at detection of whole chromosome aneuploidies.

3.6.1 Testing of protocol on genomic DNA

The initial protocol developed was assessed on genomic DNA derived from three fibroblast cell lines [47,XY,+13; 47,XY,+18; 'Pink' (46,XX)]. The two aneuploid cell lines were tested for all 96 real-time PCR assays, while 'Pink' cell line was only tested using the assays that interrogate loci on chromosomes X and Y. Two options existed for analysis of results with 'CopyCaller Software (v1.0)'; perform analysis using a calibrator (i.e. a sample of known chromosome copy number to act as a reference) or without calibrator. If selected, the calibrator would have to be amplified along with every new batch of samples tested in order for the software to be able to provide results. Eventually, it was decided to analyse samples without using a calibrator in order to reduce the cost of the test and allow for more samples to be processed in parallel.

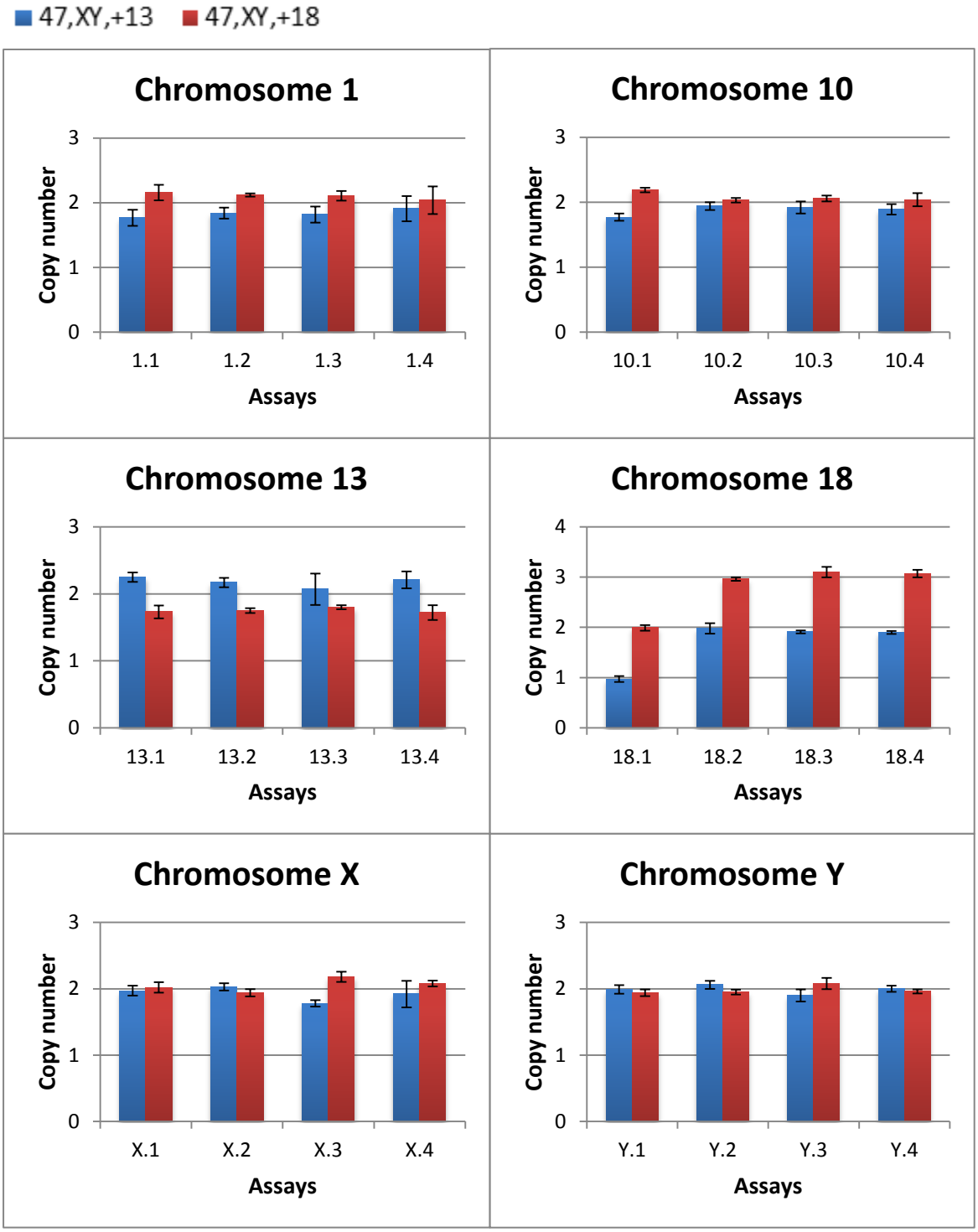
The overall accuracy rate for the assays used in the experiment was 87.9%. A total of 192 assays were tested and 190 amplified (assay 5.3 failed to amplify for both samples). Of the 190 amplified assays, 23 gave incorrect chromosome copy number results. All assays used for chromosome 13 failed to detect the extra chromosome in the sample derived from cell line 47,XY,+13 (Figure 3.25). Detection of trisomy 18

was more successful, with 3/4 assays localised to chromosome 18 correctly assigning a copy number of 3 for the sample derived from cell line 47,XY,+18. Regarding sex chromosomes, all assays used erroneously assigned a copy number of 2 for both the X and Y chromosomes (Figure 3.25).

In order to assess whether using a calibrator sample would make any difference in copy number calculation of sex chromosomes, results were re-analysed using 47,XY,+18 sample as a calibrator for 47,XY,+13 sample. As seen in Figure 3.26, when a calibrator was used the copy number of sex chromosomes was correctly identified.

Furthermore, in order to examine what samples (male or female) can be used as calibrator, an experiment was carried out using genomic DNA derived from cell lines 47,XY,+13, 47,XY,+18 and 'Pink' (46,XX). Results obtained indicated that when a female sample is used as calibrator then the copy number of Y chromosome is not correctly assigned. Conversely, when a male sample is used as calibrator copy number of sex chromosomes is correctly identified (Figure 3.27).

Figure 3.25: Copy number assigned from assays tested for 6 chromosomes of two samples derived from aneuploid cell lines 47,XY+13 and 47,XY,+18.



Notes: Assays used for chromosomes 1 and 10 correctly identified a copy number of 2 for both samples. Assays used for chromosome 13 failed to detect the extra chromosome in sample 47,XY,+13. For chromosome 18, 3/4 assays gave a correct copy number for both samples. All assays used for chromosomes X and Y falsely assigned a copy number of 2 for both samples instead of 1.

Figure 3.26: Copy number of chromosomes X and Y assigned for a sample derived from cell line 47,XY,+13 by the 'CopyCaller Software (v1.0)' with and without calibrator (47,XY,+18).

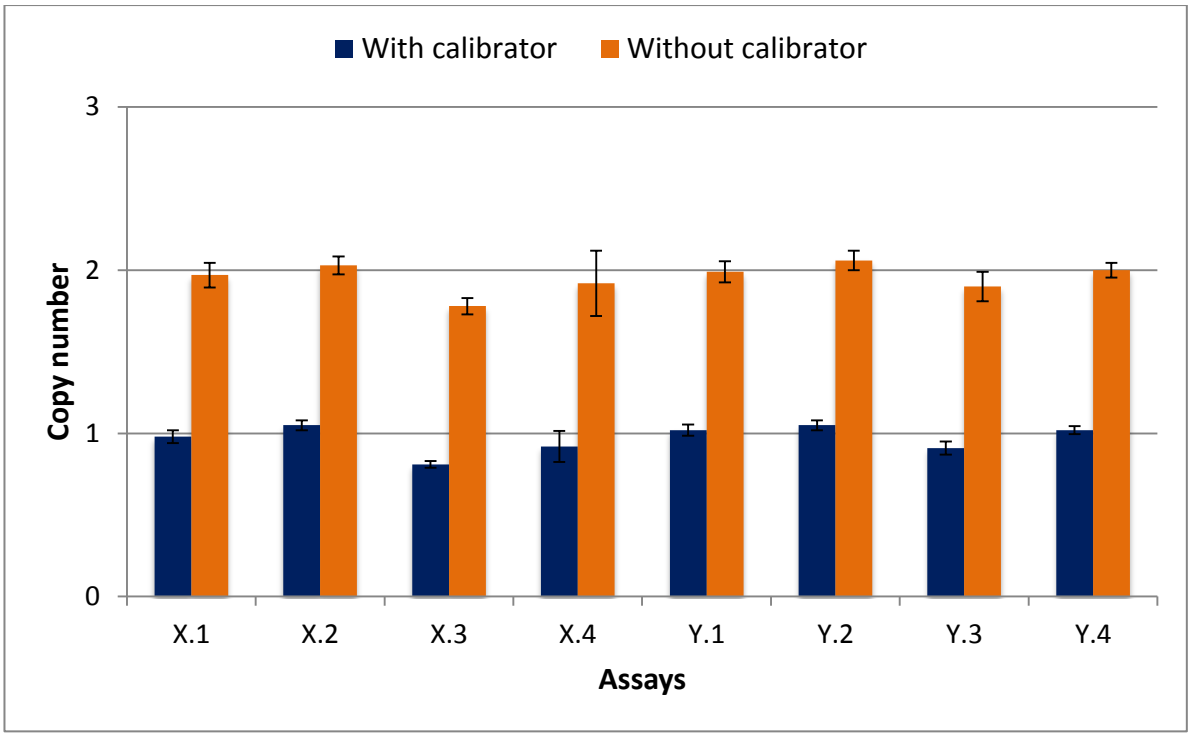
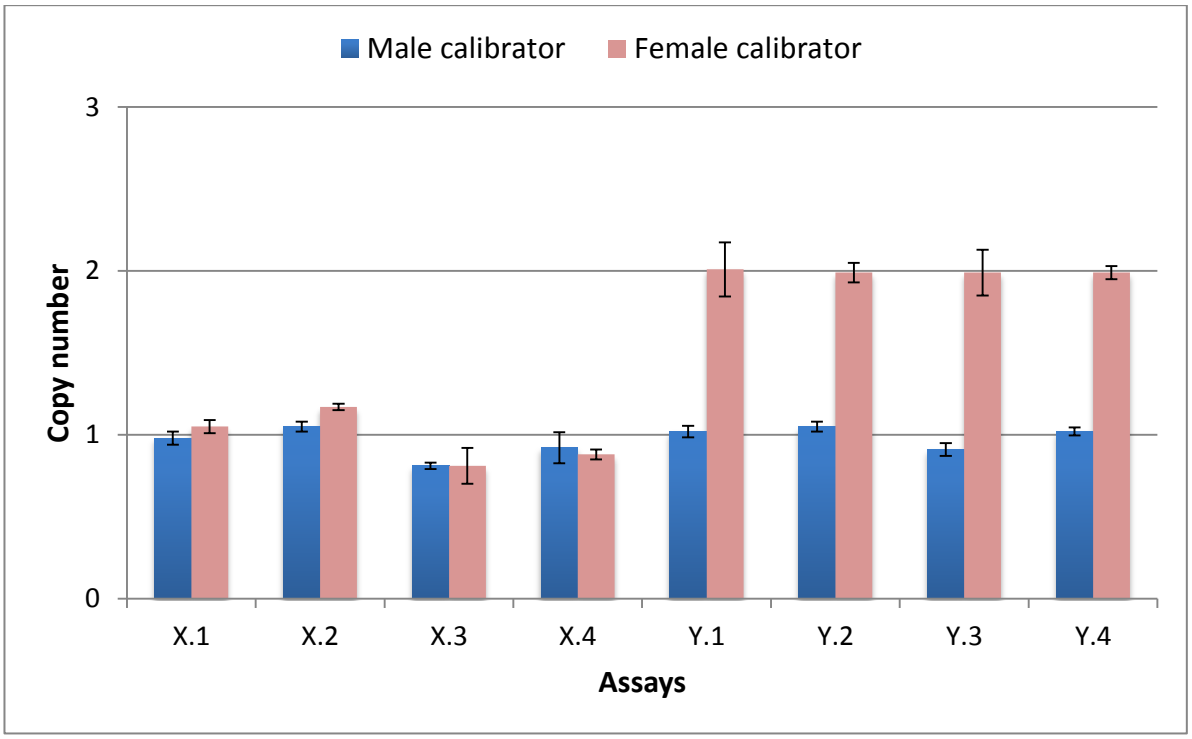


Figure 3.27: Copy number assigned to chromosomes X and Y for DNA sample (47,XY,+13) when male or female calibrator was used.



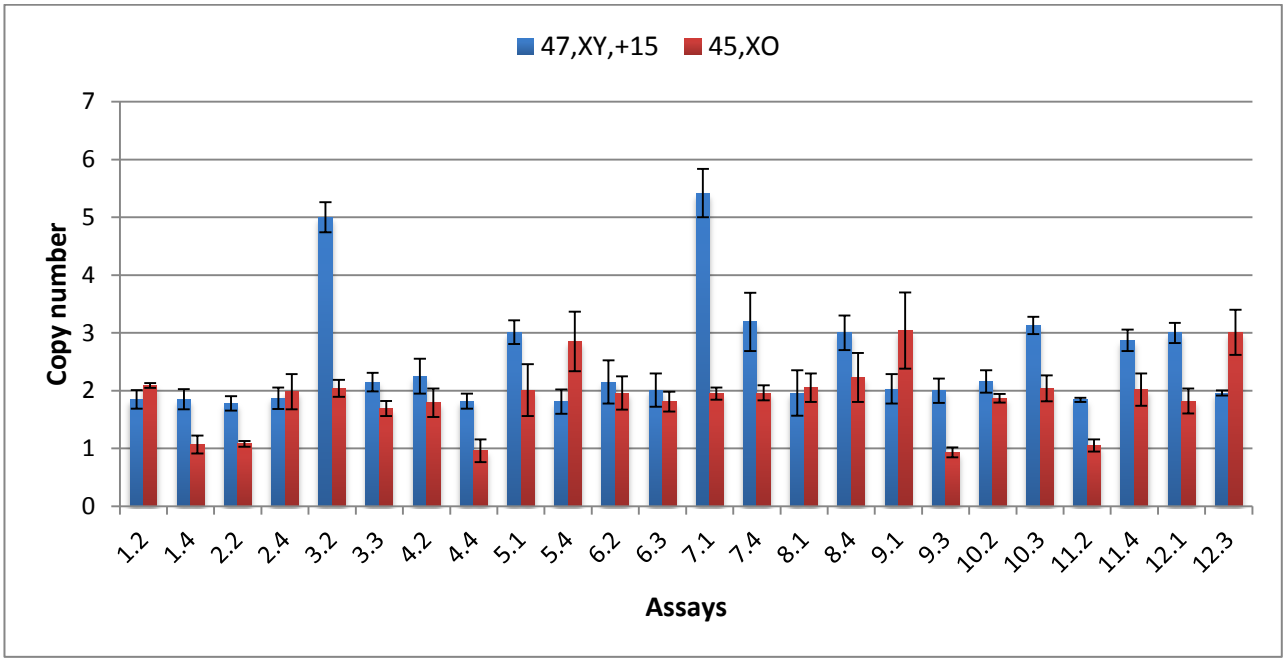
3.6.2 Testing of protocol on single cells

The two best performing assays for each chromosome from the experiment carried out on genomic DNA were selected for assessment of the protocol on single cells. Two single cells derived from cell lines 47,XY,+15 and 45,XO were tested (i.e. one cell from each cell line). Although it was determined that usage of a calibrator for analysis of results would be beneficial (section 3.6.1), results were analysed without using a calibrator. This was because if a calibrator was to be used it had to be from a sample of similar DNA quantity (prior to amplification), therefore a single cell. However, single cells (as shown below) provide highly erroneous results and usage of such a sample as a calibrator would cause the assignment of wrong chromosome copy numbers to the sample under assessment.

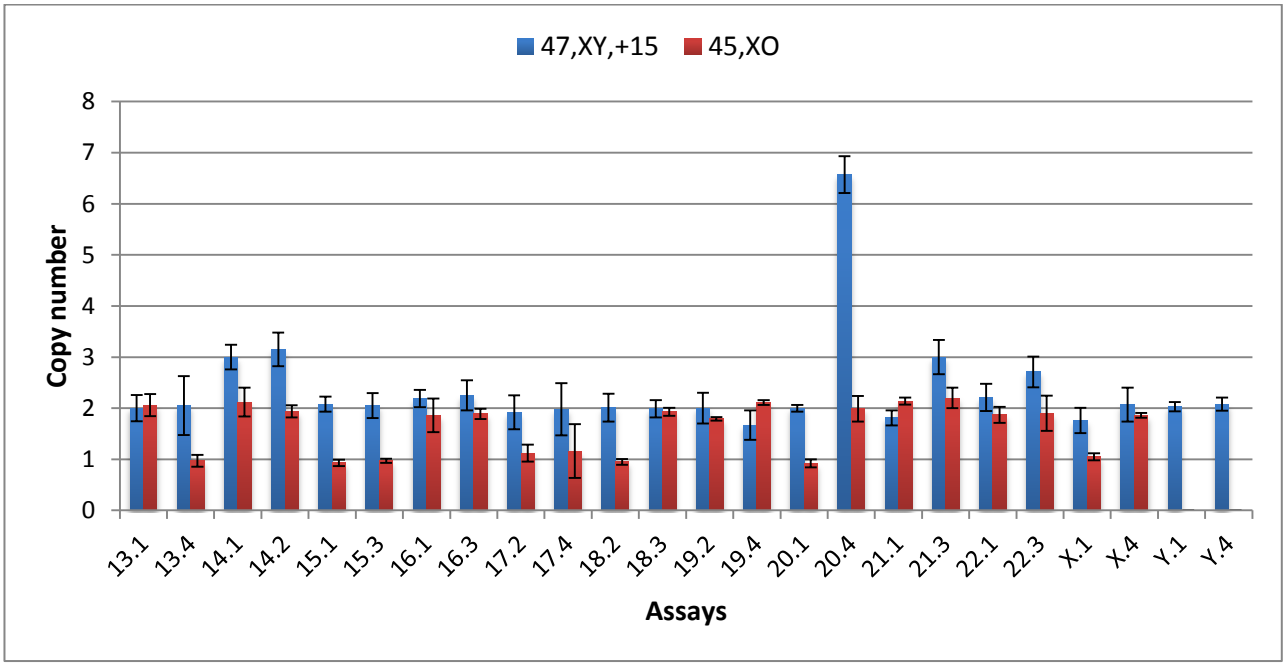
In total, 48 assays were used to assess each single cell. Overall accuracy rate, calculated from results obtained from both cells after dividing the number of assays that provided a correct copy number by the overall number of assays used, was 36.5%. Nineteen assays provided a wrong result for one of the cells and 16 for the other (Figure 3.28). Of interest is the fact that the assays which provided a wrong copy number for one of the cells were, in their majority, different from the assays with incorrect call for the other cell. Only, three assays provided erroneous copy number in both cells. Amongst these three assays were assays 15.1 and 15.3 which failed to detect the extra copy number in the single cell derived from cell line 47,XY,+15. Regarding chromosomes X/Y, apart from assays X.1, Y.1 and Y.4 that correctly identified the copy number for sample 45,XO, the rest gave wrong chromosome copy number assignments. Overall, 21 assays gave a higher copy number than expected and 14 gave a lower copy number.

Figure 3.28: Copy number assigned to chromosomes of two single cells derived from cell lines 47,XY,+15 and 45,XO. **A)** Assays for chromosomes 1 - 12. **B)** Assays for chromosomes 13 - X/Y.

A) Assays for chromosomes 1 - 12



B) Assays for chromosomes 13 - X/Y



3.6.3 Testing of protocol on clumps of cells

The protocol was also tested on clumps of cells, specifically clumps of 3 cells. For these experiments all 96 assays were used. The clumps of cells were intended to simulate a trophectoderm biopsy, which typically consist of several cells. Six euploid samples were tested; 3 male and 3 female. Analysis of samples was carried out without using a calibrator for reasons explained before (section 3.6.2).

Overall accuracy rate for all samples tested was calculated to be 92.2%. Sixty-seven out of the 96 assays tested gave a correct copy number in all 6 samples. The rest of the assays (29) assigned a wrong copy number for at least 1 of the samples. The accuracy rate of these 29 assays ranged from 33.3% to 83.3%. Apart from three assays however (14.3, 20.4, X.3), the rest had an accuracy rate ranging from 66.7% to 83.3%. For most of the inaccuracies seen (71.1%) a wrong copy number of one more or one less was assigned. Regarding chromosome X, apart from assay X.3 which had an accuracy rate of 33.3%, the rest of the assays were 100% correct in their assignment of copy number. All assays for chromosome Y had an accuracy rate of 50%, correctly identifying that there were no Y chromosome copies in female samples, but wrongly assigning a copy number of 2 for the specific chromosome in male samples.

3.7 Protocols developed for DNA fingerprinting of clinical samples

Protocols were developed for DNA fingerprinting of sperm samples, single cells and WGA products and also for contamination detection. For the development of these protocols 12 highly informative SNPs were utilized. The SNPs were amplified and genotyped using real-time PCR.

3.7.1 DNA fingerprinting of sperm samples

A fast, inexpensive and reliable protocol was sought for DNA fingerprinting of sperm samples received in IVF units. In order to achieve this, 3 different DNA extraction methods were optimised and assessed for their ability to extract DNA from neat sperm samples. The three methods were the alkaline lysis extraction method, the TaqMan Sample-to-SNP kit and the QIAamp DNA blood Mini kit.

Using the 3 methods, DNA was extracted from aliquots obtained from a sperm sample derived from the same man. Extracted DNA was genotyped for the 12 selected SNPs. Results obtained from the QIAamp DNA blood Mini kit were used as positive control to which results from the other two methods were compared to. This was because the specific kit is an optimised, commercially available kit that is used for DNA extraction from blood and other body fluids. For the sperm aliquot that had DNA extracted using alkaline lysis, a result was obtained for all 12 SNPs assessed (AE = 100%), while DNA extraction using the TaqMan Sample-to-SNP kit was only associated with results for 5/12 SNPs (AE = 41.7%). Compared to results obtained from the control, results provided from the TaqMan Sample-to-SNP kit were only

accurate for 60% of the amplified SNPs, whereas for the alkaline lysis method, accuracy rate was calculated to be 91.7%.

Although the alkaline lysis method was not found to be 100% accurate in SNP genotyping as was the control method (QIAamp kit), it was still highly accurate and it was selected over the QIAamp kit method for development of the final protocol for two main reasons: a) it involved a lower number of experimental steps and it was therefore faster (required ~15min compared to ~25min needed by the QIAamp kit), b) it was considerably cheaper than the QIAamp DNA blood Mini kit - approximate price per sample: £0.007 for alkaline lysis vs. £2.4 for QIAamp kit.

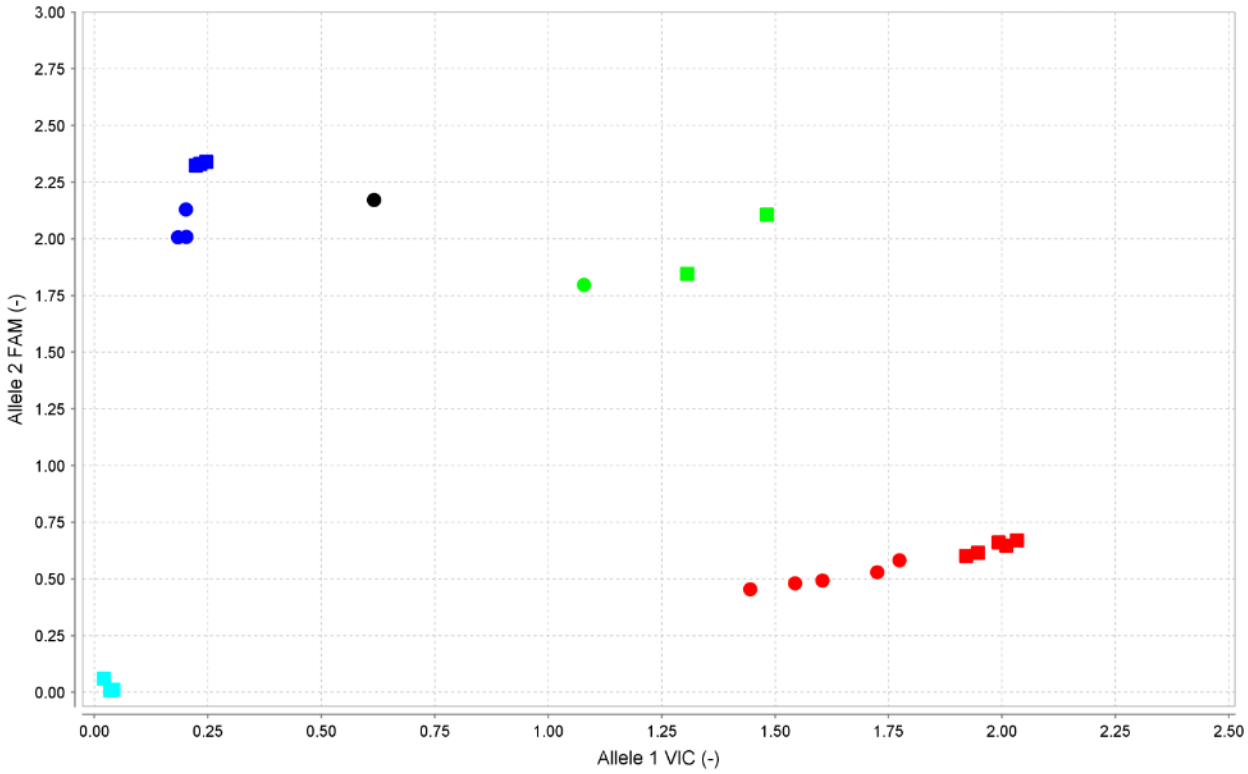
3.7.1.1 Validation of final protocol

In order to validate and assess the final protocol 10 sperm samples derived from 10 different men were used. The concentration of sperm in the neat samples ranged from low to high levels (8.6-168 million/ml). Apart from DNA extraction using the alkaline lysis method, DNA extraction was also carried out using the QIAamp DNA blood Mini kit for each sample, the latter once again serving as a control.

Amplification efficiency of the developed protocol was calculated to be 99.2% (119/120), while the accuracy was 100%. No ADO, GOH and COH phenomena were observed. Results obtained from the alkaline lysis extraction method agreed completely with results obtained from the positive controls for all samples and all SNPs tested. This accuracy rate was calculated when using default settings of the 'TaqMan Genotyper Software (v1.0.1)' for 'genotyping quality value'. 'Genotyping quality value' is a value given to each sample analysed (for each SNP tested) and

refers to the quality of genotype determination provided by the software. As a default, the software has a threshold of ‘genotyping quality value’ of 0.95 - with maximum value being 1.0. Samples with values <0.95 are not given a genotype and remain undetermined. During validation of the developed protocol, 2 SNPs from 2 different samples were assigned a ‘genotyping quality value’ of <0.95 and were therefore left undetermined (Figure 3.29). When the ‘genotyping quality value’ threshold was set lower than 0.95 (specifically 0.83) in order for the software to be able to analyse these two SNP calls, the results obtained were incorrect (i.e. they did not agree with results from controls). This indicated that in order for analysis of results to be accurate for the developed protocol, the threshold for ‘genotyping quality value’ should remain as default (0.95).

