Development of a DNA Microarray for Detection of Aneuploidy in Single Blastomeres

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Table of Contents

Abstract

Comparative Genomic Hybridisation (CGH) using metaphase chromosome spreads to screen all human chromosomes for aneuploidy in preimplantation embryos is hindered by the time required to perform the analysis. It takes at least three days to analyse a single cell using metaphase CGH, resulting in the need for the embryos to be cryopreserved rather than being able to be transferred in the maternal cycle that created them. Array CGH can also detect aneuploidy and requires much shorter hybridisation times. Microarrays manufactured to date are not purported to be able to analyse the very limited amount of genetic material (around 6pg) in a single cell; generally they require a DNA sample (0.5-1.0μg) far in excess of that contained in a single cell.

This thesis describes the development of a novel approach to the manufacture of a DNA microarray for CGH for the detection of aneuploidy in single cells. Human chromosome-specific libraries, which were depleted of repetitive sequences, were spotted on glass slides. Array CGH experiments were conducted on these arrays using either single male and/or single female lymphocytes. For the autosomes, the mean normalized ratios were all close to the expected ratio of 1.0 with overall 97% of the normalized ratios falling within the expected range 0.75-1.25. It was possible to deduce the correct copy number of X chromosomes in 93% of separate array CGH experiments, but the Y chromosome in only 29%. Array CGH was initially performed on a single fibroblast from each of three cell lines containing a specific chromosome aneuploidy (trisomy 13, 15 or 18) and in each case this method was able to obtain a diagnosis based on the fact that the aneuploid chromosomes gave the highest ratios (1.32, 1.27 and 1.27 respectively) with the ratios of all other chromosomes falling within the range 0.75-1.25. Finally, a small number of blastomeres removed from human cleavage-stage

embryos were analysed using this method. Results suggest that some blastomeres had a normal karyotype, whereas others were aneuploid or chaotic. This array CGH approach produces results within 30 hours, making it potentially more suitable for PGD aneuploidy screening than metaphase CGH.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institute and, to the best of my knowledge and my belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Dong Gui Hu

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Publications arising from this thesis

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Patents arising from this thesis

Provisional patent (No. 2003901671), to Hussey N.D. and **Hu D**. (2003) on microarray Comparative Genomic Hybridization

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Dong Gui Hu, Graham Webb and Nicole Hussey (2003). Aneuploidy detection in single cells using DNA array-based comparative genomic hybridization. North Western Adelaide Health Services Research Day, Adelaide, South Australia, 17 October. Abstract 15.

D. Hu, G.C. Webb, R.J. Norman, N.D. Hussey (2002). Detection of trisomies 13 and 18 in single cells using DNA array/CGH. The Fertility Society of Australia 21st Annual Scientific Meeting, Gold Coast, Queensland, Australia, 22-26 October. Page 44.

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Glossary/Abbreviations

21_{vys}	WCP 21q SpectrumOrange (Vysis, USA)
X_{vys}	WCP X SpectrumGreen (Vysis, USA)
ADO	Allele drop-out
AMA	Advanced maternal age
Aneuploidy	Numerical chromosomal abnormalities
Array CGH	DNA microarray/comparative genomic hybridisation
> %B532 + 2 SD	The percentage of feature pixels with intensities more than two standard deviations above the background pixel intensity, at wavelength #1 (532 nm, for Cy3)
> %B635 + 2 SD	The percentage of feature pixels with intensities more than two standard deviations above the background pixel intensity, at wavelength #2 (635 nm, for Cy5)
BAC	Bacterial artificial chromosome
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
°C	Degrees Celsius
CF	Cystic fibrosis
CSL	Chromosome-specific DNA library
DAPI	4,6-diamidino-2-phenyl indole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid

DOP-PCR	Degenerate oligonucleotide-primed PCR
Dia.	The diameter in μm of the feature-indicator
DMD	Duchenne muscular dystrophy
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
F532 % Sat	The percentage of feature pixels at wavelength #2 (for Cy3) that are saturated
F635 % Sat	The percentage of feature pixels at wavelength #1 (for Cy5) that are saturated
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
g	Gram
GAL file	GenePix Array List file
GPR file	GenePix Results format
HEX	Hexachlorofluororescein
HLA	Human Lymphocyte Antigen
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IRS-PCR	Interspersed repetitive sequence
IVF	In vitro fertilisation
JPEG	Joint Photographic Experts Group
kb	Kilobase pairs
LA-PCR	Linker-adaptor PCR
LOH	Loss of heterozygosity
Min	Minute(s)
NaAC	Sodium acetate

PAC	P1-derived artificial chromosome
PB2	Second polar body
PBS	Phosphate-buffered Saline
PCC	Premature chromosome condensation
PCR	Polymerase chain reaction
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
PGD-AS	PGD for aneuploidy screening
PMT	Photomultiplier tube
RIF	Recurrent implantation failure
RM	Recurrent miscarriage
R/T ratio	Fluorescence intensity ratios of reference to test
QL-PCR	Quantitative fluorescence PCR analysis
S:B ratio	Signal:background ratios
SEP	Selectively-Enhanced Primer-extension-preamplification
SDS	Sodium dodecyl sulphate
SKY	Spectral karyotyping
SNR532	The signal-to-noise ratio at wavelength #1 (532 nm, for Cy3), defined by (Mean Foreground 1 minus Mean Background 1)/(Standard deviation of Background 1)
SNR635	The signal-to-noise ratio at wavelength #2 (635 nm, for Cy5), defined by (Mean Foreground 1 minus Mean Background 1)/(Standard deviation of Background 1)
SRY	Sex-determining Y region
SSCP	Single strand conformation polymorphism analysis
SSM	Site specific mutagenesis

STR	Short tandem repeat
TIFF	Tagged Image File Format
T-PCR	Tagged random primers PCR
TR	Texas Red
Tris	Tris(hydroxymethyl)-aminomethane
T/R ratio	Fluorescence intensity ratios of test to reference
μ	Micro (10^{-6})
WCP	Whole chromosome paint
WGA	Whole genome amplification

Chapter 1

Literature Review

1.1 Introduction

Preimplantation genetic diagnosis (PGD) is a very early form of prenatal diagnosis that is performed on human preimplantation embryos created by *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). PGD is based on the assumption that the results from genetic analysis of the biopsied polar bodies or single blastomeres reflect the genetic status of the corresponding embryos. PGD provides an alternative to conventional prenatal diagnosis to avoid pregnancies involving affected offspring by only transferring unaffected embryos. Since the first PGD pregnancies were achieved (Handyside et al. 1990), PGD has been successfully used to diagnose a large number of single-gene defects, chromosomal abnormalities and non disease traits such as HLA type or gender for social reason (Verlinsky et al. 2001b; ESHRE PGD Consortium Steering Committee 2002; Malpani and Modi 2002). It has been estimated that by 2002, more than 4000 PGD cycles had been performed worldwide (Kuliev and Verlinsky 2002).

The two main PGD strategies currently used are the polymerase chain reaction (PCR)-based PGD protocol and the fluorescence *in situ* hybridization (FISH)-based PGD protocol. The former has been carried out for PGD of single-gene defects, mainly Mendelian diseases, and the latter for PGD of chromosomal abnormalities, especially aneuploidy and balanced reciprocal translocations (Handyside et al. 1990; Verlinsky and Evsikov 1999b; Wells 1999). PGD for aneuploidy screening (PGD-AS) has been increasingly performed for IVF patients with a poor prognosis such as advanced maternal age (>37 years old) (Munne et al. 1995; Kahraman et al.

2000), repeated implantation failure (Pellicer et al. 1999; Kahraman et al. 2000), and for couples with recurrent miscarriage (Pellicer et al. 1999). These studies indicated that PGD for aneuploidy screening not only reduced the rates of miscarriage and trisomic liveborns but also increased the rates of implantation and healthy delivery of babies for the some groups of patients with a poor IVF prognosis (Bahce et al. 1999; Gianaroli et al. 1999).

Currently, almost all the successful PGD cycles for chromosomal abnormalities have been achieved by FISH analysis. However, only five to nine different chromosomes per embryo are generally analysed by FISH (Munne et al. 1998b; Gianaroli et al. 1999; Munne et al. 2000b). Many of these abnormalities, such as monosomies and nullisomies, were never or rarely seen in clinical abortuses or newborns (Voullaire et al. 2000; Wells and Delhanty 2000). Chromosomal abnormalities found in these studies involved almost every one of the 24 human chromosomes. Obviously, FISH is not capable of detecting many of these abnormalities. Comparative genomic hybridization (CGH) (Voullaire et al. 1999; Wells et al. 1999) and interphase conversion (Verlinsky and Evsikov 1999b; Willadsen et al. 1999) can detect all of these potential aneuploidies. However, the techniques have not been widely used for clinical PGD applications due to either a long experimental duration of CGH or difficulties in obtaining adequate analysable metaphase preparations for interphase conversion.

New PGD methods are needed to increase the number of chromosomes analysed per embryo. Microarray-based comparative genomic hybridization (array CGH) may be the most promising one. Array CGH performs CGH analysis on individual probes spotted on glass slides instead of metaphase chromosomes and has proved to be reliable for aneuploidy detection using genomic DNA samples (Solinas-Toldo et al. 1997; Pinkel et al. 1998; Snijders et al. 2001). Hybridization to microarrays for CGH takes about 20 hours (overnight) compared to that of 48-72 hours

required by metaphase CGH (Pinkel et al. 1998; Bruder et al. 2001; Takeo et al. 2001). Such a short duration makes array CGH attractive for PGD aneuploidy screening. However, the limitation of array CGH is that it requires large amounts of sample DNA (normally 0.5-1.0μg) (Solinas-Toldo et al. 1997; Veltman et al. 2002), thereby making it impossible for single-cell analysis, as required for PGD. This problem might be solved as has been done for single cell metaphase CGH by the use of DOP-PCR (degenerate oligonucleotide-primed PCR) to amplify the DNA of a single cell prior to labelling and hybridisation (Voullaire et al. 1999; Wells et al. 1999). Array CGH is normally conducted using DNA arrays composed of PAC (P1-derived artificial chromosome) and BAC (bacterial artificial chromosome) clones (Snijders et al. 2001). This differs from the approach developed in this study where DNA libraries depleted of repetitive sequences were spotted on slides for array CGH analysis.

This study aimed to develop an array CGH approach that would be reliable for detecting aneuploidy in single cells and further to test the feasibility of using this array CGH approach in PGD for anueploidy screening. The following discussion is a review of the literature on topics relevant to this thesis: biopsy procedures for PGD, PGD of single-gene defects, PGD of chromosomal abnormalities, comparative genomic hybridization, array CGH, and human chromosome-specific DNA libraries (CSLs).

1.2 Biopsy procedures for PGD

PGD testing relies on obtaining genetic material from the egg or embryo. The by-products of maternal meiosis I and II are expelled as discrete cellular bodies into the space between the shell of the egg (the zona pellucida) and the oocyte and are called the first and second polar bodies respectively. Multiple oocytes are generated through ovarian stimulation protocols, and generally harvested via transvaginal ultrasound guided aspiration. The oocytes are surrounded by a large number of cumulus cells, which are tiny in comparison to the large oocyte. They are still attached to the oocyte and provide the oocyte with the correct environment to develop. Embryos are created using standard insemination with 50,000 sperm via *in vitro* fertilization (IVF) or a single sperm injected directly into the oocyte via Intracytoplasmic Sperm Injection (ICSI). The remnants of female meiosis I, the first polar bodies, provide genetic information of Meiosis I and can be removed prior to fertilisation. The second polar body, which contains information about Meiosis II, is not produced until after fertilisation. Unlike meiosis in the male, female meiosis is not completed until after fertilisation.

The earliest stage that embryonic material can be removed is considered to be day 3 post fertilisation. The human preimplantation embryo has a mass of around 6-10 cells (blastomeres) and the removal of one or occasionally two blastomeres is termed embryo biopsy. Later on day 5 post fertilisation another opportunity arrises to obtain embryonic material this time at the blastocyst stage from the trophectoderm as it is allowed to herniate through a hole in the zona pellucida. More cells are removed at this stage generally 5-20 cells. In this section, the three main procedures to obtain genetic material for PGD testing are reviewed and their advantages and disadvantages are discussed.

1.2.1 Polar body biopsy

The first polar body can generally be removed about 4 hours after oocyte retrieval whereas the second polar body can only be obtained after fertilization (2-3 hours for IVF and 1-8h for ICSI) (Strom et al. 1998a; Gianaroli 2000; Lee and Munne 2000; Strom et al. 2000). Both polar bodies can be biopsied simultaneously from fertilized zygotes (Rechitsky et al. 1999; Verlinsky et al. 1997a; Verlinsky et al. 1997b) but this approach is recommended for use of PGD for chromosomal abnormalities only as it can be difficult to distinguish which is the first and which is the second thereby losing valuable information for the diagnosis of specific genetic defects (Verlinsky and Kuliev 2000).

The removal of the polar bodies may not result in any adverse effects on later embryo development. This assumption was demonstrated by one clinical study where no specific defects and no significant decrease in birth length and live weight were found among a total of 109 infants derived from PGD following polar body diagnosis (Strom et al. 2000). Both polar bodies can be available for analysis within 24 hours after oocyte retrieval, thus offering at least two more days for subsequent genetic analysis compared to cleavage-stage biopsy. Consequently, the use of time-consuming methods for genetic analysis such as metaphase CGH in PGD becomes possible if polar bodies are used (Wells et al. 2002). After polar body biopsy spare embryos can be frozen for transfer in later natural menstrual cycles (Lee and Munne 2000).

The main disadvantage of polar body diagnosis (Strom et al. 2000) is that only maternally inherited genetic defects can be analysed and therefore with respect to the embryo formed only a partial genetic diagnosis can be made. For specific genetic defects blastomere analysis is either essential (eg for a male carrying Huntington disease) or preferable (eg for cystic fibrosis). The

analysis of polar bodies for recessive disorders such as cystic fibrosis leads to an unfavourable situation in that only oocytes (and the resultant embryos) shown not to carry the mutation are considered for transfer and all those carrying the mutation are discarded. This is a disadvantage, as half of the oocytes discarded would have been fertilised by sperm not carrying the mutation and therefore were healthy carriers. Furthermore this approach does not allow the preferential transfer of non carrier embryos as half of the oocytes free of the mutation are fertilised by sperm carrying the mutation resulting in carriers. Nevertheless some centres prefer polar body biopsy as they feel it is less invasive. However, for many couples the discarding of healthy carrier embryos is either ethically unacceptable or in practical terms reduces their chance at pregnancy to unacceptably low levels. In contrast blastomere biopsy allows the detection of affected embryos (not oocytes) to be discarded and therefore all carrier embryos are potentially identified and available for transfer. Similarly the disadvantage of using polar bodies for aneuploidy screening is that chromosomal malsegregations occurring in maternal meiosis II (in the case of first polar body analysis) and postzygotic divisions, and any paternally derived meiotic errors will be undetected (Gianaroli 2000; Lee and Munne 2000).

1.2.2 Cleavage-stage biopsy

Cleavage-stage biopsy involves the removal of one or two blastomeres from a cleavage-stage embryo normally containing at least 6 to 8 cells. In this approach, the first step is to make a hole in the zona pellucida by use of either acid Tyrode's solution, partial zona dissection, or laser technology. The second step is to remove one blastomere through this hole using an aspirating pipette (Handyside et al. 1990; Chen et al. 1998; Inzunza et al. 1998; Gianaroli 2000). Embryos start to compact on day 3 and thus decompaction using Ca^{++}/Mg^{++}-free medium to disrupt the cell to cell junctions will facilitate the biopsy procedure (Grifo et al. 1990; Santalo et al. 1996;

Kahraman et al. 2000). Studies have shown that cleavage-stage biopsy does not obviously affect embryo developmental potential (Hardy et al. 1990; Holding et al. 1993). Surplus embryos can be frozen before or after biopsy but even good quality embryos have a 70% chance at surviving the freeze/ thaw procedure. As a consequence, many PGD centres have reported a very low IVF implantation and pregnancy rate following the transfer of biopsied embryos that have been frozen (Joris et al. 1999; Magli et al. 1999; Lee and Munne 2000; ESHRE PGD Consortium Steering Committee 2002; Frydman et al. 2003).

Cleavage-stage biopsy is suitable for detecting genetic defects of maternal, paternal, and postzygotical origins. One problem of this approach is that the biopsied blastomeres may not be representative of the rest of the corresponding embryo. This is termed mosaicism. Another problem is the existence of anucleated blastomeres in human preimplantation embryos (Hardy and Handyside 1993; Cui and Matthews 1996). If such a blastomere is biopsied there can be no resulting diagnosis of the corresponding embryo. A false or misleading genetic result can result in a misdiagnosis. Some centres try to reduce this problem by removing and analysing two separate blastomeres for each embryo. Their rationale is that mosaic embryos will be identified by discordant results between the two blastomeres (Van de Velde et al. 2000; Lewis et al. 2001). Although this approach is sound for specific genetic disorders, the resultant reduction in implantation potential of embryos diagnosed purely to increase implantation rate by detecting chromosomal abnormalities is unlikely to confer a net benefit to the majority of patients.

A disadvantage of cleavage stage biopsy is the reduction in time to analyse the blastomere, however with the application of extended culture to IVF it is now possible to transfer embryos on day 5 post fertilisation giving centres at least 2 days to obtain the genetic information needed (Pehlivan et al. 2003; Rubio et al. 2003).

1.2.3 Blastocyst biopsy

Blastocyst biopsy removes a few cells from the embryo at the blastocyst stage, and is normally performed on day 5 after fertilization. Blastocysts normally contain a few hundred cells already differentiated into either trophectoderm cells (destined to become the placenta) or inner cell mass (ICM) which goes on to form the fetus proper. The advantage of this approach is that numerous cells (10-30) can be biopsied from the trophectoderm of the blastocyst (Dokras et al. 1990; Veiga et al. 1997). One disadvantage for this approach in clinical PGD applications is that only half of the embryos or fewer reach the blastocyst stage *in vitro* (Hardy et al. 1989). The recent application of sequential media allows more embryos to be cultured up to the blastocyst stage *in vitro* (Gardner 1998; Gardner 2000). Even so, only a limited number of successful clinical PGD cycles have been so far performed by blastocyst biopsy (Plachot et al. 2000; Menezo et al. 2001). A second disadvantage of this technique for chromosomal disorders is that trophectoderm biopsies may carry some chromosomal abnormalities that are not present in the fetus proper due to mosaicism (Ruangvutilert et al. 2000b).

1.3 PGD tests for single-gene defects

1.3.1 Single-gene defects detectable by PGD

PGD for single-gene defects initially focused on Mendelian inherited diseases, including classical autosomal dominant disorders such as Marfan syndrome (Harton et al. 1996), autosomal recessive diseases such as cystic fibrosis (Ao et al. 1996), and X-linked diseases such as Duchenne muscular dystrophy (DMD) (Liu et al. 1995). With the accuracy of PGD for single-gene defects being greatly increased during the last few years, many more classical Mendelian inherited diseases have been added to the list available for PGD testing (Table 1.1) including alloimmune thrombocytopenia (Van den Veyver et al. 1994), Rhesus haemolytic disease (Avner et al. 1996), cancer predispositions (Rechitsky et al. 2002) and late-onset disorders with genetic predisposition. A significant but yet surprisingly controversial diagnosis was carried out for HLA matching to an existing sibling affected with Fanconi anaemia in the hope that this affected child might obtain stem cell donation from a child born following PGD diagnosis(Verlinsky et al. 2001b). In addition, PGD is now being used for non-disease diagnoses such as HLA matching without concomitant disease testing and social sex selection (Malpani and Modi 2002).

1.3.2 Single-cell PCR for the PGD of single-gene defects

Using PCR, Handyside and colleagues successfully amplified one Y-specific locus from single male blastomeres removed from preimplantation embryos at risk for X-linked diseases. This pioneering work allowed them to obtain the first PGD monitored female pregnancy worldwide in 1990 (Handyside et al. 1990). Although gender determination is more often carried out by FISH, PCR is still the best way to detect specific genetic defects such as single nucleotide

changes, small insertions, deletions or repeat number differences. Therefore where couples wish to be able to preferentially transfer non-carrier females PCR methodology is still the only option.

Much improvement has been made during the last decade to increase the efficacy of single-cell PCR. Firstly, the sensitivity of single-cell PCR was increased by nested PCR (Li HH 1988; Holding and Monk 1989; Handyside 1990), and recently further improved by more sensitive detection systems such as fluorescent PCR (Findlay et al. 1996; Goossens et al. 2000). The biggest advantage of fluorescent PCR is its ability to detect more extreme preferential amplification compared to less sensitive means such as ethidium bromide/agarose gel electrophoresis. In contrast to nested PCR, fluorescent PCR has the advantage of requiring a lesser number of amplification cycles, making a second round unnecessary and thus the whole procedure quicker and less prone to contamination. Fluorescent PCR is also capable of precise fragment sizing, thus facilitating the linkage analysis of polymorphic repetitive sequences commonly performed in PGD of single-gene defects (Findlay et al. 1996; Findlay et al. 1999). The accuracy of PGD for single-gene defects was greatly increased by the use of multiplex PCR which allows the diagnosis of more than one locus such as linked polymorphic markers (Ray et al. 2001; Hussey et al. 2002). Finally, with the application of whole genome amplification (WGA) to single blastomeres, much more genetic information of embryos can be potentially obtained via the analysis of not just specific genetic loci but also chromosomal content (Wells et al. 1999). It is perhaps not exaggerating to suggest that further development in this direction will finally lead to "designer babies" in the future.