Figure 3.29: Scatter plot created using 'TaqMan Genotyper Software (v1.0.1)' showing results obtained from SNP assay 12 for all sperm samples and positive controls.



Notes: Allele 1 (*x*-axis) represents the normalized fluorescence intensity of the allele with nucleotide C, labelled with VIC (red). Allele 2 (*y*-axis) represents the normalized fluorescence intensity of the allele with nucleotide T, labelled with FAM (blue). Light blue squares represent negative controls, red circles and squares represent samples homozygous for allele 1, blue circles and squares represent samples homozygous for allele 2, while green circles and squares show heterozygous samples (allele 1/allele 2). Black circle indicates undetermined sample. Red-green-blue circles indicate samples under assessment, while red-green-blue squares indicate positive controls. A sample/control homozygous for allele 1 would only display fluorescence from VIC dye, shown with red colour in the scatter plot. A sample/control homozygous for allele 2 would only display fluorescence from FAM dye, shown with blue colour in the scatter plot. A heterozygous sample/control would express fluorescence from both dyes (VIC/FAM) and would be shown in the scatter plot with green colour.

3.7.2 DNA fingerprinting of single cells

Single cells were assessed for amplification of the 12 selected SNPs in order to determine whether the fingerprinting method could also be used for embryo identification and contamination detection during PGD cycles. In total, 20 single fibroblasts were assessed and 240 loci were examined.

The 'genotyping quality value' threshold was set at 0.90 for analysis of single cell results. Leaving the threshold at default setting (0.95) would have excluded 6 SNP results from analysis that were correctly called otherwise (i.e. when threshold was at 0.90). Therefore, it was concluded that for single cells a threshold of 0.90 is the most appropriate, allowing all of the SNPs to be genotyped without compromising accuracy.

The overall call rate (i.e. amplification efficiency) was found to be 99.6% (239/240) (Table 3.11). Accuracy of protocol was determined to be 97.9% (234/239). The small percentage of inaccuracies observed was mostly due to ADO. Allele dropout was seen at a rate of 4% (4/100). GOH was also observed for a single locus in one sample (1/139). It is highly likely that this single GOH event was caused due to contamination in the specific sample tube. Having this in mind, it is important to note that all of the negative controls used in these experiments (i.e. reactions performed with no DNA template included) showed no contamination. No COH events were observed (0%).

Table 3.11: Results obtained from genotyping the 12 SNPs after direct amplification and WGA of single cells.

	Call rate (%)	Non-determined (%)	Identical Calls (%)	ADO (%)	GOH (%)	COH (%)
Direct amplification	99.6	0	97.9	4	0.7	0
MDA	100	14.4	87.6	38.9	0	0
SurePlex	89.7	6.4	80.2	41.7	0	1.4

3.7.3 DNA fingerprinting of WGA products

For assessment of WGA products, 11 MDA samples (132 loci) and 13 SurePlex samples (156 loci) were used. All samples were derived from whole genome amplification of single fibroblasts.

Analysis of results was carried out using the default threshold for 'genotyping quality value' (0.95). The call rate of MDA method was 100% (132/132) (Table 3.11). This was found to be significantly higher ($P<0.001$) than the call rate of SurePlex WGA (89.7%). However, MDA was found to have a significantly higher rate ($P<0.05$) of undetermined SNP calls compared to SurePlex. Therefore, taken altogether, the percentage of SNP loci available for genotyping was similar ($P>0.05$) amongst the two methods - 85.6% for MDA compared to 84% for SurePlex. Regarding SurePlex, one of the SNP assays (assay 9) failed to amplify for almost all of the samples tested (12/13) indicating that the locus which includes the specific SNP is not amplified by SurePlex WGA. Excluding this SNP locus from calculations, overall call rate of SurePlex WGA for the remaining 11 SNP loci is determined to be 97.2%.

From the results available for analysis it was determined that the accuracy of genotyping for MDA products was 87.6% (Table 3.11). This was found to be similar ($P>0.05$) to the accuracy calculated for SurePlex (80.2%). Allele dropout was found to be relatively high for both WGA methods. Specifically, MDA had an ADO rate of 38.9% (14/36), while, SurePlex had an ADO rate of 41.7% (25/60). No significant difference was calculated between the two methods ($P>0.05$). None of the two methods showed GOH while COH was observed only on one occasion for SurePlex WGA (1.4%). Importantly, this incidence of COH was observed for the only locus that amplified for SNP assay 9 for SurePlex samples, indicating that amplification of this locus is poor and unreliable.

All of the WGA samples were also analysed using a lower threshold for 'genotyping quality value' (0.43) that allowed for analysis of all amplified loci. Using this threshold the accuracy rate for MDA samples was found to decrease considerably from 87.6% to 78.8%. Accuracy rate for SurePlex samples also slightly decreased from 80.2% to 79.3%. Although no significant differences were calculated for any of the methods ($P>0.05$), it was demonstrated that having a 'genotyping quality value' threshold of 0.95 for WGA samples is the best option, particularly for MDA samples.

3.8 Using PLCζ gene to explain infertility problems in males involved in IVF treatment

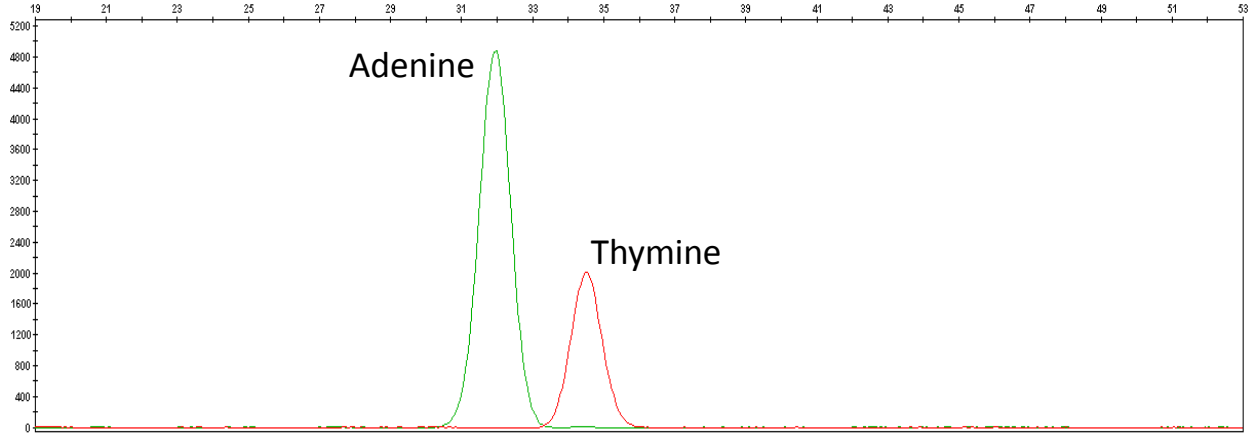
In addition to the H398P mutation previously identified in a male patient (Heytens *et al.* 2009), a new mutation in the PLCζ gene was identified through sequencing of DNA samples. Specifically, DNA extracted from 9 infertile patients whose sperm lacked oocyte activation ability, had undergone sequencing (section 2.9.1) and the nucleotide sequence of the PLCζ gene for each of these patients was determined. The new mutation identified was causing the substitution of histidine to leucine at position 233 of the amino acid sequence (H233L). The new mutation was identified in the same patient that the H398P mutation was detected. No mutations were detected in the other patients screened.

3.8.1 Mutation detection in single sperm and genomic DNA

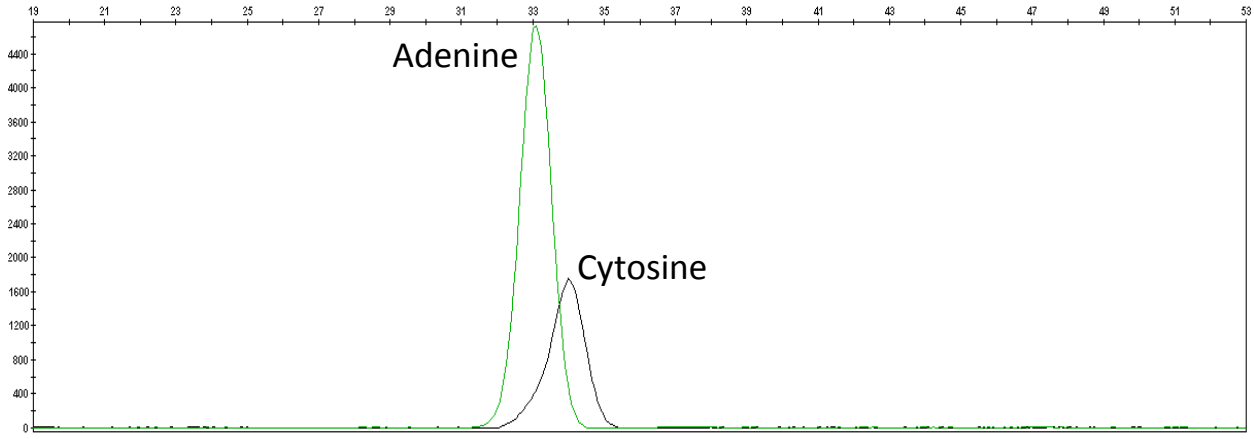
After mutation H233L was detected in patient^{H398P} it was decided to carry out minisequencing on DNA extracted from the patient and some of his family members (mother, father, half-brother, daughter) and also on single sperm isolated from the patient. Minisequencing carried out on patient^{H398P}'s DNA confirmed results obtained through sequencing, identifying the single base substitution (adenine to thymine) at position 698 of the coding DNA sequence (c.698A>T; H233L) and the single base substitution (adenine to cytosine) at position 1193 of the coding DNA sequence (c.1193A>C; H398P) (Figure 3.30). The presence of two peaks after minisequencing, one indicating the normal allele and one representing the mutant allele, for each locus (H233L, H398P) showed that the patient is heterozygous for the two mutations.

Figure 3.30: Electropherograms showing minisequencing results from screening patient^{H398P} for mutations H233L and H398P. **A)** H233L mutation. **B)** H398P mutation.

A) H233L mutation



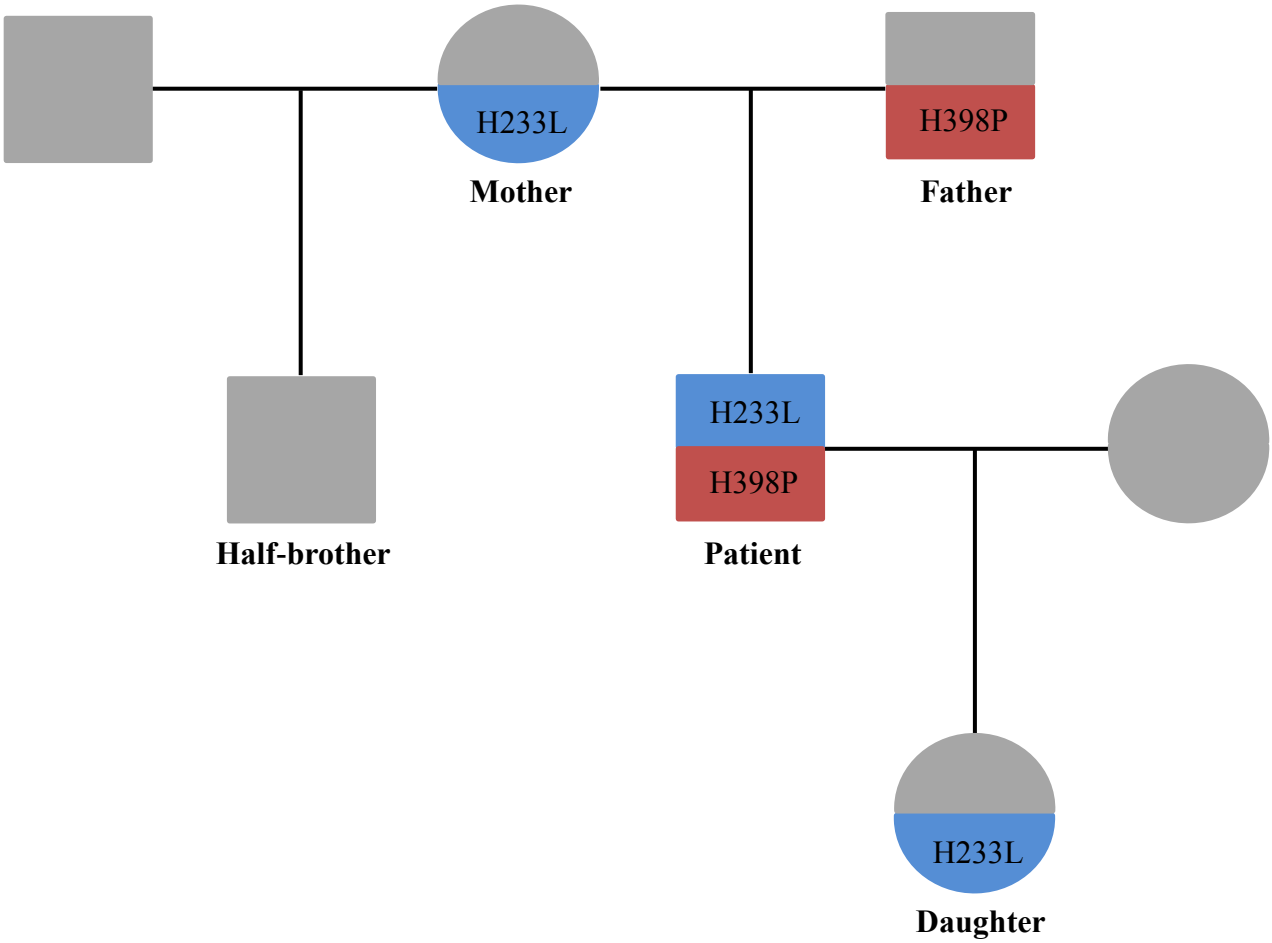
B) H398P mutation



Notes: Results show patient^{H398P} to be heterozygous for mutations H233L and H398P. Electropherogram A shows presence of adenine and thymine for locus H233L, indicating that the patient is a heterozygous carrier of the specific mutation (c.698A>T). Electropherogram B shows presence of adenine and cytosine for locus H398P, indicating that the patient is also a heterozygous carrier of this specific mutation (c.1193A>C). Green peak: adenine; red peak: thymine; black peak: cytosine.

In addition, screening of patient's family identified his father as the carrier of the H398P mutation and his mother as the carrier of the H233L mutation (Figure 3.31). The half-brother of the patient was not found to possess either of the mutations, while his daughter (conceived through assisted reproductive technology and the use of assisted oocyte activation), was identified as carrier of the H233L mutation. The conclusion drawn from the results above was that each of the mutations was found on a different copy of the PLCζ gene in patient's DNA and not on the same copy of the gene (i.e. the patient did not possess any normal copies of the gene).

Figure 3.31: Pedigree showing the inheritance of H233L and H398P mutations in the patient and his family.



This conclusion was further confirmed by the results received from minisequencing of patient's single sperm. Minisequencing showed that each single sperm possessed one of the two mutations. Specifically, H233L mutation was detected in 7/12 single sperm and H398P mutation in 5/12 (Table 3.12). The two mutations were never inherited together. The results confirmed that the patient does not possess any normal copies of the PLCζ gene and that inevitably he would pass on a mutant copy of the PLCζ gene to any offspring he has - as happened with his existing daughter.

Table 3.12: Results received from minisequencing of single sperm derived from patient^{H398P}.

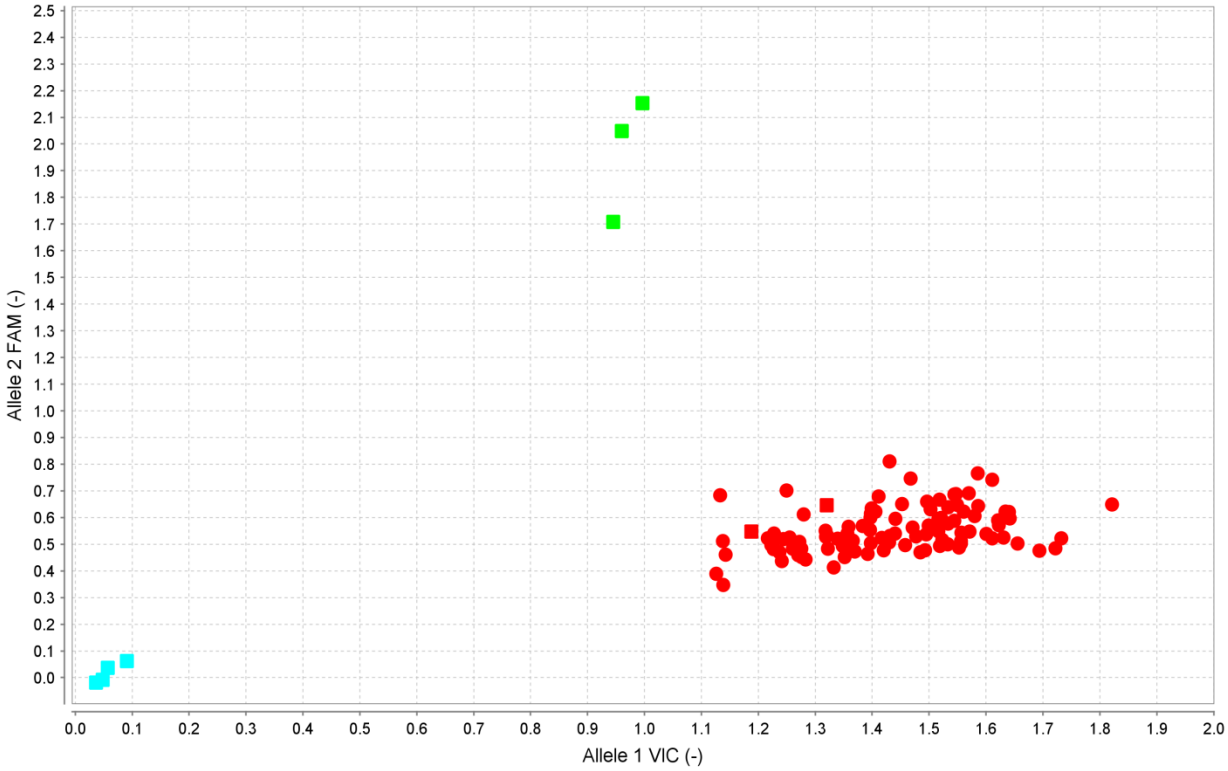
	Single Sperm											
	1	2	3	4	5	6	7	8	9	10	11	12
H233L	—	+	—	—	+	—	—	+	+	+	+	+
H398P	+	—	+	+	—	+	+	—	—	—	—	—

Notes: plus (+) indicates presence of mutation; minus (-) indicates absence of mutation.

3.8.2 Screening of 100 individuals for H398P and H233L mutations

In order to determine prevalence of the H398P and H233L mutations, 100 fertile individuals (50 couples) undergoing PGD were anonymously assessed. Neither H398P mutation nor H233L mutation (Figure 3.32) were detected in the individuals tested. Results obtained from patient's DNA and his family member's DNA confirmed previous observations (section 3.8.1).

Figure 3.32: Scatter plot created using 'TaqMan Genotyper Software (v1.0.1)' showing results obtained from H233L SNP assay for 100 individuals tested and also positive controls (patient^{H398P} and family members).



Notes: Allele 1 (*x*-axis) represents the normalized fluorescence intensity of the normal allele for H233L locus, labelled with VIC (red). Allele 2 (*y*-axis) represents the normalized fluorescence intensity of the mutant allele for H233L locus, labelled with FAM (blue). Light blue squares represent negative controls, red circles and squares represent samples homozygous for allele 1, while green squares show heterozygous samples (allele 1/allele 2). Red circles indicate samples under assessment, while red and green squares indicate positive controls. A sample/control homozygous for allele 1 would only display fluorescence from VIC dye, shown with red colour in the scatter plot. A sample/control homozygous for allele 2 would only display fluorescence from FAM dye, shown with blue colour in the scatter plot. A heterozygous sample/control would express fluorescence from both dyes (VIC/FAM) and would be shown in the scatter plot with green colour.

4) Discussion

4.1 Performance of PGD clinical cases using novel protocols based upon conventional technology

Since its first application in 1989, PGD has progressed significantly and has become an established approach for preventing genetic disorders (Fiorentino *et al.* 2006). The growing awareness amongst patients of the existence of PGD and the increasing appreciation of the validity of the method from genetic counsellors and clinical geneticists has led to a steady rise in the number of PGD cycles performed every year along with an increase in the number of infertility clinics offering PGD (Gutierrez-Mateo *et al.* 2009; Harper *et al.* 2012).

In this thesis a total of 46 PGD cycles, carried out using conventional methods (i.e. PCR), are reported. These cycles were performed over a period of 32 months and highlighted the advantages and limitations of traditional PGD technology. Most of the cycles carried out involved the development of unique, family-specific PGD protocols for the analysis of single cells from embryos.

4.1.1 Diagnosis, amplification efficiency, contamination and clinical outcome

Most IVF cycles produce only a handful of embryos and in the majority of cases only one or two have the potential for producing a child; specifically, it has been estimated that only about 5% of fresh oocytes retrieved during IVF cycles result in the production of a baby (Patrizio and Sakkas 2009). The addition of PGD to the equation means that the number of embryos available for transfer will be further reduced, since

some will inevitably be excluded after an unfavourable diagnosis. Additionally, embryos that fail to receive a diagnosis are usually excluded from transfer as their disease status remains unknown. Therefore, in order to maximise the chances of a pregnancy, a diagnosis must be given to as many biopsied samples as possible. In this study diagnosis using conventional methods produced results from 86.7% of the assessed cells from embryos. This rate compares well with other published studies reporting data from large numbers of PGD cycles: 84.4% (Gutierrez-Mateo *et al.* 2009), 80.7% (Pickering *et al.* 2003) and 83% (Feyereisen *et al.* 2007). Also, this diagnosis rate is similar to the rate reported by the ESHRE PGD Consortium after ten years of data collection that ranged from 83% to 90% (Harper *et al.* 2012).

The main reasons for no diagnosis being returned were total amplification failure and contamination. Although stringent precautions were taken when carrying out the PGD protocols for clinical cases and a specialised 'single cell' room was used (section 2.1), contamination still occurred for 3.9% (9/233) of the embryos tested. The nature of PGD and the way it is carried out makes the complete elimination of contamination impossible. The high degree of amplification required in order to render sequences from a single genome detectable creates a pronounced contamination problem (Wells and Sherlock 1998) since even a single copy of extraneous DNA can potentially be amplified at detectable levels. Furthermore, IVF laboratories usually do not have access to specialised clean-rooms for the processing of biopsied material (Gutierrez-Mateo *et al.* 2009). This increases the risk of DNA found in the environment entering sample tubes and causing contamination. In order to keep contamination levels to a minimum, it is extremely important that procedural guidelines are followed very carefully (Harton *et al.* 2011b). It is of interest to note that for 6/9 of the contamination incidences observed, contamination was only detected in the sample-

specific negative control; analysis of hyper-variable polymorphisms did not identify any non-embryonic DNA in the corresponding sample tube. This emphasises that detection of DNA in the negative control does not necessarily mean that the associated sample is contaminated (Harton *et al.* 2011b). It is possible that one or more of these samples were not contaminated and biopsied cells were falsely left undiagnosed, reducing the number of embryos available for transfer. This stresses the importance of detecting contamination in the tube containing the biopsied sample. One way this could be achieved is through utilisation of methods that allow accurate DNA fingerprinting of samples (sections 4.3.5 and 4.7.1). While the allelic pattern produced by the use of hypervariable STR polymorphisms in the current study is too simplistic to be considered a true 'DNA fingerprint', it nonetheless led to the identification of DNA contaminants in three samples. On each occasion the corresponding negative control was clean. If the STRs had not detected the contamination in the sample tube in these cases, a misdiagnosis could have occurred.

Regarding total amplification failure, this took place in 20 out of the 225 single cells (polar bodies, blastomeres) tested. This corresponds to a rate of 8.9% and it is similar to TAF rates (4.4% - 18.9%) observed in another study reporting on a high number of PGD cycles (Gutierrez-Mateo *et al.* 2009). Overall amplification efficiency determined when results from all PGD cycles were summed together was calculated to be 90.3%. When cells that showed TAF were excluded from calculations the rate increased to 98.5%. The overall ADO rate was calculated to be 5.2%. These rates are in accordance with the amplification efficiency rate (>90%) and ADO rate (<10%) recommended by the ESHRE PGD Consortium (Harton *et al.* 2011b).

The overall high amplification efficiency obtained from the developed PGD protocols and the generally low ADO rate, allowed for diagnosis of 202/233 embryos. Thirty-

six embryos were transferred leading to initiation of 12 single pregnancies (implantation rate 33%, clinical pregnancy rate 27.8%). These results are comparable to those obtained during other large studies (Fiorentino *et al.* 2006, Grace *et al.* 2006) and also to data reported recently from the ESHRE PGD Consortium (Goossens *et al.* 2012).

Of the 12 embryos that initiated a pregnancy five miscarried. Although the number of pregnancies is too small to draw any statistical conclusions, the loss rate seems high given that all of the patients achieving a pregnancy were <37 years of age. The reason for miscarriage in one case was determined to be aneuploidy (trisomy 22). For the rest of the embryos no information was available, but it is quite possible that chromosome abnormalities were involved in these cases also. It is well known that aneuploidy is present in a large percentage (>50%) of early spontaneous abortions (Boue *et al.* 1975; Fritz *et al.* 2001; Hassold *et al.* 1980; Menasha *et al.* 2005). Overall, aneuploidy involving the chromosome upon which the mutant gene was situated was detected in 7.4% of diagnosed embryos, revealed by analysis of polymorphic microsatellites. However, as only one chromosome was considered, there is little doubt that this greatly underestimates the true occurrence of chromosomal imbalance. Little data exists concerning aneuploidy rates for embryos generated by PGD patients (the majority of whom are fertile), but for infertile patients undergoing IVF more than two-thirds of cleavage stage embryos are found to be aneuploid (Voullaire *et al.* 2000; Wells and Delhanty 2000). Pregnancy loss rates observed during this study suggest that aneuploidy screening might be a desirable addition to single gene PGD protocols, potentially enhancing IVF success rates and reducing miscarriage risk.

4.1.2 Methodology used for development and performance of PGD protocols

4.1.2.1 Transport PGD

Most of the PGD cycles carried out during this project were of the 'transport' PGD category. Specifically, 35/46 cycles involved the transportation of samples from clinics up to 75 miles away. The time taken to transport the biopsied samples from the IVF clinics ranged from 1.5-3 hours, with an average of 2 hours. The overall high amplification efficiency and the low ADO rate obtained from processing the biopsied samples suggest that transportation does not affect the performance of tests. The general conclusion is that 'transport' PGD is an acceptable and safe way of performing PGD, confirming results obtained by other centres (Fiorentino *et al.* 2006; Gutierrez-Mateo *et al.* 2009).