There are two stages for PGD of single-gene defects. The first is to amplify the target sequences using PCR, the second is to analyse the amplicons to determine the genetic information contained within them.

1.3.2.1 Allele drop-out (ADO) and preferential amplification

One very important difference between PGD analysis and conventional prenatal diagnosis is the phenomenon of Allele Drop-Out (ADO) (Thornhill et al. 2001). An understanding of ADO is critical in determining what sort of accuracy a particular molecular test has. Allele drop-out (ADO) is defined as the failure in single-cell PCR to detectably amplify one of the two alleles in a heterozygous locus. Another phenomenon similar to ADO is preferential amplification, in which the two alleles of one locus are both successfully amplified but one product is severely under-represented compared to the other. The phenomenon of ADO can presumably result in the failure to amplify a product from a hemizygous locus where just one allele is present in an individual. The cause of ADO could be double stranded breaks in the DNA, incomplete lysis, or could be due to mosaicism where the second chromosome is actually not even present in the single cell. Lymphocytes have very low levels of ADO around 1-2 % whereas blastomeres from embryos have much higher levels around 15% and poorer quality embryos are more likely to display ADO (Hussey et al. 2002). Preferential amplification is due to one allele lagging in the amplification stage such that no amplification takes place for a number of cycles, thus it is under-represented in the final mix. Again incomplete lysis and poorly optimised PCR conditions can contribute to this (Thornhill et al. 2001). If a low sensitivity mutation detections system is used then some preferential amplification will be missed and classified as ADO (Findlay et al. 1996).

1.3.2.2 Strategies of PGD for single-gene defects

The strategy used for designing single cell PCR protocols depends on the type of disorder (dominant/recessive, autosomal/sex linked) and if mutations are known or unknown. The

acceptability of any PCR-based PGD test will depend on the couples' wishes: what rate of recombination risk is acceptable to them, whether they are happy to transfer carrier embryos or not, and what sort of level of accuracy they need to make the inconvenience and expense of PGD acceptable.

Dominant disorders require a different type of analysis compared to conventional prenatal diagnosis where the absence of the mutation is sufficient to say the fetus is healthy. In single cell PCR due to the fact that any allele including the disease-carrying allele, can fail to be amplified (ADO) we must prove that the embryo has inherited the non-disease carrying allele from the affected parent. This necessitates using at least one linked polymorphic marker for which the couple are preferably completely informative. In comparison, for recessive disorders it is usually sufficient to show that the embryo has inherited at least one normal copy of the gene.

If both the mother and father carry different mutations in the same gene, but too far apart to be included in the same PCR product, then an analysis of the two individual mutations is not sufficient to eliminate a misdiagnosis due to ADO. This is because a double ADO event will give normal PCR products in both reactions but the embryo could still carry two mutations. In this case as well as in the situation where one or both mutations are unknown linkage must be used to obtain the highest accuracy. If a molecular test cannot be devised to detect ADO events in these scenarios (eg if an informative marker cannot be found), then the couple need to be well informed of the risk.

1.3.2.3 Genetic analysis after single-cell PCR of the biopsy sample

If the embryo transfer must take place on day 3 or 4 then only 24-36 hours for the whole PGD diagnosis procedure is available and only quick procedures can be used. If extended culture with transfer on day 5 or 6 is available then more lengthy procedures can be employed. A wide variety of methods for mutation detection have been published, including: heteroduplex analysis (Handyside et al. 1992; Gibbons et al. 1995b), site specific mutagenesis (SSM) (Strom et al. 1998b; Sermon et al. 2000), restriction enzyme digestion (Strom et al. 1998a; Kuliev et al. 1999; Ray et al. 1999; Xu et al. 1999), denaturing gradient gel electrophoresis (DGGE) (Kanavakis et al. 1999), single strand conformation polymorphism analysis (SSCP) (Sutterlin et al. 1999), allele specific amplification (Wells and Sherlock 1998), sequencing amplicons (Hussey et al. 2002), and fluorescent PCR (Findlay et al. 1996; Goossens et al. 2000). For PGD of a specific single-gene defect, however, the method adopted depends on the characteristics of the mutation studied. Almost all methods listed above are simple so that embryo transfer can take place on day 3 or 4 of embryo development, compatible with the current IVF practice.

1.3.2.4 Contamination of single-cell PCR

Contamination of the PCR reaction by either intrinsic factors such as cumulus cells, polar bodies, sperm, serum DNA or from extrinsic factors such as cells from staff members or PCR products lying around especially from previous PCRs is one of the most common reasons why a well designed PGD test can give a misdiagnosis. Sperm contamination can be eliminated by the use of ICSI (single sperm injection) as the sperm, which failed to fertilise the oocyte, are not attached to the zona pellucida. Maternal cumulus cells can be carefully removed from the oocyte and this is usually done prior to fertilisation. Sperm contamination can be avoided by use of

ICSI, which is now increasingly applied in PGD of single-gene defects (ESHRE PGD Consortium Steering Committee 2002). Contamination of previously amplified PCR products can be avoided by separation of the area for single-cell PCR from that for analysing PCR products.

Strategies for identifying contamination in PGD of single-gene defects should be used. These include analysis of two independent blastomeres per embryo and testing the medium surrounding the biopsied embryo or the washing buffer for negative controls. Linkage analysis by multiplex-PCR can also be used and it can be an effective means of tracing the source of a contamination (De Vos et al. 1998; Harper and Bui 2002).

1.3.2.5 Amplification failure of single-cell PCR

Amplification failure in single-cell PCR is defined as the failure to amplify the target sequence in a single cell, thereby reducing the number of embryos suitable for transfer. PCR failure can occur in many different ways. Firstly, PCR may fail if the single cell is not completely lysed and/or PCR protocols are not completely optimized (Gibbons et al. 1995a; Ray et al. 1998). Secondly, PCR amplification can fail if the target DNA template does not exist in the PCR tube due to the loss of the biopsied cell during the transfer procedure or its nucleus because of cell lysis during the biopsy procedure (Inzunza et al. 1998; Kahraman et al. 2000). Finally, amplification can also fail simply because of the tested cell is anucleated (Dreesen et al. 1998; Goossens et al. 2000).

1.3.3 Misdiagnoses in PGD for single-gene defects

Misdiagnoses in PGD of single-gene defects have been reported and can be classified into two subgroups: false positive and false negative misdiagnosis (Grifo et al. 1994; Lissens and Sermon 1997; Kanavakis et al. 1999; Kuliev et al. 1999). A false positive misdiagnosis, in which a normal embryo is misdiagnosed as affected, results in no clinical consequences but reduces the number of embryos suitable for transfer. By contrast, a false negative misdiagnosis, in which an abnormal embryo is misinterpreted to be normal, leads to an affected pregnancy or offspring. PGD misdiagnoses can be caused by ADO (Section 1.3.2.1) or contamination (Section 1.3.2.4) of the single-cell PCR reaction. Following PGD, conventional prenatal diagnosis via chorionic villus sampling or amniocentesis is recommended by all PGD centres to couples who are prepared to terminate an affected pregnancy (Lissens and Sermon 1997; ESHRE PGD Consortium Steering Committee 2002).

Table 1.1 Single-gene defects detectable by PGD (Current to June 2003)

Single-gene defect	Reference
Autosomal dominant disease	
Alzheimer disease caused by V717L mutation	(Verlinsky et al. 2002b)
Central core disease	(ESHRE PGD Consortium Steering Committee 2002)
Charcot-Marie-Tooth disease type 1A (CMT1A)	(De Vos et al. 1998; Lofgren et al. 1999)
Crouzon syndrome	(Abou-Sleiman et al. 2002)
Familial amyloidotic polyneuropathy (FAP)	(Carvalho et al. 2001)
Huntington's disease	(Sermon et al. 2002; Stern et al. 2002)
Marfan syndrome	(Harton et al. 1996; Sermon et al. 1999a; Loeys et al. 2002)
Myotonic dystrophy	(Sermon et al. 1998; Harper et al. 2002)
Osteogensis imperfecta type I and type IV	(De Vos et al. 2000)
Retinitis pigmentosum	(Strom et al. 1998b)
Stickler syndrome	(ESHRE PGD Consortium Steering Committee 2002)
Tuberous sclerosis	(ESHRE PGD Consortium Steering Committee 2002)
Autosomal recessive disease	
Ataxia Telangiectasia	(Hellani et al. 2002)
β-thalassemia	(Kanavakis et al. 1999; Kuliev et al. 1999; Hussey et al. 2002)
Congenital adrenal hyperlasia(CAH)	(Van de Velde et al. 1999)
Cystic fibrosis(CF)	(Handyside et al. 1992; Harper et al. 2002)
Epidermolysis bullosa	(ESHRE PGD Consortium Steering Committee 2002)
Fanconi anaemia	(Verlinsky et al. 2001b)
Gaucher's disease	(ESHRE PGD Consortium Steering Committee 2002)
Herlitz junction epidermolysis bullosa	(Cserhalmi-Friedman et al. 2000)
Hyperinsulinaemic hypoglycaemia PHH1	(ESHRE PGD Consortium Steering Committee 2002)
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	(Verlinsky et al. 2001c)
Medium- chain acyl-CoA dehydrogenase (MCAD) deficiency	(Sermon et al. 2000)
Sickle Cell Anemia	(Xu et al. 1999; Chamayou et al. 2002)
Spinal muscular atrophy	(Dreesen et al. 1998; Daniels et al. 2001)
Tay-Sachs disease (TSD)	(Gibbons et al. 1995b)
Von Hippel-Lindau syndrome (VHL)	(Rechitsky et al. 2002)
X-linked disease	
Agammaglobulinaemia	(ESHRE PGD Consortium Steering Committee 2002)
Alport syndrome	(ESHRE PGD Consortium Steering Committee 2002)
Barth's syndrome	(Grifo et al. 1994)
Bloch-Sulzberger syndrome	(Pettigrew et al. 2000)
Duchenne Muscular Dystrophy (DMD)	(Liu et al. 1995; Lee et al. 1998; Hussey et al. 1999)
Fragile Xa Syndrome	(Sermon et al. 1999b; Apessos et al. 2001; Platteau et al. 2002)
Haemophilia A	(Handyside 1998)
Hurter's syndrome	(ESHRE PGD Consortium Steering Committee 2002)
Lesch-Nyhan syndrome(LN)	(Ray et al. 1999)
Ocular albinism I	(Handyside 1998)
Ornithine transcarbamylase deficiency	(Ray et al. 2000)
Oro-facial-digital syndrome type 1	(ESHRE PGD Consortium Steering Committee 2002)
Pelizaeus-Merzbacher syndrome	(Grifo et al. 1994)
Severe combined immunodeficiency	(Handyside 1998)
X-linked hydrocephaly	(Drury et al. 1996)
X-lnked spinal and bulbar muscular atrophy (SBMA)	(Georgiou et al. 2001)
Inherited cancer predisposition	
Familial posterior fossa brain tumour (hSNF5)	(Rechitsky et al. 2002)
Familial adenomatous polyposis coli (FAP)	(Rechitsky et al. 2002)
familial dysautonomia	(Rechitsky et al. 2003)
Neurofibromatosis (NF1/NF2)	(Harper et al. 2002; Rechitsky et al. 2002)
p53 tumour suppressor gene mutations	(Verlinsky et al. 2001d)
p53 mutations in Li-Fraumeni syndrome	(Simpson 2001)
p53 muntations in cancer predisposition	(Rechitsky et al. 2002)
Retinoblastoma	(Sutterlin et al. 1999; Rechitsky et al. 2002; Girardet et al. 2003)

1.4 PGD of chromosomal abnormalities

1.4.1 Chromosomal abnormality in oocytes

Cytogenetic studies on chromosomal abnormalities in human oocytes have been extensively performed on oocytes which failed to fertilize in clinical IVF programs (Kumar and Khuranna 1995; Lim et al. 1995; Roberts and O'Neill 1995; Plachot 2001; Honda et al. 2002; Pellestor et al. 2002). The more recent of these studies (Table 1.2) have shown that 45-87% of unfertilized oocytes had metaphase chromosome spreads suitable for analysis. Of those analysed, 22.1-47.6% were found to be chromosomally abnormal. Of all abnormalities, aneuploidy was always the most common aberration, with a frequency ranging from 10.8% to 34.8%; Aneuploidies found in these studies included hyperhaploidy, hypohaploidy, and extensive aneuploidy. Other aberrations also observed comprised diploidy, tetraploidy, higher hyperploidy, and some structural abnormalities. It must be remembered that these oocytes failed to fertilise and therefore may not be representative of oocytes that do fertilise.

Chromosomal aberrations of oocytes have also been investigated by polar body analysis conducted for PGD of aneuploidy screening using FISH for patients with advanced maternal ages or some other indications. In the most recent of these studies, three (13, 18, and 21) or five (13, 16, 18, 21, and 22) chromosomes were screened per polar body by interphase FISH analysis (Table 1.3). Aneuploidy was the most common aberration found in these studies, with its frequency ranging from 43% to 52% (Verlinsky et al. 1997a; Kuliev et al. 2003). Kuliev et al. (2003) found that of all aneuploidies observed 41.8% could be derived from errors in meiosis I, 30.7% from errors in meiosis II, and 27.6% from errors in both meiotic divisions. They also found that of the aneuploidies originating from meiosis I: 15.4% contained extra chromatids,

48.1% missing chromatids, 0.5% extra chromosomes, 5.9% missing chromosomes, and 30.1% complex abnormalities. Aneuploidies from meiosis II contained 41.2% extra chromatids, 36.6% missing chromatids, and 22.1% complex abnormalities (Kuliev et al. 2003).

Malsegregation of chromosomes in meiotic divisions, including nondisjunction and predivison, are considered to be the major cause for the generation of human oocyte aneuploidy (Hassold and Chiu 1985; Angell et al. 1991). Nondisjunction of bivalent chromosomes in maternal meiosis I can cause two half-bivalents to migrate to the same pole, resulting in oocytes gaining an extra chromosome or missing a chromosome. Such abnormalities have been observed in first polar bodies as well as metaphase-II oocytes (Dailey et al. 1996; Verlinsky et al. 2001a; Kuliev et al. 2003). Non-disjunction is regarded as one of the major mechanisms resulting in aneuploidy, especially aged-related trisomies (Hassold and Chiu 1985; Angell 1994).

Predivision, segregation of chromosomal univalents at maternal meiosis MI division instead of MII division, was first proposed by Angell (1991) to explain the frequent observation of single chromatids in human first polar bodies and metaphase-II oocytes (Angell et al. 1991; Angell 1994; Dailey et al. 1996; Angell 1997; Verlinsky et al. 2001a; Kuliev et al. 2003). Abnormalities of extra or missing chromosomes in first polar bodies and metaphase-II oocytes, presumably derived from non-disjunction, was found to be very rare or much less than that of extra or missing chromatids, which presumably originate from predivision (Angell et al. 1991; Angell 1994; Verlinsky et al. 2001a; Kuliev et al. 2003). These findings suggest that predivision rather than non-disjunction may be the major mechanism for the formation of aneuploidy in human oocytes generated through ovarian stimulation protocols in clinical IVF program (Angell et al. 1991; Angell 1997; Wolstenholme and Angell 2000).

Table 1.2 Chromosomal abnormalities in oocytes which failed to fertilize in a clinical IVF program

Oocyte		Method		Normal and abnormal oocyte (%)								Study
No. of oocytes Obtained	No. of oocytes Analysable	Type of Method	No. of Chromosome analysed per oocyte	Overall Normal	Overall abnormal	aneuploidy[a]	chromatid	diploidy	tetraploidy	hyperploidy	Structural abnormality	
286	233 (81%)	Karyotype	all	77.7	22.3	12.0		6	4..3			(Roberts and O'Neill 1995)
263	179 (68%)	Karyotype	all	72.6	27.4	12.3	4.5	10.1			2.8	(Lim et al. 1995)
121	69 (57%)	Karyotype	all	52.4	47.6	34.8		7.2		2.8	2.8	(Kumar and Khuranna 1995)
?	2, 434	Karyotype	all	74.5	26.5	21.4		3.5			1.6	(Plachot 2001)
208	183 (87%)	FISH	3 (18, 21, X)	91	9	3		6				(Honda et al. 2002)
3, 042	1,397(45%)	Karyotype	all	77.9	22.1	10.8	3.8	5.4			2.1	(Pellestor et al. 2002)

[a]including hyperhaploidy, hypohaploidy, and extensive aneuploidy

?details not reported, but estimated to be 3000

Table 1.3 Chromosomal abnormalities in oocytes of PGD patients with advanced maternal ages revealed by polar body diagnosis

Oocyte	FISH	Source and type of chromosomal abnormalities in oocytes (%)										Study
No. of Oocytes Obtained/analysable/abnormal	Chromosome analysed	Errors in both meiosis I and II	Abnormalities due to errors in meiosis I						Abnormalities due to errors in meiosis II				
			Overall	Chromatid extra	Chromatid missing	chromosome extra	chromosome missing	others[a]	Overall	Chromatid Extra	Chromatid missing	complex abnormality[b]	
8,382/6,733/3,509 (52%)	13,16,18,21,22	27.6	41.8	15.4	48.1	0.5	5.9	30.1	30.7	41.2	36.6	22.1	(Kuliev et al. 2003)
5,590/4,596/2,077 (45%)	13,16,18,21,22	24.9	44.9	16.3	51.3	0.7	8.2	23.5	30.2	39.1	44.1	16.8	(Verlinsky et al. 2001a)
3,943/3,217/1,388 (43%)	13,18,21		35.7						26.1				(Verlinsky et al. 1999)
3,651/2,952/1,271 (43%)	13,18,21												(Verlinsky et al. 1997a)
116/87/29 (33%)[c]	13,16,18,21,22												(Munne et al. 2000b)

[a]complex abnormalities

[b]involving missing or extra chromatids of different chromosomes

[c]first polar body analysed only

1.4.2 Chromosomal abnormality in preimplantation embryos

1.4.2.1 Chromosomal abnormality in cleavage-stage embryos

Cytogenetic studies using G-banding (Michelmann and Mettler 1985; Angell et al. 1986; Plachot et al. 1987; Papadopoulos et al. 1989; Jamieson et al. 1994; Almeida and Bolton 1998) have revealed that chromosomal abnormalities frequently occur in human cleavage-stage embryos (Table 1.4). Abnormalities frequently found in these studies had trisomy, monosomy, nullisomy, hyperploidy, hypoploidy, haploidy, triploidy, tetraploidy, pentaploidy, heptaploidy, structural aberrations (chromosome gap or break, fragment, deletion, acentric fragment, dicentric chromosome, single chromatid), and mosaics (trisomic/diploid, trisomic/polyploid, hypodiploid/hyperdiploid). The overall frequency of chromosomal abnormalities (Table 1.4) in cleavage-stage embryos was found to be as high as 90% (106/118) but varied largely in different studies, probably due to the differences in the quality of embryos investigated (Pellestor et al. 1994) or the culture conditions (Harvey et al. 2002). The most common aberration was always aneuploidy, but in some cases it could be polyploidy (Pellestor et al. 1994) or structural anomalies (Papadopoulos et al. 1989).

Cytogenetic studies can detect all sorts of chromosomal abnormalities. However, This approach can only be suitable for the one third of embryos, which have at least one blastomere with an analysable metaphase chromosome spread, thereby resulting in no diagnosis for the majority of embryos studied. In addition, determination of mosaics requires analysing at least two blastomeres per embryo but only a limited number of embryos contain more than one blastomere suitable for cytogenetic analysis. Therefore, the frequency of mosaics among

cleavage-stage embryos cannot be reliably assessed by cytogenetic methods (Michelmann and Mettler 1985; Angell et al. 1986; Plachot et al. 1987).

Studies using FISH on interphase nuclei have shown that chromosome abnormalities in IVF-created human cleavage-stage embryos could be generally classified into four different categories: (1) normal embryos, consisting only of diploid blastomeres; (2) uniformly abnormal embryos, with the same abnormal karyotype in all blastomeres, such as aneuploidy, haploidy, and polyploidy; (3) mosaic embryos, either containing normal diploid cells and another abnormal cell line or with all cell lines being abnormal; (4) chaotic embryos, with every cell having a different abnormal chromosomal complement (Munne and Cohen 1993; Schrurs et al. 1993; Munne et al. 1994; Munne and Weier 1996; Kahraman et al. 2000; Bielanska et al. 2002; Rubio et al. 2003). Similar patterns of aberrations (Table 1.5) were also observed in single-cell comparative genomic hybridisation (CGH) analysis of human cleavage-stage embryos (Voullaire et al. 2000; Wells and Delhanty 2000). Both of these studies found that only one quarter of embryos (3/12) were entirely normal diploid; most of the remaining embryos (7/9 and 8/9) were mosaic but the majority of them had at least one normal cell (Table 1.5). Recently, Rubio *et al.* (2003) found that the overall frequency of chromosomally abnormal embryos for IVF couples was: maternal age <37 years, 33% (37/111); maternal age ≥37 years, 58% (60/104), and recurrent miscarriages, 70% (395/559).

Comparative studies showed no significant difference in the rate of chromosomal abnormalities, which could be observed between embryos created by IVF and ICSI (Palermo et al. 1996; Bonduelle et al. 1998).