4.1.2.2 Lysis method

It has been demonstrated through different studies carried out that the method used to lyse cells affects amplification efficiency and ADO rates of single cells (Kim *et al.* 2009; Thornhill *et al.* 2001). In this study, the proteinase K lysis method was used initially to develop PGD protocols and also to carry out two clinical cases. Eventually however, it was decided to use the alkaline lysis method since it was found to be much faster (10min vs. 70min), simpler (i.e. required no oil overlay to be added to samples) and it was shown by experiments carried out in the laboratory of Reprogenetics UK (data not shown), and also most published studies, to be the method with the highest amplification efficiency and the lowest ADO rate (El-

Hashemite and Delhanty 1997; Gitlin *et al.* 1996; Kim *et al.* 2009; Thornhill *et al.* 2001; Tsuchiya *et al.* 2005).

4.1.2.3 Fluorescent-multiplex PCR, nested PCR and minisequencing

In this study, all protocols developed for performing PGD for single gene disorders utilised fluorescent-multiplex PCR, allowing direct mutation detection to be combined with analysis of STRs. As well as assisting in the detection of DNA contaminants, the use of linked STR markers serves as a tool for indirect mutation detection, providing a means of detecting ADO and therefore avoiding misdiagnosis (Rechitsky *et al.* 1999).

Coupled with STR analysis of each sample, most protocols (27/31) utilised direct mutation detection. Nested PCR was used to amplify the mutation sites and then minisequencing was used to detect the specific mutation in each case. Essentially the same minisequencing protocol (section 2.2.3.3.3) was used in every protocol, differing only in the DNA primer used to designate the nucleotide position to be interrogated, demonstrating that this technique is very adaptable and appropriate for a wide range of mutations. The high efficiency and flexibility of this technique have also been demonstrated by other studies (Fiorentino *et al.* 2003, 2006).

4.1.2.4 Genetic analysis of gametes: testing of polar bodies for aneuploidy screening of oocytes and of single sperm for haplotype construction

4.1.2.4.1 Combination of comprehensive aneuploidy screening with single gene testing

On one occasion PGD for a single gene disorder was combined with comprehensive aneuploidy screening (section 2.2.3.4.2). Aneuploidy screening was carried out through examination of 1st PBs using microarray CGH, while single gene testing (using conventional techniques) was performed on single blastomeres obtained from day-3 embryos. This is one approach that can be used to efficiently combine single gene testing with comprehensive aneuploidy screening. Aneuploidy screening of IVF embryos, through assessment of polar bodies, is an approach currently utilised by multiple PGD centres worldwide (Fishel *et al.* 2010; Fragouli *et al.* 2010b; Kuliev *et al.* 2003). However, a major drawback of this approach is that any chromosomal abnormalities contributed by the sperm, and also any errors that occur post-zygote formation, cannot be detected. Furthermore, combined assessment of polar bodies and blastomeres increases the cost of the IVF/PGD cycle significantly since more biopsies are needed and also a larger number of samples will need to be processed. An alternative approach of combining aneuploidy screening with single gene analysis, capable of circumventing these drawbacks, is desirable. Results obtained from this study suggest that WGA is a technique that could be used towards this end (discussed in section 4.3).

4.1.2.4.2 Usage of single sperm for haplotype construction

For some PGD cycles DNA from members of the extended family is not available. In addition, there are occasions when a couple with no previous pregnancies, carrying a *de novo* mutation, requests PGD. In both of these situations, the strategy of using linked markers to supplement direct mutation detection is difficult to apply, since this approach depends on the ability to construct an STR haplotype, by working out the phase of alleles through analysing relatives' DNA.

One way that this hurdle can be overcome, in cases where the male is the carrier of the mutation, is through utilisation of single sperm. Spermatozoa obtained from the carrier male can be used for simultaneous amplification of linked STRs and the mutation site in order to construct a haplotype. This strategy was followed in two PGD cycles for this project and haplotypes were successfully constructed indicating that this approach is a viable alternative to conventional means of determining the phase of linked markers, at least in cases where the male is the carrier of a mutation. This approach has also been demonstrated in a few other studies (Altarescu *et al.* 2006; Verlinsky *et al.* 2005).

4.1.2.5 Validation of PGD protocols

Since it is not possible to obtain large numbers of single blastomeres with the same alleles/mutations as patients requesting PGD, the protocols developed were validated using single lymphocytes from patients. Single lymphocytes can be easily obtained through processing of blood samples provided by the patients and this approach is used by many PGD centres before clinical application (Fiorentino *et al.* 2006;

Girardet *et al.* 2009; Kakourou *et al.* 2010). After analysis of results obtained from this project, it was concluded that AE and ADO rates between single blastomeres and single lymphocytes were similar ($P>0.05$) for 27 different loci assessed for both types of cells. These outcomes provide reassurance that results obtained from amplification of single lymphocytes are representative of results likely to be obtained from single blastomeres during actual clinical cases.

Furthermore, results obtained from validation of PGD protocols were used to assess various DNA fragments with the aim of determining whether specific characteristics of the amplicons influenced diagnostically important factors such as AE and ADO rates. Fragment length was found to be directly proportional to ADO rates, increasing fragment length correlated with increasing ADO rates ($P<0.05$). Amplification efficiency was less affected by fragment length showing only a minor decrease with increasing fragment length, which was not found to be statistically significant. These results are in accordance with those obtained from a previous study carried out by Piyamongkol and colleagues (2003). It can be concluded from these data that in order to maximise efficiency and accuracy of PGD protocols, amplification of large fragments - especially larger than 300bp - should be avoided.

4.1.3 Current techniques - Pitfalls

The protocols developed for PGD for single gene disorders were found to be highly robust and accurate. The high diagnostic accuracy (estimated at 95-99% for almost all cycles), the acceptable amplification efficiency (90.3%) and ADO rates (5.2%) obtained from the developed protocols support this conclusion. The low contamination rate observed (3.9%) and the fact that no misdiagnosis has been

reported to date further provide evidence of the reliability of the protocols. Collectively, these results indicate that the conventional techniques used currently in PGD are efficient and accurate in providing a diagnosis for the assessed embryos. This conclusion was also reached by other substantial studies reporting outcomes and performance of PGD cycles (Fiorentino *et al.* 2006; Gutierrez-Mateo *et al.* 2009).

However, PGD for single gene disorders using conventional methodology has some important limitations. Although every effort was made to standardise development of PGD protocols (e.g. usage of multiplex PCR, nested PCR and minisequencing for almost all cases), a high degree of patients-specific tailoring of methods was still required. Consequently, the preparation of protocols remained a lengthy and expensive procedure, requiring an average of 3 months for completion. The length of time needed for development of protocols and the high expense of the procedure, as well as the fact that the birth of a child is far from guaranteed, might discourage some patients from choosing PGD in its current form. Additionally, even though aneuploidy screening of polar bodies was successfully combined with single gene testing of blastomeres in one PGD cycle, this was not used in subsequent PGD cycles due to the limitations of this approach described (section 4.1.2.4.1).

The development and utilisation of techniques that would make optimisation of protocols easier and less expensive and also that would allow for comprehensive aneuploidy screening to be carried out in parallel with single gene analysis is of great importance for the future of PGD. Whole genome amplification is one technique that has the potential, at least in theory, to circumvent many of the disadvantages of conventional techniques. For this reason, different WGA methods were extensively assessed in this study for their applicability in PGD (discussed in section 4.3).

4.2 PGD for HLA typing

It has been 11 years since the first clinical application of PGD for HLA-typing (coupled with testing for Fanconi anaemia) was reported (Verlinsky *et al.* 2001). Since then, PGD for HLA typing with or without testing for a single gene disorder has been carried out for a large number of PGD cycles (Harper *et al.* 2012) and the performance of PGD for this indication is increasing year by year (Goossens *et al.* 2008b, 2009; Harper *et al.* 2010).

In this study, a widely applicable PGD test was developed for usage in clinical cases requiring HLA typing of embryos. The test involves the simultaneous amplification of 19 STR markers found interspersed throughout the human HLA region. The usage of STR markers avoids the need for design of locus-specific primers in order to perform direct genotyping of the HLA region, as was carried out by early HLA typing studies (Fiorentino *et al.* 2004; Verlinsky *et al.* 2001). Currently, HLA typing through STR analysis alone is found to be the strategy of choice for many PGD centres around the world, achieving concordant results with direct genotyping methods (Fiorentino *et al.* 2005; Rechitsky *et al.* 2004; Van de Velde *et al.* 2004). Importantly, usage of STR markers allows for detection of recombination events within the HLA region, a serious problem since it can alter the HLA-type of the resulting individual. The large size of the region (>4 Mb) (Robinson *et al.* 2011) means that recombination is not uncommon and if undetected can cause misdiagnosis (Rechitsky *et al.* 2004). Recombination has been reported by published PGD/HLA typing studies to occur at a rate of 1.9-4.8% (Fiorentino *et al.* 2005; Rechitsky *et al.* 2004; Van de Velde *et al.* 2009).

4.2.1 Validation and clinical application of the novel HLA-typing protocol

The developed protocol was validated on lymphocytes and displayed high amplification efficiency (99.7%), while the overall ADO rate was low (3.1%). Amplification efficiency and ADO rates were also calculated for each STR marker individually. The rates were found to be similar for all tested markers (Figure 3.5). These results indicated that the protocol was highly accurate and efficient and that it could be used reliably to carry out clinical cases for PGD/HLA-typing.

Ultimately, 3 clinical cases were carried out by the Reprogenetics laboratory in the USA using the developed protocol, two for HLA typing alone and one for HLA typing combined with testing for an autosomal recessive disease (Mucopolysaccharidosis type 1) and also gender determination. Development of the protocol for the Mucopolysaccharidosis type 1 case involved the addition of 6 more loci (3 loci for single gene testing and 3 loci for gender determination) to those included for HLA typing, creating a multiplex-PCR composed of 26 loci. Importantly, addition of these extra loci did not affect in any way the performance of the HLA-typing protocol. The only optimisation needed was regarding amplification of the newly added loci. This observation is of particular significance since it shows that the novel HLA-typing protocol can be easily combined with testing of a single gene disorder without needing to make any changes to it.

From the 13 embryos tested in clinical cases only 10 were given a diagnosis since three samples showed TAF. Two samples were found to be chromosomally abnormal (haploid or monosomic for chromosome 6). The seven embryos assessed in cases performed for the purpose of HLA matching only, yielded only one that was

compatible with an existing affected sibling. This compatibility rate (14.3%) was lower than the expected rate of 25% but similar to the one observed in other published PGD/HLA typing cases (Kahraman *et al.* 2011). This emphasizes the importance of obtaining a sufficient number of embryos in each PGD/HLA typing cycle in order for the cycle to be successful (i.e. identification and transfer of at least one viable, euploid, HLA-matched embryo). It also emphasises that adequate patient counselling is of paramount importance. The management of patient expectations is vital in all PGD cycles, but this is particularly true for cycles involving HLA matching, where the likelihood of success is relatively low and patients may be desperate.

4.2.2 Comparison of the new protocol with published protocols

The novel HLA-typing protocol is found to have advantages in comparison with previously published methods. In contrast to other reports (Fiorentino *et al.* 2005; Rechitsky *et al.* 2004; Van de Velde *et al.* 2004), this protocol avoids the need for selection of new STR markers with every new referral. In other words, the protocol is expected to be applicable to a wide range of patients. This is because it includes a high number of STR markers that are of high heterozygosity (most have >0.75 heterozygosity rate; Appendix 4). The almost universal applicability of the protocol reduces the time needed for preparation of the case to an absolute minimum. This is important since any delay to stem cell transplantation could have adverse effects to the health of the ill and deteriorating affected child. Also, as shown from the clinical case carried out, the protocol developed in this study can be easily combined with single gene testing, again helping to reduce the waiting time for patients to a minimum. Furthermore, in contrast to other protocols (Fiorentino *et al.* 2005;

Rechitsky *et al.* 2004), which require nested singleplex reactions to amplify the STR markers to a detectable level, the developed protocol carries out amplification of STR markers in just three multiplex reactions. This makes the procedure less cumbersome, significantly decreasing the time needed for setting up a clinical case and substantially reducing the expense of the procedure. Additionally, minimising the number of reactions leads to a considerable reduction in the opportunities for contamination to occur.

4.3 Usage of whole genome amplification in PGD

As determined from this study, conventional methods (e.g. multiplex PCR) used to perform single gene testing are accurate and robust, yet have some important disadvantages (discussed in section 4.1.3). One technique that can be used to overcome some of these limitations is whole genome amplification. In theory, WGA methods amplify the entire genome, allowing the product to be treated similarly to a genomic DNA sample, providing material for multiple different downstream applications. Therefore usage of WGA methods is expected to make optimisation of protocols easier and less time consuming compared to conventional techniques used in PGD (i.e. single cell multiplex PCR). Also, optimisation of protocols using WGA methods is expected to be less expensive, since avoiding the challenges associated with single cell DNA amplification would mean that fewer experiments are needed before the final optimised protocol is obtained. Furthermore, usage of WGA methods for development of PGD protocols offers the potential for combining CCS with single gene testing through utilisation of molecular cytogenetic approaches (e.g. CGH).

WGA methods such as MDA and GenomePlex have been studied before in regards to their applicability in single cell testing and PGD (Chen *et al.* 2008; Glentis *et al.* 2009; Handyside *et al.* 2004, 2010; Hellani *et al.* 2005; Treff *et al.* 2010a, 2010b, 2011a). However, only very few studies exist directly comparing different WGA methods and examining their usage in PGD (Gutierrez-Mateo *et al.* 2011; Treff *et al.* 2011b). Furthermore, the only published studies regarding the SurePlex WGA method concern its utilisation for the purpose of microarray-CGH for aneuploidy screening (Alfarawati *et al.* 2011a; Fiorentino *et al.* 2011; Geraedts *et al.* 2011; Gutierrez-Mateo *et al.* 2011).

This project aimed to provide an extensive validation of three of the most popular WGA methods, assessing their usage for both cytogenetic and molecular genetic diagnostic applications. To the knowledge of the author, this is the most comprehensive study carried out to date regarding WGA methods and their usage in PGD.

4.3.1 Concentration measurement and fragment length

In accordance with previous studies, the length of MDA products was found to range from 1kb to >10kb (Dean *et al.* 2002; Hellani *et al.* 2004). This is considerably larger than the length of amplified fragments produced by the two PCR-based methods, GenomePlex and SurePlex. It has been suggested that the production of large fragments can be advantageous for some downstream applications such as restriction fragment length polymorphism and sequencing (Dean *et al.* 2002).

Regarding DNA yield after WGA, this was found to be significantly higher ($P<0.05$) in MDA-unprocessed products than any other product from any of the three methods assessed (Table 3.4). However, it is expected that not all the DNA measured using the Nanodrop spectrophotometer will have been synthesised from the DNA of the single cell. It is estimated that some of the DNA represents primer directed non-specific products, while other DNA is amplified from contamination leftover from the host organism used to produce the enzyme (Coskun and Alsmadi 2007; Spits *et al.* 2006b). Processing of WGA samples was found to significantly reduce ($P<0.05$) the amount of amplified DNA, especially when column purification was used, with potential consequences for subsequent analyses. Processing methods are used to remove leftover reaction components (e.g. primers, nucleotides, DNA polymerase, salts) and

also any cellular components such as carbohydrates and proteins. Purification of WGA samples is important for optimal application on DNA microarrays. Based on recommendations from DNA microarray manufacturers regarding 260/280 and 260/230 ratios (Agilent Technologies 2007; Roche NimbleGen 2009), it was concluded that Sigma column purification and precipitation are the most appropriate methods for purification of WGA products derived from single cells, if these are to be applied on DNA microarray platforms. Additionally, DNA yielded after MDA was found to be of high purity and therefore may not require any kind of processing before application on DNA microarrays, potentially saving time and money. Reduced costs are an important consideration for PGD/PGS procedures, which are an expensive addition to the already costly process of IVF, while reductions in procedure length are desirable given the restricted time available for PGD.

4.3.2 STR analysis of WGA products

In order to assess amplification characteristics of the three WGA methods, 10 clinically relevant STR loci were amplified through PCR. The 10 STR loci selected were linked to different genes associated with inherited disorders including: Huntington disease, Myotonic dystrophy type 1, Sandhoff syndrome and Hereditary multiple exostoses.

During analysis of results various phenomena were observed such as preferential amplification and DNA polymerase slippage. Increased rates of preferential amplification, in comparison with that seen using direct PCR of single cells, were observed in all three WGA methods. This is an important phenomenon since in extreme cases it is possible that the under-amplified allele might not be detected

(essentially this would be scored as ADO), reducing the diagnostic efficiency of the test. Preferential amplification has been reported before in a number of studies regarding MDA (Glentis *et al.* 2009; Handyside *et al.* 2004; Hellani *et al.* 2004; Renwick *et al.* 2006; Spits *et al.* 2006a) and also PCR-based WGA methods (Paunio *et al.* 1996; Wells *et al.* 1999). DNA polymerase slippage, generating false 'alleles', has also been reported before in several WGA studies (Glentis *et al.* 2009; Renwick *et al.* 2007; Wells *et al.* 1999). In this study, slippage was seen for 0.9% of the loci tested regarding MDA; a rate, significantly lower ($P<0.05$) than the slippage rates obtained with the two PCR-based methods. This is in accordance with previous studies performed with MDA determining very low levels or no presence at all of Φ29 DNA polymerase slippage (Glentis *et al.* 2009; Handyside *et al.* 2004; Hellani *et al.* 2004; Spits *et al.* 2006a). The tight binding of the Φ29 DNA polymerase to the template prevents the dissociation of the enzyme when obstacles caused by DNA primary or secondary structure are encountered. As a result, the rate of slippage of Φ29 DNA polymerase at microsatellite loci is greatly reduced (Dean *et al.* 2002; Jiang *et al.* 2005). Furthermore, slippage of DNA polymerase is inversely related to strand displacement efficiency. The Φ29 DNA polymerase shows a high strand displacement activity and therefore is not prone to slippage (Canceill *et al.* 1999). On the contrary, PCR-based WGA methods - especially those that use thermophilic polymerases without strand displacement activity [e.g. *Taq*, *Pyrococcus furiosus* (*Pfu*)] - are found to be linked to increased rates of polymerase slippage (Handyside *et al.* 2004; Viguera *et al.* 2001; Wells *et al.* 1999).

4.3.2.1 Amplification efficiency and ADO rates

MDA was found to have significantly higher ($P<0.001$) total amplification efficiency than the other two methods (95.8% of loci successfully amplified), in agreement with other studies which had reported AE rates of 89.5% - 98.5% (Handyside *et al.* 2004; Ren *et al.* 2007; Renwick *et al.* 2006; Spits *et al.* 2006a). This suggests that MDA achieves greater/more reliable coverage of the genome than the alternative PCR-based methods. In terms of ADO, SurePlex was found to have the lowest rate (20.5%), slightly less than that obtained for MDA (26.8%; $p=0.053$) and significantly lower than the rate determined for GenomePlex (53.3%).

In this study, the effect that the different processing methods might have on AE and ADO rates was examined. This is one parameter that has not been studied before and is of significant importance since published studies are found to use different methods of processing WGA samples (Hellani *et al.* 2004; Le Caignec *et al.* 2006; Ling *et al.* 2009; Treff *et al.* 2010b) without considering what effect this might have on results. Data from this study suggest that column purification negatively affects MDA products and should be avoided if products are to be used for PCR amplification. It is noteworthy that a significant reduction in DNA concentration of MDA products was seen with the usage of column purification methods (from ~2,116ng/µl to ~127ng/µl and ~22ng/µl) (Table 3.4). Considering these results, a possible explanation for worse AE and ADO rates is that during column purification apart from removal of leftover components a considerable amount of MDA material is also lost, including templates vital for PCR. Regarding GenomePlex, Sigma column purification was found to improve AE and ADO rates. This method of processing also seemed to be beneficial for SurePlex, although differences in ADO and AE were not statistically significant ($P>0.05$). It is possible that purification of WGA products received from these two

PCR-based methods facilitates the removal of reaction components and/or organic compounds that interfere with PCR amplification and therefore provides better AE and ADO rates. Furthermore, it is likely that the small fragments created by the two PCR-based methods are more efficiently purified than the much larger MDA products, which may become stuck in the matrix of the purification column. As a result, the proportion of PCR-amplified fragments lost during the purification process might not be as great as the amount lost when processing MDA products and therefore, no vital PCR templates are lost.

When the different loci amplified with each method were considered, it was determined that MDA provided high AE rates across all 10 loci with low inter-locus variation, confirming the conclusion drawn by Lage *et al.* (2003) that production of large amplicons results in more uniform representation of different sequences. The ADO rate was found to be relatively high for all loci tested, ranging from 20% - 42.9%. This high ADO rate is expected to affect the accuracy and efficiency of any PGD protocols developed. It is therefore important to include an appropriate number of STR markers in each protocol developed to compensate for this and achieve sufficient accuracy levels. Regarding GenomePlex, low AE rates across all loci were observed and very variable ADO rates, ranging from 0% to 100% were seen. GenomePlex relies on random fragmentation of the DNA, prior to ligation of adapters and PCR amplification (Sigma-Aldrich 2010), which possibly destroys some copies of the target DNA. If the starting material is just a single cell, then there is a significant risk that breakage could destroy the only copies of the template present, precluding amplification. SurePlex on the other hand, was found to have very high AE for some loci, lower efficiency for other loci. Although, SurePlex works in the same way as GenomePlex (i.e. LA-PCR), from the results obtained in this study

SurePlex seems to fragment the DNA in a more specific way and therefore, some loci are always amplified while other loci consistently amplify at very low rates. The ADO rates obtained from SurePlex were found to be relatively low for most of the loci tested. These results indicate that SurePlex can be used for development of PGD protocols, however extra caution should be taken on selection of loci to be amplified in the protocol and also careful validation should be carried out to determine if AE and ADO rates of these loci are satisfactory before performing a clinical case.

4.3.3 Application of WGA products on DNA microarrays

The evaluation of WGA products as templates for PCR indicated their suitability for use in conjunction with PGD of single gene disorders. In order to assess the applicability of WGA methods for PGD of cytogenetic abnormalities (and for the purpose of PGS), products were applied to oligonucleotide and SNP arrays.

4.3.3.1 NimbleGen oligonucleotide array

A NimbleGen oligonucleotide array was used to assess the three WGA methods for their ability to detect aneuploidy in single cells through the usage of array-CGH. Results obtained identified SurePlex as the most appropriate method for usage in array-CGH for aneuploidy screening of single cells. Specifically, SurePlex samples were found to provide accurate identification of chromosomal abnormalities and also gender for almost all samples tested. On the contrary, results obtained using MDA and GenomePlex samples provided a high degree of background noise which made detection of aneuploidies impossible. The high incidence of artefactual gain and loss

of chromosomal material observed for MDA in this study, has been also detected previously when MDA was applied on single cells and array-CGH was performed (Tan *et al.* 2010).

After SurePlex was determined to be the best method for usage in combination with the NimbleGen array, more experiments were performed to assess applicability of the procedure in PGD. Column purified SurePlex products were found to be as reliable as the other SurePlex products tested but were determined to be the least expensive approach and were therefore selected for application on the array. Furthermore, a reduction in hybridisation time was attempted. It was concluded that usage of a shortened protocol had no significant effect on the performance of the array. The shortened protocol makes the procedure more applicable to PGD/IVF since, it allows the entire procedure (including amplification) to be completed in about 24-25 hours depending on the number of samples.

From results obtained during this study, it was concluded that the specific NimbleGen oligonucleotide array could be potentially used in PGD for detection of aneuploidy in a clinical setting. However, it should be emphasised that before this happens further assessment and extensive validation of the array are required. Currently, only six distinct aneuploidies have been tested, whereas any chromosome can potentially be affected by aneuploidy in human oocytes and preimplantation embryos. Additionally, the ability of the microarray to detect segmental abnormalities (affecting chromosomal fragments rather than whole chromosomes) needs to be evaluated since such anomalies are relatively common, particularly at the cleavage stage (Wells and Delhanty 2000; Voullaire *et al*. 2000; Vanneste *et al.* 2009a).

4.3.3.2 Illumina SNP array

4.3.3.2.1 SNP genotyping

Almost all studies that have been carried out to date comparing MDA with PCR-based WGA methods have utilised input DNA quantities that are larger than those found in a single cell (Barker *et al.* 2004; Lovmar *et al.* 2003; Park *et al.* 2005). Only one study has employed SNP arrays to make comparisons between different WGA methods at the single cell level (Treff *et al.* 2011b). However, this specific study only compared MDA and GenomePlex WGA methods and had not used SurePlex, while it also did not study the effect that the different processing methods might have to genotyping performance.

Results obtained from this project had identified MDA as the method with the highest genome coverage - 78.7% to 81.2%, depending on the method of purification. Furthermore, MDA had a significantly higher rate ($P<0.001$) of correct calls (i.e. identical to unamplified genomic DNA) (90.1% - 91.2%) than GenomePlex (35.4% - 49.7%) and SurePlex (83.5% - 85.5%). Additionally, MDA showed significantly lower GOH and COH rates than the two PCR-based methods. These rates were found to be at very low levels indicating that MDA rarely introduces errors during amplification. In fact, it has been documented that Φ29 DNA polymerase used in commercially available MDA kits has a very low error rate ($< 3 \times 10^{-6}$) (Esteban *et al.* 1993; Nelson *et al.* 2002). In contrast *Taq* polymerase, which is used in many PCR-based methods, shows an error rate 10-fold higher (3×10^{-5}) (Lovmar and Syvanen 2006).

Although MDA had shown the best genotyping performance for most of the values assessed with the SNP array, ADO was seen at a relatively high level (36.5% -

50.6%). SurePlex was found to give the lowest ADO rates amongst the three methods (31.1% - 41.3%). These observations mirror the ones made when STR analysis of WGA products was carried out (section 4.3.2), which identified MDA as the method with the highest genome coverage and SurePlex as the method with the lowest ADO rate. Furthermore and as was observed with STR analysis, processing of WGA products has an effect on genotyping performance of the WGA methods. Column purification and precipitation were found to negatively affect the genotyping performance of MDA. Contrary, for the two PCR-based methods, Sigma column purification was found to be beneficial, providing the best genotyping values.

In conclusion, MDA unprocessed products are found to be the most appropriate for application on SNP arrays. The high genome coverage (81.2%), the high accuracy rate (91.8%), the relatively low ADO rate (36.5%) and the extremely low GOH and COH rates obtained with this method, render it highly appropriate for usage in SNP genotyping of single cells. Furthermore, this method was the one that provided the highest percentage of consistent-correct SNP calls.

4.3.3.2.2 Aneuploidy Screening

The two methods found to be the best in terms of genotyping performance (MDA-Sigma column purified, MDA-unprocessed) were assessed for their ability to detect aneuploidy through utilisation of the Illumina array. Two types of references were used for analysis of results - unamplified genomic DNA and MDA DNA derived from single cell samples. The results clearly demonstrated that amplified DNA derived from single cells was the most appropriate reference. Only a low degree of background noise was observed when amplified single cell DNA was used as a

reference and results were readily interpretable, providing accurate detection of all aneuploidies attempted. This conclusion was also reached by another study which assessed aneuploidy detection after application of single cell MDA products on SNP arrays (Ling *et al.* 2009). It has been demonstrated by a number of studies that MDA produces representational bias, especially in the centromeric and telomeric regions of chromosomes (Lovmar and Syvanen 2006). Biases were found to be systematic rather than random and it was therefore suggested that amplified DNA is used as a reference in order to normalise results and correct these discrepancies (Lage *et al.* 2003; Ling *et al.* 2009; Paez *et al.* 2004). The findings of this study agree with this suggestion.

Results regarding the two MDA methods assessed were similar, although MDA-Sigma column purified products were found to produce slightly less background noise. Consequently, usage of column purified products might be advantageous when the SNP array is only going to be used for aneuploidy detection. However, in cases where the SNP array is going to be used for aneuploidy screening coupled with single gene testing, usage of unprocessed products might be more appropriate since this approach provided better genotyping performance.

Restricting the SNP loci used for aneuploidy detection to those that provided correct genotype data was found to provide no improvement in chromosome screening results. Therefore, this kind of analysis method is not necessary and can be omitted from utilisation in clinical cases.

4.3.4 PGD for single gene disorders using WGA - clinical cases

Five clinical cases for PGD of single gene disorders were performed using MDA and SurePlex WGA methods. Three of these cases were combined with comprehensive aneuploidy screening. Based on results obtained from STR analysis (section 4.3.2) it was decided to use unprocessed SurePlex and MDA products for amplification of the disease-relevant loci. Omission of processing methods is advantageous since the time and cost of the procedure are reduced, while most importantly, the risk of contamination is decreased.

The time needed for development of the PGD protocols was found to be significantly decreased compared to the time needed when conventional methods were used (15-30 days vs. 3 months). The large amount of DNA available after WGA avoided the need for development of complex, highly-optimised single cell multiplex PCR protocols. Instead, singleplex reactions were used to amplify loci, reducing the degree of optimisation required to an absolute minimum.

Usage of SurePlex WGA allowed for combination of single gene testing with comprehensive aneuploidy screening employing microarray-CGH via the 24sure BAC array (BlueGnome Ltd). Although the combination was successful, it is important to note that AE was decreased when SurePlex samples were used as a template for PCR. The AE was found to be 86.2% for single blastomeres after excluding cells with TAF and was significantly lower than AE rates observed with either direct multiplex PCR (98.5%) or MDA (98%). Lower amplification efficiency will result in an increased risk that some embryos will remain undiagnosed, reducing the number of embryos available for transfer and negatively impacting pregnancy rates. Therefore, extensive validation of any protocol based on SurePlex WGA remains essential prior to clinical

application. MDA on the other hand showed a consistently high AE and a relatively low ADO rate (10.3%). It is determined from this study that MDA is a highly reliable and efficient method for use in PGD for single gene disorders; this is concordant with previous studies (Chow *et al.* 2009; Hellani *et al.* 2005; Lau *et al.* 2010; Lledo *et al.* 2006, 2007; Ren *et al.* 2009).

The use of comprehensive aneuploidy screening in combination with single gene testing was found to provide additional genetic information of potential relevance to clinical outcome, revealing that 5/8 embryos that were eligible for transfer based upon the results of single gene PGD were actually aneuploid. One of the adverse outcomes of transferring an aneuploid embryo is miscarriage. Miscarriage following transfer of an embryo diagnosed normal for the familial single gene disorder can be particularly distressing for patients undergoing PGD, since so much (emotionally and financially) has been invested in the procedure. This unfortunate outcome, confirmed to have been caused by a chromosome abnormality, had occurred in one of the PGD cases in this study which had not included simultaneous aneuploidy detection. This result emphasises the importance of utilising methods that will be able to efficiently combine aneuploidy screening with single gene testing in PGD.

4.3.5 Karyomapping

Karyomapping is an analysis method that has been developed in order to process SNP genotyping results obtained after analysis of PGD samples using SNP arrays (Handyside *et al.* 2010). Through analysis of parental genotypes and the genotype of other appropriate family members, it is possible to identify informative loci for each of the four parental haplotypes and utilise this information to identify the parental

origin of each inherited chromosome or chromosome segment in an embryo. Therefore, karyomapping can theoretically diagnose any single gene disorder in an embryo via linkage analysis. Furthermore, the genotyping information obtained can also reveal chromosomal abnormalities such as trisomies of meiotic origin and monosomies.

In this study, MDA products obtained from amplification of single blastomeres derived from two clinical cases were applied unpurified to SNP arrays. Karyomapping analysis was performed on the genotyping results obtained. Since this was the first ever clinical application of karyomapping, a conventional PGD test, assessing the same MDA products, was run in parallel. Results received from karyomapping were in complete accord with those obtained using traditional PGD techniques (direct mutation detection plus analysis of linked STRs) (section 3.3.5). Furthermore, for the Smith-Lemli-Opitz syndrome case, which had included microarray-CGH analysis of the polar bodies for the identification of oocyte aneuploidy, all chromosomal abnormalities of maternal origin were detected by karyomapping. In addition to maternally derived chromosomal abnormalities, karyomapping also identified several other abnormalities predicted to be of paternal origin in the embryos tested. It should be noted however, that although karyomapping is able to identify the parent of origin of a chromosomal abnormality, it is not able to identify if the error occurred during meiosis or mitosis (i.e. after zygote formation). This applies specifically to abnormalities involving loss of chromosomal material (e.g. monosomies). Regarding gain of chromosomal material (e.g. trisomies), the aneuploidies that can be detected by karyomapping will always be of meiotic origin; in these occasions the aneuploidies detected would involve chromosomes derived from different parental haplotypes. Any gains of chromosomal material that arise during mitosis would involve duplication of

a chromosomal segment and this would be undetectable by karyomapping since duplicated segments would have identical genotypes. The only way of detecting such abnormalities would be through quantitation of fluorescence emitted from SNP probes; a feature not included in karyomapping analysis.

from duplication of chromosomes (such as the ones that occur during mitosis after zygote formation) will not be detected because the genotype of the duplicated chromosomes will be identical.

Results obtained from this study indicate that karyomapping is an efficient and reliable method for preimplantation genetic diagnosis of single gene disorders and suggest that it is now ready for clinical application. The protocol used in this study allowed for completion of diagnosis in approximately 36 hours. This is considerably less than the time suggested in the manufacturer's protocol (~3 days required). The shortened duration of the protocol allows for transfer of any suitable embryos (biopsied on day-3) on day-5, avoiding the need for cryopreservation. However, cryopreservation of embryos following biopsy will still be needed if this approach is applied at the blastocyst stage.

In general, karyomapping is advantageous since it provides a universal protocol for the majority of patients requesting PGD for a single gene disorder, eliminating the need for extensive patient-specific protocol design and optimisation, while also combining single gene testing with comprehensive aneuploidy screening. As has been well documented, a high number of human preimplantation embryos are affected by chromosomal abnormalities of meiotic or postzygotic origin (Cohen *et al.* 2007; Delhanty *et al.* 1997; Vanneste *et al.* 2009a). The use of a method that allows selection of embryos for uterine transfer based upon chromosomal as well as single

gene status may be beneficial in terms of improving success rates of IVF-PGD cycles, increasing the likelihood that a viable embryo will be chosen. However, this remains to be conclusively proven in properly controlled randomised studies.

The karyomapping approach provides additional potential advantages. Highly accurate DNA fingerprinting of embryos would be possible allowing for confirmation of parental origin, reducing the risk of a laboratory error that could lead to the transfer of embryos to the wrong patient. Furthermore, DNA fingerprinting would allow identification of which of the transferred embryos (in cases of multiple embryo transfer) actually resulted in birth. This provides a very powerful tool for research studies regarding identification of factors that might affect implantation potential of embryos (Treff *et al.* 2010a, 2010c; Wells *et al.* 2008). Moreover, DNA fingerprinting would make possible the detection of contamination derived from accidental inclusion of extraneous cells in the sample tube and would eliminate the need for assessment of blank (negative control) samples. Testing of blanks does not guarantee detection of contamination in the tube containing the sample (Gutierrez-Mateo *et al.* 2009) while it might increase the number of undiagnosed embryos by falsely suggesting that a sample is contaminated (section 4.1.1).

As mentioned above, the use of karyomapping/SNP arrays for single gene PGD has the advantage that a single protocol is utilised for most patients. However, one major limitation of this approach, is the need for analysis of at least one additional family member (other than the parents) in order to identify which of the parental haplotypes are linked with the disease-causing mutation/s. Premature death of affected individuals in the previous generation, unwillingness of the patients to involve other relatives, and disease caused by *de novo* mutation can all result in unavailability of the

essential DNA samples. This approach is therefore not applicable to all single gene PGD cases.

4.4 Development of a customised oligonucleotide array

Identification and transfer of euploid embryos is expected to improve pregnancy rates (Munne *et al.* 1993; Yang *et al.* 2012). However, on many occasions even after identification and transfer of a morphologically good-quality and euploid embryo, no implantation occurs. Furthermore, when more than one embryo is found to be euploid and of good quality, a decision is often made by IVF centres to transfer two or more embryos in order to increase the possibilities of achieving a pregnancy. This is especially true in the United States and certain other countries. However, adoption of this strategy greatly increases the chances of multiple gestations. Multiple gestations are associated with a rise in health risks such as disability of the newborn and also mortality (Keith *et al.* 2000; Luke and Brown 2007). Taking these facts into consideration it is concluded that it is of ultimate importance for the field of ART to be able to reliably select the embryo with the best chance of forming a viable pregnancy for transfer. This will allow elective single embryo transfers to be performed without decreasing pregnancy rates, avoiding the financial and medical complications associated with multiple gestations.

In response to the need for a method that will be able to reliably determine the best embryo for transfer, the development of a unique microarray platform was undertaken in this study. This project was initiated before there were any commercially available microarrays for the purpose of PGD/PGS. The novelty of the specific array developed resides in the fact that it does not only include probes for aneuploidy detection but also probes for relative quantification of mitochondrial DNA and telomere length, two factors of potential biological and clinical significance. Mitochondrial DNA quantity is assessed in order to estimate the number of mitochondria in the processed cell,

which may provide an indirect insight into the bioenergetic capacity of the biopsied embryo.

Validation of the developed microarray in terms of aneuploidy detection demonstrated a diagnostic concordance rate of 95.1% relative to results obtained using the most well-established microarray available (BlueGnome, 24sure). Chromosome 19 was found to be responsible for almost half of the discrepancies observed. Problematic interpretation of chromosome 19 aneuploidies when using CGH has been observed before by different studies (Gutierrez-Mateo *et al.* 2004; Moore *et al.* 1997; Voullaire *et al.* 2000). After this observation was made, the microarray was further optimised in order to be able to accurately determine chromosome 19 numerical status, a protocol that involved selection of a new set of probes for this chromosome. Taking into consideration the results obtained from initial validation and also the correction made regarding chromosome 19 detection, it is estimated that the aneuploidy detection rate would rise to 97.6%. Given that many embryos contain more than one aneuploidy the accuracy of diagnosis rate for abnormal embryos is even higher, reaching 99%. However, these figures assume that the 24sure microarray, to which the novel microarray was compared, always gives a correct aneuploidy assessment. While generally of high accuracy, the 24sure microarray is not infallible as shown by follow-up analysis performed during the current study using FISH. All in all, these results indicate that the developed microarray is highly accurate (although not 100%) in determining the chromosomal status of samples obtained for PGD.

Further validation of the microarray demonstrated that it is accurate in determining the relative amount of mitochondrial DNA in SurePlex amplified samples. On the contrary, the microarray was not found to be reliable in determining the relative amount of telomeric DNA in amplified samples. It was observed that the telomere

specific probes were oversaturated (i.e. loss of dynamic range due to excessive fluorescence on telomere-specific probes). Another factor that might have contributed to false results is amplification of samples with SurePlex WGA (discussed in section 4.5).

Apart from aneuploidy screening and relative quantification of telomeric and mitochondrial DNA, the possibility of including SNP probes on the specific array was assessed. The results were littered with many genotyping errors, suggesting that the SNPs initially chosen might not represent an optimal selection, many being poorly amplified by SurePlex. Nonetheless, it was clear that when SNP calls from an embryo were compared to those from the correct parents the number of correct SNP calls (i.e. genotypes consistent with inheritance from the parents) was considerably higher than when an embryo was compared to the wrong set of parents. Furthermore, the number of SNP calls showing Mendelian inconsistencies (genotypes incompatible with inheritance from the two parents) was considerably lower when the correct set of parents was used. This set of results indicates that with further optimisation it should be possible to perform reliable embryo fingerprinting in combination with microarray-CGH and evaluation of mitochondrial DNA copy number. As discussed above (section 4.3.5), the ability to perform embryo fingerprinting has multiple potential benefits.

4.4.1 Relative quantitation of mtDNA in relation to chromosomal status and maternal age

Relative mtDNA quantities were considered for PBs, single blastomeres and trophectoderm samples applied on the novel microarray developed. When results obtained from all sample types regarding mtDNA concentration and chromosomal status were considered, no correlation could be identified between these two variables. These results suggest that the incidence of aneuploidy in preimplantation embryos is not correlated with the bioenergetic capacity of the mitochondria in the embryo, although specific analysis of mitochondrial activity would be needed to confirm this. However, a non-significant trend was seen towards decreased quantities of mtDNA for aneuploid oocytes/embryos. Further work should be undertaken and the sample size increased to confirm whether or not there is a genuine association. Low mtDNA concentration in human oocytes has been linked to inefficient formation of birefringent meiotic spindles (Zeng *et al.* 2007), which is unsurprising given the energy requirements of spindle formation. This might provide an explanation for the trend seen since absence of an appropriately formed meiotic spindle could lead to aneuploidy.

Furthermore, results obtained from this study determined PBs derived from women of AMA to have significantly lower ($P<0.05$) mtDNA concentration than PBs derived from women of younger reproductive age. Other studies carried out on human oocytes had also shown that mtDNA copy numbers decrease with advancing maternal age (Chan *et al.* 2005; de Boer *et al.* 1999). This phenomenon could be due to an inherent property of oocytes recruited later in a woman's reproductive lifespan (de Boer *et al.* 1999). The so called 'production line' hypothesis suggests that the first oocytes to be

generated during foetal life are also the first to be ovulated and have a superior potential for the production of viable embryos (Henderson and Edwards 1968; Polani and Crolla 1991).

Contrary to the results obtained with PB samples, the relative mtDNA quantities found in blastomeres and trophectoderm samples were similar between the different age groups ($P>0.05$). In order to be able to explain this divergence, it is important to note that the PBs tested using the novel microarray provide information about the earliest stage of preimplantation life (fertilised oocytes). Having this in mind, a possible explanation for the variance seen between the different sample types is that, generally, some of the fertilised oocytes derived from women of AMA contain critically low mtDNA quantities; this translates to critically low numbers of mitochondria (if the assumption that mtDNA quantity is reflective of the actual number of mitochondria is correct). The number of mitochondria in the oocyte is likely to be directly related to the bioenergetic capacity of the embryo to develop normally after fertilisation (Van Blerkom 2009). As a result, fertilised oocytes with greatly reduced numbers of mitochondria - and also mtDNA - might not have the ability to develop to day-3 embryos. Therefore, when day-3 and day-5 embryos are assessed the significant differences seen with oocytes regarding mtDNA quantity and maternal age, no longer exist. Another possible explanation for the divergence observed is that after fertilisation mtDNA replication takes place and therefore, any differences that might have existed at the oocyte stage are no longer present at day-3 cleavage stage. However, this is unlikely since different studies in mammals have indicated that mtDNA replication is generally absent during preimplantation development up to and including the morula stage (reviewed in St John *et al.* 2010).

It is important to note that any results obtained from PB analysis should be treated with great caution. Not only is the division of mtDNA between oocytes and polar bodies disproportionate due to their greatly differing cytoplasmic volume, but active mechanisms to increase the proportion of mitochondria retained by the oocyte also appear to exist. Ultimately, the mitochondria passed on to polar bodies represent only 1-10% of the total amount found in oocytes (Steuerwald *et al.* 2000). It was concluded that the concentration of mtDNA observed for polar bodies might not be representative of that found in the corresponding oocyte (Gigarel *et al.* 2011; Steuerwald *et al.* 2000). Similarly to PBs, there is the possibility that mtDNA quantity determined in the trophectoderm of blastocyst stage embryos is not representative of the inner cell mass. This conclusion is drawn after considering research carried out in mice which indicated that the number of mitochondria in trophectoderm is considerably larger than in the inner cell mass (Houghton 2006). However, even if this is determined to apply to human blastocysts, quantification of mtDNA concentration - and therefore indirect estimation of potential ATP supply - in trophectoderm could still be of clinical relevance. Increased supply of ATP in trophectoderm is assumed to be required for important processes such as maintenance of the blastocoel cavity, hatching of the embryo in order to initiate implantation (Van Blerkom 2011) and possibly subsequent invasion of cells into the endometrium. In fact, assessment of mtDNA quantity in trophectoderm samples might even be advantageous compared to assessment in single blastomeres. This is because on some occasions, distribution of mitochondria amongst blastomeres is found to be disproportionate (Van Blerkom *et al.* 2000) and therefore quantification of mtDNA of a single blastomere might not be indicative of the entire embryo.

4.5 Measurement of telomere length in SurePlex WGA samples

Since telomere analysis of oocytes and embryos using microarrays had proven problematic, it was decided to develop a method allowing quantification of telomere DNA (an indicator of mean telomere length) from SurePlex WGA products. Using this strategy, part of the SurePlex product could be used for telomere assessment, while another aliquot would be used for microarray CGH using the well-validated 24sure microarray (a platform optimised to use DNA produced by SurePlex amplification).

Results obtained from applying the optimised protocols on genomic DNA extracted from five cell lines indicated that the different protocols evaluated provided variable results. Variability between the two protocols using 'telo 2' primer set was less than between the two protocols developed using 'telo 1' primer set. Actually, results obtained from usage of 'telo 2' primer set were almost in complete accordance with the two endogenous controls. Furthermore, it was concluded that results obtained using the 'telo 1' primer set were different from results obtained from usage of 'telo 2' primer set, no matter which endogenous control was used. This is an unexpected result since these two primer sets ('telo 1'='tel1-tel2', 'telo 2'='telg-telc') had been validated by studies to be accurate in determination of telomere length in genomic DNA samples (Cawthon 2002, 2009) and would therefore be expected to provide identical results. However, the two primer sets were determined to function with differing efficiencies. Specifically, the protocol developed using 'telo 2' primer set (Cawthon 2009) was found to be more accurate than the protocol developed using 'telo 1' primer set (Cawthon 2002). If the actual differences in telomere length of the

cell lines assessed in this study were small, then it is possible that the less accurate primer set was not able to correctly determine the relative quantities of telomeric DNA, causing the discrepancy seen between the two primer sets.

The developed protocols were also applied to WGA products derived from SurePlex amplification of single cells. Results obtained showed that relative telomere quantities provided did not agree between amplified and unamplified samples, for either set of telomere primers. In fact, results obtained from WGA single cells were considerably different than results received from unamplified genomic DNA. One conclusion that can be drawn from these results is that like other WGA methods (Ballantyne *et al.* 2007; Berthier-Schaad *et al.* 2007; Iwamoto *et al.* 2007; Lage *et al.* 2003), SurePlex does not efficiently replicate the telomeric regions of the human genome thereby resulting in under-representation of these regions in the amplified product. This might account for the large discrepancies seen in this study between amplified and unamplified samples. However, it has also been reported that the GenomePlex method under-represents telomeric regions (Ballantyne *et al.* 2007) but, nevertheless, was successfully used to developed a real-time PCR test for accurate relative quantification of telomere DNA in single cells (Treff *et al.* 2011c). Since SurePlex resembles GenomePlex in many technical aspects (both are LA-PCR methods developed by Rubicon Genomics, USA) it seems likely that a telomere quantification method using SurePlex products could be developed. Furthermore, as determined from this study (section 4.3), in general SurePlex is a superior WGA method to Genomeplex when it comes to genome coverage and accurate representation of the original genome. However, in order to produce an accurate protocol it is clear that further optimisation of the protocols presented in this study is required.

4.6 Development of a quantitative PCR method for comprehensive chromosome screening of human embryos

A variety of methods exist for performance of comprehensive aneuploidy screening of human embryos including CGH, array-CGH and SNP arrays. These methods are all found to be accurate enough and sufficiently reliable to be used in clinical cases (Fishel *et al.* 2010; Schoolcraft *et al.* 2010, 2011). One of the limitations of these methods however, is that the minimum time required to perform CCS exceeds 12 hours. Although this might not be of high importance when considering application of these methodologies to polar bodies or blastomeres, it is of great relevance when application to blastocysts is considered. The time needed by these methodologies to perform aneuploidy screening would possibly necessitate cryopreservation of the embryos to allow enough time for analysis to take place and for transfer to the uterus at a time of appropriate synchrony between embryo and endometrium (Treff *et al.* 2012; Van Voorhis and Dokras 2008). Although the efficiency of cryopreservation methods applied to embryos has improved considerably (i.e. usage of vitrification) (Keskintepe *et al.* 2009; Liebermann 2009; Schoolcraft *et al.* 2010), some risks remain. Furthermore, most patients would prefer to avoid the additional expenses and waiting time associated with a frozen IVF cycle.

This project aimed at the development of a method, through utilisation of quantitative PCR, which would be able to provide accurate comprehensive screening of whole chromosomes (i.e. not partial chromosomal aberrations) in a rapid and relatively inexpensive way. With the methodology used in this project performance of comprehensive aneuploidy screening is achievable in less than 5 hours allowing for transfer of any euploid embryos on the same day of the biopsy. Furthermore, the

expense of the procedure is calculated to be 1/2 to 2/3 of the expense of the microarray-CGH test currently used by most PGD laboratories.

Usage of quantitative PCR to perform aneuploidy screening in a PGD context has only been rarely reported in the past and only concerned a limited number of chromosomes (Martinhago *et al.* 2010; Yu *et al.* 2007). Only one study has been published, very recently, reporting comprehensive screening of human blastocysts using this approach (Treff *et al.* 2012). It is important to note that the project reported in this thesis was initiated long before that specific study was published. Furthermore, contrary to the published study which only reports analysis of blastocyst biopsies (3-10 cells), this study also investigated performance of quantitative PCR applied to single cells.

4.6.1 Application of a novel protocol for the comprehensive chromosomal analysis of genomic DNA, single cells and clumps of cells

Initial application of the protocol on genomic DNA samples identified its potential for detecting aneuploidies (i.e. trisomy 18), while it also revealed the need for utilisation of male samples as a reference (calibrator) in order for the correct copy number to be assigned to chromosomes X and Y.

When the developed methodology was applied on single cells, the overall accuracy rate obtained (number of assays giving a correct copy number divided by the total number of assays used) was considerably lower than that observed from usage of genomic DNA (36.5% vs. 87.9%). Preferential amplification and ADO were very

prominent in single cells tested and were responsible for the significant decrease observed in the overall accuracy rate. The results received indicate that due to the inherent problems of single cell PCR, accurate and reliable comprehensive chromosome analysis of single cells using real-time PCR would be extremely difficult if not impossible. Furthermore, it was concluded that usage of just two copy number assays per chromosome is insufficient to perform CCS on single cells. If this methodology is going to be applied on single cells a minimum of 96 assays (four per chromosome) are required; possibly more.

The problems observed at the single cell level, led to the conclusion that the real-time PCR approach to chromosome enumeration should not be applied to single blastomeres or polar bodies. However, the question of the methods suitability for analysis of trophectoderm biopsies (which usually consist of ~5 cells) remained open. Application of the real-time PCR protocol to clumps of three cells yielded a much improved accuracy rate (92.2%) relative to single cells. These results were in agreement with the study published by Treff *et al.* (2012) which showed a high accuracy rate from assessment of clumps of 5 cells and demonstrated that this methodology can be applied to blastocyst biopsied material with high accuracy. However, contrary to Treff *et al.* (2012) this study did not use any reference samples (i.e. calibrator) for analysis of results. It is highly likely, that usage of a reference file containing results from a number of samples (as performed by Treff *et al.* 2012) would enhance significantly the accuracy of the test described in this study and bring it closer to 100%.

4.7 Development of protocols for DNA fingerprinting of sperm samples and samples used for PGD

Amongst the procedures carried out in embryology laboratories and IVF clinics are the collection of sperm samples, retrieval and fertilisation of oocytes through IVF or ICSI, biopsy of embryos for PGD purposes and transfer of appropriate embryos to the uterus of the woman. In order for these actions to be carried out successfully, it is important that they are coordinated and monitored in a highly precise manner. For this reason strict rules and detailed guidelines have been published by different authorities (Human Fertilisation and Embryology Authority 2009). Despite the guidelines that are in place and are used by IVF clinics and despite the utilisation of automated equipment (e.g. bar-coding technology) errors still occur in ART (Adams and Carthey 2006). In the past a number of 'mismatching' cases had been reported including fertilisation of the correct oocytes with sperm from the wrong man/donor and transfer of wrong embryos into a woman's uterus (reviewed in Bender 2006). Risks are even greater in the case of PGD/PGS, as not only do the gametes and patients need to be matched, but individual oocytes and embryos need to be carefully tracked so that the correct embryo(s) can be transferred based upon the diagnostic results obtained.

Research carried out aimed at the development of a method that would be able to identify sample mix-ups in a rapid, inexpensive and accurate way without significantly disrupting the current routine followed in IVF/PGD laboratories. For the purposes of this project 12 TaqMan assays were selected based upon the rates of heterozygosity for the SNPs that they interrogate (>0.45 in all human populations for which data was available). Through usage of the mean minor allele frequencies of the 12 selected SNPs (Appendix 7) and through utilisation of the Hardy-Weinberg

equation and the SNP Identity Percentage Calculation (Nicklas and Buel 2008), it was determined that the possibility of two random individuals having the same genotype using the selected 12 SNPs is ~1 in 118,000.

4.7.1 Prospective clinical application

One of the procedures routinely carried out in IVF clinics is sperm collection. Witnessing is not feasible during sperm collection allowing for potential sample mix-up errors to occur. Additionally, although extremely rare, there has been at least one recorded case where a patient deliberately presented a sperm sample from another individual as their own, with the aim of later accusing the clinic of making an error and attempting to claim compensation (Dr. Dagan Wells personal communication). In an attempt to enhance the witnessing system of IVF clinics and avoid accidental (or deliberate) sperm sample mix-ups, a DNA fingerprinting protocol using the SNPs described above was developed for semen analysis.

It was considered critical that the test developed should be fast and compatible with standard processes going on in the IVF laboratory. It should also be simple to use, preferably avoiding electrophoresis and other techniques unfamiliar to embryologists, allowing for easy application in the IVF clinic. Finally, the method should not add significantly to the cost of an IVF procedure and provide an adequate discrimination of different individuals. The protocol developed is intended for use immediately following sperm collection and before insemination takes place, in order to confirm that no sample mix-up has occurred. It is essential for identification purposes that a DNA sample (e.g. through buccal swab or blood sample) is also obtained from the sperm donor. The collection of that sample must be witnessed and can be performed

on the day the sperm sample is collected or weeks earlier. Results obtained from the two types of samples are compared to confirm a genetic match and therefore determine whether or not any errors have occurred. Ultimately, a protocol was developed that requires a minimal amount of semen (5µl) and takes less than 2 hours, allowing for testing of a sperm sample without cryopreservation or any significant delay to the IVF procedure. Furthermore, validatory work demonstrated that the developed protocol is widely applicable since it was able to provide SNP genotyping for samples with sperm concentrations ranging from low to high levels. Moreover, the protocol was very efficient (AE=99.2%) and highly accurate (accuracy rate=100%). These results clearly indicate that the protocol is appropriate for clinical usage and could help reduce sperm sample mix-ups in IVF clinics.