Table 1.4 Chromosomal abnormalities in cleavage-stage embryos revealed by cytogenetic analysis

Embryo		Type of chromosome aberration	Study
No. of embryos Analysed	No. of abnormal Embryos (%)		
8	8 (100%)	triploidy and polyploidy	(Michelmann and Mettler 1985)
22	8 (36%)	trisomy, monosomy, polyploidy, haploidy, nullisomy, mosaics, hypodiploidy, structural aberrations	(Angell et al. 1986)
68	25 (37%)	trisomy, polyploidy, haploidy, nullisomy, mosaics, structural aberrations	(Plachot et al. 1987)
35	14 (40%)	hypodiplody, mosaics (trisomic/diploid, trisomic/polyploid, hypodiploid/hyperdiploidy), Structural abberration (chromatid break, chromatid gap, chromosome gap, chromosome fragment)	(Papadopoulos et al. 1989)
189	51 (27%)	Aneuploidy, hyperploidy, hypoploidy, polyploidy, structural aberration	(Jamieson et al. 1994)
118	106 (90%)	hypodploidy, hyperdiploidy, hypo/hyper, haploidy, polyploidy (triploidy, tetraploidy, pentaploidy, heptaploid), mosaics, structural aberration (chromosome gap, break or fragment, deletion, acentric fragment, dicentric chromosome, single chromatid)	(Pellestor et al. 1994)
171	108 (63%)	trisomy, hyperploidy, hypoploidy, polyploidy, structural aberration	(Almeida and Bolton 1998)

Table 1.5 Chromosome abnormality in cleavage-stage embryos revealed by single-blastomere CGH analysis

Embryos					Aberrant embryos			Aberrations of blastomeres	Study
No. of embryo analysed	No. of blastomere studied/analysed	Type of embryo analysed	No. of embryo (normal)	No. of embryo with normal cells	Aneuploidy	Mosaics	Chaotic[a]		
12	73/64	Fresh (Grade 1-2)	3	9 (75%)	1	6	2	trisomy, monosomy, intensive aneuploidy, nullisomy, partial gain or loss of chromosome	(Wells and Delhanty 2000)
12	56/63	Frozen (Grade 1-3)	3	8 (67%)	1	7	1	trisomy, monosomy, intensive aneuploidy, partial gain or loss of chromosome	(Voullaire et al. 2000)

[a]embryos with each cell containing a different array of chromosome abnormalities

1.4.2.2 Chromosomal abnormalities in human blastocysts

Studies of G-banding and FISH on cells isolated from spare IVF blastocysts indicated that some 5- to 8-day-old human blastocysts were uniformly diploid but others had a variety of chromosomal abnormalities (Clouston et al. 1997; Evsikov and Verlinsky 1998; Ruangvutilert et al. 2000b; Clouston et al. 2002). Abnormalities found in these studies included mosaics, polyploidy (mainly tetraploidy), and aneuploidy (mainly trisomy), with the mosaicism being the most common aberration. Most mosaic embryos generally had diploid cells as their main cell lines. Mosaics could be further classified as mosaic/aneuploid for sex chromosomes, mosaic/aneuploidy for autosomes, mosaic/haploid, mosaic/polyploid (mainly diploid/tetraploid), and chaotic mosaicism. These studies found that the common chromosomal abnormalities present in the first trimester could already be seen in human blastocysts (Clouston et al. 2002).

Surprisingly, some aberrations frequently found in cleavage-stage embryos such as haploidy, monosomy, and nullisomy were very rare in human blastocysts. Embryos with such aberrations might be eliminated prior to blastocyst formation probably due to a poor developmental potential (Clouston et al. 1997; Evsikov and Verlinsky 1998; Clouston et al. 2002). Further analysis of mosaicism in the inner cell mass (ICM) showed that the average degree of mosaicism in ICM was similar to the overall blastocyst mosaicism, indicating that preferential allocation of chromosomally normal cells to ICM and abnormal cells to trophectoderm may not exist (Evsikov and Verlinsky 1998).

1.4.2.3 Causes of chromosome abnormality

Meiotic errors in gametogenesis, abnormal fertilization, and postzygotic mitotic errors are thought to be responsible for the formation of chromosomal abnormalities observed in human preimplantation embryos. Malsegregation of chromosomes such as predivision, nondisjuction, and anaphase lagging in maternal and paternal meiosis could lead to the generation of the aneuploidies commonly seen in embryos. Similarly, errors in the postzygotic mitosis could lead to the mosaicism commonly in human embryos. Predivision and non-disjunction were previously discussed (Section 1.4.1). Anaphase lagging of chromosomes can result in missing chromosomes; such errors in meiosis and postzygotic mitotic divisions could generate monosomic and mosaic embryos, respectively (Sugawara and Mikamo 1980a; Sugawara and Mikamo 1980b). In addition, abnormal fertilization, such as dispermy, could produce triploidy or even tetraploidy (Coonen et al. 1998). Chaotic embryos may result from the absence of some cell cycle checkpoints during meiosis (Delhanty and Handyside 1995 and LeMaire-Adkins et al. 1997) or a low mitochondrial membrane potential in oocytes (Wilding et al. 2003).

1.4.2.4 Consequences of chromosomal abnormalities

As previously mentioned (Table 1.5), only a small proportion (about 25%) of human IVF-created preimplantation cleavage-stage embryos consist of only normal diploid cells (Voullaire et al. 2000; Wells and Delhanty 2000). These chromosomally normal embryos would have a superior developmental potential and are obviously the best for IVF transfer. Some aberrations, such as monosomy, nullisomy, haploidy, multiple aneuploidy and chaotic mosaicism, observed in human IVF-created embryos were rarely or never seen in newborns, foetuses subjected to prenatal diagnosis and spontaneous abortions. These findings suggest that the majority of

embryos with such chromosomal aberrations would be eliminated prior to implantation or shortly after implantation (Boue et al. 1985; Boue 1990; Wells and Delhanty 2000). However, mosaic embryos with some normal diploid blastomeres may lead to normal viable foetuses by three different mechanisms. In the first mechanism, the abnormal blastomeres degenerate shortly after their formation, leaving only the diploid blastomeres to become the foetus. In the second mechanism, the diploid cells divide much faster than the abnormal cells, leading to the formation of a core of normal cells, from which a normal foetus may be generated. The third mechanism is preferential allocation of abnormal blastomeres to form the trophectoderm and the normal cells to generate the inner cell mass (ICM) during the formation of blastocysts (Crane and Cheung 1988; James and West 1994).

1.4.3 Methodology of PGD for chromosomal abnormality

Chromosomal aberrations of preimplantation embryos have been successfully analysed by many different methods, including metaphase cytogenetics (Michelmann and Mettler 1985; Santalo et al. 1995), interphase FISH (Munne and Cohen 1993; Munne et al. 1995), primed *in situ* labelling (Pellestor et al. 1996), spectral imaging (SKY) (Marquez et al. 1998), comparative genomic hybridization (CGH) (Voullaire et al. 2000; Wells and Delhanty 2000), and interphase conversion (Willadsen et al. 1999; Evsikov et al. 2000). Among them, the three methods, interphase FISH, interphase conversion, and CGH, have been so far successfully used in clinical PGD application. However, almost all of the successful PGD screening for chromosomal abnormalities has been achieved by FISH analysis (ESHRE PGD Consortium Steering Committee 2002; Kuliev et al. 2003).

1.4.3.1 Interphase FISH for PGD of aneuploidy screening

In interphase FISH for PGD of aneuploidy screening, chromosomal probes used for analysis are always those of clinical relevance. The five probes, for chromosomes X, Y, 13, 18, and 21, are most often included in order to avoid aberrations commonly seen in livebirths, such as XO, XXY, and trisomies 13, 18, and 21 (Gianaroli et al. 1997a; Kahraman et al. 2000). Initially, no more than 5 chromosomes could be reliably analysed per blastomere. This limitation was overcome by sequential FISH analysis on single interphase nucleus (Martini et al. 1997; Bahce et al. 2000; Harrsion et al. 2000; Rubio et al. 2003). This new approach allows another four chromosomes, 14, 15, 16, and 22, to be analysed along with those five described above. Inclusion of these four chromosomes in PGD screening gives a chance to avoid pregnancies affected with trisomies 14, 15, 16, and 22, which are frequently observed in spontaneous abortions (Gianaroli et al. 1999; Bahce et al. 2000). With three sequential hybridizations, it has been reported that a total of 13 different chromosomes, 2, 3, 4, 11, 13, 15, 16, 17, 18, 21, 22, X, and Y, can be reliably analysed per blastomere (Abdelhadi et al. 2003). It has been suggested that all 24 different types of chromosomes can be assessed per blastomere by use of four sequential hybridizations, with each round of hybridization to assess 6 different chromosomes (Bahce et al. 2000; Munne et al. 2002). However, one technical limitation of this method is the decrease of hybridization efficiency with each round of hybridisation (Liu et al. 1998a; Liu et al. 1998b; Gianaroli et al. 1999).

Liu et al. (1998a) used Whole Chromosome Paints (WCP) (Vysis, USA) to test sequential FISH analysis and found that the rate of nuclear loss, presence of signals, and absence of signals was: 3%, 96%, and 4%, respectively, after the first round of FISH; 4%, 93%, and 7%, respectively, after the second round of FISH; and 6%, 87%, and 13%, respectively, after the

third round of FISH analysis. Many studies suggested that the efficiency might be improved by progressively increasing the duration of each round of hybridisation. The disadvantageous consequence of this modification is that embryo transfer might then have to take place on day 4 (Grifo et al. 1998) or day 5 of embryo development (Pehlivan et al. 2003; Rubio et al. 2003).

1.4.3.2 Interphase FISH for PGD of structural abnormalities

PGD of structural chromosomal abnormality using interphase FISH analysis was most often performed for translocations, but with quite a few cases for inversions (Cassel et al. 1997; Iwarsson et al. 1998; Van Assche et al. 1999; Weier et al. 1999; Coonen et al. 2000; Munne et al. 2000a; Escudero et al. 2001; Fridstrom et al. 2001; Menezo et al. 2001; Scriven et al. 2001; Emiliani et al. 2003). DNA probes used so far included carrier-specific breakpoint-spanning probes (Cassel et al. 1997; Weier et al. 1999), probes distal to the breakpoints such as telomeric probes (Iwarsson et al. 1998; Pierce et al. 1998), chromosome-specific satellite probes (Cassel et al. 1997), and probes proximal to the breakpoint such as centromeric probes (Munne et al. 2000a). Using breakpoint-spanning probes, interphase FISH can differentiate between embryos with normal and balanced karyotypes, which are both predictably clinically normal, and embryos with unbalanced karyotypes, which are expected to be clinically abnormal. However, the limitation of this method is the difficulty of development of patient-specific probes, which is always expensive and time-consuming (Cassel et al. 1997; Weier et al. 1999). Similarly, to find specific probes distal and/or proximal to the breakpoints of translocations and inversions is also labour-intensive and sometimes impossible. In these cases commercial chromosome telomeric- and centromeric-specific instead of patient-specific DNA probes can often be used. Obviously these protocols are suitable for all translocations and inversions except for Robertsonian

translocations, where chromosome enumerator probes are required to detect aneuploid embryos (Conn et al. 1998; Munne et al. 2000a).

1.4.3.3 Limitations of interphase FISH analysis

Interphase FISH analysis mainly relies on observing the calculated number of hybridization signals in the interphase nuclei of blastomeres or polar bodies. In principle, one target DNA sequence produces only one signal. In practice, the presence or absence of a FISH signal is frequently affected by some other factors, including signal overlapping, splitting, loss of micronuclei during fixation, and hybridization failure (Verlinsky and Kuliev 2000; Gianaroli et al. 2001). Signal splitting is more likely to occur in cells, which are in S and G_2 phases than in cells in G_1; for example, a normal embryo could be misdiagnosed as monosomic or trisomic due to signal overlapping or splitting resulting in a false diagnosis of abnormality. By contrast, a monosomic or trisomic embryo might be interpreted as normal owning to signal splitting or overlapping leading to a false negative diagnosis. Obviously, a normal embryo could be misdiagnosed as chaotic in the cases of signal overlapping and splitting simultaneously involving many different chromosomes. False monosomies appear to be the most common type of misdiagnosis (Gianaroli et al. 1999; Gianaroli et al. 2001); a rather obvious interpretation of this finding is that the missing signals were the result of technical failure of the FISH. The overall frequency of such FISH misdiagnosis was up to 15% when only 9 chromosomes (X, Y, 13, 14, 15, 16, 18, 21, and 22) were analysed per blastomere (embryo) using two consecutive rounds of hybridizations (Munne et al. 1998a). .

The accuracy of interphase FISH per probe per cell has been estimated to be 91-96% for euploid samples and less for trisomic samples (Ruangvutilert et al. 2000a). The cumulative

experimental artefacts of FISH tend to increase as more probes are employed, as evidenced by an increase in the discordant results among cells of the same normal embryos. This could lead to more embryos being misdiagnosed as mosaic, leading to overestimating the frequency of mosaics in IVF-created preimplantation embryos.

1.4.3.4 Interphase conversion

Interphase conversion, also called nuclear transfer, is a technique, by which the interphase nucleus of a second polar body (PB2) or blastomere is induced into the metaphase stage after being fused with an oocyte or zygote. This method enables full karyotyping single PB2 and blastomeres, and is thus highly valuable for PGD of chromosomal rearrangements (to date only translocations). In contrast to blastomere conversion suitable for both maternal and paternal translocations, PB2 conversion can only be applied for translocations of maternal origins (Table 1.6).

One critical step of this method is the generation of hybrid cells, which could be human PB2-human oocyte (Verlinsky and Evsikov 1999a), human blastomere-bovine oocyte (Willadsen et al. 1999), human blastomere-mouse zygote (Verlinsky and Evsikov 1999b). Bovine and mouse oocytes used in these studies were either intact or enucleated but human oocytes were always enucleated prior to cell fusion (Table 1.6). Hybrid cells can be created by either microinjection, similar to ICSI (Willadsen et al. 1999) or agglutination using phytohaemagglutinin (Verlinsky et al. 2002a). Once generated, hybrid cells were first treated by electrofusion, then cultured *in vitro* until the disappearance of the pronuclei, and finally fixed at mitosis. Prior to fixation, induction of premature chromosome condensation (PCC) by exposure of hybrid cells to okadaic acid is necessary for some heterokaryons with persisting pronuclei (Verlinsky et al. 2002a). The

Table 1.6 Karyotyping of human individual blastomeres or polar bodies following interphase conversion

Biopsy sample	Recipient	No. of cells studied	No. of cells analysable	Aberration analysed	Methods for diagnosis	Study
Blastomere	Bovine intact oocyte	87	64 (73%)	Translocation	Karyotype and FISH	(Willadsen et al. 1999)
Second polar body	Human enucleated oocyte	38	25 (66%)	Translocation	Karyotype and FISH	(Verlinsky and Evsikov 1999a)
Blastomere	Mouse enucleated or intact zygote	69	63 (91%)	Translocation Aneuploidy	Karyotype and FISH	(Verlinsky and Evsikov 1999b)
Blastomere	Mouse enucleated or intact zygote	437	383 (88%)	Translocation	Karyotype and FISH	(Verlinsky et al. 2002a)

metaphase chromosome spreads obtained can be used to detect translocations by G-banding analysis, sometimes in combination with metaphase FISH using whole chromosome painting probes (WCP) (Willadsen et al. 1999). However, full karyotyping may be difficult or impossible in some instances due to chromosome overlapping or aposition (Verlinsky and Evsikov 1999b; Verlinsky et al. 2002a).

Interphase conversion is now conducted in a few laboratories worldwide probably due to the laboratories having sophisticated skills such as micromanipulation and electrofusion. Since its first introduction in 1999, only a limited number of live births with a normal or balanced chromosome complement has been obtained for PGD couples with translocations by this method (Willadsen et al. 1999; Verlinsky et al. 2002a). The efficiency of this method of interphase conversion has been variable to date, varying from 66 to 91% of cells analysable (Table 1.6).

1.4.4 Indications for PGD of chromosomal abnormality

1.4.4.1 Reciprocal and Robertsonian translocations

Reciprocal and Robertsonian translocations are one type of the most common structural chromosomal abnormalities. Affected couples usually experience high rates of recurrent miscarriage, mainly due to a high proportion of their gametes having unbalanced chromosome complements. Reciprocal translocations occur in 0.2% of the general population, 0.6% of infertile couples, 3.2% of couples with repeated IVF failures, 9.2% of couples with high rates of repeated miscarriage, and 2-3.2% of male patients undergoing ICSI (Testart et al. 1996; Meschede et al. 1998; van der Ven et al. 1998; Stern et al. 1999). The use of PGD for these couples allows embryos with a normal or, less preferable, balanced chromosome complement to

be identified and selectively transferred, thus enhancing the chance of IVF implantation as well as reducing recurrent spontaneous abortions. An advantageous reduction in spontaneous abortion, from 95% in natural cycles to 13% in PGD cycles, has been reported (Munne et al. 2000a).

1.4.4.2 Advanced maternal age (AMA)

It is well known that numerical chromosomal abnormality (mainly aneuploidy) increases with maternal age (Boue et al. 1985; Hassold and Chiu 1985). Many studies have shown that chromosomal abnormalities are very common in preimplantation embryos derived from IVF couples with AMA. Of all abnormalities observed in these studies, aneuploidy was always the most common aberration, including monosomies, trisomies, and aneuploidies involving more than one chromosomes. The main objective of these studies was to improve implantation and reduce spontaneous abortions and trisomic offspring for IVF couples with AMA undergoing aneuploidy screening by PGD (Munne et al. 1998a; Gianaroli et al. 1999; Kahraman et al. 2000; Kuliev et al. 2003). Some of these studies carried out by blastomere diagnosis (Table 1.7, rows 1-2) can be compared with studies performed by polar body diagnosis (Table 1.3).

Comparative studies showed that spontaneous abortions and trisomic offspring were significantly decreased for couples with AMA following PGD of aneuploidy screening. Such a decrease in the frequency of spontaneous abortion from 23% to 11% or trisomic offspring from 3.2% to 0.8 % (2/262) was recently reported (Munne 2002). However, the predictable increase in the implantation and pregnancy rates was less obvious than the decrease in spontaneous abortions and trisomic offspring (Gianaroli et al. 2001; Munne 2002). Based on these findings, Gianaroli et al. (2001) was prompted to advise that aneuploidy screening by PGD should not be

used as a routine practice until more conclusive evidence of its benefits became available. Nevertheless, it seems clear that aneuploidy screening by PGD may not only decrease trisomic abortions and offspring but also improve IVF implantation for couples with maternal ages of ≥ 37 years if more than eight or nine chromosomes can be reliably screened for each embryo (Munne 2002).

1.4.4.3 Recurrent implantation failure (RIF)

Recurrent implantation failure (RIF) is defined as three or more failed IVF cycles. As high as 70% of preimplantation embryos of patients with RIF have been found to be chromosomally abnormal (Table 1.7, rows 3-7), mainly aneuploidy and mosaics (Gianaroli et al. 1997b; Munne et al. 1998a; Gianaroli et al. 1999; Kahraman et al. 2000; Voullaire et al. 2002; Pehlivan et al. 2003). One main objective of these studies was to improve the IVF implantation and pregnancy rates for patients with RIF by transferring chromosomally normal embryos after aneuploidy screening by PGD. Interphase FISH was used in most of these studies with metaphase CGH in just one study (Voullaire et al. 2002). Most of these studies failed to achieve a significant increase in the implantation and pregnancy rates for patients with RIF, especially for older patients (>37 years old). Some of the studies with FISH results and clinical IVF outcomes have been summarised (Table 1.7).

In the study reported by Pehilvan *et al.* (2003) a pregnancy rate of 34.0% and an implantation rate of 19.8% for patients with RIF was found: quite similar to the 33.3% pregnancy and 24.1% implantation rates found in normal fertile controls undergoing PGD for X-linked diseases. Even better pregnancy and implantation rates, 40.7% and 24.6%, respectively, were observed in this study if the patients with RIF were <37 years old. Such high implantation and pregnancy rates

might be mainly due to the combination of biopsy on day 3 with blastocyst transfer on day 5 adopted in this study. This strategy allowed the selection of both chromosomally and morphologically normal embryos for IVF transfer with very favourable timing. Results from this study are encouraging but larger series of studies with proper controls should be conducted to assess the benefits of aneuploidy screening by PGD for patients with RIF.

1.4.4.4 Recurrent miscarriage (RM)

Recurrent miscarriage (RM), usually defined as three or more spontaneous pregnancy losses, is a common reproductive problem. Cytogenetic studies have shown that chromosomal abnormalities are very common in abortuses of patients with RM, with an overall frequency of abnormalities ranging from 50% to 70% (Boue et al. 1975; Hassold 1980; Plachot et al. 1989; Eiben et al. 1990; Stephenson et al. 2002). Aneuploidy (mainly trisomies) has always been found to be the most common aberration in these studies. For example, of all abnormalities observed in the miscarried foetuses, up to 66.5% (131/225) were found to be aneuploid (Stephenson et al. 2002). Many studies have shown that chromosomal abnormalities also frequently occur in preimplantation embryos of patients with RM (Simon et al. 1998; Pellicer et al. 1999). These studies also showed that an increase in the IVF pregnancy rate could be achieved for patients with RM following aneuploidy screening by PGD. Similar results were also obtained in a recent study but this study found that such an increase was only observed in younger patients of $\leq$35 years with RM (Egozcue et al. 2002). Most recently, Rubio et al. (2003) compared the results of aneuploidy screening by PGD (Table 1.7, rows 8-10) between the patients with RM and normal fertile controls undergoing PGD for X-linked diseases. No significant differences in the pregnancy rate were observed between the patients and the controls. These results suggest that PGD could improve the pregnancy rate of patients with RM

up to the level similar to the normal fertile controls, thus highlighting the usefulness of aneuploidy screening by PGD for patients with RM.