The methodology used to develop the protocol for assessing sperm samples was also attempted on single cells to assess whether the method could be used for embryo fingerprinting. The idea is for application of the developed protocol on samples used for PGD purposes (i.e. single gene testing), either directly on single cells (multiplex PCR) or after WGA. Incorporation of such technology in PGD procedures would provide an extra means of avoiding sample/embryo mix-ups, while also offering other advantages that come with embryo fingerprinting, such as contamination detection and identification of the embryos succeeding in implanting and forming a pregnancy (section 4.3.5). Considering calculations made by Nicklas and Buel (2008) and also considering the mean minor allele frequencies of the 12 selected SNPs (Appendix 7), the possibility of 2 sibling embryos having an identical genotype when the SNP fingerprinting protocol presented in this study is used, would be slightly higher than 0.2%.

Direct application of the protocol on single cells revealed a call rate of 99.6% and an accuracy rate of 97.9%. These results indicate the protocol is very reliable and could be used clinically for embryo fingerprinting. Usage of WGA products on the other hand, did not show such a high reliability. The accuracy rates and call rates (including undetermined calls) obtained from application of the protocol on MDA and SurePlex products were considerably lower than direct application of the protocol to single cells. Consequently, since the number of SNPs included in the protocol is not large, the power of discrimination of the protocol when applied on WGA products is greatly compromised. Furthermore, the high error rate (i.e. ADO) observed would provide erroneous results that might lead to false conclusions. Usage of the protocol on WGA products should therefore be avoided.

4.8 Phospholipase C zeta and infertility

Over the last two decades, the role of genetics in ART has primarily focused on detection of aneuploidy in embryos. However, it is becoming increasingly clear that other aspects of genetics may be relevant to the diagnosis and treatment of infertility. It has been proposed that mutations and polymorphisms affecting individual genes might influence factors ranging from embryo quality to miscarriage. In terms of infertility, a particularly interesting gene is PLCζ. Mounting evidence suggests that abnormal forms of PLCζ might be responsible for male factor infertility associated with oocyte activation deficiency (Heytens *et al.* 2009; Yoon *et al.* 2008). Previous studies have shown that in some couples with a history of OAD the sperm show absent or reduced amounts of PLCζ (Heytens *et al.* 2009; Kashir *et al.* 2011; Taylor *et al.* 2010; Yoon *et al.* 2008). However, little is known regarding genetic mechanisms associated with PLCζ that might play a role in OAD.

The first indication of a genetic link between PLCζ and male infertility was established from a study carried out by Heytens *et al.* (2009). In that study, a mutation (H398P) in the coding sequence of the PLCζ gene was identified in a male whose sperm was deficient of oocyte activation. This mutation was predicted through computer modelling to disrupt the protein structure, while further experiments on mice indicated that the mutation severely affected or abolished the ability of PLCζ to trigger Ca^{2+} oscillations (Heytens *et al.* 2009; Nomikos *et al.* 2011). It was suggested that the mutant PLCζ protein could be acting in a dominant-negative fashion (Heytens *et al.* 2009). However, further investigation of the same patient's DNA presented in this thesis revealed a second mutation resulting in a substitution of histidine to leucine at position 233 of the PLCζ amino-acid sequence (H233L). Dr. Junaid Kashir went on

to perform micro-injection of PLCζ^{H233L} cRNA into mouse oocytes and established that it caused abnormal Ca^{2+} oscillation profiles which were not sufficient for oocyte activation. This confirms that the specific mutation affects the activity of the protein and contributes to the infertility of the patient (Kashir *et al.* 2012).

Initially it was not clear whether the H233L and H398P mutations detected in this patient are on the same chromosome or whether the two copies of the gene are each affected by a different mutation. This has implications for understanding the mode of inheritance of PLCζ inactivation and inherited forms of OAD. Ultimately, genotyping of the patient's family members and also, minisequencing of single sperm derived from the patient, conclusively demonstrated that the two mutations are located on different copies of the PLCζ gene (i.e. the patient possess no normal copies of the gene) and that one mutation was inherited from each of his parents. Taking these results into consideration, along with the fact that although the patient's father was a carrier of the H398P mutation he was still able have a child without the use of ART, it can be concluded that the PLCζ mutations (at least those described in this study) function in a rather recessive manner.

To the best of the author's knowledge, this is the first study that reports a maternally inherited autosomal mutation that affects male fertility. As expected, the daughter of the patient who was conceived through means of AOA, was found to have inherited one of the two PLCζ mutations (H233L). Consequently, there is a 50% chance for any male children she has in the future to inherit the specific mutation. It has been demonstrated that sperm derived from patient$^{H398P+H233L}$ exhibits significantly reduced amounts of PLCζ protein or even absence of the protein (Heytens *et al.* 2009; Kashir *et al.* 2011). It is possible that males carrying only one PLCζ mutation will express subfertility. Given the fact that the incidence of subfertility is increasing and that

sperm defects are the single most common cause (Publicover *et al.* 2007), it is important that more studies are carried out to determine the role of mutations affecting the PLCζ gene in this condition. In this study the incidence of the two mutations was investigated in 100 DNA samples derived from a fertile population (i.e. more than 200 copies of the gene analysed). Neither of the mutations was detected in any of the individuals assessed. This indicates that the mutations are rare or even unique to the specific family. Furthermore, the occurrence of any mutation affecting the PLCζ gene was investigated in 8 patients (other than patient$^{H398P + H233L}$) whose sperm was affected with OAD. No mutations were detected in any of the patients.

Although these mutations were not detected in the additional patients tested (100 fertile patients, 8 OAD patients), no definitive conclusions can be drawn since the sample size used in this study was small and most patients tested were derived from a fertile population. In order to critically assess the involvement of PLCζ mutations in male infertility and subfertility, it is essential that more individuals are screened with emphasis given to patients whose sperm displays a deficit of oocyte activation.

4.9 Concluding remarks and future work

In conclusion, this study developed a large number of novel PGD protocols using conventional technologies, highlighting the strengths and weaknesses of current strategies and suggesting avenues for research into improved methods. A variety of new methodologies, which could potentially be applied in PGD and PGS in order to enhance their performance, were assessed. Furthermore, the role that genetic factors other than aneuploidy (e.g. PLCζ gene, telomeres and mitochondrial DNA) might have in infertility was investigated.

The 46 clinical PGD cases were associated with high rates of diagnosis and accuracy and have led to the birth of six healthy children. Apart from PGD of single gene disorders, a universal HLA-typing protocol was developed. Validation of the protocol and its application in clinical cases, have demonstrated its high efficiency, versatility and reliability in providing a fingerprint of the human HLA region. As discussed earlier (section 4.2.2), the developed protocol is advantageous (in many aspects) over other published PGD/HLA-typing protocols.

While the conventional methods used in this study performed exceptionally well, the limitations observed (discussed in section 4.1.3) led to the consideration of alternative techniques, especially whole genome amplification. From results obtained, it was concluded that these methods have much to offer in terms of accelerating the speed of protocol development and their potential to extend the types of diagnosis possible on a single cell (e.g. combining testing for single gene disorders and aneuploidy). However, factors not previously considered to be of great importance, such as post-amplification processing of WGA products, were found to be critical for obtaining the maximum benefits from WGA protocols.

Alternative WGA methods were found to have different benefits and drawbacks. For example, MDA offered the highest genome coverage and the most accurate genotyping amongst the three methods assessed, making it ideal for single gene PGD applications, whereas SurePlex was superior when applied to aneuploidy screening through array-CGH. Results obtained from this study indicated that MDA amplified DNA is an excellent substrate for karyomapping, providing highly accurate genotyping of single cells and also detecting aneuploidies. The data suggests that this linkage-based method is now ready for clinical usage in PGD/PGS. Currently, the use of MDA in combination with SNP arrays and karyomapping analysis seems to be the most appropriate method for successful performance of PGD for single gene disorders coupled with comprehensive aneuploidy screening, in a time frame compatible with IVF procedures (~36 hours). However, further optimisation of the procedure will be needed if it is to be applied to cycles involving blastocyst analysis and fresh transfer, reducing the length of the protocol to a maximum of 24 hours. The principal limitation of linkage-based approaches such as karyomapping is the need for assessment of DNA derived from at least one family member additional to the couple requesting PGD so that the phase of alleles with respect to the disease can be determined. The current study has shown that in cases of male inheritance the phase of linked markers can sometimes be determined by the testing of single sperm. Furthermore, work reported in this thesis confirms that MDA products are a suitable substrate for direct mutation analysis. This means that in cases where no additional family DNAs are available an aliquot of MDA product can be taken for PCR of the mutation site and mutation detection, while the remainder of the MDA product is used in parallel for karyomapping, revealing aneuploidies and potentially allowing the

phase of linked markers to be determined during the case (i.e. from the analysis of the embryos).

The research described in this thesis also addressed the question of aneuploidy detection in human preimplantation embryos by developing a novel microarray platform. Results obtained from validation indicated that the final, fully optimised version of the microarray can be used to reliably determine the chromosomal status in single cells. Additionally, the microarray allows relative quantitation of mtDNA a factor of potential clinical and scientific significance.

Assessment of mtDNA quantity in 89 clinical samples tested using the microarray revealed some interesting biological information regarding the relation of mtDNA concentration to chromosomal status of embryos and also to maternal age. However, the possibility that measurement of the relative mtDNA quantity could assist the selection of the most appropriate embryos for transfer – and therefore enhance IVF success rates - requires more work in order to be proven. Specifically, a valuable study to perform would involve comparison of relative mtDNA quantities in embryos that successfully implant with embryos that fail to implant, revealing if any significant differences exist between the two. If mtDNA quantity is relevant to embryo viability it might be possible to determine a minimum amount required for normal embryo development, viability and implantation. Previous studies performed on human and mouse oocytes/embryos suggest that critical thresholds of ATP and mtDNA do exist (Van Blerkom *et al.* 1995; Wai *et al.* 2010).

As well as providing information on chromosomes and mitochondria the microarray also demonstrated that simultaneous DNA fingerprinting of embryos might be possible, reducing the risk of sample mix-ups. However, further optimisation and

validation is required (e.g. selection of SNP probes that consistently provide correct calls) before such an approach can be utilised for clinical samples.

The use of aCGH and karyomapping were not the only technologies assessed for the purpose of chromosome screening. In this study, a method that involves PCR to amplify multiple loci from each chromosome in a quantitative manner, providing comprehensive chromosome screening, was developed. Utilisation of this method is found to have a number of advantages when compared to currently available methods of comprehensive chromosome screening, including greatly accelerated speeds of diagnosis, a particularly important factor for blastocyst analysis (discussed in section 4.6). Application of this methodology to single cells and also clumps of cells (representing trophectoderm biopsies) demonstrated that it could be successfully used for blastocysts but in its current form it is not suitable for polar bodies or single blastomeres. Further experiments are needed before it is determined if this methodology could ever be used for single cells, but it is highly likely that the number of assays per chromosome would need to be significantly increased.

Using real-time PCR combined with sequence detection probes, protocols were developed for DNA fingerprinting of sperm samples and embryos. The developed protocol was found to be highly efficient in providing an accurate DNA fingerprint for sperm samples. Utilisation of this protocol would cause minimal disturbance to the routine of IVF laboratories and is expected to help decrease mix-up errors. Application of the protocol to single cells demonstrated a high reliability regarding its usage at this level too and indicated that it could be used clinically to perform embryo fingerprinting. This would provide an extra means for detection of sample mix-ups and would also make detection of contamination more precise. Future experiments

regarding this project should involve a move from a research setting to a clinical trial of the technology in sperm and embryos.

The importance of genetics for the field of reproductive medicine extends beyond PGD and PGS. There is increasing evidence that mutations and polymorphisms may exist that affect fertility and/or modulate the response to assisted reproductive treatments, influencing success rates and affecting levels of risk for complications such as ovarian hyperstimulation syndrome (AlSheikh *et al.* 2012; Altmae *et al*. 2010; Fang *et al.* 2011; Gerasimova *et al.* 2010; Hanevik *et al.* 2011; Miyamura *et al.* 2011). As part of the current research, the PLCζ gene, which produces a sperm-specific protein essential for oocyte activation was investigated. Contrary to a previous study which suggested that PLCζ mutations act in a dominant fashion (Heytens *et al.* 2009), the results of this study indicated that a recessive mode of inheritance is more likely, requiring both copies of the gene to be dysfunctional in order for male infertility to occur. This is an important finding which is expected to guide research in the field along new paths. So far, the specific mutations assessed in this study appear to be unique (not seen in more than 200 additional chromosomes assessed). Future research needs to assess larger numbers of individuals for the presence of PLCζ sequence variants (especially more males producing sperm with OAD). Although this is just an assumption at the moment, if mutations in the PLCζ gene are concluded to play an important role in infertility and subfertility then, just like aneuploidy screening a test that screens for mutations in the specific gene could be developed and used to provide answers to patients while also revealing, indirectly, the kind of treatment that should be followed.

New technologies with the potential to revolutionise embryo selection are just beginning to be introduced (e.g. next generation sequencing, metabolomics and time-

lapse evaluation). In parallel, improved understanding of the genetics underlying problems such as fertilisation failure, response to ovarian stimulation and miscarriage may soon allow more precise diagnosis of infertility and the tailoring of treatments to individual patients, enhancing results and safety. Results presented in this thesis highlight developments of each of these important areas, describing new methods of aneuploidy detection, revealing variation in key biological factors such as mitochondria during the preimplantation phase, and confirming the part that individual genes (e.g. PLCζ) play in infertility. Together these data and that derived from other studies published in recent years indicate that we are at the cusp of an exciting new era of research in the field of reproductive medicine.

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Supplementary appendices

Appendix 1: Primers used to carry out PGD clinical cases

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
A/D Hyper IgE Syndrome	*STAT3*	D17S1299	F - TAGCACTTGAGCACACATGG R - GTGCATTATGGGGACCATTA	HEX	188-208	NCBI UniSTS
		D17S1861	F - GCAGCAGTCCTGTAGACAAAAC R - AAGAGGCTGGAGAAGGAAAT	6-FAM	194-200	Altarescu *et al.* 2008
		c.1281+1G>A Outer	F - AGCAGAGATGTGGGAATGG R - GAAATGTTCCAAGCCAGAGAG		234	---
		c.1281+1G>A Inner	F - * R – CATGTCACTTTGGCCTGAA		188	---
		c.1281+1G>A Mini	R – TTCATCCCCAACAAAACTTA		21	---
A/D Polycystic Kidney Disease	*PKD1*	D16S3082	F – CCTGCGGAAATAACGGTGA R – GTTTCGAGGACAGCCCTGG	6-FAM	180-212	NCBI UniSTS
		D16S291	F - GCAGCCTCAGTTGTGTTTCCTAATC R - AGTGCTGGGATTACAGGCATGAACC	6-FAM	158-167	NCBI UniSTS
		D16S3399	F – ACCTAGATCCCTCCAGGTTT R - GGGCCATTATTCAGCCAATC	HEX	174-185	NCBI UniSTS
		16PTEL06	F – GAACAGGACATGGCTGTCAT R - TCTACATGTGCCTAGAAGAC	6-FAM	144-168	NCBI UniSTS
Alpha-1-antitrypsin Deficiency	*SERPINA1*	D14S553	F – TACAAAGCCACAAGGGAGT R – AGCTATGTTTGTGCCATGG	6-FAM	247-294	NCBI UniSTS
		D14S62	F – ATCACACAACTGGAGGCTTC R - CAAACCAGCACCACTGTTAG	6-FAM	118-127	NCBI UniSTS
		c.1096G>A Outer	F – CCTGGGATCAGCCTTACAAC R - CATGAAGAGGGGAGACTTGG		300	---
		c.1096G>A Inner	F – GTGCATAAGGCTGTGCTGAC R - *		194	---
		c.1096G>A Mini	F – AGGCTGTGCTGACCATCGAC		21	---

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
Branchio-oto-renal Syndrome	*SIX1*	D14S1038	F – GATCCATCTTAGCCATTAAGG R - CAGTCAGGTGTCCATCTAAAAC	6-FAM	197-243	NCBI UniSTS
		D14S997	F - TGGTCTTGGCAACCCTATAAAAATC R - CAAAGGAGCATGTTTCCATAGC	HEX	204-220	NCBI UniSTS
		c.386A>G Outer	F – AGTTCTCGCCTCACAACCAC R – GTCTCTTTGCCTCCGGTTC		317	---
		c.386A>G Inner	F – TTACGTGGAGGCCGAGAAG R – GCGATGGGTAGGGATTGTG		179	---
		c.386A>G Mini	F – GGACGGCGAGGAGACCAGCT		21	---
Cystic Fibrosis	*CFTR*	D7S486	F - CCCACTGAGAAAGTAAACATAAAAG R - GCCCAGGTGATTGATAGTGC	HEX	113-125	RGUSA
		D7S2847	F - TCACCTTCAGAAAGTATTGCCTAA R - TTCTCCAAGCTCTGTTCCTCA	HEX	173-189	RGUSA
		c.1521_1523delCTT	F - GTTTTCCTGGATTATGCCTGGCAC R - GTTGGCATGCTTTGATGACGCTTC	HEX	92-95	RGUSA
Dominant Dystrophic Epidermolysis Bullosa	*COL7A1*	D3S1581	F – CTCTTCCCAGTCCCTGTATC R - GATTGCACTATGTCTCCAGG	6-FAM	150	NCBI UniSTS
		D3S2409	F - GGTGACAGAGACTCTTGTCTCA R - CATTCTGGTTGGGGAACATA	HEX	115-127	NCBI UniSTS
		c.6501+1G>C Outer	F – GCAAGAAGGTGGCTCTCACA R - ATGCACACACACAGCAGCA		272	---
		c.6501+1G>C Inner	F – GACTCTGTCTAGGGGGATGG R - *		195	---
		c.6501+1G>C Mini	F – CTGGGCCTGAAGGGAAGCCG		21	---
Duchenne Muscular Dystrophy	*DMD*	DXS1214	F – TAGAACCCAAATGACAACCA R - AAGATAGCAGGCAACAATAAGA	6-FAM	210-222	NCBI UniSTS

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		DXS1236	F – CTCTTCGTCGTTTACCAGCTC R - CTGGAGTGGGAAGTGGTCAG	6-FAM	161-178	NCBI UniSTS
		DXS997	F – TGGCTTTATTTTAAGAGGAC R – GTTTTCAGTTTCCTGGGT	6-FAM	108-117	NCBI UniSTS
Ehlers Danlos Syndrome type IV	*COL3A1*	D2S389	F - TAAAGCCTAGTGGAAGATCATC R - GCTGAGTTAACAGTTATCAACAATT	HEX	189-219	NCBI UniSTS
		c.2492G>A Outer	F - GCTGAGAGATTGCTGTTGTTGTTG R - GAGAGGGAGAGAGAGAGGGAGAGA		228	---
		c.2492G>A Inner	F - GCATGTAGGGACAGAATGGTGAA R - GGAAGGAACTTACAGCAGGTCCA		154	---
		c.2492G>A Mini	R – GGCCTCCTTCACCTTTCTCA		21	---
Hereditary Multiple Exostoses	*EXT1*	D8S592	F - TAGATAGATGATGTTGTTGTTGATG R - GACTGAATATATGTCCTGTTGGC	6-FAM	148-162	NCBI UniSTS
		c.811 T>G Outer	F – TTCAACACCATCCCTCCTCT R - GCGAGAATCCTTGTGCTTTT		177	---
		c.811 T>G Inner	F - GCTGGTATTCAAGGGGAAGAG R - CAGGTGGTGAGGAGCACAA		105	---
		c.811 T>G Mini	F – TGGTATTCAAGGGGAAGAGG		21	---
Huntington Disease	*HTT*	HD	F - GGCGGCTGAGGAAGCTGAGGA R - ATGGCGACCCTGGAAAAGCTGATGA	FAM	148-242	RGUSA
		D4S136	F - CTGACTTGATCCAATCCAAAGGAAAG R - TTGAACCTAGTAGGCGGAAGTTGCAC	HEX	223-250	RGUSA
		D4S127	F – GCAATCCATTTTGCACTACGG R – CCTGGCTCTCTCCCTCTTTC	FAM	130-136	RGUSA
		D4S412	F – ACTACCGCCAGGCACT R - CTAAGATATGAAAACCTAAGGGA	FAM	237-249	NCBI UniSTS

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		I1CAHD	F - TATGCCACTACACTACAACCTGGGC R - ACCAGCATGTGGTATTGTCAAAGTG	TAMRA	135-175	Moutou *et al.* 2004
Krabbe Disease	*GALC*	D14S67	F - TCACTACGCCTCTACAATTCTATG R - TAGTCAGGGTTTGCCAGAGA	6-FAM	133-167	NCBI UniSTS
		D14S68	F – GAGAGGTGGTTTTCAGTGGT R - TCAGGGATAGTTGGTGGGTA	HEX	148-172	NCBI UniSTS
		c.1180delA Outer	F – AGTGCATACGGCCATTTCTT R - TGGCTTCTGTCCTGCTAGATT		255	---
		c.1180delA Inner	F - * R - TCGGAAGACAGAAGACACCA		226	---
		c.1180delA Mini	R – AGATCCCTTAAGAACAAAGG		21	---
Marfan Syndrome	*FBN1*	D15S143	F - CCTAAGGAGGCAACAGCAAAG R - GTAAAGACTGGTATCTGTAGCAC	HEX	186-195	NCBI UniSTS
		D15S196	F – GACCTGTAGCTGAAGGGAAG R - ATAAAAGTGGTGGGGAAGGATG	FAM	264-275	NCBI UniSTS
		D15S659	F - GTGGATAGACACATGACAGATAGG R - TATTTGGCAAGGATAGATACAGG	6-FAM	174-206	NCBI UniSTS
		c.1904A>G Outer	F – TCCTATCTTCCCCATTTTCAAG R - TTTTCTTACCAACACACACACG		234	---
		c.1904A>G Inner	F – AGTGCCTTTCTCTGCCACA R – AACACACACACGGCCATC		142	---
		c.1904A>G Mini	F – CGTCAACACTGATGGCTCCT		21	---
		c.5721C>G Outer	F – GGCCTGGTGAACCCTAAAA R - TGCACACGCACCTATACAGTC		225	---
		c.5721C>G Inner	F - AATGTGAAAGAGATGCCTGTG R - GTCATTGTTGTGAGAAAGGATG		101	---

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		c.5721C>G Mini	F – GGGAATGGAACTTGCCGGAA		21	---
		c.6569G>A Outer	F – TTCTGTTGGCAATCCTTGTG R - TTCCCAGGATCAGTACACGTAA		157	---
		c.6569G>A Inner	F – ACCTGCAAGAATGTGATTGG R - *		129	---
		c.6569G>A Mini	F – TGTGATTGGAGGTTTTGAAT		21	---
		c.235C>T Inner	F – TGGATGGAAAACCTTACCTG R - CAGTTACAAAAGGCCACATTC		131	---
		c.235C>T Mini	F – AAACCTTACCTGGCGGAAAT		21	---
		c.2097T>A Outer	F – GGGGGTTCTCATCTGTTTGA R - AAGACCTCAATGGTGGCAGA		269	---
		c.2097T>A Inner	F - AAGAGAGGCCAGTGTATCAAACC R - *		177	---
		c.2097T>A Mini	F – GGGGAACCTTGCCAGCCGTG		21	---
Mitochondrial DNA Depletion syndrome	*POLG*	D15S979	F – TGCTGCCCAACATCCT R – CAGTGCTACATCCACGGAA	6-FAM	135-167	NCBI UniSTS
		D15S116	F – AGCTTCCAACTNCGCCCTCC R - AGGGGTGTTACATCGCGGGT	HEX	164-184	NCBI UniSTS
		c.1399G>A Outer	F – CATGCTGGAGATGGGTGTC R – TCCACTAGGGCAGGGCTAA		247	---
		c.1399G>A Inner	F – CCAGCGGGAGATGAAGAAG R - *		160	---
		c.1399G>A Mini	F – AGAAGTCGTTGATGGATCTG		21	---

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		c.2542G>A Outer	F - GGAGAACTGAGGTGAGGTGGT R - ACAGACCTGGGAGAGGAAGAG		247	---
		c.2542G>A Inner	F – CCCCGACTATGATGAGGAAG R - *		165	---
		c.2542G>A Mini	F - TGCCCCAAGTGGTGACTGCC		21	---
Mucopolysaccharidosis type I	*IDUA*	D4S2936	F - AGACTCCGTCTGAAAACAAAC R - TGGCACATCACCAACAACTG	6-FAM	143-157	RGUSA
		D4S3038	F - AAGACCAGCATTCGGAAGAC R - GTTCTTTTCTCTGTGTCTGAGT	HEX	148-169	RGUSA
		D4S3360	F - CTAGCTTTGATTCTATTGACC R - GGTCTAAATCAATGACCTAAGC	6-FAM	174-199	NCBI UniSTS
Neurofibromatosis type I	*NF1*	D17S783	F - AGGACTCGAAATGCTTTCAT R - TAACAGAAAACTTGGAGCCG	HEX	237-255	NCBI UniSTS
		D17S1294	F - TGGCATGCAATTGTAGTCTC R - TTCTTTCCTTACTAAGTTGAGAACG	HEX	248-272	NCBI UniSTS
		D17S1880	F - AGGGATTGCTTGAGCC R - TGACAGAATTTGAACACTTTG	6-FAM	154-196	NCBI UniSTS
		D17S1800	F - CTAAACTAGGTTGGGTTGAAATCTC R - TCTGGCACAAAGACCTGAG	6-FAM	268-284	NCBI UniSTS
		D17S1824	F - ACTGAAAACTCACTCTTGTTCTGG R - GATGTAAGTAGCATTGCCTCCC	6-FAM	116-134	NCBI UniSTS
		IVS38GT	F - CAACAGAGCAAGACCCTGTCTC R - CCATTAGCACCCCTCCTAACAT	HEX	171-187	Spits *et al.* 2005
		IVS2728	F - GTTCTCAACTTAAATGTAAGT R - GAACATTAACAACAAGTACC	6-FAM	207-219	Lazaro *et al.* 1994

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		c.5499-2A>G Outer	F - CCAATCTCTTAATCTCTGAAGGAG R - TGTTGCACTGGTTTTGATGA		280	---
		c.5499-2A>G Inner	F - TTGATTAGGCTGTTCCAATGA R - *		142	---
		c.5499-2A>G Mini	F – GTTATTTTCCTTCTTCAACT		21	---
		c.3916C>T Outer	F - TGTGTGTATGTGTGTGCTGAGG R - GCGGTTCTATGTGAAAAGATGAC		247	---
		c.3916C>T Inner	F - TCGTGCATTTCTGTAGGTATATGG R - *		165	---
		c.3916C>T Mini	R - AGAGGATGTGATCACAATTC		21	---
		c.3457-3460delCTCA	F - GCAAACAGGTGGCAGGAA R - TCTTGTCTGGAGATCCTTGTGG		274	---
Otopalatodigital Syndrome	*FLNA*	DXS1073	F - ATGCCCTCTCCGAGTTATTAC R - GATTGGTGGCCTTTGAAACAC	FAM	128-142	RGUSA
		DXS8061	F - GCTTGAAGTGTCCATGAGGTATC R - AGAAGCTGATGTGCTCCCTG	HEX	125-153	NCBI UniSTS
		c.1664C>A Outer	F - TCTTGGCAGAGGGAGAGGAG R - ACACAGGGAACACCGAGGA		294	---
		c.1664C>A Inner	F - * R - GAGTGTGCAGAGCTGGGAGA		173	---
		c.1664C>A Mini	R - CGATGTTCTGACCACCCCAC		21	---
Phenylketonuria	*PAH*	STR-3	F - TGTGGAAAGCAGAAAGAC R – TCATAAGTGTTCCCAGACA	HEX	219-232	Verlinsky *et al.* 1999
		VNTR-13	F - CTTGATTTAATCATTTTACAAT R - CTCAGAGAAGCACATCTTTT	6-FAM	256-403	Verlinsky *et al.* 1999