Table 1.7 Aneuploidy screening by PGD in couples with a poor IVF prognosis

Patient		FISH results							IVF results (%)			Study
Maternal age (years)	IVF cycles Conducted	chromosome analysed	No. of Embryos analysed	No. of embryos Abnormal	Aneuploidy monosomy	trisomy	haploidy	polyploidy	mosaic	Implantation rate	Pregnancy rate	Miscarriage rate	
39.2 ± 2.2 AMA1	73	X, Y, 13, 14, 15, 16, 18, 21, and 22	432	64.2%	21%	23%	0.4%	0.36%	44.4%	25.8	39	4	(Gianaroli et al. 1999)
37.9 ± 2.1 AMA2	49	X, Y, 13, 18, and 21		40.4%	85.2%[a]		4.5%	10.3%		Data not available	25.5	Data not available	(Kahraman et al. 2000)
32.2 ± 2.3 RIF1	27	X, Y, 13, 14, 15, 16, 18, 21, and 22	138	53.6%	18.9%	5.4%	6.7%	3.5%	55.4%	17	25	20	(Gianaroli et al. 1999)
30.3 ± 3.1 RIF2	23	X, Y, 13, 18, and 21		43.2%	85.5%[a]		6.2%	8.3%		Data not available	30.4	Data not available	(Kahraman et al. 2000)
33.7 ± 1.6(<37) RIF3	27	X, Y, 13, 18, 21, and 22	272	65.4%	47.5%[a]		1.5%	4.5%	20.5%	24.6	40.7	1	(Pehlivan et al. 2003)
39.5 ± 1.6 (≥37) RIF4	22	X, Y, 13, 18, 21, and 22	171	70.7%	54.5%[a]		3.0%	2.4%	18.9%	12.2	25	1	(Pehlivan et al. 2003)
36.2 ± 3.3 RIF5[b]	49	X, Y, 13, 18, 21, and 22	443	67.4%	50.2%[a]		2.1%	3.8%	19.9%	19.8	34.0	2	(Pehlivan et al. 2003)
33.2 ± 2.1(<37) RM1	63	X, Y, 13, 16, 18, 21, and 22	426	70.7%	78.7%[a]		6.6%	2.3%		30.8	38.8	10.5	(Rubio et al. 2003)
33.2 ± 2.1(≥ 37) RM2	23	X, Y, 13, 16, 18, 21, and 22	133	70.7%	84%[a]		4.2%			19.4	22.2	25	(Rubio et al. 2003)
35.6 ± 3.0 RM3[c]	86	X, Y, 13, 16, 18, 21, and 22	559	70.7%	80%[a]		6.0%	1.7%		28.0	34.3	13	(Rubio et al. 2003)

AMA: advanced maternal age

RIF: recurrent implantation failure

RM: recurrent miscarriag

[a] including monosomies, trisomies and complex aneuploidies

[b] results of RIF3 and RIF4 pooled together

[c] results of RM1 and RM2 pooled together

1.5 Comparative genomic hybridisation using metaphases

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique by which the entire genome can be screened for differences in DNA sequence copy number in a single hybridization between test and reference DNA samples on normal metaphase chromosome spreads. Without the need to culture the test samples, as is required for G-banding analysis, CGH generates a "copy-number-karyotype" for the test sample, displaying the variations in the DNA copy number across the whole genome of the specimen (Kallioniemi et al. 1994).

Since its advent in 1992, CGH has been mainly applied to investigate genomic imbalances in tumour samples, especially amplifications of oncogenes in solid tumours (Kallioniemi et al. 1992). These studies have facilitated the identification and characterization of pathological genomic aberrations in cancer, the understanding of tumour development and progression, or the analysis of tumour genetic heterogeneities (Joos et al. 1995; Houldsworth et al. 1996; Kim et al. 2000; Clausen et al. 2001; Umayahara et al. 2002). Furthermore, recurrent aberrations revealed by CGH may be used as diagnostic, prognostic, or even classification tools for tumours (Iwabuchi et al. 1995; Verhagen et al. 2000; Hirose et al. 2001; Harada et al. 2002; Junker et al. 2003a; Junker et al. 2003b).

CGH has also been useful in prenatal diagnosis (Yu et al. 1997; Lapierre et al. 2000; Thein et al. 2000; Marton et al. 2001; Chen et al. 2003), in the analysis of spontaneous abortions (Daniely et al. 1998; Daniely et al. 1999; Lomax et al. 2000; Ostroverkhova et al. 2002), in PGD for aneuploidy screening (Voullaire et al. 2000; Wells and Delhanty 2000), and some other fields of human genetic diseases (Levy et al. 1998; Lomax et al. 1998; Levy et al. 2002).

1.5.1 CGH protocols

In CGH, test and reference DNA samples are first labelled by different fluorochromes, normally with green for the test sample and red for the reference sample. Labelled test and reference probes are then mixed together and comparatively hybridized to a normal metaphase chromosome spread in the presence of human Cot-1 DNA (a mixture of all highly repetitive sequences) at 37°C for 2-4 days. The Cot-1 DNA hybridizes very rapidly to the probes and any hybridization to the repetitive sequences on the chromosomes is therefore suppressed. After hybridization and washing, a fluorescence microscope is applied to capture the hybridized metaphase images, which are then analysed by dedicated CGH software. Individual metaphases are analysed separately. Quantification of the signal intensities of both fluorochromes from the p to q telomeres (pter to qter) of each chromosome is performed. The average green/red ratio of fluorescence intensity for the entire metaphase is then normalized to 1.0, assuming that the total fluorescence intensities are the same in both channels. Averaging the results from multiple (5-20) metaphases produces the mean ratio profiles for each chromosome, by which the relative copy number of DNA sequences in the test genome can be predicted, compared to a normal diploid genome contained in the reference sample (Kallioniemi et al. 1992; Karhu et al. 1997).

Simply put, metaphase CGH compares the amount of DNA from a specific chromosome in the test sample with the amount of DNA in the same chromosome in the reference sample using the chromosome at the metaphase as an isolating mechanism.

1.5.2 CGH ratio profiles

1.5.2.1 Ratio profiles of autosomes

Theoretically, CGH should give a ratio value of 1 for all autosomes and 0.5 for the X chromosome in male (green)/female (red) comparisons. Practically, deviations from such ideal values are frequently observed (Karhu et al. 1997). Ratios of autosomes always fluctuate around the expected value of 1.0 but should not exceed a selected threshold range, such as 0.85 to 1.15 (Kallioniemi et al. 1994), which might vary in different studies. Other threshold ranges used so far could be symmetrical ones, such as 0.75 to 1.25 (Voullaire et al. 1999) or 0.8 to 1.2 (Hirose et al. 2003), or asymmetrical, such as 0.85 to 1.20 (Isola et al. 1994) or 0.85 to 1.17 (Larramendy et al. 1998). Ratios of autosomes falling within the selected thresholds always serve as an internal negative control to indicate that the CGH protocols used are optimized and reliable.

When a test sample labelled with green is under investigation, gains of DNA sequences in the test sample can be indicated by ratios greater than the higher threshold value and losses of DNA sequences by ratios less than the lower threshold value (Larramendy et al. 1998). Ratios of >2.0 are indicative of high-level amplification (Daigo et al. 2001a; Franke et al. 2001).

1.5.2.2 Ratio profiles of gonosomes

For the X chromosome, the expected ratio value of 0.5 is rarely obtained in male/female comparisons (Karhu et al. 1997). However, the X chromosome can produce a ratio in the range of 0.60 to 0.70, which is less than the lower threshold values applied to the autosomes. Consequently, the copy-number of the X chromosome can always be correctly predicted (Isola

et al. 1994; Kallioniemi et al. 1994; Larramendy et al. 1998). Kallioniemi et al. (1992) demonstrated a linear correlation between X chromosome copy number (from 1 to 5) and their corresponding ratios although the obtained ratios always underrepresent the true copy number of X chromosome. Ratios of the X chromosomes far below the lower threshold value and approaching the theoretical value of 0.5 in male/female comparisons are always used as an internal positive control to show that single copy-number changes such as trisomies and monosomies can be reliably detected by the CGH protocols used.

For the Y chromosome, ratios of the euchromatic region but not the heterochromatic regions (especially the distal part of its q-arm) always exceed the ratio thresholds of autosomes in male/female comparisons. As a consequence, determination of the copy number of the Y chromosome relies on the ratios generated by the euchromatic regions (Kallioniemi et al. 1992; Speicher et al. 1995; Voullaire et al. 1999). In order to avoid false diagnoses of the Y chromosome, its heterochromatic regions (Speicher et al. 1995) or even the entire Y chromosome (Franke et al. 2001) are always excluded from CGH analysis.

1.5.2.3 Chromosomal region- or band-specific ratio fluctuations

False overrepresentations or underrepresentations always appear for some chromosomal regions, including centromeric, pericentromeric, heterochromatic, and telomeric regions. Hybridization signals on these regions are always very weak due to the suppression of signals by Cot-1 DNA. As a result, signal:background ratios (S:B ratio) of these regions may be below the minimal acceptable level of 3.0 (Karhu et al. 1997). In practice, these problematic regions are always excluded from CGH analysis (Kallioniemi et al. 1992; Isola et al. 1994; Kallioniemi et al. 1994).

False overrepresentations of 1p33-pter, 16p, 17p, 19, 22, and p-arms of acrocentric chromosomes have also been well documented in the case of the test samples labelled by FITC (Speicher et al. 1993; Kallioniemi et al. 1994; Kim et al. 1995; Malmgren et al. 2002). Overrepresentations of 7q21, 9q34, 16q 17q, and chromosome 20 as well as underrepresentation of chromsome 20 were also reported in one study (Larramendy et al. 1998). Such CGH artefacts can be reduced by direct labelling using FITC-dUTP and TR-dUTP instead of biotin-14-dATP and digoxigenin-11-dUTP (Mohapatra et al. 1995) or even eliminated by reverse labelling, in which the test sample is labeled by TR-dUTP and the reference sample by FITC-dUTP (Larramendy et al. 1998). However, some other false overrepresentations at 4q13-q21, 11q21-23, 13q21-qter, and Xq21-q22, as well as the false underrepresentation at 19p, were observed in the reverse labelling approach (Larramendy et al. 1998). In order to avoid any false positive results due to these artefacts, these problematic regions or chromosomes are commonly excluded from CGH analysis (Isola et al. 1994; Kallioniemi et al. 1994; Karhu et al. 1997). If one does suspect unbalanced aberrations at these regions, it has been advised that CGH using reverse labelling should be performed to confirm these findings (Mohapatra et al. 1995; Marchio et al. 1997; Larramendy et al. 1998).

1.5.3 Resolution of CGH analysis

Under optimal experimental conditions, the minimal size of deletions reliably detectable by CGH has been estimated to be in the range of 3-8 Mb (Larsen et al. 2001). And the minimal amount of DNA amplification (defined as the size of an amplified DNA sequence multiplied by its copy number) detected by CGH has been demonstrated to be at least 2Mb (Joos et al. 1993; Piper et al. 1995). The ability to detect such small amounts of deleted or amplified DNA by CGH is somewhat surprising since the resolution of changes to G-banded prophasic

chromosomes, showing the maximum number, is widely regarded as 6 Mb of DNA, assessed by comparison of homologous chromosomes under the highest magnification possible with visible light microscope (GC. Webb, personal communication). In a blind trial, 3 Mb of transgenic DNA could not be detected using FISH in mouse chromosome 11, even though the exact point of insertion was known (GC. Webb, personal communication).

1.5.4 Limitations of CGH analysis

Firstly, genomic imbalances in the problematic regions described above cannot be reliably identified because the ratio profiles are inadmissible for CGH analysis. Secondly, CGH cannot detect balanced aberrations (such as balanced translocations or inversions and polyploidies), point mutations, and some other small intragenic rearrangements (Kallioniemi et al. 1994). Thirdly, contamination of tumour samples by normal cells from surrounding tissue could lead to the failure of CGH to detect copy number changes of genomic DNA present only in neoplastic tumour cells. In early tumours, such contamination is almost inevitable due to the infiltrative nature of the tumour. However, it could be minimized by microdissection of the samples, and the resulting small DNA samples are enough for reliable CGH analysis after being preamplified with whole genome amplification (WGA) (Hirose et al. 2003).

1.5.5 CGH combined with whole genome amplification

1.5.5.1 Whole Genome Amplification (WGA)

WGA is a strategy used randomly to amplify small amounts of DNA prior to any specific genetic analyses. The amplified products are thought to be representative of the initial sample, thereby permitting numerous genetic loci to be analysed. WGA is performed using either degenerate (partially or completely) or non-degenerate primers. The former includes degenerate oligonucleotide-primed PCR (DOP-PCR) (Telenius et al. 1992), primer extension preamplification (PEP) (Zhang et al. 1992), and tagged random primers PCR (T-PCR) (Grothues et al. 1993). A low-stringency amplification step applied to these methods allows the random primers to anneal efficiently to the target DNA template.

Non-degenerate WGA methods include linker-adaptor PCR (LA-PCR) (Vooijs et al. 1993) and PCR using interspersed repetitive sequence (IRS-PCR) (Ledbetter et al. 1990). In the LA-PCR approach, the DNA sample is first digested by a restriction endonuclease, an oligonucleotide adaptor is then ligated to both ends of all cleaved DNA fragments, and all ligated DNA fragments are finally amplified by PCR using a primer complementary to the adaptor. The IRS-PCR approach amplifies the DNA sample by using primers complementary to Alu or L1 elements, which are conserved, interspersed repetitive elements found throughout the human genome. In contrast to LA-PCR, IRS-PCR is simpler but it leads to biased amplification owning to the uneven distribution of Alu and L1 elements across the genome.

1.5.5.2 DOP-PCR-CGH for analysis of microdissected tumour tissues

CGH combined with microdissection and DOP-PCR enables reliable CGH analysis to be performed on microdissected neoplastic tissues or cells (Kuukasjarvi et al. 1997; Aubele et al. 1998; Franke et al. 2001). Precise microdissection can minimize, or even eliminate, the contamination by normal cells of the tumour samples, making CGH analysis more accurate. Therefore, this approach has become an effective method for cytogenetic investigation in invasive tumours (Kim et al. 2000; Hirose et al. 2001; Harada et al. 2002; Umayahara et al. 2002; Hirose et al. 2003; Junker et al. 2003b).

1.5.5.3 WGA-CGH for analysis of single cells from preimplantation embryos

CGH combined with whole genome amplification using either DOP-PCR (DOP-PCR-CGH) (Voullaire et al. 1999; Wells et al. 1999; Hussey and Metthews 2000) or LA-PCR (LA-PCR-CGH) (Klein et al. 1999) has proved to be reliable for CGH analysis of single cells. Both approaches have been successfully performed on single blastomeres of preimplantation embryos with a success rate ranging from 97% (63/65) (Voullaire et al. 2000) to 70% (66/94) (Malmgren et al. 2002). Two studies using DOP-PCR-CGH have been previously summarized (Table 1.5), in which fresh embryos (Wells and Delhanty 2000) or frozen embryos (Voullaire et al. 2000) were analysed. Only one quarter (3/12) of the analysed embryos were found to be entirely normal diploid embryos; the remainder was mostly mosaic. Such low frequencies of normal embryos may explain the low success rates of human conceptions in nature as well as in clinical IVF practice (Wells and Delhanty 2000). The high incidence of mosaics suggests that malsegregation of the chromosomes in postzygotic divisions is a major cause of numerical chromosomal abnormalities in preimplantation embryos. In addition, the chromosomal

aberrations found in blastomeres in these studies included monosomies, nullisomies, extensive aneuploidies, chaotic aberrations, and structural abnormalities. Many of these abnormalities are never or rarely seen in spontaneous abortion and foetuses subjected to prenatal diagnosis (Boue et al. 1975; Hassold 1980; Stephenson et al. 2002).

Using LA-PCR-CGH, Malmgren et al. (2002) found that all 28 embryos analysed were mosaic. Most of embryos (22/28) analysed in this study were obtained from patients undergoing PGD for chromosomal translocations, and were all diagnosed as unbalanced by FISH diagnosis. LA-PCR-CGH confirmed FISH results in many cases, and further revealed that a variety of numerical chromosomal abnormalities were also present in these embryos. These findings indicate that LA-PCR-CGH can be reliably used for PGD of patients with translocations, and further suggest that full karyotyping is required in these cases to detect any potential aneuploidies.

1.5.5.4 DOP-PCR-CGH in PGD of aneuploidy screening

Because of the ability to analyse all chromosomes in single cells, DOP-PCR-CGH analysis using chromosome spreads has been suggested to be an effective method for PGD aneuploidy screening. However, this method normally takes 4 to 6 days to complete, which is incompatible with the current PGD practice with blastomere biopsy on day 3 followed by embryo transfer no later by around day 5 of embryo development. Wilton *et al.* (2001) froze the embryos after blastomere biopsy, and this strategy allowed them to obtain the first healthy baby after PGD screening for chromosomal abnormalities by DOP-PCR-CGH analysis (Wilton et al. 2001). One disadvantage of this approach is that the freezing-thawing process could reduce the developmental capacity of the embryo (Joris et al. 1999; Magli et al. 1999). A strategy to avoid

this problem (Wells et al. 2002) used first polar body biopsy instead of blastomere biopsy, thereby obtaining at least two more days for DOP-PCR-CGH analysis. This strategy was performed for one couple with repeated IVF failures. In this study, one out of the 11 first polar bodies analysed was found to be chromosomally normal and the corresponding normal embryo was subsequently transferred but there was no resulting pregnancy. The major limitation of this method is the inability to detect chromosomal aberrations of paternal and postzygotic origins (Wells et al. 2002).

1.6 DNA microarray-based comparative genomic hybridization

For isolation of comparatively-hybridized DNA, a micro-dot of DNA from a specific chromosome can be substituted for the relevant metaphase chromosome. Solinas-Toldo et al. (1997) performed CGH analyses on DNA microarrays (array CGH) instead of metaphase chromosome spreads (metaphase CGH) in the hope of increasing the resolution of metaphase CGH and to simplify its procedure. This study proved it possible to obtain reliable ratio profiles of copy-number changes for individual defined DNA probes applied to the DNA arrays, thus permitting direct mapping of unbalanced aberrations onto genomic DNA sequences for tumour samples. This modification greatly enhances the resolution of CGH analysis, with a detection limit of 40 kb for high-level amplifications such as oncogenes and 40~130 kb for low-copy-number-changes such as heterozygous and homozygous deletions (Solinas-Toldo et al. 1997; Pinkel et al. 1998; Bruder et al. 2001); again (Section 1.5.3), this is unexpectedly good resolution (GC Webb, personal communication). With such a high resolution and the other advantages of high throughput, genome-wide screening and short duration of hybridization (normally overnight), this new CGH approach, now generally termed array CGH, has been increasingly used during the last few years, instead of metaphase CGH, to investigate genomic DNA imbalances, especially in cancer (Albertson and Pinkel 2003). Some DNA arrays suitable for CGH analysis are commercially available, such as the chips for analysis of oncogenes from Vysis (USA) (Daigo et al. 2001).

1.6.1 Applications of array CGH

The majority of array CGH studies published so far have been performed for tumour samples. The main objective of these studies focused on detection of oncogene amplifications (Daigo et

al. 2001; Hui et al. 2001; Takeo et al. 2001; Ishizuka et al. 2002) and deletions of tumour-suppressor genes (Bruder et al. 2001). Other objectives included differential diagnosis of subtypes in tumours (Wilhelm et al. 2002) and detection of loss of heterozygosity (LOH) (Dumur et al. 2003). However, the most fundamental applications of array CGH in cancer so far might be to narrow down amplicon boundaries (Weiss et al. 2003) or even identify the oncogenes (Albertson et al. 2000).

Array CGH has also been useful in detection of constitutional chromosomal aberrations. Snijders *et al.* (2001) correctly diagnosed aneuploidies such as trisomies and monosomies by use of DNA arrays covering the whole genome, thus highlighting the usefulness of array CGH in prenatal or even preimplantation diagnosis. Small telomeric genomic imbalances are one of the common reasons resulting in mental retardation, congenital anomalies, and miscarriages. Such aberrations involving any of the whole suite of telomeres, such as deletions and duplications, can be detected using an array consisting of all of the chromosome (sub)telomere-specific DNA probes (Veltman et al. 2002). More recently, Veltman *et al.* (2003) successfully defined a critical region of 5 Mb on 18q22.3-23 for congenital aural atresia by use of a high-resolution chromosome-18-specific DNA array, thus highlighting the value of array CGH analysis in characterization of disease genes (Veltman et al. 2003a).

1.6.2 DNA arrays used for Array CGH

DNA arrays, cDNA arrays, and oligonucleotide arrays have all been successfully used for array CGH experiments (Albertson and Pinkel 2003). The DNA array CGH approach allows reliable detection of single-copy changes to be performed on individual DNA clones. By contrast, using cDNA and oligonucleotide arrays, detection of such aberrations is less reliable

and always relies on a 'moving average' of ratios from symmetric three to five adjacent array spots mainly due to array elements of cDNA and oligonucleotide being much shorter than PAC and BAC clones used in DNA arrays (Lin et al. 2002; Pollack et al. 2002). DNA arrays have been applied in almost all array CGH studies, and therefore the remainder of this section will focus on a review of DNA array CGH studies.

DNA arrays always contain large insert clones such as those cloned in the P1-derived artificial chromosome (PAC), bacterial artificial chromosomes (BAC), and cosmids. Initially, PAC and BAC clones were directly spotted on slides. This approach requires large-scale preparation of cloned DNAs, and furthermore, spotting of PAC and BAC clones at sufficient concentration is always difficult, due to the high viscosity of the very long DNA sequences in the spotting solution (Solinas-Toldo et al. 1997; Pinkel et al. 1998). The use of WGA strategies to produce representations of tiny amounts of primary clone DNA has been used to solve these problems. Methods used so far include LA-PCR (Lucito et al. 2000; Snijders et al. 2001; Wessendorf et al. 2002; Weiss et al. 2003), IRS-PCR (Geschwind et al. 1998), and DOP-PCR (Veltman et al. 2002). However, these WGA approaches could co-amplify the DNA of the vector *Escherichia coli*, which is of necessity, a common contaminant present in the DNA samples derived from BAC and PAC clones. Feigler *et al* (2003) solved this problem using three modified DOP-PCR primers capable of preferential amplification of the inserted DNA in the PAC and BAC (Fiegler et al. 2003).

In DNA array CGH, the coverage of the genome varied among different studies. It ranged from a specific region of a chromosome (Albertson et al. 2000; Bruder et al. 2001; Weiss et al. 2003), an entire chromosome arm (Weiss et al. 2003), a whole chromosome (Pinkel et al. 1998), to the entire genome (Snijders et al. 2001). To what extent the genome should be covered

depends on the specific aims of individual studies. For instance, genome-wide coverage might be the preferred strategy for screening of genomic imbalances in cancer (Cai et al. 2002). By contrast, a high-resolution array, covering only the region under investigation, might be the best choice in cancer studies for defining the boundaries of deletions and amplifications (Veltman et al. 2003a) and even identifying the relevant oncogenes (Albertson et al. 2000).

1.6.3 Normalization of array CGH data

For interpretation of array CGH results, the fluorescence intensity ratios of test to reference (T/R ratios) are always chosen. Systematic biases of such ratios frequently occur due to a number of variables inherent to DNA array experiments. These include unequal quantities of initial DNA sample between test and reference, and differences in efficiency of labeling and scanning between the two fluorescence dyes used (Bilban et al. 2002). The process of normalization is aimed at removing or at least minimising such biases prior to analysis. Many methods of normalization can be used for this purpose (Quackenbush 2002). The most frequently used is global normalization, which assumes that the total fluorescence intensity of the entire array should be, in theory, the same for both dyes. Hence the mean T/R ratio of the entire array is can be adjusted to one. Practically, signal intensities may be higher for some spots in one channel than the other. But such fluctuations may disappear or at least be extremely reduced when summing up hundreds of thousands of spots (Bilban et al. 2002). After normalization, T/R ratios should fluctuate around one, and if necessary can be combined or even compared among different array CGH experiments (Pinkel et al. 1998; Veltman et al. 2002; Veltman et al. 2003a; Veltman et al. 2003b).

Array CGH data can also be normalized by use of control spots arrayed on the same slides. Such spots always contain DNA with the same copy-number in both test and reference samples. This approach of normalization is likely to be the preferred choice for array CGH analysing a small number of DNA probes (Solinas-Toldo et al. 1997; Geschwind et al. 1998; Pinkel et al. 1998).

1.6.4 Transformation of array CGH data

T/R ratios are sometimes transformed into Logarithmic values to various bases in array CGH prior to normalization. One main objective of log transform is to bring the ratio values closer to homogeneity of variance and normal distribution required for global normalization. In this case, ratios of individual spots are normalized to an average log ratio of zero for the entire array (Hedenfalk et al. 2003).

Log ratios are very useful for dealing with up- and down-regulated genes in microarray studies of gene expressions because numbers and their reciprocals are treated symmetrically. For example, using a $\log_2$ transform, genes up-regulated by a factor of 2 have a $\log_2$ ratio of 1, and those down-regulated by a factor of 2 give a $\log_2$ ratio of -1. Both values of 1 and -1 are symmetrically disposed around the value of zero for those genes expressed at a same level between test and reference samples (Quackenbush 2002).

Recently, log ratios have been increasingly applied in array CGH, particularly for analysing high-copy-number amplifications in cancer (Wessendorf et al. 2002; Hedenfalk et al. 2003). They are also helpful for displaying the ratios of low-copy-number changes, such as heterozyous and homozygous deletions (Snijders et al. 2001). In the cases of trisomic duplications, before

transformation, the T/R ratio and its R/T ratio are 1.5 (3/2) and 0.67 (2/3), respectively. Both ratios are not symmetrically disposed around the expected ratio of 1 (2/2), applicable to no changes of copy-number. This means that thresholds which are symmetrically disposed around the expected value of 1 such as 0.75-1.25 (or 0.85 –1.15) do not give the same result when the fluorescent dyes are swapped. Thus the threshold of 0.75 is closer to the theoretical value of 0.67 than 1.25 is to the theoretical value of 1.5. This can lead to misleading results when thresholds are being applied. In contrast, this problem is resolved by using the $\log_2$ of the ratio. In these cases the transformed T/R and R/T ratios are 0.58 [$\log_2(3/2)$] and -0.58 [$\log_2(2/3)$], respectively. Obviously, both ratios are symmetrically disposed around the ideal ratio of zero [$\log_2(2/2)$], representing no changes of copy-number between the test and reference samples. Although the later approach is somewhat easier to interpret it is not used in this thesis since the majority of the literature relating to the field of aneuploidy detection outside of the cancer field does not use it.

1.6.5 Ratio profiles of array CGH

1.6.5.1 Ratio thresholds

A total number of 5 to 8 independent array CGH analyses in male/female (or vice versa) comparisons are normally carried out to assess the normal ratio deviations of array probes (Pinkel et al. 1998; Hui et al. 2001; Veltman et al. 2002; Wilhelm et al. 2002). Ratio thresholds for detecting genomic imbalances should be broader than the normal deviations observed but should not exceed the ratios for X chromosome probes. Probes with ratios always falling outside of ratio thresholds in these control trials are normally replaced or even discarded. Thresholds used so far might vary in different studies, including a range of 0.85 to 1.15 (Cai et al. 2002), 0.82 to 1.18 (Hui et al. 2001), 0.80 to 1.20 (Veltman et al. 2002), and 0.75 to 1.25 (Wessendorf

et al. 2002). In the case of 0.75 to 1.25, ratios of >1.25 ($\log_2$ 0.32) or < 0.75 ($\log_2$ -0.41) indicate gains or losses of DNA copy-number in the test sample (Wessendorf et al. 2002). Ratios of X chromosome probes outside of the thresholds are normally used to demonstrate the reliability of array CGH for detecting single-copy changes such as trisomy and monosomy (Pinkel et al. 1998; Wessendorf et al. 2002). The validation of array CGH can be further tested using some cell lines with known genomic imbalances (Solinas-Toldo et al. 1997; Snijders et al. 2001).

1.6.5.2 Ratios of X chromosome probes

In female/male comparisons, ratios ranging from 1.49 to 1.52 were obtained in one study for X chromosome probes compared to the expected value of 2 (Hui et al. 2001). In another similar study, $\log_2$ ratios of X chromosome probes were found to be 0.72 ± 0.08 in contrast to the ideal value of 1 ($\log_2$ 2) (Snijders et al. 2001). In male/female comparisons, ratios of X chromosome probes were reported to be 0.69 ± 0.05 (Pinkel et al. 1998) or 0.59 ± 0.004 (Bruder et al. 2001), compared to the theoretical value of 0.5. Such underrepresentation of the true copy number of X chromosome probes could be caused by a number of reasons (Pinkel et al. 1998; Pollack et al. 1999; Fiegler et al. 2003). Among these reasons, the most important may be incomplete suppression of the repetitive sequences on the X chromosome (probably those not included in the Cot-1 DNA) and inaccuracy in background substruction. Other reasons may include probe autofluorescence, crosshybridization due to homology of X-Y or X-autosomes, and the effects of inactivation of one female X chromosome. Despite these deviant findings, a linear correlation between the copy number of X chromosome and the ratio was observed when normal female DNA was compared to DNA samples of cell lines containing 1 to 5 copies of X chromosome (Pinkel et al. 1998). However, the slope of the relation observed was 0.37, much lower than its ideal value of 0.5, and somewhat in agreement with the results described above.

1.6.5.3 Detection of low-copy-number changes

Low-copy-number changes include heterozygous and homozygous deletions and duplications, as well as low-copy number amplifications especially in cancer. Using homozygous diploid samples as reference, Wessendorf et al. (2002) found that ratios for low-copy-number gains and losses in cancer ranged from 1.3 to 1.5 and 0.60 to 0.70, respectively. In some other studies, ratios were found to be 0.59 ± 0.05 (Bruder et al. 2001) or 0.67 (Veltman et al. 2002) for heterozygous deletions, 0.26 for homozygous deletions (Bruder et al. 2001), and 1.28 for trisomic duplications (Veltman et al. 2002). Snijders AM *et al.* (2001) reported a $\log_2$ratio of <-2 for homozygous deletions. Obviously, deviation from the ideal values occurred in all cases, indicating that underrepresentation of the true copy number of array probes is a common phenomenon in array CGH analysis probably due to reasons similar to those for the X chromosome mentioned above (Section 1.6.5.2).

To test the reliability of detecting deletions in array CGH, so far, most studies have used the comparisons of Turner's syndrome sample (XO) versus normal female (XX) for a limited number of X-linked loci. Such reliability should be further tested on more autosome-linked loci using samples with known autosomal deletions (Pinkel et al. 1998; Daigo et al. 2001).

1.6.5.4 Detection of high-copy-number amplifications

Log_2 ratios greater than 1 ($\log_2 2$) indicate high-level amplifications of genomic sequences in cancer (Wessendorf et al. 2002). This threshold is the same as that used in metaphase CGH (Section 1.5.2.1). For example, a $\log_2$ ratio of >6 was found for amplified *MYC* in COLO320 with an amplification level of ~ 70 fold (Snijders et al. 2001).

1.6.6 Replicas of probe spots

The reliability of metaphase CGH is greatly enhanced by use of ratios averaged from several metaphases of good quality (Section 1.5.1). For the same purpose, replicas of individual probes are always spotted for array CGH analysis. Triplicates are most frequently applied (Daigo et al. 2001; Snijders et al. 2001; Veltman et al. 2002). Others used so far include duplicates (Fiegler et al. 2003), quadruplicates (Pinkel et al. 1998), five replicates (Bruder et al. 2001), six replicates (Cai et al. 2002), eight replicates (Solinas-Toldo et al. 1997), and even 10 replicates (Wessendorf et al. 2002). In addition, the availability of replicates allows the selection of spots of good quality for analysis, a process similar to that of choosing metaphases of good quality for analysis in metaphase CGH.

1.6.7 Exclusion of spots from analysis

Spots may be excluded from array CGH analysis due to printing artefacts such as misplacement, irregularity of morphology, evenness or size, overlying debris, nearby background and complete failure to print the spots (Pollack et al. 1999). Spots may also be eliminated because of hybridization problems. Spots with low fluorescence intensity approaching the background, high intensity saturating the detectors, or low signal/background ratios (S:B ratio) should be excluded from analysis. Wessendorf et al (2002) discarded spots with a S:B ratio of < 2. Exclusion of some probes may also be required if the standard deviation of their ratios exceed the acceptable values (such as $> \pm 0.30$) (Wessendorf et al. 2002). The criteria for exclusion of spots from analysis always differ in different studies, but the critical decision is that only spots with fluorescent intensity in both Cy3 and Cy5 channels statistically

significantly different from background can be included in the final analysis (Snijders et al. 2001; Veltman et al. 2002).

1.6.8 Reverse labelling

Wessendorf et al. (2002) found that ratio deviations of some probes could not be overcome by repetition of the labelling and hybridization but could be corrected by reversing the labelling of the test and reference. Based on these findings, they proposed that this might be due to the difference in affinity of certain classes of DNA sequences, such as GC-rich or poor sequences, between the Cy3 and Cy5 dyes. In order to overcome this problem, Wessendorf et al. (2002) performed each of their experiments twice with a comparison of Cy3-labelled test versus Cy5-labelled reference and another of Cy5-labelled test versus Cy3-labelled reference; They then obtained precise ratios by averaging the two experiments. Obviously, reverse labelling should be used to confirm positive findings of array CGH analysis, as is commonly recommended for metaphase CGH (Section 1.5.2.3).

1.6.9 DOP-PCR array CGH

Array CGH normally requires starting DNA materials of 0.5-1.0 μg for each of test and reference samples. In order to overcome this limitation, Daigo *et al.* (2001) used DOP-PCR to preamplify tiny amounts of DNA from microdissected tumour samples (~ 500 cells) and further achieved reliable array CGH analysis using such DOP-PCR products for breast cancer cell lines as well as for formalin-fixed, paraffin-embedded tissue samples. Comparative studies showed that ratios derived from DOP-PCR-array CGH were highly concordant with, but sometimes underestimated, the ratios resulting from nick translation-based array CGH, particularly for

high-level amplifications: eg., using a ratio threshold of 1.2 for the diagnosis of amplifications, DOP-PCR-based array CGH showed a 98.4% positive predictive value, 97.6% negative predictive value, 90.6% sensitivity, and 99.6% specificity compared with the results of nick translation-based array CGH. Daigo *et al* (2001) also tested a cell line containing a trisomy 7, DOP-PCR-based array CGH produced a mean ratio of 1.3, ranging from 1.22 to 1.45. Furthermore, a ratio of 0.69 was observed in this study for X chromosome probes in male/female comparisons. Overall, the results of Daigo *et al.* (2001) showed that the use of DOP-PCR-based array CGH was equivalent to nick translation-based array CGH for detection of single-copy changes (Pinkel et al. 1998; Daigo et al. 2001).

1.7 Human chromosome-specific DNA libraries

Human chromosome-specific DNA libraries have been established by many methods, including the cloning of digested flow-sorted chromosomes (Vooijs et al. 1993), PCR amplification of single human chromosomes from human/rodent hybrid cells (Ledbetter et al. 1990; Lichter et al. 1990), and PCR amplification of human microdissected chromosomes (Meltzer et al. 1992; Guan et al. 1994). One objective of these studies was to make whole chromosome painting probes for FISH analysis. Using microdissection, Guan *et al.* (1996) developed a whole set of human chromosome arm-specific probes, including 24 long arms and 19 short arms (excluding the p arms of all five acrocentric chromosomes 13, 14, 15, 21, and 22) (Guan et al. 1996). Bolzer *et al.* (1999) generated a complete set of repeat-depleted, PCR-amplifiable, human whole chromosome paints by affinity chromatography. These paints could produce specific signals in metaphase FISH analysis without the addition of Cot-1 DNA (Bolzer et al. 1999).

1.8 Hypothesis of this project

The requirement for clinical application of PGD for detecting chromosomal abnormalities is to screen as many chromosomes as possible to reduce the probability of transferring an embryo affected with a chromosomal abnormality, especially aneuploidy. Using DNA arrays with dots representing all human chromosome-specific DNA libraries, array CGH technology should be capable of detecting aneuploidies for all the 24 human chromosomes. If successfully developed for clinical application of PGD, the technology may increase the rates of implantation and decrease the rates of miscarriages and trisomic offspring, by transferring preimplantation embryos least likely to contain aneuploidy. This new approach would particularly benefit IVF couples with a poor prognosis such as advanced maternal age, recurrent miscarriage, and probably repeated IVF implantation failure.

1.9 Aims of this project

The first aim of this project is to design and make a DNA array with dots representing all human chromosome-specific DNA libraries. In this section, a suitable set of all the human 24 chromosome-specific DNA libraries will be chosen and spotted on the arrays as specific representatives for all human chromosomes by DNA microarray facilities. The resulting array will mimic the spreads of metaphase chromosomes previously used to produce the results of metaphase CGH analysis (Fig. 1.1).

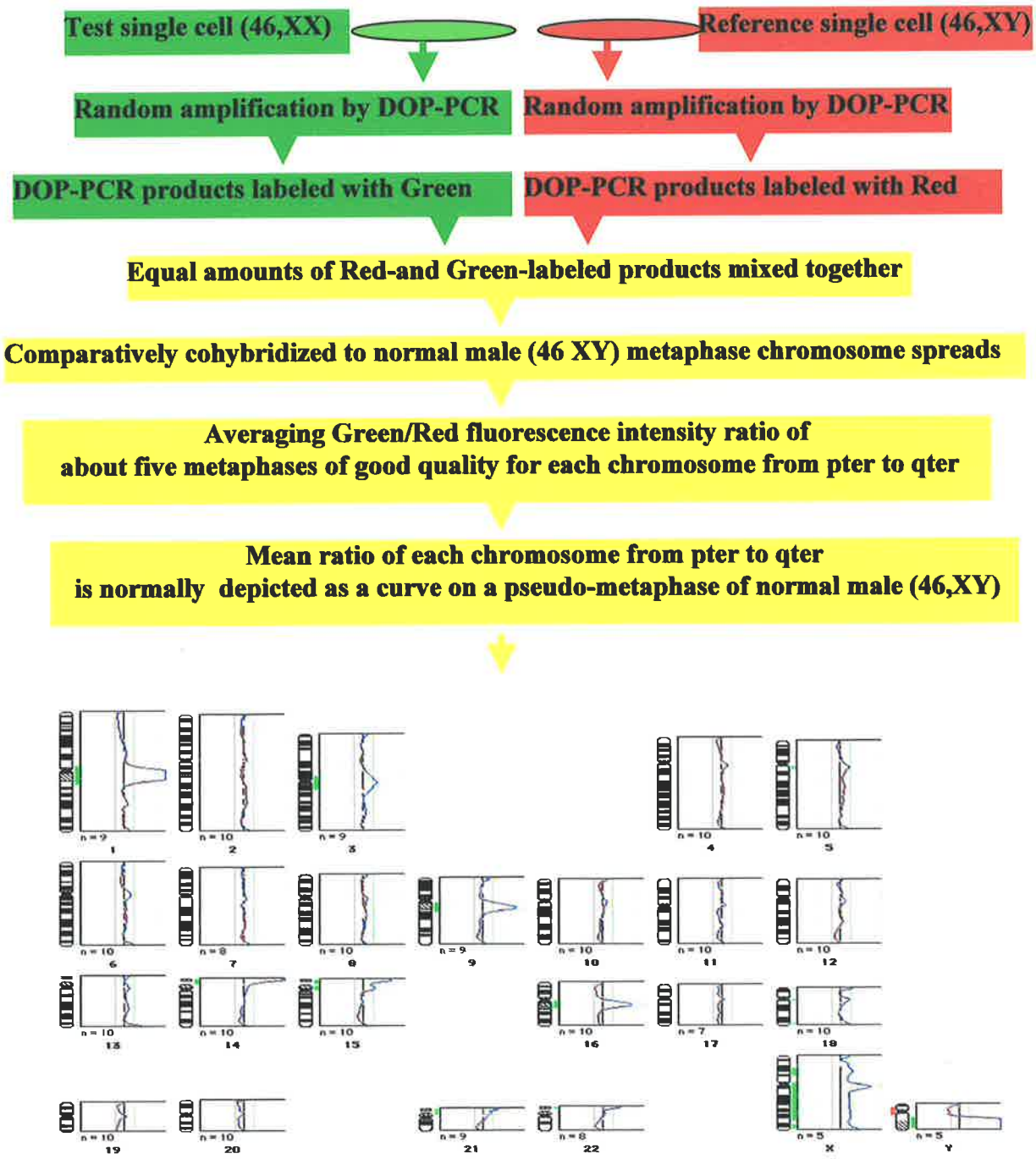


Figure 1.1. Flow diagram of single-cell metaphase comparative genomic hybridization. One normal female single cell labeled with Green compared with one normal male single cell labeled with Red. The gender of the female cell can be predicted by the ratio of the X chromosome exceeding the higher threshold value of 1.25 (green line) as well as the ratio of the Y chromosome beyond the lower threshold value of 0.75 (red line). False overrepresentation at centromeric and telomeric regions can also be observed for some chromosomes (Hussey and Metthews 2000).

The second aim of this project is to develop the protocols of single-cell array CGH analysis. In this section, a number of array CGH experiments of normal female/normal male comparisons will be carried out using our arrays. The objectives of these experiments would include (1) establishment and optimization of single-cell array CGH protocols; (2) standardisation of methods for data analysis suitable for single-cell array CGH experiments; (3) assessment of the normal ratio deviation for each chromosome and subsequent definition of the ratio threshold for diagnosis of autosomal aneuploidy such trisomies and monosomies; (4) examination of the possibility of using the autosomal ratio threshold for gender determination with the obtained ratios for X and Y chromosomes.

The third aim of this project is to test the feasibility of array CGH for diagnosis of aneuploidy using single cells containing known chromosomal abnormalities such as trisomies 13, 15,18, 21, plus the sex chromosomes anomalies.

The fourth aim of this project is to test array CGH on single blastomeres removed from preimplantation embryos. Although single cell diagnoses cannot be independently verified the results will be compared to those obtained by FISH and metaphase CGH. It is hoped that chromosomally normal and abnormal blastomeres will be identified. In order to identify mosaic embryos, it will be preferable to test all blastomeres from the same embryos, at least in some cases for comparison with published studies.

The fifth aim is to test the possibility of using DOP-PCR from single blastomeres for both array CGH analysis and gene-specific PCR analysis. This approach will allow PGD to be performed for both chromosomal and single-gene defects using the same biopsied polar body or blastomere.

Chapter 2

Materials and Methods I: General methods

2.1 Materials

2.1.1 Ordered reagents

DNA markers from GeneWorks Pty Ltd, SA, Australia

(1) *SPP*-1 Phage DNA/*Eco*RI (Cat. No. DMW-S1)

Fragments (Size): 1(8,557), 2(7.427), 3(6,106), 4(4,899), 5(3,639), 6(2,799), 7(1,953), 8(1.882), 9(1.515), 10(1.412), 11(1.164), 12(992), 13(710), 14(492), 15(359), 16(81).