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		c.194T>C Inner	F - ACCCTCCCCATTCTCTCTTC R - AGGCAGGCTACGTTTATCCA		122	---
		c.194T>C Mini	F - TGATGTAAACCTGACCCACA		21	---
		c.1241A>G Inner	F - GTGGTTTTGGTCTTAGGAACTTTG R - ATCTTAAGCTGCTGGGTATTGT		112	---
		c.1241A>G Mini	F - TCGGCCCTTCTCAGTTCGCT		21	---
POMGNT1 related muscular dystrophy	*POMGNT1*	D1S2677	F - GTTCTGGAGTTTGCCTCTAA R – AGCTGTGATTGTNCCACT		135-153	NCBI UniSTS
		D1S2797	F - ATCACATCACACACAATGACTGTGG R - TGTCCATTCAAAGGATTGGTCTC		144-180	NCBI UniSTS
		c.1539+1G>A	F - TTTCCCGATCCTACCACTTT R - CTTCAGAGTCCATGTTCTTGTTC		125	---
		c.1539+1G>A Mini	R – CTCCCTGCCTCCCACTCA		19	---
		c.1738C>A	F - TGCAGTGAGGCTGAGGTTCT R - CAGGGTGGGCATGGTATTG		166	---
		c.1738C>A Mini	R - TGAAGTCATCATCTTTCTCCATTC		25	---
Sandhoff Disease	*HEXB*	D5S1988	F - AGCTTACTTCACTTGGCATAA R - GTCCACCGATGGATGAATG	FAM	200-207	Kuliev *et al.* 2006
		D5S2003	F - AGCCTAAGTGACAAAGTGAGACA R - TAGAGTCCTTTTCATTGCCAA	HEX	116-136	Kuliev *et al.* 2006
		c.115delG	F1 - CTGTTGGCGACACTGCTG R1 – ATTGGGGCTGTGGCTGAT	HEX	201	---
		c.115delG	F1 - CTGTTGGCGACACTGCTG R2 - GGTTCGGGGTCATCTTCAC	HEX	154	---

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
Sickle Cell Anaemia	*HBB*	D11S1760	F - ATCTCAAGTGTTTCCCCACAAC R - CTGCATCATGACTTGAAAAACG	HEX	90-105	Fiorentino *et al.* 2006
		D11S4181	F - CTGGGCAACAAGAGTAAGTCTCT R - CCTTAAGAACTGAGACCAAGAACA	FAM	109-116	Fiorentino *et al.* 2006
		D11S2362	F - TGGACTATAGGACCCCCTTC R - GAGAACAGCCTGTCACACCT	HEX	209-230	NCBI UniSTS
		c.20A>T Outer	F - AGTCAGGGCAGAGCCATCTA R - GTCTCCACATGCCCAGTTTC		229	RGUSA
		c.20A>T Inner	F - * R - CCTCACCACCAACTTCATCC		158	RGUSA
		c.20A>T Mini	R - CAGTAACGGCAGACTTCTCC		21	RGUSA
Spinal Muscular Atrophy (type 1)	*SMN1*	D5S610	F - GGCAGTGTCCTAAAATCTTTTG R - CCTAAACTGAACTTTCAAAGCTG	6-FAM	127-144	Girardet *et al.* 2008
		D5S629	F - CAGTGAGCCGAGATCTCGTC R - CCGGTTTGTTCCTGTGATCAG	6-FAM	190-197	Girardet *et al.* 2008
		D5S637	F - GAGTGTGAATTAATTGCCAAACAC R - CATTTAATACTGCAATGAAAAAGTC	HEX	235-237	Girardet *et al.* 2008
		Exon 7 Outer	F - AGCCTAATAATTGTTTTCTTTGGGAT R - AATGCTGGCAGACTTACTCCTTAAT		---	Girardet *et al.* 2008
		Exon 7 Inner	F - * R - CACCTTCCTTCTTTTTGATTTTGTTT		235	Girardet *et al.* 2008
		Exon 8 Outer	F - GCAATGTGAAATATTTTACTGGAC R - CAGCCATGTCCACCAGTTAGA		---	Girardet *et al.* 2008
		Exon 8 Inner	F - * R - TGCCACATACGCCTCACATAC		287	Girardet *et al.* 2008

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
Thalassaemia B	*HBB*	c.142+5G>C Outer	F - CTGTCATCACTTAGACCTCA R - TGGTCTCCTTAAACCTGCTTG		~310	Fiorentino *et al.* 2004
		c.142+5G>C Inner	F - CATCACTTAGACCTCACCCTGT R - TCTCCTTAAACCTGTCTTGTAACC		~290	Fiorentino *et al.* 2004
		c.142+5G>C Mini	R – TGTCTTGTAACCTTGATA		19	---
Tuberous Sclerosis 1	*TSC1*	D9S149	F - GATTGACCTGTGAATTTGTACAGC R - TGTTATGCCTTGCTGTTGCT	FAM	146-176	Henske *et al.* 1993
		PM4	F - TTTTAATGGAAACAACTTGACC R - TAAATGGTGCAGCCACTGT	HEX	150-170	Parry *et al.* 2000
		c.912T>G Outer	F - ATGTCACCACCAGCCCTTAT R - CAGGTAGCAGACCAAGGACA		248	---
		c.912T>G Inner	F - AGCCCTTATGCTGACACACAG R - GAAAATGGAGGGACATGCAA		183	---
		c.912T>G Mini	F - GCTGACACACAGAATAGCTA		21	---
		c.1525C>T Outer	F - GGATGCCACTTTTTCTCCTC R - GAGTGTAAAGGCTCAGGGTTC		299	---
		c.1525C>T Inner	F - ACCCAAACTGCCTAGTCTTTC R – TCTTCCGCTGAGAACCTG		188	---
		c.1525C>T Mini	F - GCTTTGACTCTCCCTTTTAC		21	---
X-linked Hydrocephalus	*L1CAM*	DXS1227	F - AGAGGTCCGAGTCTTCCAC R - ATAAGGGTTTACTCCCCCAA	6-FAM	174-186	NCBI UniSTS
		c.551G>A Outer	F - TGTTAGCGGGGGTGTCTTC R - GGAGGTCAATGGGTTCCTTC		218	---
		c.551G>A Inner	F - * R - TCTGAGTGGTTGTCGGAGGT		153	---

Appendix 1 (continued)

Disease	Gene	STR markers / mutation detection primers	Primer sequences (5' to 3')	5' label	Product size (bp)	References**
		c.551G>A Mini	F - GCACATCAAGCAGGACGAGC		21	---

* The outer F or the outer R primer was used in conjunction with the inner primer in order to perform the inner reaction. In these occasions a hemi-nested approach was used.

** The primer sequences used to amplify the STR markers were selected from 3 different sources. Some were from published papers. Others were provided from Reprogenetics LLC (Livingston, New Jersey, USA) - noted as 'RGUSA'. Some others were found in the NCBI 'UniSTS' database - noted as 'NCBI UniSTS' (http://www.ncbi.nlm.nih.gov/unists). Most of the primer sequences used for mutation detection were designed using 'Primer3' software while the rest were selected from published papers or were provided from 'RGUSA'.

Appendix 2: Loci used in PGD cases for Gender Determination

Locus	Primer sequences (5' to 3')	5' label	Product size (bp)	References
SRY	F – AGCAGTCAGGGAGGCAGATCA R – CCCCCTAGTACCCTGACAATGTATT	6-FAM	95	Drobnic 2006, Lo *et al.* 1998
AMEL	F – CCCTGGGCTCTGTAAAGAATAGTG R – ATCAGAGCTTAAACTGGGAAGCTG	HEX	105-111	RGUSA
HPRT	F – ATGCCACAGATAATACACATCCCC R – CTCTCCAGAATAGTTAGATGTAGG	HEX	263-299	Edwards *et al.* 1992
X22	F – TAATGAGAGTTGGAAAGAAA R – CCCATTGTTGCTACTTGAGA	6-FAM	189-242	Cirigliano *et al.* 1999

Appendix 3: 24sure Test

The 24sure test (BlueGnome Ltd, UK) was used to carry out full chromosomal screening of oocytes (through PBs) and embryos (through biopsied blastomeres and trophectoderm cells). Both gains and losses of chromosomes were detected at the single cell level. The test included a number of steps: cell lysis, whole genome amplification of samples, fluorescent labeling and hybridisation of the samples and 'reference DNA' samples, post-hybridisation washes and finally scanning and analysis of images.

Cell lysis and WGA of samples were carried out using the SurePlex DNA amplification system (Rubicon Genomics, USA) according to manufacturer's instructions. After WGA was completed, 5µl of each sample were used to carry out gel electrophoresis (1% agarose gel) as described in section 2.1.2, in order to check if amplification was successful.

The Fluorescent Labeling System (BlueGnome Ltd) was used to label the amplified samples and commercially available control male DNA samples (46,XY) (BlueGnome Ltd). Specifically, the sample and control were labeled with Cy3 (green)

and the Cy5 (red) flurophores, respectively, using random primers. The control DNA sample (SureRef male), which was well-matched to amplified single cells, was used as a hybridisation control. Labeling was carried out as instructed by the manufacturer. Amplified samples and control samples were incubated at 37°C for 3 hours in order for labeling to occur.

After labeling, test and reference DNA co-precipitation [using a centrifugal evaporator (miVac, Genevac, UK)], their denaturation, array hybritisation and post-hybridisation washes were carried out according to manufacturer's instructions.

A two channel, laser scanner (InnoScan 710, Innopsys, France) was used to excite the hybridized fluorophores and read and store the resulting images of the hybridisation. The 'MAPIX' software (Innopsys) was used to scan the microarray slides. The resulting images were analysed with the help of the 'BlueFuse Multi Analysis' software (BlueGnome Ltd).

Appendix 4: Primers used in the final HLA-typing protocol developed

STR markers	Heterozygosity	Primer sequences (5' to 3')	5' label	Product size (bp)
D6S105	0.79-0.81 [A B]	F - AACAAGAGCAAAACTCCGTCTC R – TCACCTTGATATCTTATTACCCTGG	6-FAM	134-155
D6S2443	0.78 [C]	F - CCATACCAAAGTAAAACCCAGTG R – CATTTGATACTGAGGATGAAGGG	VIC	180-207
D6S1629	0.79 [A C]	F - CACAGTGACTTGTACTGAAAGCTCA R – GGCTCCCCAATTATCTCTGC	VIC	154-165
LH-1	0.71 [B]	F - GCTAGTCTGTGCCAAGGAACTC R – ACCTTACTGGGCACAAATTCAC	NED	126-160
MOG-CA	0.77 [B]	F - AGATCACCTCGAGTGAGTCTCTT R – TTGACCATGGGTAACTGAAGC	NED	205-236
D6S1560	0.84-0.85 [A C]	F - TCCTTGGTGGTAGTGTTTCTAA R – TGAGTCAAGTGAGAAACAGAGAG	PET	130-146
D6S265	0.76-0.79 [A B]	F - TCGTACCCATTAACCTACCTCTCT R – TCGAGGTAAACAGCAGAAAGATAG	6-FAM	110-125
62	0.82 [B]	F - GATTTCATCCAGCCACAGGA R – TCCAATCACCTCTGCTCACC	6-FAM	140-170
D6S439	0.58-0.60 [A B]	F - CCCCTATTCTCCACCCACTAGA R – CAGCCTCAGGGAAGACACATT	VIC	116-130
DRA-CA	0.84 [B]	F - ACTTTCCTAATTCTCCTCCTTC R – GCATGAGTAAACTATGGAATCTC	NED	122-140
MIB	0.82 [B]	F - CGTTTTCAGCCTGCTAGCTTAT R – CCACAGTCTCTATCAGTCCAGATTC	NED	155-187
D6S388	0.30-0.75 [A C]	F - GCTGATGGAGAATGAAATATGG R - GGTTAGACGTAGCTTAAGAGAGAAT	PET	150-156
D6S1666	0.87 [A C]	F - GTTGGGCAGCATTTGTAGATTTC R – ACCCAGCATTTTGGAGTTGTGT	6-FAM	112-142
D6S1611	0.64-0.68 [A C]	F - GGATTTCTTGCAAAACAAACCC R – AAGGGCTGAGTTTCTTCTTGGG	6-FAM	176-185
D6S510	0.74 [B]	F - TTTGTCTTTCCCAATGTACTACAC R – GCTACTACTTCACACCAATTAGGA	6-FAM	140-161
HLABC-CA	0.78 [B]	F - GTCAAGCATATCTGCCATTTGG R – ACTTGGGCAATGAGTCCTATGA	VIC	113-149
D6S1683	0.70-0.72 [A C]	F - ACATGTATCCGAGAACTTAAAGT R – AAGTAGAGACAGGATTTCTTGT	NED	169-178
D6S258	0.77-0.83 [A C]	F - GCAAATCAAGAATGTAATTCCC R – GCTTTGTAGTTCTTTTTGTGGA	NED	121-131
D6S1568	0.86-0.87 [A C]	F - AGATATCCCCACCAAGGCAG R – AGCTAGGCCAGGCCGTGT	PET	123-152
DQCAR*	0.78 [B]	F - CTGCATTTCTCTTCCTTATCACTTC R – TGGCCAATCAGAATCTTTCCTA	VIC	150-179

* DQCAR is not part of the final HLA-typing protocol developed. It was only used in one of the protocols developed for a clinical case (section 2.3.3.2).

[A] From Broman *et al.* 1998 - Mammalian Genotyping Service (http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp)

[B] From Fiorentino *et al.* 2004

[C] From Fiorentino *et al.* 2005

Appendix 5: TaqMan copy number assays used for development of the Real-Time PCR protocol for CCS of human embryos

Assay No.	Assay ID	Gene Symbol	Gene Name	Chromosome	Cytogenetic Band
1.1	Hs03193256_cn	RCC1	regulator of chromosome condensation 1	1	1p35.3b
1.2	Hs00876448_cn	TMCO4	transmembrane and coiled-coil domains 4	1	1p36.13a
1.3	Hs03178347_cn	SRP9	signal recognition particle 9kDa	1	1q42.12c
1.4	Hs07226270_cn	DISC1	disrupted in schizophrenia 1	1	1q42.2b
2.1	Hs04692736_cn	GTF3C2	general transcription factor IIIC, polypeptide 2, beta 110kDa	2	2p23.3a
2.2	Hs04658573_cn	IAH1	isoamyl acetate-hydrolyzing esterase 1 homolog (S. cerevisiae)	2	2p25.1d
2.3	Hs04716535_cn	LASS6	LAG1 homolog, ceramide synthase 6	2	2q24.3f
2.4	Hs01702970_cn	DCAF17	DDB1 and CUL4 associated factor 17	2	2q31.1c
3.1	Hs01278165_cn	NGLY1	N-glycanase 1	3	3p24.2a
3.2	Hs00493458_cn	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	3	3p25.3b
3.3	Hs03491014_cn	ZBTB38	zinc finger and BTB domain containing 38	3	3q23c
3.4	Hs02815441_cn	SMC4	structural maintenance of chromosomes 4	3	3q25.33b
4.1	Hs05959876_cn	CORIN	corin, serine peptidase	4	4p12a
4.2	Hs05920128_cn	PGM2	phosphoglucomutase 2	4	4p14d
4.3	Hs02075730_cn	USP53	ubiquitin specific peptidase 53	4	4q26f
4.4	Hs01271024_cn	ZNF827	zinc finger protein 827	4	4q31.21c
5.1	Hs01044784_cn	SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	5	5p15.31c
5.2	Hs02178036_cn	SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	5	5p15.31c
5.3	Hs07226234_cn	ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	5	5q35.3d
5.4	Hs07226236_cn	ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	5	5q35.3d

Appendix 5 (continued)

Assay No.	Assay ID	Gene Symbol	Gene Name	Chromosome	Cytogenetic Band
6.1	Hs04902228_cn	PHACTR1	phosphatase and actin regulator 1	6	6p24.1a
6.2	Hs02988007_cn	C6orf145	chromosome 6 open reading frame 145	6	6p25.2a
6.3	Hs06798399_cn	LAMA4	laminin, alpha 4	6	6q21h
6.4	Hs02761573_cn	CENPW	centromere protein W	6	6q22.32b
7.1	Hs04339214_cn	THSD7A	thrombospondin, type I, domain containing 7A	7	7p21.3b
7.2	Hs02418225_cn	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	7	7p22.1b
7.3	Hs03114237_cn	NA	NA	7	7q34e
7.4	Hs03113881_cn	NA	NA	7	7q34e
8.1	Hs00275884_cn	MAK16;C8orf41	MAK16 homolog (S. cerevisiae) chromosome 8 open reading frame 41	8	8p12c
8.2	Hs06163596_cn	MSRA	methionine sulfoxide reductase A	8	8p23.1c
8.3	Hs00310789_cn	TMEM71	transmembrane protein 71	8	8q24.22b
8.4	Hs01241456_cn	TMEM71	transmembrane protein 71	8	8q24.22b
9.1	Hs00956247_cn	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	9	9p21.1a
9.2	Hs00113964_cn	DMRT1	doublesex and mab-3 related transcription factor 1	9	9p24.3b
9.3	Hs02334579_cn	FAM78A	family with sequence similarity 78, member A	9	9q34.13a
9.4	Hs01254139_cn	ARRDC1	arrestin domain containing 1	9	9q34.3f
10.1	Hs01210989_cn	BEND7	BEN domain containing 7	10	10p13d
10.2	Hs05189556_cn	UCN3	urocortin 3 (stresscopin)	10	10p15.1c
10.3	Hs07226388_cn	C10orf90	chromosome 10 open reading frame 90	10	10q26.2a
10.4	Hs07226389_cn	C10orf90	chromosome 10 open reading frame 90	10	10q26.2a
11.1	Hs01644358_cn	FAM160A2	family with sequence similarity 160, member A2	11	11p15.4c
11.2	Hs01423651_cn	OR51F1	olfactory receptor, family 51, subfamily F, member 1	11	11p15.4d
11.3	Hs03194732_cn	TIMM8B	translocase of inner mitochondrial membrane 8 homolog B (yeast)	11	11q23.1c

Appendix 5 (continued)

Assay No.	Assay ID	Gene Symbol	Gene Name	Chromosome	Cytogenetic Band
11.4	Hs03176196_cn	TIMM8B	translocase of inner mitochondrial membrane 8 homolog B (yeast)	11	11q23.1c
12.1	Hs03194239_cn	TPI1	triosephosphate isomerase 1	12	12p13.31d
12.2	Hs01557728_cn	ENO2	enolase 2 (gamma, neuronal)	12	12p13.31d
12.3	Hs00213327_cn	GOLGA3	golgin A3	12	12q24.33d
12.4	Hs01436960_cn	GOLGA3	golgin A3	12	12q24.33d
13.1	Hs00614394_cn	DZIP1	DAZ interacting protein 1	13	13q32.1b
13.2	Hs00216560_cn	COL4A2	collagen, type IV, alpha 2	13	13q34a
13.3	Hs00201890_cn	PROZ	protein Z, vitamin K-dependent plasma glycoprotein	13	13q34c
13.4	Hs03861872_cn	MIR19A;MIR20A; MIR17HG	microRNA 19amicroRNA 20aMIR17 host gene (non-protein coding)	13	13q31.3b
14.1	Hs02269694_cn	MARK3	MAP/microtubule affinity-regulating kinase 3	14	14q32.32b
14.2	Hs02277899_cn	TMEM179	transmembrane protein 179	14	14q32.33b
14.3	Hs02330595_cn	KIAA0284	KIAA0284	14	14q32.33c
14.4	Hs02968347_cn	KIAA1409;COX8C	KIAA1409 cytochrome c oxidase subunit 8C	14	14q32.12b
15.1	Hs05351793_cn	BLM	Bloom syndrome, RecQ helicase-like	15	15q26.1c
15.2	Hs00689935_cn	CHD2	chromodomain helicase DNA binding protein 2	15	15q26.1e
15.3	Hs02820990_cn	FAM169B	family with sequence similarity 169, member B	15	15q26.3a
15.4	Hs05389092_cn	ANPEP	alanyl (membrane) aminopeptidase	15	15q26.1b
16.1	Hs01480069_cn	MGRN1	mahogunin, ring finger 1	16	16p13.3c
16.2	Hs03055169_cn	CREBBP	CREB binding protein	16	16p13.3d
16.3	Hs00150593_cn	CCDC113	coiled-coil domain containing 113	16	16q21a
16.4	Hs03934353_cn	NA	NA	16	16q24.1c
17.1	Hs02194326_cn	BCL6B	B-cell CLL/lymphoma 6, member B	17	17p13.1d
17.2	Hs03181823_cn	OR1E2	olfactory receptor, family 1, subfamily E, member 2	17	17p13.2c

Appendix 5 (continued)

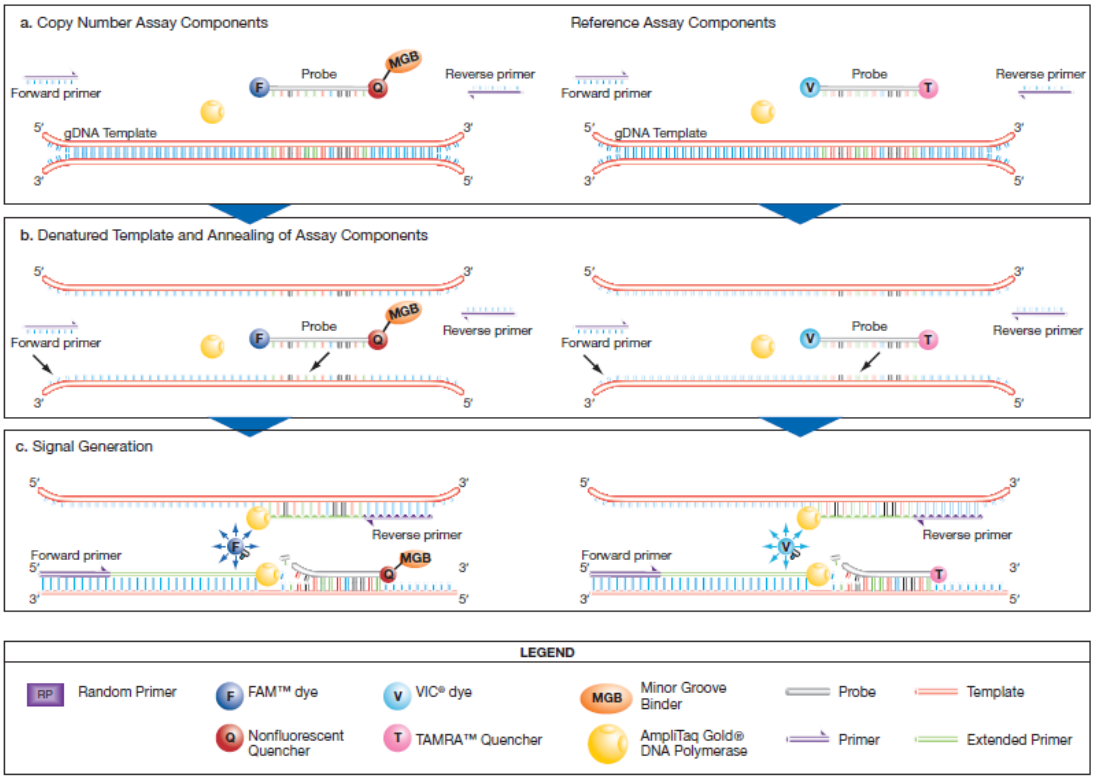
Assay No.	Assay ID	Gene Symbol	Gene Name	Chromosome	Cytogenetic Band
17.3	Hs03181459_cn	H3F3B	H3 histone, family 3B (H3.3B)	17	17q25.1c
17.4	Hs03184552_cn	H3F3B	H3 histone, family 3B (H3.3B)	17	17q25.1c
18.1	Hs02106900_cn	EPB41L3	erythrocyte membrane protein band 4.1-like 3	18	18p11.31b
18.2	Hs00488766_cn	USP14	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	18	18p11.32c
18.3	Hs01306949_cn	MBP	myelin basic protein	18	18q23b
18.4	Hs06498356_cn	ATP9B	ATPase, class II, type 9B	18	18q23d
19.1	Hs01413810_cn	ZNF77	zinc finger protein 77	19	19p13.3f
19.2	Hs01339946_cn	ZNF77	zinc finger protein 77	19	19p13.3f
19.3	Hs02995438_cn	ZSCAN4	zinc finger and SCAN domain containing 4	19	19q13.43c
19.4	Hs00566496_cn	ZNF776	zinc finger protein 776	19	19q13.43c
20.1	Hs07203821_cn	TASP1	taspase, threonine aspartase, 1	20	20p12.1d
20.2	Hs00358577_cn	ANKRD5	ankyrin repeat domain 5	20	20p12.2b
20.3	Hs00624199_cn	B4GALT5	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5	20	20q13.13d
20.4	Hs02620199_cn	PFDN4	prefoldin subunit 4	20	20q13.2c
21.1	Hs04068763_cn	UMODL1	uromodulin-like 1	21	21q22.3b
21.2	Hs02427310_cn	UBASH3A	ubiquitin associated and SH3 domain containing A	21	21q22.3b
21.3	Hs04498861_cn	PDXK	pyridoxal (pyridoxine, vitamin B6) kinase	21	21q22.3c
21.4	Hs01167169_cn	PRDM15	PR domain containing 15	21	21q22.3a
22.1	Hs02065601_cn	ATXN10	ataxin 10	22	22q13.31c
22.2	Hs02311370_cn	ATXN10	ataxin 10	22	22q13.31c
22.3	Hs05561659_cn	PPP6R2	protein phosphatase 6, regulatory subunit 2	22	22q13.33b
22.4	Hs07226295_cn	FBLN1	fibulin 1	22	22q13.31c
X.1	Hs03194350_cn	TMSB4X	thymosin beta 4, X-linked	X	Xp22.2b

Appendix 5 (continued)

Assay No.	Assay ID	Gene Symbol	Gene Name	Chromosome	Cytogenetic Band
X.2	Hs00982082_cn	ARHGAP6	Rho GTPase activating protein 6	X	Xp22.2c
X.3	Hs02022459_cn	SLITRK4	SLIT and NTRK-like family, member 4	X	Xq27.3a
X.4	Hs02497197_cn	MAGEA12	melanoma antigen family A, 12	X	Xq28e
Y.1	Hs01090783_cn	ZFY	zinc finger protein, Y-linked	Y	Yp11.31b
Y.2	Hs01026408_cn	SRY	sex determining region Y	Y	Yp11.31c
Y.3	Hs01079454_cn	UTY	ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	Y	Yq11.221a
Y.4	Hs05704387_cn	NLGN4Y	neuroligin 4, Y-linked	Y	Yq11.221c

Notes: The assays utilized in the project were amplifying small parts of different genes spread across all 24 human chromosomes. Some assays were amplifying different parts of the same gene (e.g. assays 22.1 and 22.2). 'NA' stands for Not Available.