(2) *pUC*19 DNA/*Hpa*II (Cat. No. DMW-P1)

Fragments (Size): 1(501), 2(489), 3(404), 5(242), 6(190), 7(147), 8(111), 9(110), 10(67), 11(34), 12(34), 13 (26).

PCR reagents from Applied Biosystems, CA, USA

(1) AmpliTaq DNA polymerase (Cat. No. N808-0172): 1000 Units (U), 5 U/μL along with 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl) and 25 mM $MgCl_2$ solution

(2) dNTP solutions (Cat. No. N808-0007): 10 mM of each of all four dNTPs.

DNA blocking agents from Gibco BRL

(1) Salmon sperm DNA solution (Cat. No. 15632-011): sheared DNA with majority of DNA $\leq$ 2000 bp and a concentration of 10 mg/ml.

(2) Human Cot-1 DNA (Cat. No. 15279-011): 1 mg/ml

DNA purification kits from *Mo Bio* Laboratories, CA, USA

(1) UltraClean™ GelSpin DNA purification kit (Cat. No. 12400-100)

(2) UltraClean™ PCR Clean-up kit (Cat. No. 12500-250)

Fluorescent dNTPs for FISH or array CGH analysis

dNTPs	Excitation (nm)	Emission (nm)	Company	Cat. No.	Colour	Application
SpectrumGreen dUTP	497	524	Vysis	30-803200	Green	FISH
SpectrumRed dUTP	587	612	Vysis	30-803400	Red	FISH
FluoroLink™Cy3 (Cy3-AP3-dUTP)	550	570	Amersham Phamacia Biotech	PA 53022	Green	Array CGH
FluoroLink™Cy5 (Cy3-AP3-dUTP)	649	670	Amersham Phamacia Biotech	PA 55022	Red	Array CGH

Reagents for DNA extraction

(1) RNase A (Cat. No. 109142, Roche Diagnostics GmbH, Manniheim, Germany)

(2) Proteinase K (Cat. No. 161 519, Roche Diagnostics GmbH, Manniheim, Germany)

(3) Phenol/ Chloroform/Isoamyalcohol (25/24/1) (Cat. No. P-2069, Sigma, USA)

(4) Chloroform/Isoamyalcohol (24/1) (Cat. No. C-0549, Sigma).

Reagents for cell culture from Invitrogen (US)

(1) Minimum Essential Medium (MEM) (1X) (Cat. No. 10370-021)

(2) Trypsin-EDTA [(0.25% Trypsin + 1 mM EDTA.4Na (1X)] (Cat. No. 25200-056)

(3) Phosphate-buffered Saline (PBS) pH 7.2 (1X) (Cat. No. 20012-027)

(4) Dulbecco's phosphate-buffered Saline (D-PBS) (1X) (Cat. No. 14190-144)

(5) L-Glutamine, 200 mM (100X) (Cat. No. 25030-149)

(6) Fetal Bovine Serum, certified (US) (Cat. No. 16000-036)

Whole chromosome paints (WCP) from Vysis, Inc., IL, USA

(1) WCP 21q SpectrumOrange (Cat. NO. 33-120021): containing fluorophore-labelled painting probe and blocking DNA in Tris-EDTA buffer. This probe hybridises to the q arm of human chromosome 21 (band region 21q21-q22), resulting in fluorescence with moderate to bright intensity along the length of the 21q arm. This probe does not hybridise to the p arm or the centromere of chromosome 21. WCP 21 q may also hybridise to and fluoresce with weak intensity at the centromere of chromosome 13.

(2) WCP X SpectrumGreen (Cat. NO. 33-122023): containing fluorophore-labelled painting probe and blocking DNA in Tris-EDTA buffer. This probe hybridises to the Xp arm, Xq arm, and to the centromere of human chromosome X (band region Xp11.1–Xq11.1). The hybridised probe fluoresces with moderate to bright intensity along the length of chromosome X, with slightly brighter intensity at the X centromere. The region in the vicinity of band Xq13 may occasionally appear slightly less intense. When viewed with an orange-specific filter set, WCP X may also hybridise to and fluoresce with weak intensity to the pseudoautosomal region at band Yp11.32 on the human Y chromosome (Vysis catalogue, 1999).

2.1.2 Prepared solutions

Chemicals used to make the following solutions were purchased from various suppliers and were all of analytical grade. Generally, solutions were made up with MilliQ H_2O and sterilised by either autoclaving at 103 kPa, 121 °C for 20 min for large-scale preparation, or by filtering through a 0.22 μm MILLEX®-GP Filter Unit (Cat. No. P25390, MILLIPORE, Bedford, MA, USA) for small-scale preparation. The solutions were:

0.5 M EDTA: 93.05 g of EDTA dissolved in 500 ml of H_2O, pH to 8.0

1X TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA

5X TBE: 1 M Tris-HCl, 0.9 M boric acid, 0.2 M EDTA, pH 8.3

20X SSC: 3 M NaCl, 0.30 M sodium citrate, pH 7.0

50X Denhardt's solution: 1% Ficoll400, 1% Polyvinylpyrrolidone, 1% BSA

6X gel loading buffer: 0.25% bromophenol blue, 40%(W/V) sucrose

Ethidium Bromide (10 mg/ml): 0.2 g of ethidium bromide was dissolved in 20 ml of MilliQ H_2O and stored at room temperature.

10% Sodium Dodecyl Sulphate (SDS): 10 g of SDS was dissolved in 100 ml of H_2O

3 M Sodium Acetate (NaAC) (pH 5.2): 49.2 g of NaAC was dissolved in 200 ml of H_2O and adjusted to pH 5.2 using 10 M NaOH.

Phosphate-buffered saline: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 pH 7.0

Lysis solution: 200 mM KOH, 50 mM dithiothreitol DTT

Neutralization solution: 300 mM KCl, 900 mM Tris-HCl, pH 8.3, 200 mM HCl

10X K^+-free PCR Buffer: 1 mg/ml gelatin in 100 mM Tris-HCl

Deionized Formamide: 100 ml of Formamide (Cat. No. 47671, Sigma-Aldrich) was mixed with 7-10 g of AG® 501-X8 (D) Resin (Cat. No. 143-6425, Bio-Rad) and then magnetically stirred for 3-4 hours, filtered through a 0.22 μm Filter Unit (MILLEX®-GP), and stored at 10-15°C no longer than one month prior to use.

Array CGH hybridization solution: 50% deionized formamide, 0.1% SDS, 5X Denhart's solution, 3X SSC, 10% Dextran sulfate. This solution could be stored at -20°C for up to six months.

FISH hybridization solution: 50% deionized formamide, 0.1% SDS, 5X Denhardt's solution, 2X SSC, 10% dextran sulfate. This solution could be stored at -20°C for up to six months.

2.1.3 Equipments

PCR machines

(1) Minicycler (Cat. No. MC009144MJ Research, USA)

(2) PC 960C (Corbett Research, Australia)

(3) PTC-100 Thermocyler (Cat. No. 3229, MJ Research, USA)

Digital Photography of DNA agarose gel

(1) Kodak digital camera DC120 (Amersham)

(2) ID Kodak digital science software (Amersham)

Olympus Fluorescence microscope for FISH

AHBT3 (Olympus, Tokyo, Japan): DAPI images of chromosomes, SpectrumGreen signals, and spectrumRed signals were visualized and captured under fluorescent filter cubes of Blue, Triple (green, blue, UV together, balanced for less UV than the colours), and UV (ultraviolet), respectively.

Scanner and software for Array CGH

(1) GenePix 4000B scanner for array scanning (Axon Instruments, Union City, CA, USA)

(2) GenePix Pro software for data analysis (Axon Instruments, Union City, CA, USA)

2.2 Methods

2.2.1 Preparation of single cells

2.2.1.1 Preparation of single lymphocytes

Single normal lymphocytes used in this study were isolated from peripheral blood samples, which were donated from one normal female (46,XX) and two normal male (46,XY): Male Donor 1 and Male Donor 2.

2.2.1.1.1 Isolation of lymphocytes from peripheral blood

Fresh blood samples (~4 ml) were centrifuged at 1700 rpm (600 g) for 10 min in a Beckman TJ-6 centrifuge. The upper plasma layer was transferred into a 1.5 ml Eppendorf tube and then treated as in Section 2.2.1.1.2, the remaining blood was diluted up to a final volume of about 8 ml with PBS buffer. Approximately 2 ml of Ficoll Hypaque (Crown Scientific) was then carefully layered under the diluted cells to ensure a sharp interface, and the tube was centrifuged at 1700 rpm for 20 min. The lymphocyte layer (white ring) was transferred into a fresh 10 ml tube, then diluted to a volume of 10 ml with PBS and then centrifuged at 1700 rpm for 10 min. The supernatant was discarded and the lymphocyte pellet

was then rewashed twice with PBS. After washing, the lymphocyte pellet was resuspended in the residual PBS (300~500 μl) and the resultant lymphocyte suspension was ready for single-cell sorting (Section 2.1.1.3) or DNA extraction (Section 2.2.2).

2.2.1.1.2 DNase I treatment of plasma

The plasma was centrifuged at 14,000 rpm for 20 min. The upper layer of plasma was filtered into a fresh Eppendorf tube through a 0.22 μm filter (MILLIPORE, USA) and debris at the bottom of the tube such as red blood cell pellet was discarded. 2 μl of DNase I (10 U/μl) (Cat. No. 776785, Boehringer Mannheim, GmbH, Germany) was mixed with 25 μl of filtered plasma in a 0.5 PCR sterilized tube. The DNase I/plasma mixture was incubated at 37°C for one hour followed by inactivation of the enzyme at 65°C for 10 min and then used for cell sorting.

2.2.1.1.3 Single lymphocyte sorting

50 μl of lymphocyte suspension (Section 2.2.1.1.1) was added to the 25 μl of DNase I-treated plasma mixture and mixed thoroughly prior to cell sorting. Cell sorting (Fig. 2.1) was performed on a superfrost microscope glass slide (Menzel-Glaser, Germany) using an inverted light microscope under a 20 X 10 magnification (CK2, Olympus, Japan). Briefly, slides were washed thoroughly with sterilized 70% ethanol (Delta West Pty Ltd, west Australia, Australia) and mounted onto the microscope. 100 μl of RPMI medium (Sigma) was pipetted onto the left side of the slide and 5 μl of the above lymphocyte/plasma/DNase mixture was added. Another three smaller ponds (approximately 50 μl) of 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) were created to the right of the RPMI pond in sequence and designated (from left to right) as 1X PCR buffer pond #1, pond #2, and pond #3, respectively (Fig. 2.1). Using a 9", extruded, cotton-plugged glass Pasteur pipette, lymphocyte

suspension was aspirated from the RPMI pond and approximately one hundred cells were then transferred to the 1X PCR buffer pond #1. Using a fresh pipette, less than 15-20 cells were transferred into the 1X PCR buffer pond #2. 3-6 cells were aspirated with a fresh pipette from the pond #2 and transferred into the 1X PCR buffer pond #3. For washing the cells within this pond, one lymphocyte was aspirated and gently pumped in and out the end of pipette at a fresh location with a fresh pipette. After washing, this lymphocyte was aspirated into the end of the pipette with a minimal amount of PCR buffer and then transferred into a 0.5 ml sterilized PCR tube. Using the same pipette, more single lymphocytes could be isolated separately from the pond #3, and finally a small amount of 1X PCR buffer from the area which had contained the lymphocytes in pond #3 was aspirated and transferred into a PCR tube, to act as a negative control. Isolated single lymphocytes were used immediately or stored at -20°C.

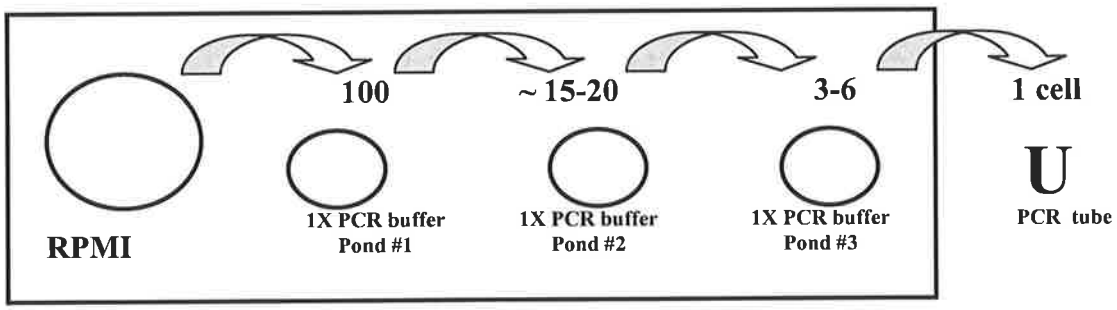


Figure 2.1 Single-cell sorting procedure. A lymphocyte suspension is diluted through four ponds created on a microscope slide up to the point where there are only 3-6 cells in the 1X PCR buffer pond #3. Single lymphocytes were washed for several times in this pond and then transferred into a 0.5 ml sterilized PCR tube. 1X PCR buffer (< 3 μl) was aspirated from the pond #3 and used for a negative control.

2.2.1.2 Preparation of single amniocytes

Cultures of trisomic amniocytes including 47,XX,+13, 47,XY,+18, and 47,XY,+21 were obtained from the Department of Cytogenetics and Molecular Genetics at the Women's and Children's Hospital, Adelaide. A monolayer of the cells was washed twice with 1X PBS. After washing, 4 ml of Tyrpsin-EDTA solution (0.25% Trypsin, 1 mM EDTA) (Invitrogen) was added into each flask which was then placed at room temperature for approx. 4 min until the cells were stripped off the flask. After stripping, the cell suspension in the flask was transferred into a sterilized 10 ml tube and centrifuged at 1700 rpm for 5 min (Beckman GPR). The supernatant was discarded and the cells washed with 5 ml of 1X PBS and centrifuged again. The resultant pellet was washed again with 1X PBS and then resuspended in 300 μl of fresh 1X PBS. Single amniocytes were then isolated from these amniocyte suspensions as previously described (Fig. 2.1) except that no DNase I-treated plasma was added to the amniocyte suspensions prior to cell sorting.

2.2.1.3 Preparation of single fibroblasts

Five fibroblast cell lines purchased from Coriell Cell Repositories (Camden, NJ, USA) were nominally: GM01359 (47,XY,+18), GM02948A (47,XY,+13), and GM07189 (47,XY+15), GM0111145 (47,XY,+9) and GM04435 (48,XY,+16,+21). The cultures were shipped in flasks, containing medium with only 5% Fetal bovine serum (FBS) and no Glutamine, to slow down cell proliferation during transportation of approx. one week from USA to our laboratory. Newly received flasks were placed cell side down in an incubator at 37°C overnight without opening to allow the cells to settle. Flasks were checked 20 hours after receipt under a microscope, and not many cells were found to have settled in all cell lines. The shipping medium was removed from the flasks and replaced with freshly prepared Minimum Essential Medium (1X) containing 15% FBS and 2 mM L-Glutamine (Invitrogen)

to support proliferation. The flasks were then examined once a day for the following two weeks, during which period half of the culture medium was changed every 3-4 days to preserve the growth factors.

The GM04435 cell line failed to proliferate since no vital cells were available upon receipt. After culturing the GM01359 and GM0111145 cell lines for two weeks and the GM02948A cell line for one month, they reached confluency and were harvested (Section 2.2.1.2). Three quarters of the resultant suspensions were subcultured in three fresh flasks for G-banding analysis and the rest were used immediately for single-cell sorting as previously described (Fig. 2.1). The GM07189 cell line grew very slowly and had very few cells after being cultured for one month. To promote cell proliferation, the cells were stripped off the original flask and transferred into a new flask containing freshly prepared culture medium which was renewed every 3-4 days. This cell line finally reached confluency about two months after receipt, and the fibroblasts were then harvested for single-cell sorting (Fig. 2.1) as well as for subculturing for G-banding analysis.

Cytogenetic analyses of G-banded metaphases from fibroblast cell lines were carried out by the Cytogenetics laboratory of the Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, Queen Elizabeth Hospital, The University of Adelaide, SA, Australia.

2.2.2 DNA Extraction

Lymphocyte pellets, prepared from ~ 5ml of peripheral blood, were digested in a volume of 2.5 ml, containing 2.275 ml of ND solution (0.075 M NaCl, 0.024 M EDTA, pH 8), 50 µl of Proteinase K (10 mg/ml, Sigma), 50 µl of RNase A (10 mg/ml, Sigma), and 125 µl of 10% SDS solution in a shaking waterbath at 37°C overnight. The digested solution was mixed with equal volume of phenol/chloroform/isoamyalcohol (25:24:1, Sigma) and gently agitated by manual shaking for 15 min. This mixture was centrifuged at 10,000 rpm for 10 min and the aqueous layer was transferred into a fresh sterilized tube. The above-described extraction by phenol/chloroform/isoamyalcohol was repeated once. The resultant aqueous layer was removed into a fresh tube and added with equal volume of chloroform: isoamyalcohol (24:1, Sigma). The resulting mixture was gently agitated by manual shaking for 15 min and then centrifuged at 10,000 rpm for 10 min. The upper aqueous layer containing DNA was transferred into a fresh tube and precipitated by the addition of twice the volume of 100% ethanol (-20°C) and 1/10 volume of 3 M NaAC (pH 5.2). Preciptated DNA pellets were spun down to the bottom of the tube at 10,000 rpm for 25 min. The resultant DNA pellet was washed twice with 70% ethanol and then dried in air or in a vaccum. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or Ultrapure H_2O (Biotech International, Perth, WA, Australia) and then stored at -20°C.

2.2.3 Agarose Gel Electrophoresis

DOP-PCR products (Section 2.2.6, below) were always run on a 1% agarose gel along with two DNA molecular markers of *SPP*-1 Phage DNA/*Eco*RI and *pUC*19 DNA/*Hpa*II (Geneworks, Australia). Normally, 5 µl of each Cy3- and Cy5-labelled DOP-PCR products was loaded into one single well of the gel. Conventional PCR products were always run on a 2% agarose along with one DNA molecular marker of *pUC*19 DNA/*Hpa*II. In this case, 10 µl

of each PCR product was loaded into one single well of the gel. The gel-loading buffer used was 6X buffer IV, containing 0.25% bromophenol blue and 40% (W/V) sucrose. Both the electrophoresis buffer of 0.5X TBE and the agarose gels always contained ethidium bromide with a concentration of 0.1-0.2 μg/ml. After electrophoresis, bands on the gels were visualised under ultraviolet radiation and captured by a Kodak digital camera DC120 (Amersham) followed by analysis using ID Kodak digital science software (Amersham).

2.2.4 Purification of DNA probes from an agarose gel

Purification of DNA probes from one 1-2% agarose gel was carried out using UltraClean™ GelSpin DNA purification kit (*Mo Bio* Laboratories, USA) according to the manufacturer's instructions. Briefly, a gel slice containing the desired DNA band was cut from the gel and its weight was determined. Up to 0.2 grams of gel was placed in the spin filter basket and 3 volumes of GelBind (NaClO4 solution) was added to the gel (0.1 gm/0.3 ml) followed by incubation at 65°C for 2 min or until the gel was completely melted. The spin filter was centrifuged at 10 000 g for 10 seconds. The liquid flow in the collection tube was mixed thoroughly and then reloaded back onto the spin filter basket. The spin filter was centrifuged again at 10 000 g for 10 seconds. The liquid flow in the collection tube was discarded and 300 μl GelWash buffer (Tris/Ethanol solution) was added into the spin filter basket. The spin filter was centrifuged one more time at 10 000 g for 10 seconds and the liquid flow was discarded. Centrifuging was resumed for another 30 seconds and then the spin filter basket was transferred to a fresh collection tube. 50 μl of Elution buffer (10 mM Tris) was added into the spin filter basket followed by centrifuging at 10 000 g for 30 seconds. The liquid flow in the collection tube contained the purified DNA.

For a gel slice over 0.2 grams in weight, more than one spin filter was used with 0.2 grams purified per spin filter.

2.2.5 Fluorescence in situ hybridization (FISH)

300-500 ng of whole chromosome-specific paints (WCPs) labelled by either SpectrumGreen-dUTP or SpectrumRed-dUTP (Vysis, USA) was mixed with 20 μg of Cot-1 DNA (GIBICO, BRL), 50 μg of Salmon sperm DNA (GIBICO, BRL) and precipitated with two volumes of 100% ethanol and 1/10 volume of 3 M NaAC (pH 5.2). The resulting probe solution was placed at -20°C for 2 hours and then centrifuged at 14,000 rpm for 25 min at 4°C. The precipitated DNA was washed once with 70% ethanol and centrifuged again. The resulting DNA pellets were dried at 60°C in an oven, and dissolved in 10 μl of hybridization solution (50% deionized formamide, 2X SSC, 0.1% SDS, 10% dextran sulfate, and 5X Denhardt's solution). The dissolved probe mixture was denatured at 80°C for 10 min and preannealed at 37°C for 30 min. In the meantime, metaphase slides were denatured in 70% deionized formamide/2X SSC, pH 7.0 for 5 min at 70°C. Slides were immediately dehydrated through an ethanol series (70%, 95%, and 100%) and then dried in the air at room temperature. Once preannealing was finished, the probes were applied to the slides, covered by coverslips, and sealed with rubber cement. Hybridization was carried out in a humid incubator at 37°C for 15~20 hours. Post-hybridization washing included twice in 2X SSC at 60°C for 10 min, twice in 0.1X SSC at 60°C for 5 min, once in 1X SSC at room temperature for 10 min, and three times of a brief rinse in MilliQ H_2O. After drying in the air in the dark, the slides were counterstained with DAPI, which was included in the antifade mountant.