Appendix 6: Schematic diagram showing how TaqMan copy number assays and reference assay work. Reproduced from Life Technologies Corporation (2010) with permission.



Notes: Each TaqMan copy number assay contains: two unlabeled primers amplifying the target sequence and one TaqMan probe for detecting the target sequence. The probe includes a FAM reporter dye attached to its 5' end and a non-fluorescent quencher and a Minor Groove Binder attached to its 3' end. The Minor Groove Binders increase the melting temperature (Tm) without increasing probe length and therefore allow for the design of shorter probes. The TaqMan copy number reference assay contains: two unlabeled primers for amplifying the reference sequence and a TaqMan probe for detecting the reference sequence. The probe includes a VIC reporter dye attached to its 5'end and a TAMRA quencher attached to its 3'end.

During PCR, the DNA template is denatured and the two unlabeled primers from each assay anneal to their target sequences. The TaqMan probe anneals to its complementary sequence which is located between the binding sites of the two unlabeled primers. When the probes are intact, the reporter dye signal is quenched. This is caused by the proximity of the quencher dye to the reporter dye. AmpliTaq Gold DNA polymerase amplifies the target and reference sequences with each round of PCR. This DNA polymerase has a 5' nuclease activity that cleaves the probes attached to target and reference sequences. This causes the quencher located on the probe to be separated from the reporter dye increasing the fluorescence emitted by the reporter. Amplification of the target and reference sequences is detected by monitoring the increase in fluorescence of each reporter dye at each PCR cycle.

Appendix 7: TaqMan SNP genotyping assays used for the development of a Real-Time PCR protocol for DNA fingerprinting of clinical samples.

Assay No.	Assay ID	SNP ID	Genotype (VIC/FAM)	Mean Minor Allele Frequency	Gene Symbol	Chromosome	Cytogenetic Band
1	C___1447248_10	rs3768460	G/A	0.4675	RBM15	1	1p13.3a
2	C___2153818_1_	rs3739005	C/T	0.4675	NPAS2	2	2q11.2e
3	C___2068055_10	rs2892830	A/G	0.4625	SYNPO2	4	4q26f
4	C____409917_10	rs9400554	C/T	0.46875	NA	6	6q16.1d
5	C___2568962_10	rs12699207	C/G	0.465	THSD7A	7	7p21.3b
6	C___3039789_10	rs12549400	A/C	0.4625	TRAPPC9	8	8q24.3b
7	C___1365626_10	rs508485	C/T	0.4725	PIWIL4	11	11q21b
8	C___1310376_10	rs7144509	A/G	0.46625	GPR137C;TXNDC16	14	14q22.1c
9	C___2890442_10	rs9788670	C/T	0.47625	VPS13C	15	15q22.2b
10	C____128983_10	rs6114327	C/G	0.4775	CST2	20	20p11.21b
11	C___2444983_1_	rs1543754	C/G	0.47125	KCNJ6	21	21q22.13b
12	C____328358_1_	rs8137254	C/T	0.4875	ZNRF3	22	22q12.1c

Notes: Minor allele frequency refers to the frequency of occurrence of the less common allele (when considering a SNP) in a certain population. The mean minor allele frequency was obtained by calculating the average value from 8 populations: Applied Biosystems African-American, Applied Biosystems Caucasian, Applied Biosystems Chinese, Applied Biosystems Japanese, HapMap YRI (Yoruba in Ibadan, Nigeria), HapMap CEU (Northern and Western European Ancestry in Utah, USA), HapMap CHB (Han Chinese in Beijing, China) and HapMap JPT (Japanese in Tokyo, Japan). 'NA' stands for Not Available.

Appendix 8: Primers designed for detection of PLCζ (H398P, H233L) mutations

Primers	Primer sequences (5' to 3')	Product size (bp)
H398P Outer	F - TGCCAAGACAATACATATCAAAA R - GTTACTTCTGCAAACAACTCAATATC	291
H398P Inner	F - * R - TCTTGGGGATTAAAATTAGAAGAG	226
H398P Mini	F - AGTCCATGAGTTTATTTTTC	21
H233L Outer	F - TTTTGCTAGTGCCCTTGTG R - AGGCTAAGCATTATAGGATTTTGA	240
H233L Inner	F - ATTGACTGCTGGGATGGA R - *	200
H233L Mini	F - AACTGTTATCCAAGCTATAC	21

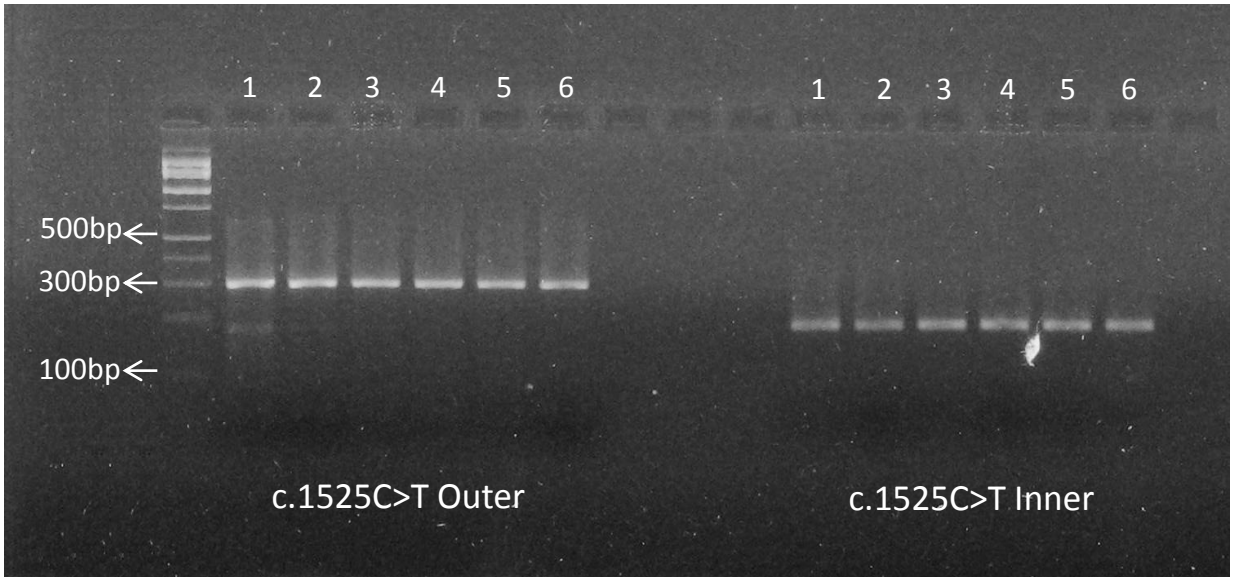
* The outer F or the outer R primer was used in conjunction with the inner primer in order to perform the inner reaction.

Appendix 9: Development of PGD protocol for Tuberous Sclerosis 1 (c.1525C>T). **A)** Design of primers for mutation detection. **B)** Gel electrophoresis of gradient PCR products obtained from amplification of the mutation site (c.1525C>T) with outer and inner primers. **C)** Electropherograms showing results obtained from gradient PCR of linkage markers D9S149 (**i**) and PM4 (**ii**). **D)** Informativity test of linkage markers D9S149 (**i**) and PM4 (**ii**). **E)** Testing developed primers for mutation detection. **F)** Optimisation of multiplex PCR. **G)** Location and genetic distance of linkage markers from *TSC1* gene.

A) Design of primers for mutation detection

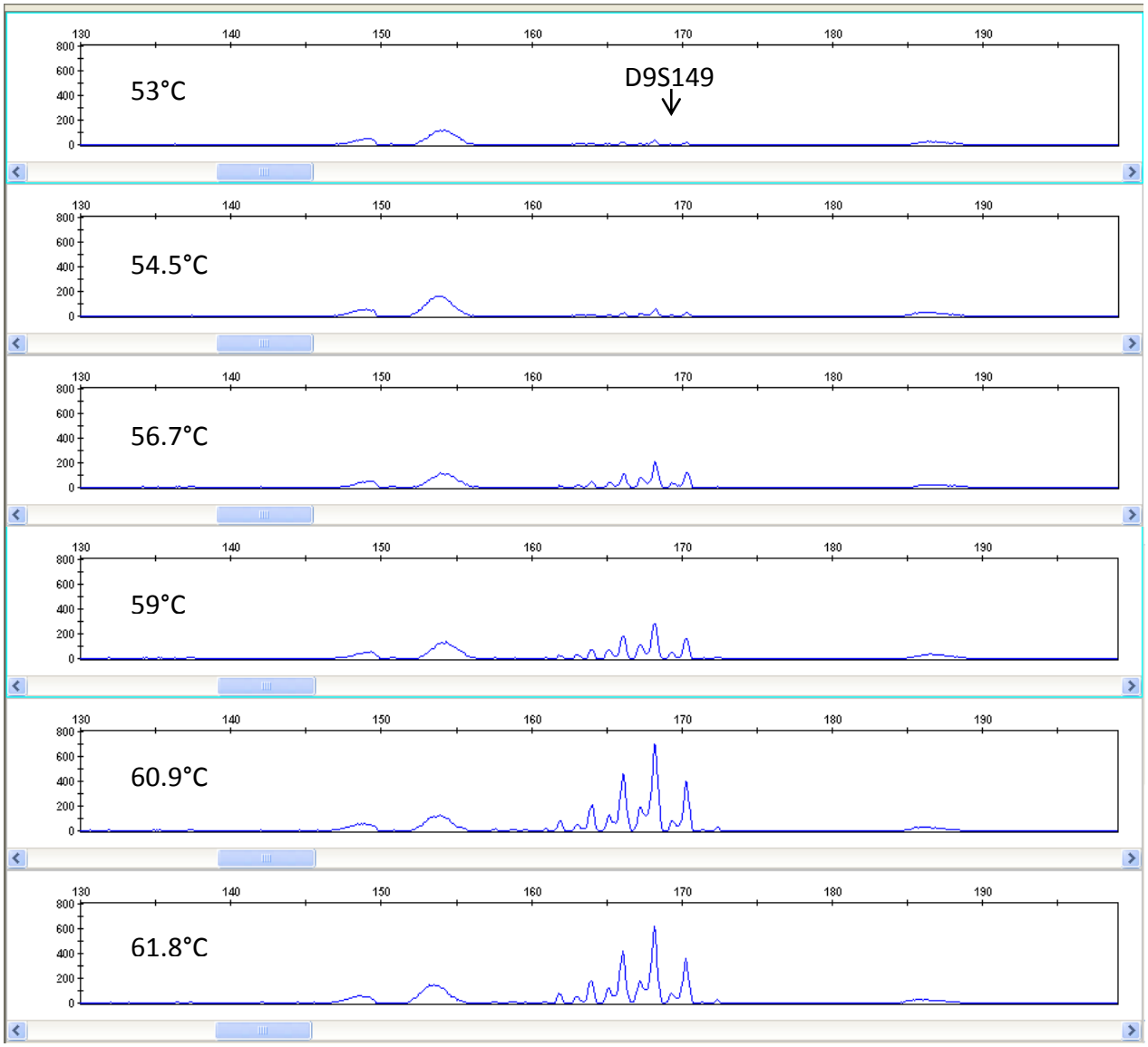
Notes: Image shows part of the genomic sequence of *TSC1* gene (NCBI Reference Sequence: NG_012386.1) that includes the c.1525C>T substitution mutation (in brackets and highlighted in red). Highlighted in different colours are the different area where the designed primers bind - yellow is for outer primers, green is for inner primers and blue for minisequencing primer.

B) Gel electrophoresis of gradient PCR products obtained from amplification of the mutation site (c.1525C>T) with outer and inner primers

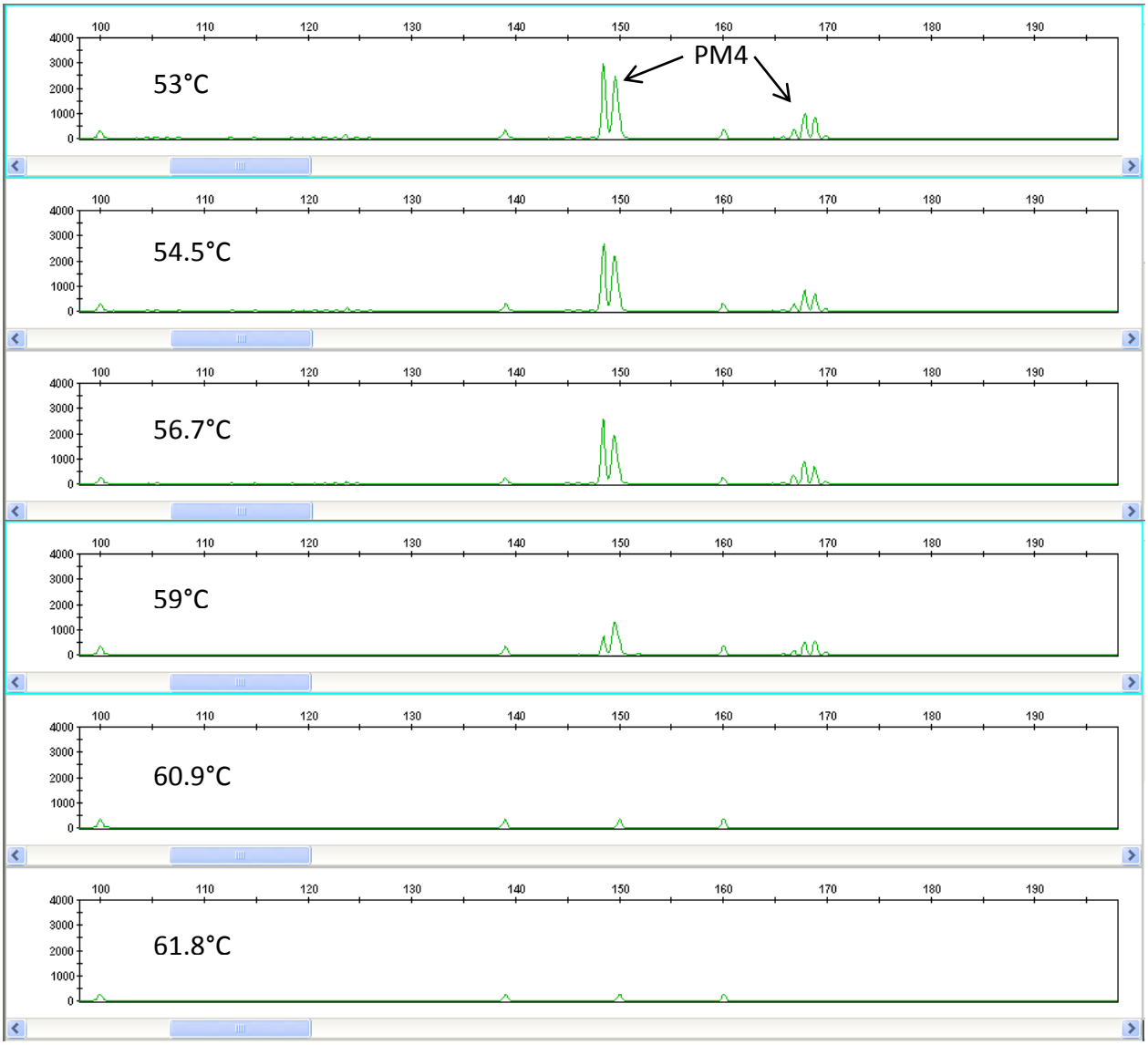


Notes: Each of numbers 1-6 denotes a different PCR annealing temperature (1=53°C, 2=54.5°C, 3=56.7°C, 4=59°C, 5=60.9°C, 61.8°C). As seen in the gel image, the two primer sets (outer and inner) amplified well giving a clear product band of expected size at all temperatures assessed.

Ci) Electropherograms showing results obtained from gradient PCR of linkage marker D9S149

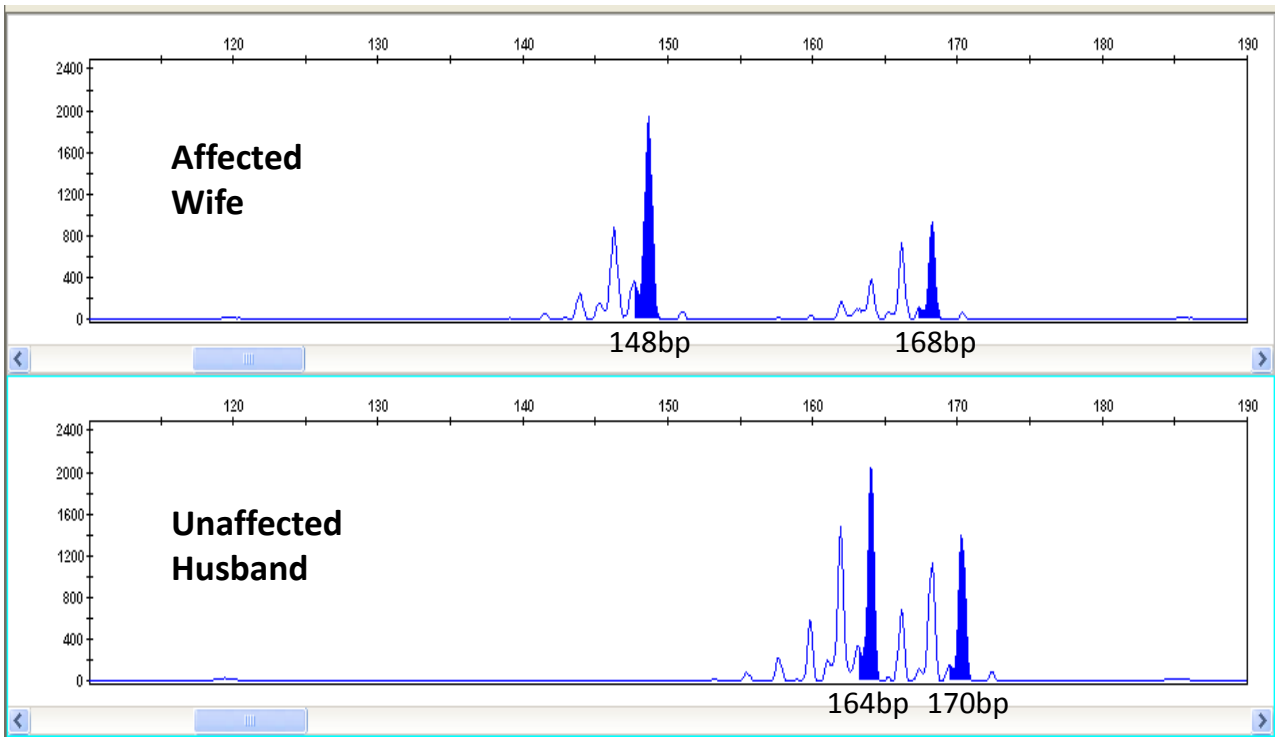


Cii) Electropherograms showing results obtained from gradient PCR of linkage marker PM4

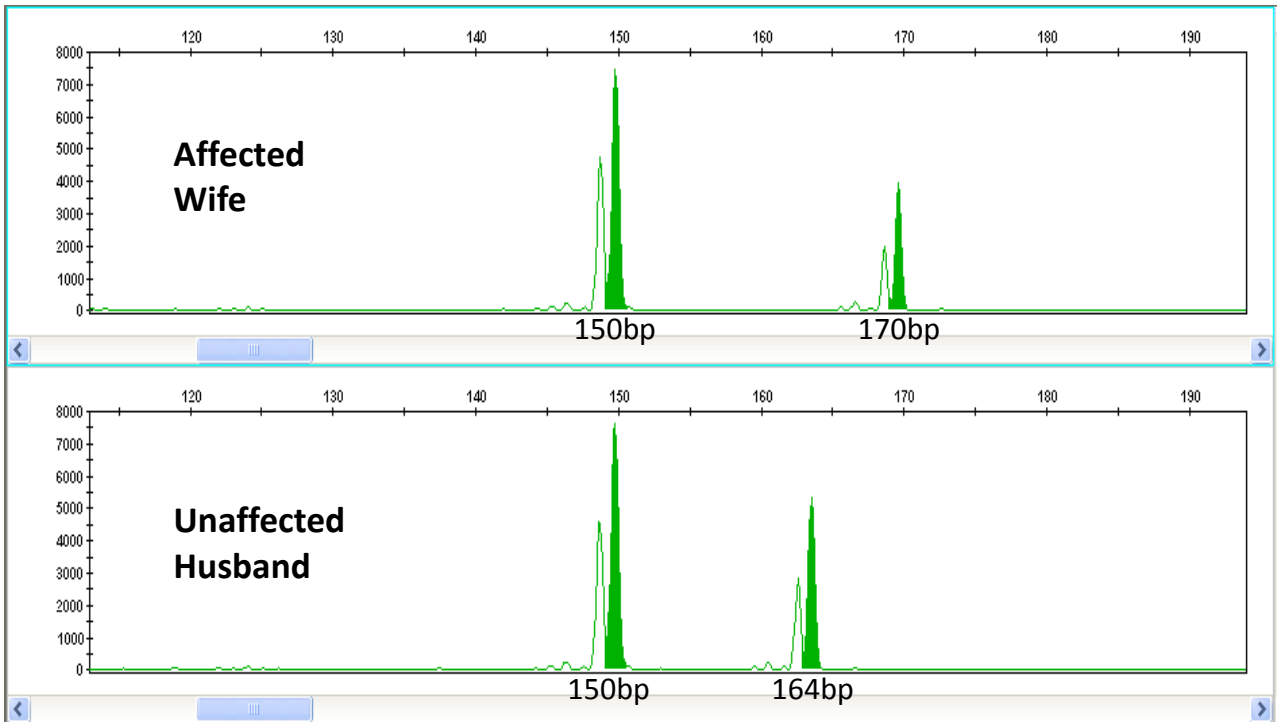


Notes: The gradient PCRs were carried out at an annealing temperature range of: 53-61.8°C. As seen, the temperatures giving detectable amplification for D9S149 were 56.7-61.8°C. The temperatures giving detectable amplification for PM4 were 53-59°C.

Di) Informativity test of linkage marker D9S149

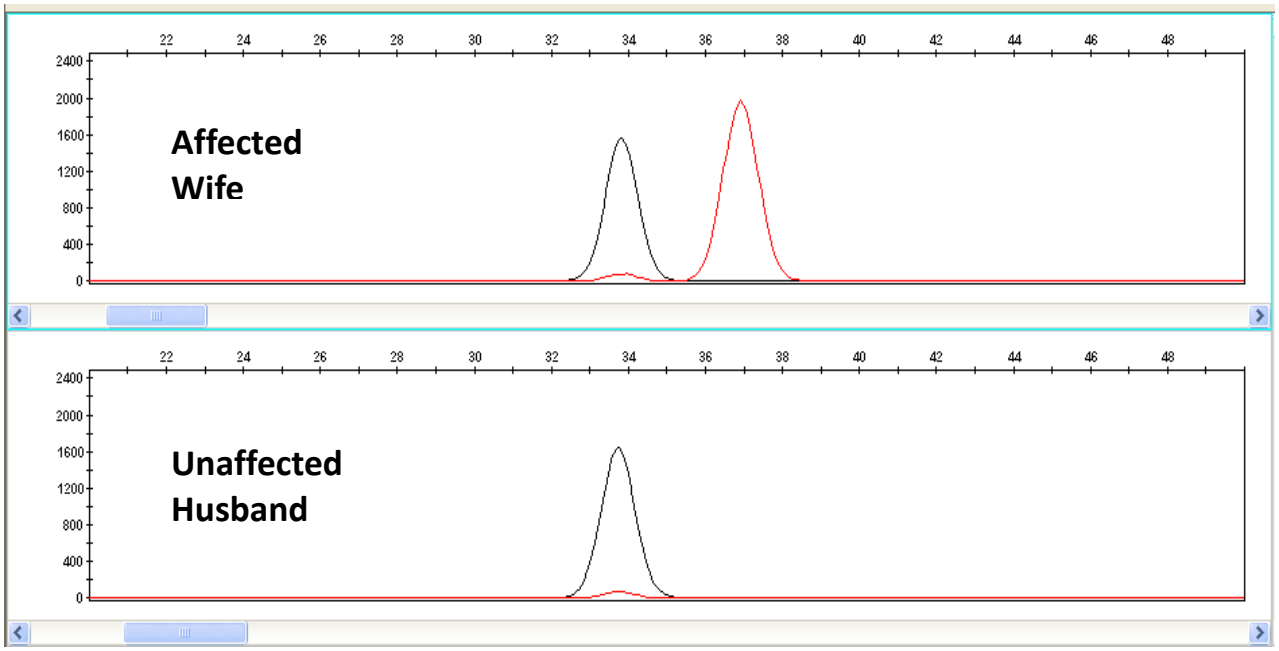


Dii) Informativity test of linkage marker PM4



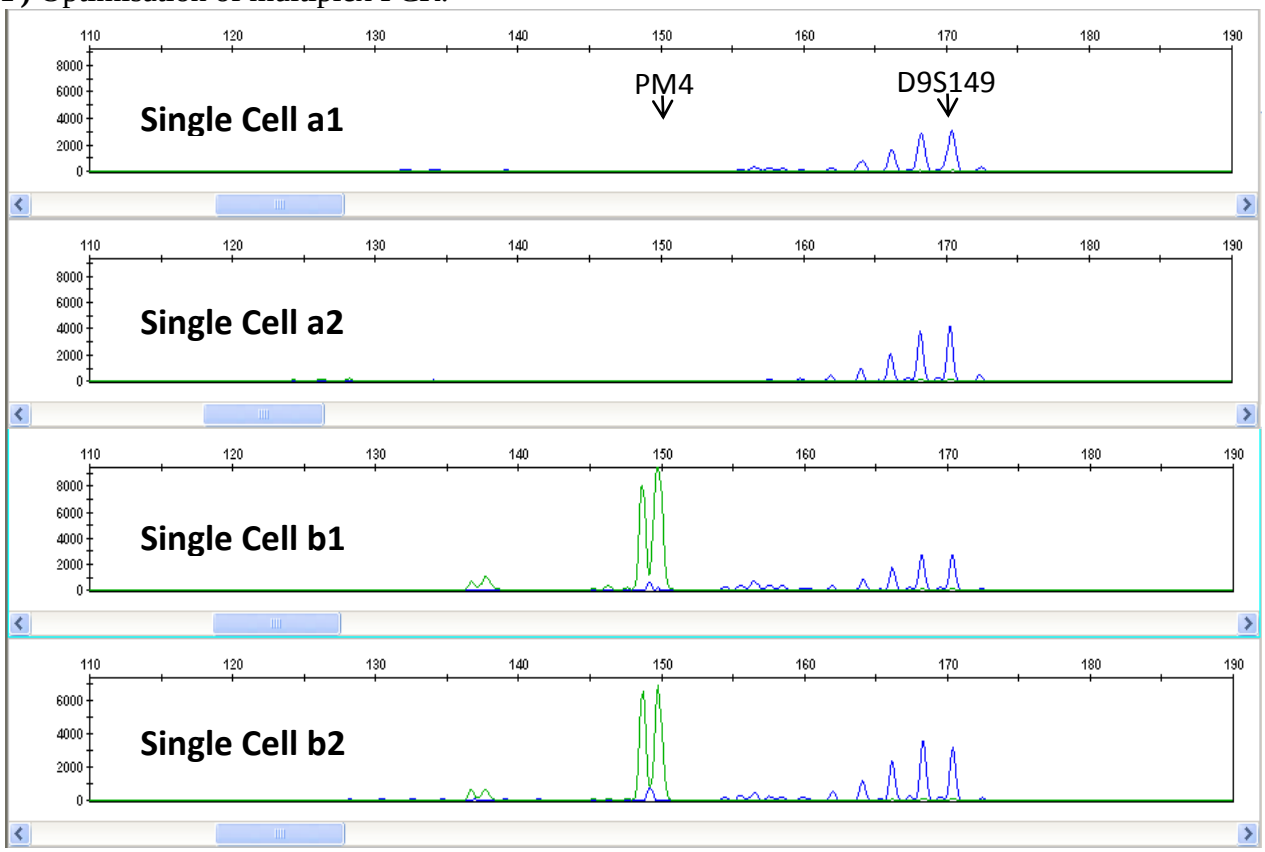
Notes: D9S149 was found to be fully informative since the prospective parents are both heterozygous and do not share any alleles between them. PM4 was found to be semi-informative since the prospective parents share one of the alleles at 150bp. Both markers were considered appropriate for usage in the final protocol.