Hybridized images were visualised and captured using an Olympus microscope (AHBT3) equipped with an Olympus Camera C-35AD-4 using Sensia II 400 film (RH-135, FUJI Photo

Film CO., Ltd., Tokyo, Japan). A DAPI image of metaphase chromosome spreads was capured in each case under UV excitation (334 nm). A second image was also taken in each case under blue excitation (490 nm) for SpectrumGreen-labelled probes or under triple excitation (simultaneously exciting at 334 nm, 490 nm, and 546 nm) for SpectrumRed-labelled probes. All films were processed in the Department of Clincal Photography, TQEH, Adelaide, SA, Australia, who also mounted films on slides, scanned images, and saved them as JPG formats.

2.2.6 Single-cell array CGH

2.2.6.1 Single-cell lysis

Lysis of single cells was carried out exactly as previously published (Cui et al. 1989). Briefly, 5 μl of lysis buffer (200 mM KOH, 50 mM dithiothreitol) was added to the 0.5 ml PCR tube containing a single cell and incubated at 65°C for 10 min followed by neutralization using 5 μl of neutralisation solution (300 mM KCl, 900 mM Tris-HCl, 200 mM HCl, pH 8.3).

2.2.6.2 First round of DOP-PCR for random amplification of single cells

First round of DOP-PCR was performed in a Minicycler (MJ Resrearch, USA) in a volume of 50 μl containing the single-cell lysed and neutralized solution (10 μl), 5 U of *Taq* polymerase (Applied Biosystems), and a final concentration of 50 mM KCl, 100 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatin, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2 μM DOP-PCR 6MW primer (5'-CCGACTCGAGNNNNNNATGTGG-3') (Telenius et al. 1992). The sample was centrifuged briefly, denatured at 95°C for 5 min, and cycled for 8 cycles of: 94°C for 1 min, 30°C for 1.5 min, 72°C for 3 min with a ramp of 1°C per 4 seconds between the annealing and the extension steps, followed by 26 cycles of 94°C for 1 min, 62°C for 1 min,

72°C for 3 min initially, but increased by 14 seconds for each cycle, and a final extension step at 72°C for 10 min.

2.2.6.3 Second round of DOP-PCR for Cy3/Cy5 labelling

First round of DOP-PCR products (5 μl) were labelled in a volume of 50 μl, containing 5 U of *Taq* polymerase (Applied Biosystems), and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 160 μM for each of dGTP, dCTP, and dATP, 120 μM dTTP, 40 μM of either Cy3-dUTP or Cy5-dUTP (Amersham Phamacia Biotech, USA), and 2 μM DOP-PCR 6MW primer. The sample was centrifuged briefly, denatured at 95°C for 4 min, and cycled for 25 cycles of: 94°C for 1 min, 62°C for 1 min, 72°C for 3 min initially but increased by 10 seconds for each cycle. An extension step at 72°C for 10 min was added at the end. Normally, 5 μl of each DOP-PCR product was run on 1% agarose gels in 0.5X TBE to check the quality of amplification (Section 2.2.3) and the remaining products were purified (Section 2.2.6.4).

2.2.6.4 Purification of Cy3- and Cy5-labelled products

Cy3-or Cy5-labelled DOP-PCR products were purified by UltraClean™ PCR Clean-up kit (*Mo Bio* Laboratories, USA) according to the manufacturer's instructions. Briefly, 5 volumes of SpinBind (Guanidine HCl/isopropanol) was added to the PCR products (45 μl) and then mixed thoroughly by pipetting. The PCR/SpinBind mixture was transferred to a spin filter unit and centrifuged at 14,000 rpm in a microcentrifuge for 10-30 seconds. The liquid flow in the collection tube was discarded and 300 μl SpinClean buffer (ethanol solution) was then added to the same spin filter unit followed by centrifuging at 14,000 rpm for 30-60 seconds. The collection tube containing the liquid flow was replaced with a fresh collection tube and 50 μl of Elution buffer (10 mM Tris, pH 8.0, DNase-free) was directly added onto the filter

membrane of the same spin filter unit followed by centrifuging 30-60 seconds at 14,000 rpm. The spin filter basket was discarded and the collection tube contained the purified Cy3- or Cy5-labeled probes. These purified probes were free of all PCR reaction components such as DOP-PCR 6MW primer, salt, *Taq* polymerase, and Cy3- and Cy5-dUTP and used immediately in array CGH or stored at -20°C at least for two months prior to array CGH analysis. 5 ul of each purified product was always run on a 1% agarose gel to check the efficiency of both labelling and purification.

2.2.6.5 Array CGH

Equal volumes (5 ~ 10 μl) of each of Cy3-labeled (test) and Cy5-labelled (reference) DOP-PCR products were mixed with 70 μg of human Cot-1 DNA (GIBCO, BRL), 20 μg of sheared salmon sperm DNA (GIBCO, BRL) and precipitated with two volumes of 100% ethanol, and 1/10 volume of 3 M NaAC (pH 5.2). The resulting mixture was placed at -20°C for 2 hours and then centrifuged at 14,000 rpm for 25 min at 4°C. The resultant DNA pellets were washed once with 70% ethanol followed by centrifuging at 14,000 rpm for 10 min at 4°C, dried either by air in the dark or at 60°C in an oven, and finally dissolved in 10 μl of hybridization solution (50% deionized formamide, 3X SSC, 0.1% SDS, 10% dextran sulfate, and 5X Denhardt's solution). After denaturation at 80°C for 10 min and preannealing at 37°C for 80 min, the probe mixture was applied to the array area and covered with a coverslip. Hybridization was carried out at 37°C for 15~20 hours in a humid incubator. After hybridization, the slides were immersed in 50% formamide/2 X SSC until the coverlips fell off by themselves (normally taking 10 min). Post-hybridization washing included twice in 50% formamide/2X SSC at 45°C for 10 min, twice in 2X SSC at 45°C for 5 min, once in 1X SSC at room temperature for 10 min, and three times of a brief rinse in MilliQ H_2O. All of the above solutions used in washing were filtered through a 0.22 μm filter (MILLIPORE, USA)

prior to washing. After washing, the slides were dried in the dark and then scanned immediately, or they could be stored in the dark at room temperature for at least 73 days.

2.2.7 Array scanning and Data analysis

GenePix 4000B is an integrated scientific instrument with a GenePix 4000B scanner for scanning slides and the software GenePix Pro for data analysis (Axon Instruments, Union City, CA, USA). GenePix 4000B lasers excite at 532 nm (green) and 635 nm (red). The emission filters are 575DF35 (green; ~557-592 nm) and 670DF40 (red; ~650-690 nm). These lasers and filters are optimized for Cy3 and Cy5. GenePix 4000B scanner scans Cy3 and Cy5 simultaneously and it takes about 5 minutes for a full scan of a standard microscope slide (25 mm x 75 mm) at a resolution of 10 microns (and under 12 minutes for a full scan at 5 microns resolution), and much less time for user-defined sub-scans.

2.2.7.1 Array scanning

Briefly, a Preview Scan (40 micron resolution) was used to locate the array on the slide and set the scanning parameters including Photomultiplier tube (PMT) voltages, scan area, and laser powers. A high-resolution (10 micron) Data Scan was then used to acquire the images for CGH analysis. Photomultiplier tube (PMT) gains (voltages) of both channels used in this study ranged from 400 to 900 whereas laser powers of both channels were always at 100% level. The primary data acquired by GenePix 4000B are the single-wavelength images, and by default these were saved as 16-bit grayscale TIFFs (Tagged Image File Format) in a single multi-image, which included the Cy5/Cy3 ratio image saved in both TIFF and JPEG (Joint Photographic Experts Group) format. TIFF files were used for analysis (Section 2.2.7.2, below) and JPEG files only for presentations.

2.2.7.2 Data analysis

As time goes by new versions of GenePix Pro come out. Two different versions including GenePix 3.0.6.81 and GenePix Pro 4.0.1.12 were used at different stages of this study, both of them perform analysis much the same way. As mentioned above, the 16-bit grayscale TIFF ratio images were used for analysis. Briefly, GenePix Pro used a GenePix Array List files (GAL file) to locate the size and position of all features. After analysis, the results were saved as GPR files (GenePix Results format), which included a header consisting of general information about image acquisition and analysis as well as the data extracted from each feature including more than 40 different parameters. In this study, the median of pixel-by-pixel ratios (Cy3/Cy5) of pixel intensities with the median background subtracted was selected for interpretation.

2.2.7.3 Exclusion of dots for analysis

Seven different parameters of the GPR files were used in this study for data filtering, including:

(1) Dia.: the diameter in μm of the feature-indicator

(2) > %B635 + 2 SD: the percentage of feature pixels with intensities more than two standard deviations above the background pixel intensity, at wavelength #1 (635 nm, for Cy5)

(3) > %B532 + 2 SD: the percentage of feature pixels with intensities more than two standard deviations above the background pixel intensity, at wavelength #2 (523 nm, for Cy3)

(4) SNR635: the signal-to-noise ratio at wavelength #1 (635 nm, for Cy5), defined by (Mean Foreground 1 - Mean Background 1)/(Standard deviation of Background 1)

(5) SNR532: the signal-to-noise ratio at wavelength #2 (532 nm, for Cy3), defined by (Mean Foreground 1 - Mean Background 1)/(Standard deviation of Background 1)

(6) F635 % Sat.: the percentage of feature pixels at wavelength #1 (for Cy5) that are saturated

(7) F532 % Sat.: the percentage of feature pixels at wavelength #2 (for Cy3) that are saturated

Dots were excluded from analysis if they failed to pass any of the following parameters of: (1) Dia. > 50 μm, (2) > %B635 + 2 SD > 70, (3) > %B532 + 2 SD > 70, (4) SNR635 >3.0, (5) SNR532 > 3.0, (6) F635 % Sat. = 0, and (7) F532 % Sat.= 0. The definitions of these parameters are available from http://www.axon.com/gn_GenePix_File_Formats.html (Axon Instruments).

2.2.7.4 Ratio Normalization

The mean of ratios for each chromosome was calculated from up to 8 qualified replicates. Normalization was then carried out using the 22 means of ratios from all autosomes assuming that the mean ratio value of all autosomes in each array CGH hybridization was 1.0. This normalization method can be found at: http://www.axon.com/mr_Axon_KB_Article.cfm?ArticleID=50 (Axon Instruments) and can be briefly described as follows:

(1) the median of ratios for all included dots was averaged for each chromosome to give the raw mean

(2) the Log value for each raw mean of median of ratios value is determined

(3) the Average of all of the Log values was calculated ("Avglog")

(4) the True average was calculated ("TrueAvg"), TrueAvg=10^Avglog)

(5) the Normalization Factor (NF) was determined (NF=1/TrueAvg)

(6) the Normalization factor was applied to rescale all raw means of median of ratios (Normalized mean of median of ratios = NF times the raw mean of median of ratios) to give the normalised ratios.

2.2.8 Analyses of single blastomeres by array CGH and locus-specific PCR

Three frozen human IVF embryos used in Chapter 6 of this study were obtained from IVF Australia, Westmead, NSW, Australia: where the embryos were thawed, briefly incubated, disaggregated, and each cell was aspirated into a polymerase chain reaction (PCR) tube. These PCR tubes were placed in dry ice and posted to our laboratory by express mail. A total of 12 single blastomeres were obtained from the three frozen embryos.

2.2.8.1 Array CGH analyses of single blastomeres

Array CGH analyses of single blastomeres were carried out exactly the same as previously described in Sections 2.2.6 and 2.2.7.

2.2.8.2 Molecular analyses of single blastomeres using locus-specific PCR

2.2.8.2.1 Random amplification of single blastomeres

5 μl (1/10 vol.) of each first round DOP-PCR product of single blastomeres (Section 2.2.6.2) was used to seed a second round of DOP-PCR, which was performed in a Minicycler (MJ Research, USA) in a volume of 50 μl, containing 5 μl of each first round DOP-PCR product, 5 U *Taq* DNA polymerase, and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.0 μM 6MW primer, 2.5 mM $MgCl_2$, and 0.25 mM of each of all four dNTPs. The reaction mix was centrifuged briefly, denatured at 95°C for 4 min, and cycled for 30-35 cycles of: 94°C for 1 min, 62°C for 1 min, 72°C for 3 min for the initial cycle, and increased

by 10 seconds for each subsequent cycle. An extension step at 72°C for 10 min was applied at the end of cycling amplification.

2.2.8.2.2 First round of locus-specific PCR

First round of locus-specific PCR was separately performed for each of the four DNA fragments. PCR was performed in a Minicycler (MJ, Research) in a volume of 50 µl, containing 1/10 volume (5 µl) of each second round DOP-PCR product, 1 U of *Taq* DNA polymerase (Applied Biosystems), 10 pM each of the forward and reverse primers (Table 2.1), and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, and 0.25 mM of each of all the four dNTPs. The sample was denatured at 95°C for 4 min, and cycled for 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min followed by a final extension step of 72°C for 5 min.

2.2.8.2.3 Second round of locus-specific PCR

Second round of locus-specific PCR was also separately performed for each of the four DNA fragments. PCR was performed in a Minicycler (MJ, Research) in a volume of 50 µl, containing 3.5 µl of each first round locus-specific PCR product, 1 U of *Taq* DNA polymerase (Applied Biosystems), 10 pM each of the forward and reverse primers (Table 2.2), and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, and 0.25 mM of each of all the four dNTPs. The sample was denatured at 95°C for 4 min, and cycled for 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min followed by a final extension step of 72°C for 5 min. 5 µl of each amplified product was run on a 1% agarose gel and photographed (Section 2.2.3).

Table 2.1. Primers used in the first round of locus-specific PCR amplification following preamplification of single blastomeres of IVF-created embryos

Locus	Orientation	Primer sequences (5' → 3')	Products (bp)	Reference
DMD, Exon 17	F	GACTTTCGATGTTGAGTTACTTTCCC	416	Chamberlain et al. (1990)
	R	AAGCTTGAGATGCTCTCACCTTTTCC		
DMD, Exon 47	F	CGGTCAAGTCGCTTCATTTT	365	
	R	ATCCACATACCAGCCTCCTC		
DMD, SRT45.1	F	GCATCCCACCCATCACCACATA	345	
	R	TCAAGAAGATTTTCAAAACCAG		
SRY	F	GTTGTCCAGTTGCACTTCGCTGCA	351	Cui et al. (1994)
	R	CAGTGTGAAACGGGAGAAAACAGT		

DMD: Duchenne Muscular Dystrophy
SRY: Sex-determining region Y
F: Forward primer for PCR
R: Reverse primer for PCR

Table 2.2. Primers used in the second round of locus-specific PCR amplification following preamplification of single blastomeres of IVF-created embryos

Locus	Orientation	Primer sequences (5' → 3')	Products (bp)	Reference
DMD, Exon 17	F	GCTGTCACCACCACTCAGCCATCA	154	Chamberlain et al. (1990)
	R	CAGAATCCACAGTAATCTGCCTCTTC		
DMD, Exon 47	F	CTAATCAATAGAAGCAAAGACA	309	
	R	GAAGCACCCAGGAAACAAAA		
DMD, SRT45.1	F	CTCTTTCCCTCTTTATTCATGTTAC[a]	~160	
	R	GAGGCTATAATTCTTTAACTTTGGC		
SRY	F	CATGAACGCATTCATCGTGTGGTC	254	Cui et al. (1994)
	R	CTGCGGGAAGCAAACTGCAATTCTT		

[a] Tagged with the Hexachlorofluorescein (HEX)
~Sizes of STR45.1 PCR products change if the number of the repeats is different
Others are the same as described in the legend to Table 2.1

2.2.8.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Amplified products of STR45.1 from the second round of locus-specific PCR (Section 2.2.8.2.3) were tagged with Hexachlorofluororescein (HEX). These products were further electrophoresed on a denaturing polyacrylamide gel containing 42% Urea and 5% Acrylamide:Bis-Acrylamide (19:1) using GEL-SCAN 2000 DNA Fragment Analyzer (CORBETT RESEARCH, Australia). Genescan-350 TAMRA (Applied Biosystem) DNA markers were always included. Briefly, 1 μl of each sample was mixed with 2 μl of denaturing loading buffer containing 50% deionised formamide. Samples were denatured at 96°C for 2 min and placed on ice. 1 μl of each denatured sample was loaded into the wells of the gel followed by electrophoresis in 0.6x TBE buffer for 45 min at 1200 volts. Images of the results were saved and converted to TIFF images, which were further analysed by Genescan software for sizing and analysing the profile of bands for each sample. The GEL-SCAN 2000 DNA Fragment Analyzer is capable of accurate fragment sizing and this was used for allele analysis of the short tandem repeat STR 45.1.

2.3 Statistical analysis

Statistical analyses were performed using Excel 97 (Microsoft Corporation, Redmond, WA, USA). Differences in ratios of CSLs were analysed using a single factor analysis of variance. The usual value of $P < 0.05$ was considered to be statistically significant.

Chapter 3

Materials and Methods II: Manufacture of the DNA microarrays

3.1 Introduction

The highest priority for array CGH experiments is to manufacture functional DNA arrays, and to achieve that goal adequate DNA probes are required. DNA probes currently used in arrray CGH include cDNAs, oligonucleotides, and PAC and BAC clones (Section 1.6.3). Single-cell array CGH has not yet been reported using any of these DNA probes. One possible reason might be that these DNA probes could not produce signals sensitive enough for single-cell array CGH analysis. In this study, chromosome-specific DNA libraries (CSLs) were spotted on arrays as dots in the hope that such arrays could be used to assess copy number changes of a whole chromosome in single-cell array CGH because they could theoretically produce more sensitive hybridization signals than the probes mentioned above. Whole chromosome-specific paints (WCP) were the only commercially-available chromosome-specific DNA libraries at the beginning of this project and these were therefore tested initially in this study. However, these commercial WCPs were specifically provided for FISH studies and were therefore always labeled with either fluorophores or biotin/digoxin when purchased from the companies. CSLs might have been made by our own laboratory using microdissected or flow-sorted chromosomes but a whole set of unlabeled chromosome-specific paints was obtained (Bolzer et al. 1999) for use in this study

3.1.1 Terminology

The reader is warned that in recent terminology, the dots of DNA in arrays are termed "probes" and the sample DNA applied to them is the target. This is a reversal of the terminology used for FISH etc., where the target is the chromosomes and the probe is regarded as the DNA in the solution applied to a slide. The terminology used in this thesis is that the words: array dots, spots, and probes, are used interchangeably.

3.2 Manufacture of the first batch of arrays

Two chromosome-specific DNA painting probes of WCP 21q SpectrumOrange for chromosome 21 and WCP X SpectrumGreen for chromosome X (designated respectively as 21_{vys} and X_{vys} in this thesis) were spotted on the first batch of arrays. Both 21_{vys} and X_{vys} were amplified by DOP-PCR prior to array spotting. The aim of these arrays was to test the feasibility of diagnosis of trisomy 21 and gender determination using single-cell array CGH.

3.2.1 Array probes

Both 21_{vys} and X_{vys} contained the fluorophore-labelled painting probe and blocking DNA (Vysis, USA). 21_{vys} paints the bands 21q21-q22 of human chromosome 21, resulting in orange fluorescence along the length of the 21q arm; 21_{vys} does not hybridise to the p arm or the centromere of chromosome 21. X_{vys} hybridises to the Xp arm, Xq arm, and to the bands Xp11.1–Xq11.1 of human chromosome X, resulting in green fluorescence along the length of chromosome X.

3.2.2 Amplification of the array probes by DOP-PCR

Briefly, 1 μl of original probe was diluted into 19 μl of Ultrapure H_2O (Biotech, Australia). DOP-PCR was performed in a Minicycler (MJ Research, USA) in a volume of 50 μl, containing

5 μl of the diluted probe solution, 5 U *Taq* DNA polymerase, and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.0 μM 6MW primer, 2.5 mM $MgCl_2$, and 0.25 mM of each of all four dNTPs. The sample was centrifuged briefly, denatured at 95°C for 4 min, and cycled for 30-35 cycles of: 94°C for 1 min, 62°C for 1 min, 72°C for 3 min initially, but increased by 10 seconds for each cycle. An extension step at 72°C for 10 min was applied at the end of cycling amplification. 5 μl of each DOP-PCR product (first round) was run on a 1% agarose gel (Section 2.2.3) and the remaining was purified (Section 2.2.6.4). 5 μl of the purified products was used for another round of DOP-PCR (second round) carried out exactly as described immediately previously.

3.2.3 Array spotting

DOP-PCR-amplified 21_{vys} and X_{vys} products were dissolved in a final concentration of 500 ng/μl and then spotted on glass slides with an estimated 0.3 ng of DNA per dot by the Microarray Facility of Australian Genome Research Facility, Walter & Eliza Hall Institute of Medical Research, Victoria, Australia.

3.2.4 Post-processing of arrays

The boundaries of each array area were clearly marked on the backs of slides with a diamond scriber. Rehydration was conducted by putting the arrays face down over 1 X SSC in a humid slide chamber (Shandon-Lipshaw) until the dots glistened and swelled slightly but did not run into each other. After snap-drying on a 70-80°C inverted heat block for a few seconds, UV crosslinking DNA to glass was carried out with an Stratalinker set for 65 mJ. Chemical blocking was achieved by immersing a slide rack with the arrays in a blocking solution containing 6g succinic anhydride (Aldrich), 325 ml 1-methyl-2-pyrrolidinone (Aldrich), and 15 ml sodium borate (1 M, pH 8.0) for 15-20 min. Orbital shaking was conducted during the chemical blocking.