E) Testing developed primers for mutation detection



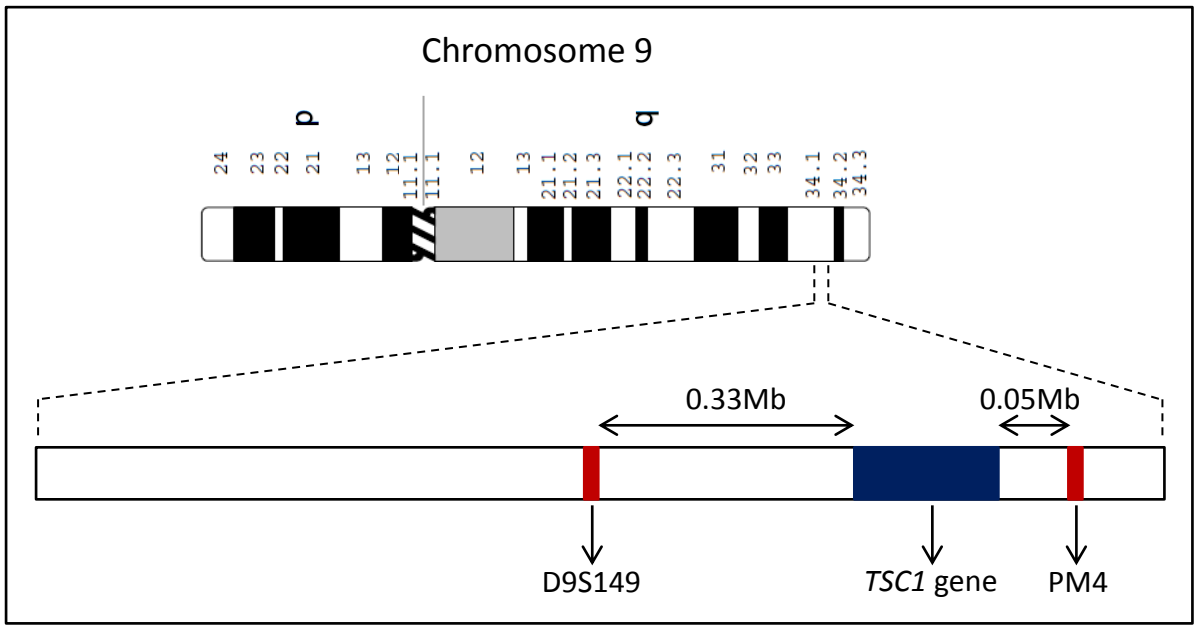
Notes: Amplification of the mutation site and subsequent minisequencing analysis to detect the sequence alteration were found to be working properly, allowing identification of the mutation (c.1525C>T). The black peak represents nucleotide C and the red peak nucleotide T. The female was correctly identified as having both alleles (normal and mutant) while the husband has only the normal allele (nucleotide C - black peak).

F) Optimisation of multiplex PCR.



Notes: Taking into consideration the results obtained from the gradient PCR experiments (shown above) carried out for the loci included in the outer multiplex reaction (c.1525C>T Outer, D9S149, PM4), it was decided to use a PCR annealing temperature of 59°C for development of the multiplex protocol. Before optimisation (single cells a1, a2), linkage marker PM4 was not amplified to detectable levels. After several rounds of optimisation both linkage markers (PM4, D9S149) (single cells b1, b2) and mutation site (data not shown) were amplified at detectable levels and therefore PGD protocol was ready for usage in single cells. Optimisation involved the assessment of different primer concentrations for each of the loci included in the multiplex protocol before determining the optimal ones and obtaining the final protocol. Finally, multiple single lymphocytes isolated from the mutation carrier's blood were subjected to amplification and analysis using the optimised protocol, confirming the single cell detection of the mutation and the specific polymorphic alleles present in the affected individual. This allowed amplification failure and allele dropout rates to be calculated, providing an indication of the likely accuracy of the protocol.

G) Location and genetic distance of linkage markers from *TSC1* gene

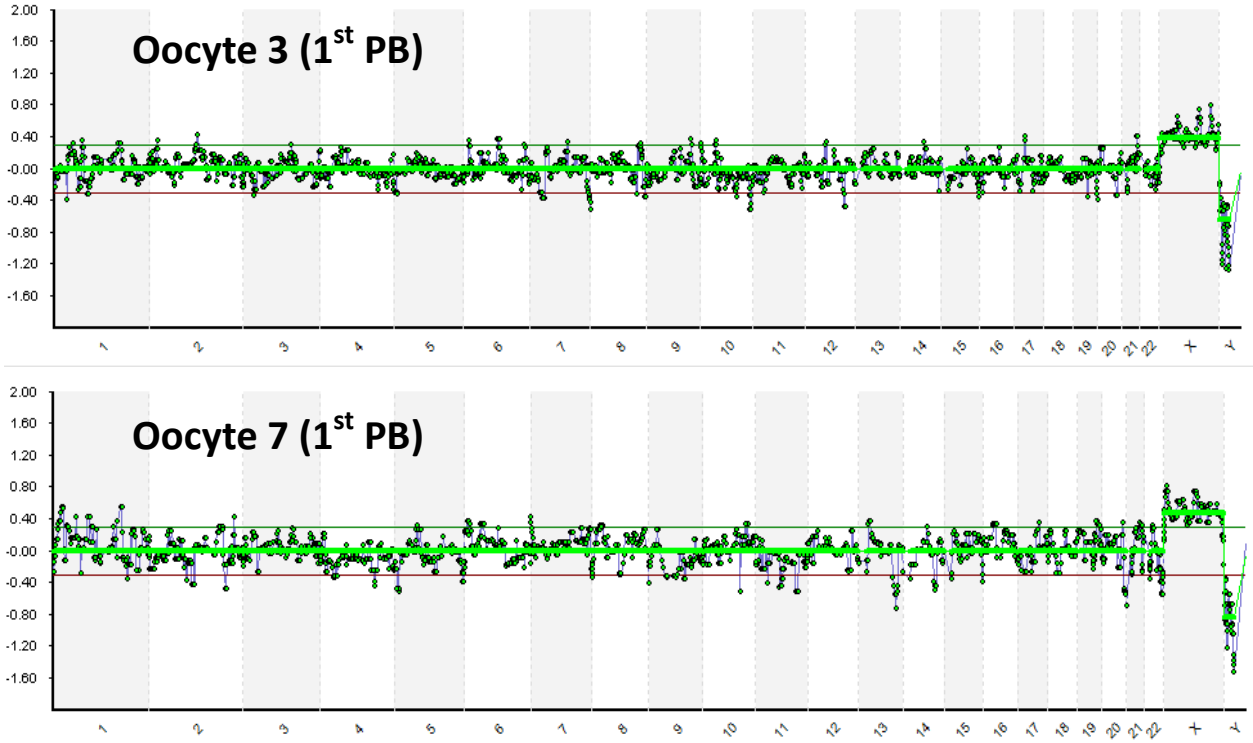


Notes: Linkage markers were selected to be either side of the *TSC1* gene and also, as close as possible to the gene in order to avoid any misdiagnosis from happening due to recombination.

* Part of this figure was adapted from Adler (1994).

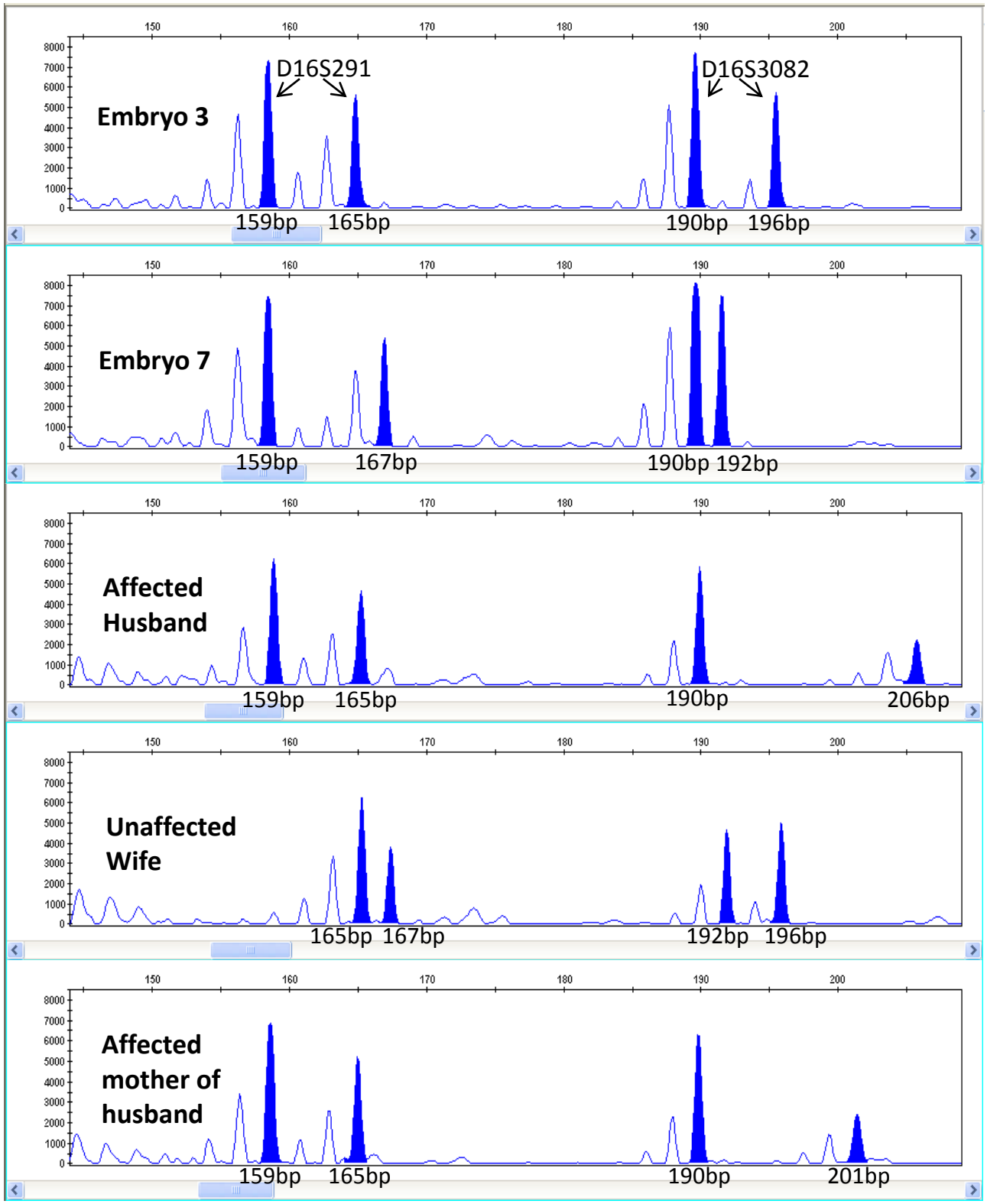
Appendix 10: Performance of PGD for autosomal dominant polycystic kidney disease. **A)** Aneuploidy testing of 1st PBs with 24sure test. **B)** Single gene testing of single blastomeres - results from D16S291 and D16S3082 linkage markers. **C)** Single gene testing of single blastomeres - results from 16PTEL06 and D16S3399 linkage markers. **D)** Pedigree Analysis. **E)** Location and genetic distance of linkage markers from *PKD1* gene.

A) Aneuploidy testing of 1st PBs with 24sure test



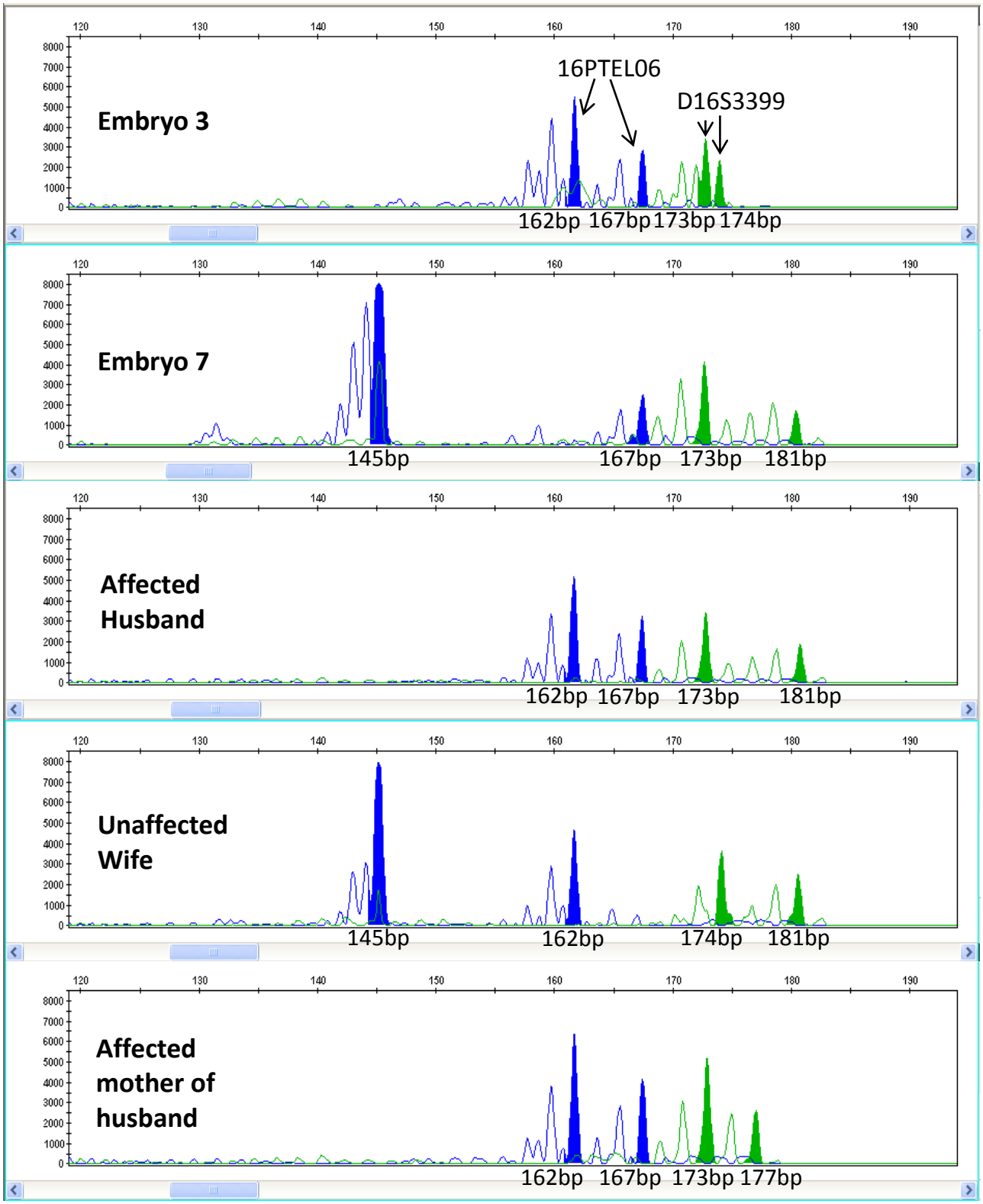
Notes: Image shows representative results from aneuploidy testing of 1st PBs derived from the 5 oocytes that eventually formed the 5 embryos tested in this case. Results from 1st PBs of oocytes 3 and 7 are shown. Both PBs are found to be euploid and have no gains or losses of chromosomal material. The gain of X chromosome and loss of Y seen are expected since the PBs were tested against a male reference DNA. All 5 PBs tested in this case were found to be euploid indicating that the corresponding oocytes were euploid although a chance remained that an error occurred during the 2nd meiotic division.

B) Single gene testing of single blastomeres - results from D16S291 and D16S3082 linkage markers.

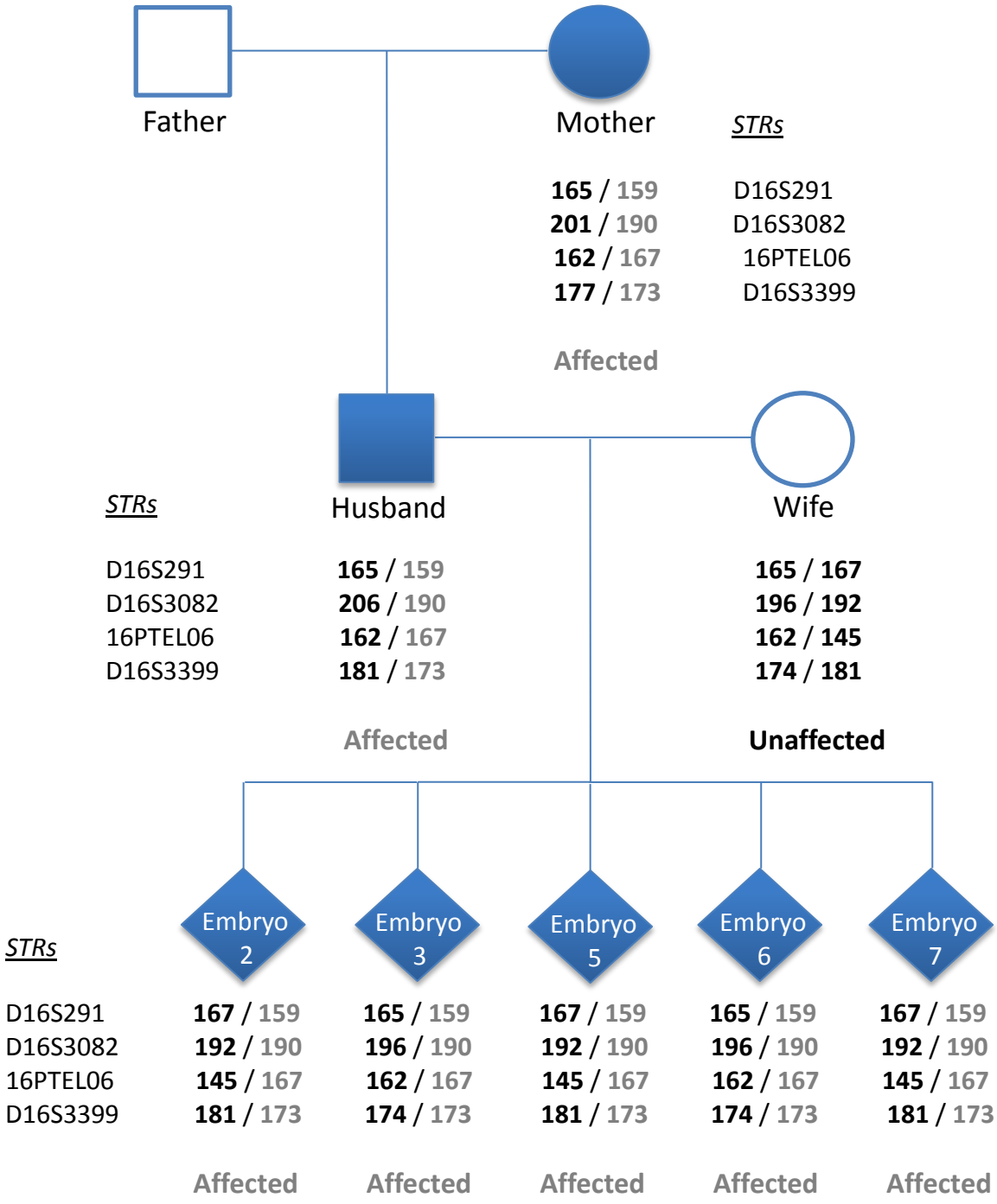


Notes: The specific mutation responsible for the disease was not identified and therefore this case was carried out using linkage analysis alone. DNA from the affected mother of the husband was used to identify which alleles of the linkage markers used in the protocol were inherited from her affected son and therefore identify the alleles linked with the mutation/disease and determine affected embryos (see 'Pedigree Analysis' below).

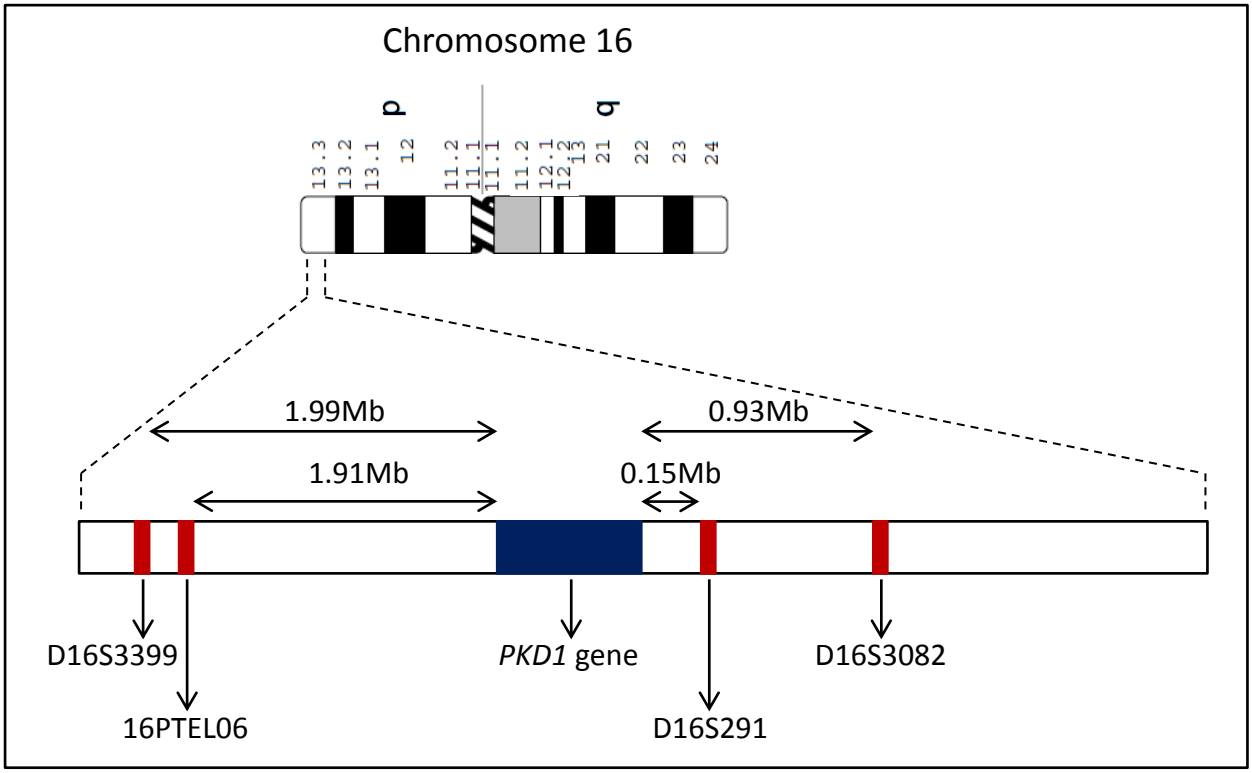
C) Single gene testing of single blastomeres - results from 16PTEL06 and D16S3399 linkage markers.



D) Pedigree Analysis



E) Location and genetic distance of linkage markers from *PKD1* gene



*Part of this figure was adapted from Adler (1994).

List of publications

Articles

- Lavery, S., Abdo, D., Kotrotsou, M., Trew, G., **Konstantinidis, M.** and Wells, D. (2013) Successful live birth following preimplantation genetic diagnosis of phenylketonuria in day 3 embryos by specific mutation analysis and elective single embryo transfer. *Journal of Inherited Metabolic Disease*, **7**, 49-54.
- Kashir, J., **Konstantinidis, M.**, Jones, C., Lemmon, B., Lee, H.C., Hamer, R., Heindryckx, B., Deane, C.M., De Sutter, P., Fissore, R.A., Parrington, J., Wells, D. and Coward, K. (2012) A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCζ) leads to male infertility. *Human Reproduction*, **27** (1), 222-231.
- Kashir, J., **Konstantinidis, M.**, Jones, C., Heindryckx, B., De Sutter, P., Parrington, J., Wells, D. and Coward, K. (2012) Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C zeta (PLCζ) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertility and Sterility*, **98** (2), 423-431.

Conference abstracts

- Wells, D., **Konstantinidis, M.**, Alfarawati, S., Hurd, D. and Fragouli, E. A novel tool allows simultaneous genomic and cytogenetic assessment of oocytes and embryos and yields unique data of scientific and clinical importance. *67th annual meeting of the American Society for Reproductive Medicine (ASRM).* 2011. Orlando, USA. [**2011 SART Prize**].
- Alfarawati, S., Fragouli, E., **Konstantinidis**, M. and Wells, D. Are patients undergoing PGD for chromosome rearrangements at increased risk of

aneuploidy affecting chromosomes unrelated to their rearrangement (interchromosomal effect)? *67th annual meeting of the American Society for Reproductive Medicine (ASRM).* 2011. Orlando, USA.

- Fragouli, E., Alfarawati, S., **Konstantinidis**, M., Jaroudi, S. and Wells, D. The progress of chromosome abnormalities from meiosis to the blastocyst stage. *67th annual meeting of the American Society for Reproductive Medicine (ASRM).* 2011. Orlando, USA.

- **Konstantinidis, M.**, Alfarawati, S., Hurd, D. and Wells, D. A novel tool for the assessment of IVF embryos. *27th annual meeting of the European Society of Human Reproduction and Embryology (ESHRE).* 2011. Stockholm, Sweden.

- **Konstantinidis, M.,** Alfarawati, S., Harton, G., Lavery, S. and Wells, D. Whole genome amplification: clinical implications in preimplantation genetic diagnosis. *Fertility 2011 Conference*. 2011. Dublin, Republic of Ireland.

- Jaroudi, S., **Konstantinidis, M.,** Gutierrez-Mateo, C., Sanchez, J. and Wells, D. Outcomes of over 400 cycles of 'transport' PGD. *Fertility 2011 Conference*. 2011. Dublin, Republic of Ireland.

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Prizes and awards

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