The array slides were then denatured at 95°C in MilliQ H_2O for 2 min, and immediately dehydrated once in 95% ethanol at room temperature. The array slides were immediately centrifuged in a plate centrifuge at 500 rpm for 5 min to avoid streaking. The slides could be used immediately for array CGH, or stored in a slide box in the dark at room temperature for up to 2 months.

3.2.5 Array CGH analysis

Array CGH experiments were carried out as previously described (Section 2.2.6) except that SpectrumGreen-dUTP and SpectrumRed-dUTP were used for labeling. Slide scanning and data analysis was also as previously described (Section 2.2.7).

3.2.6 Results from the first batch of arrays

Both original 21_{vys} and X_{vys} probes were DOP-PCR products (phone communication with Vysis, USA), and therefore they were expected to be sucessfully amplified by DOP-PCR in the present study. The amplified products showed a majority of DNA fragments ranging from 200 bp to 800 bp after runing for 45 min on a 1% agarose gel (Fig. 3.1A).

The basic layout of the first batch of arrays is shown in Fig. 3.1B. Single-cell array CGH experiments using normal male (red)/female (green) comparison was carried out (Section 3.2.5) and scanned by a GenePix 4000B scanner. The ratio (red/green) image for one experiment, saved in TIFF format, is shown in Fig. 3.1B; The corresponding JPEG image was not available due to inexperience in data management at the early stage of this study. The signal intensity of the 21_{vys} dots was uniform and unexpectedly much lower than that of the X_{vys} dots (Fig. 3.1B), so both 21_{vys} and X_{vys} could not be used reliably for either gender determination or diagnosis of ploidy of

chromosome 21 in array CGH analysis. Consequently, no more array CGH experiments were tested on this batch of arrays.

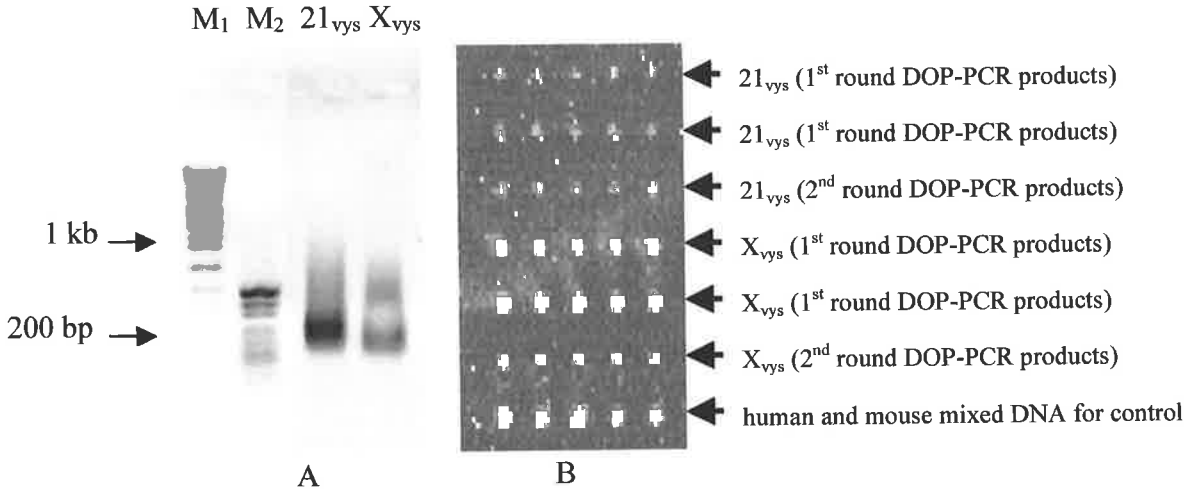


Figure 3.1. Manufacture of the first batch of arrays. A): DOP-PCR products of 21_{vys} and X_{vys} run on a 1% Agarose gel: DNA molecular markers *SPP*-1 DNA/*Eco*RI (M_1) and pUC19/*Hpa*II (M_2). The origin of samples is indicated above each lane. **B)**: Layout of the first batch of arrays: 7 rows were spotted on each array, each row had five identical replicates of the same sample. Both 21_{vys} and X_{vys} were spotted in three different rows, two of which contained first round DOP-PCR products and the third row had second round DOP-PCR products. A mixture of human and mouse genomic DNA was spotted at the bottom row as positive (internal) controls.

3.2.7 Discussion of the first batch of arrays

As previously mentioned (Section 3.2.1), the original 21_{vys} and X_{vys} were fluorophore-labeled DNA probes containing blocking DNA reagents, and therefore autofluorescence and non-specific hybridization might be problematic for accurate array CGH. However, the process of DOP-PCR amplification should have diluted the fluorochromes applied commercially to these probes to an insignificant level. If this was not the case, a drop in the intensity for the 2nd round DOP-PCR products should have been noticeable. For the 21_{vys}, the intensity of the dots containing the 2nd-round DOP-PCR products was not different from that produced by the 1st round DOP-PCR products. But for the X_{vys} dots, they showed a slight reduction (Fig. 3.1B) in the intensity of the dots containing the 2nd round DOP-PCR products, indicating that for X_{vys} perhaps the fluorochrome applied by Vysis, which was Spectrum Green, might have a high autofluorescence, even after dilution by DOP-PCR amplification (Section 3.2.2).

3.3 Manufacture of the second batch of arrays

After the failure of the first batch of arrays, a whole set of human chromosome-specific DNA paints (CSLs) was obtained, all of which were both unlabelled and repeat-depleted (Bolzer et al. 1999) and therefore qualified as the best probes suitable for this study at that time. The CSLs were designated, for this thesis, as CSL_1, CSL_2 ··· CSL_{22}, CSL_X, and CSL_Y for all the 24 human chromosomes. These CSLs were amplified by DOP-PCR and then spotted on the second batch of arrays. With all human CSLs, these arrays would be used to test the feasibility of detecting aneuploidies involving all chromosomes by single-cell array CGH.

3.3.1 Repeat-depleted chromosome-specific DNA paints (CSLs)

The whole set of CSLs was provided by Dr A. Bolzer, from the Institute für Anthropologie und Humangenetik, LMU, München, Germany, and Dr J.M. Craig, presently at the Murdoch Children's Research Institute, Melbourne, Australia. These CSLs were initially generated, either by microdissection for 15 chromosomes (1, 3, 6, 7, 9,12-15, 17, 19-22, and X) (Guan et al. 1994), or by flow-sorting, for the chromosomes 2, 4, 5, 8, 10, 11, 16, 18, and Y (Bolzer et al. 1999). In order to avoid cross-hybridisation in FISH study among the p arms of the five acrocentric chromosomes, 13, 14, 15, 21, and 22, only their q arms were microdissected to construct CSLs for these chromosomes (Guan et al. 1994). Repetitive sequences were later depleted from these CSLs by affinity chromatography in combination with negative subtraction hybridization using human Cot-1 DNA as subtractors (Craig et al. 1997; Bolzer et al. 1999). Centromere-specific repetitive sequences were further depleted from the CSLs of chromosomes 1, 3, 12, 18, 19, and X by complex procedures (Bolzer et al. 1999). In addition, repetitive sequences were removed from CSL_{14} using DNA from chromosome 14 itself as a subtractor and from CSL_{22} using DNA from both chromosomes 14 and 19 as subtractors. In order to minimize contamination derived from the

depletion process, a form of PCR termed CTA_4DOP was specifically developed to recover the repeat-depleted CSLs (Craig et al. 1997). In FISH, all these CSLs painted their entire target chromosomes or the q-arms of the five acrocentric chromosomes without the addition of Cot-1 DNA or a preannealing step prior to hybridization (Craig et al. 1997).

3.3.2 Amplification of all CSLs by DOP-PCR

1 μl of each of the CSLs was diluted with 9 μl of Ultrapure H_2O (Biotech Internationals, Australia). DOP-PCR was performed as previously described (Section 3.2.2) except that 2 μl of the diluted CSL solution was employed.

3.3.3 Amplification of all CSLs by CAT_4 DOP-PCR

CTA_4 DOP-PCR was performed in a Minicycler (MJ Research, USA). The 50 μl of PCR reaction mixture contained: 2 μl of the diluted CSL solution, 5 U *Taq* DNA polymerase, and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5-2.0 μM CTA_4DOP primer (5'-CTACTACTACTACGACTCGAG-3') (Craig et al. 1997; Bolzer et al. 1999), 2 mM $MgCl_2$, and 0.2 mM of each of the all four dNTPs. PCR conditions for CTA_4 DOP-PCR were exactly as reported and were: the reaction mixture was denatured at 96°C for 5 min followed by 4 cycles of: 96°C for 1 min, 48°C for 1.5 min, 72°C for 3 min, and then another 17 cycles of: 96°C for 1 min, 53-55°C for 1.5 min, 72°C for 3 min, with the addtion of 1 second per cycle to the extension time.

3.3.4 DOP-PCR amplification of genomic DNA

Extraction of the genomic DNA of a normal male (Male Donor 1) was carried out as previously described (Section 2.2.2). DOP-PCR was performed in a Minicycler (MJ Research, USA). The total 50 μl of PCR reaction mixture contained 10 ng of genomic DNA, 5 U *Taq* DNA polymerase, and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 0.2

mM of each of the all four dNTPs, 2.0 μM DOP-PCR 6MW primer. Cycling conditions were as previously described (Section 2.2.6.2).

3.3.5 Array spotting

26 samples spotted on slides as the second batch of arrays included all of the 24 DOP-PCR-amplified CSLs, one positive sample containing DOP-PCR products of normal male genomic DNA (Male Donor 1), and one negative control containing H_2O. After purification (Section 2.2.6.4), amplified products were dissolved at a concentration of 250 ng/μl in 3 X SSC solution, and then spotted in 8 replicates for each sample on glass slides (Polysine^{TM} microscope glass slides, Menzel-Glaser, Germany) by Microarray facility of Molecular Biosciences, Adelaide University, Australia. Post-processing of arrays was then conducted (Section 3.2.4).

3.3.6 Results from the second batch of arrays

All DOP-PCR-amplified CSLs were smears with the majority less than 1 kb after run on a 1% agarose gel at 80 Volts for 30 min (Fig. 3.2A). These smears were quite similar to those (ranging from 200 bp to 800 bp) reported by Guan et al. (1994), thereby indicating the successful amplification of all CSLs. However, smears extending up to more than 3 kb were seen for eleven chromosomes (1, 2, 4, 5, 8, 9, 10, 11, 16, 21, and Y); Among them, CSL_1, CSL_9, and CSL_{21} were initially produced by microdissection and the remaining by flow-sorting (Bolzer et al. 1999). Comprehensive optimisation of DOP-PCR protocols, including changing the temperature and time of both annealing and elongation steps, amplification cycles, quantities of templates, amounts of *Taq* polymerase, and concentrations of $MgCl_2$, failed to obtain the typical smears for these eleven CSLs; an example of these experiments conducted for CSL_9 is shown (Fig. 3.3). No products were found on the gel when only 2.5 U of *Taq* polymerase was used, indicating that a high amount of *Taq* polymerase was required for DOP-PCR due to its lengthy elongation step

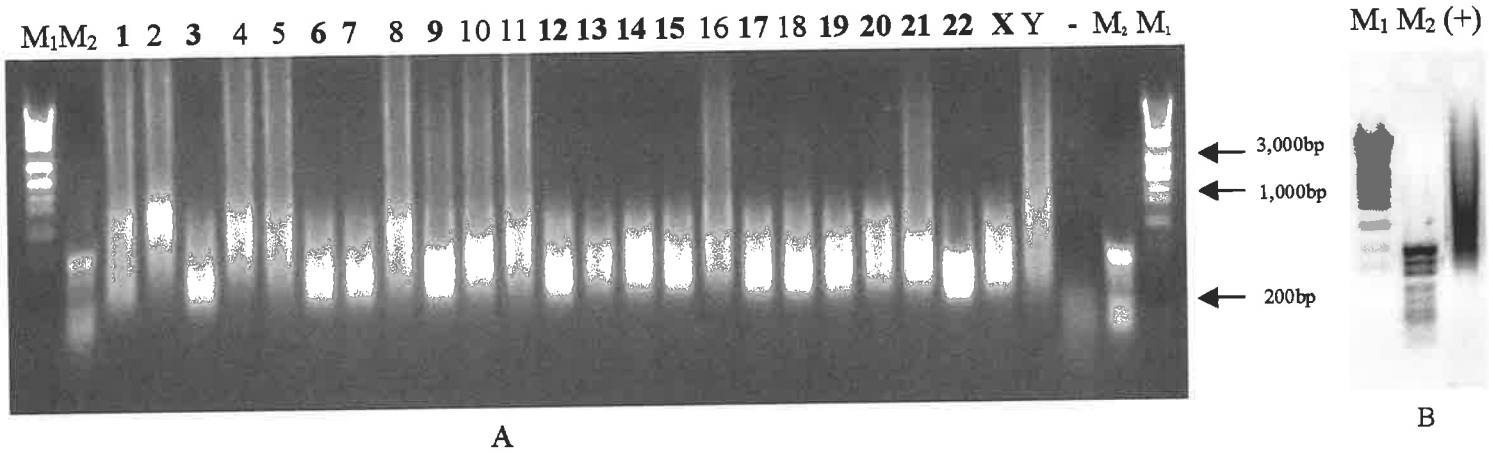


Figure 3.2. Electrophoresis of all 24 DOP-PCR-amplified CSLs (A) and the positive control (B) on a 1% agarose gel. The origin of each sample is indicated above each lane. CSLs made by microdissection are indicated in bold, the remainder were made by flow-sorting. Positive control (+) was DOP-PCR products amplified from a normal male genomic DNA (Male Donor 1), and negative control (-) had no target DNA added to the DOP-PCR reaction. All these 26 samples were spotted on the second batch of arrays. DNA markers were *SPP*-1/EcoRI (M_1) and pUC19/*HpaII* (M_2).

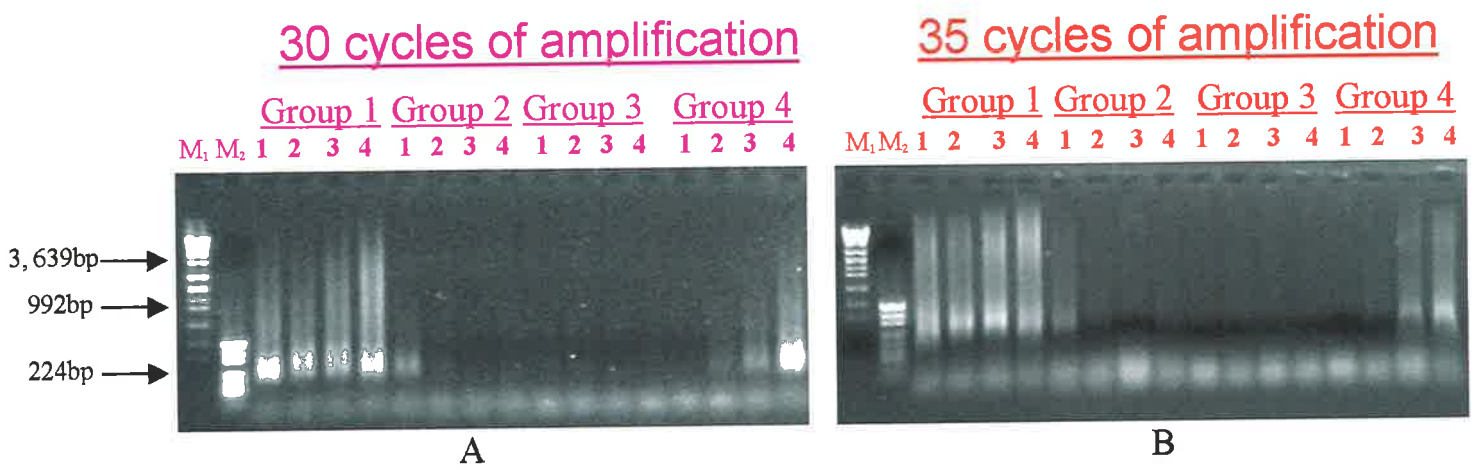


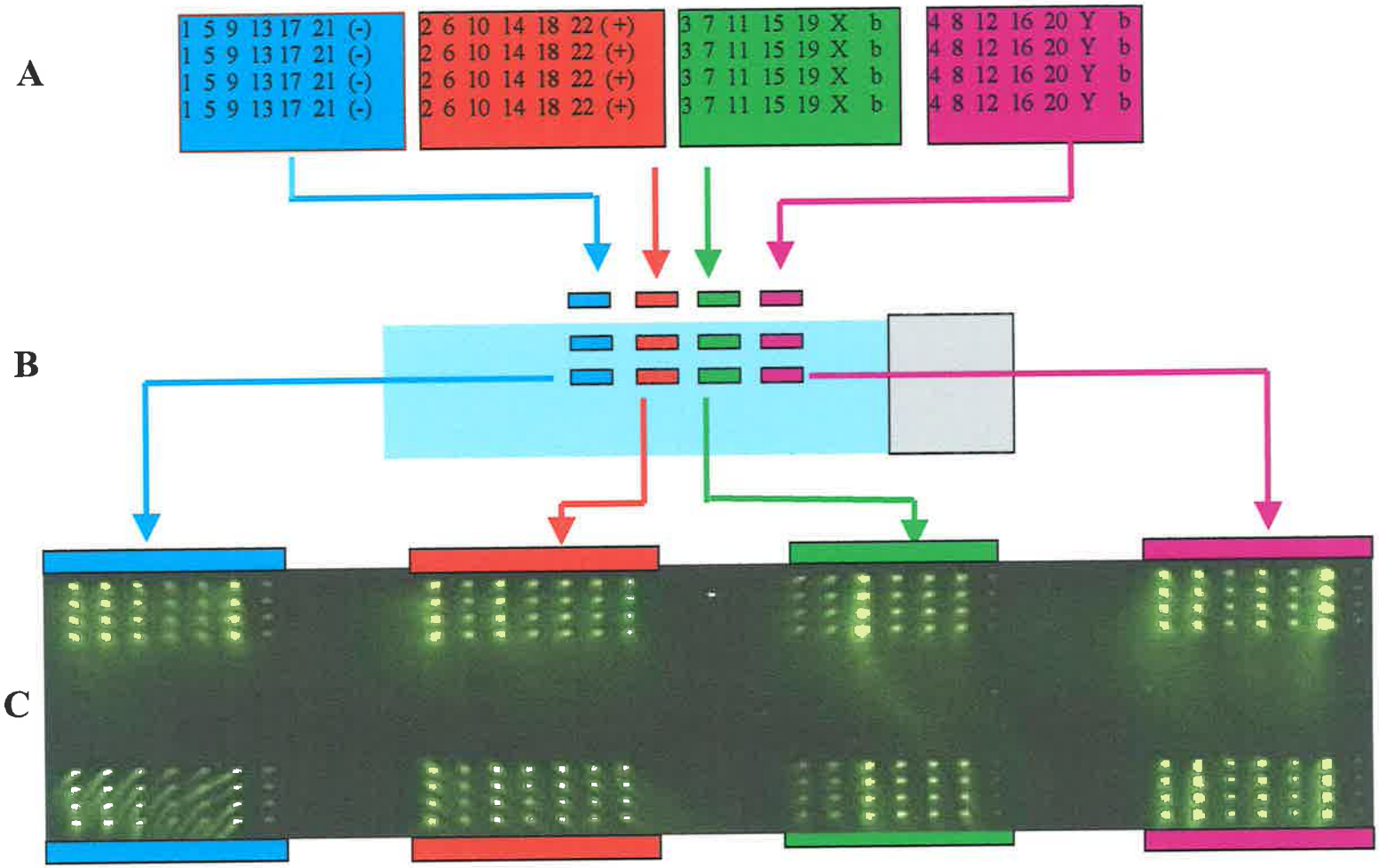
Figure 3.3. Optimisation of DOP-PCR protocols for chromosome 9-specific CSL. DOP-PCR was tested using 100 ng, 75 ng, 50 ng, and 150 ng of template CSL_9 DNA (designated as 1, 2, 3, and 4, respectively). Each level was amplified under four different PCR conditions, including Group 1 (2.5 mM $MgCl_2$ and 5 u of *Taq* polymerase), Group 2 (2 mM $MgCl_2$ and 2.5 u of *Taq* polymerase, Group 3 (1.5 mM $MgCl_2$ and 2.5 u of *Taq* polymerase), and Group 4 (1.5 mM $MgCl_2$ and 5 u of *Taq* polymerase). Consequently, a total of 16 different DOP-PCR reactions was performed. **A**): At the end of 30 cycles of amplification, 5 µl was taken out of each PCR tube and run on a 1% agarose gel. **B**): DOP-PCR was then continued for up to 35 cycles of amplification, at which stage another 5 µl was taken out of each PCR tube and run on another 1% agarose gel. The best amplification (1st condition of Group 1 in **A**) was observed when 30 cycles of amplification were carried out using 100 ng of template DNA, 5 u of *Taq* polymerase, and 2.5 mM $MgCl_2$.

(Telenius et al. 1992). The best amplification was observed when 30 cycles of amplification were carried out using 100 ng of template DNA, 5 u of *Taq* DNA polymerase, and 2.5 mM $MgCl_2$. All original CSLs were CTA_4-DOP-PCR products so that CTA_4-DOP-PCR was initially tried to amplify them but it failed to produce the typical smear for almost all CSLs (Fig. 3.4). Consequently, DOP-PCR were used to amplify the CSLs for array manufacture. DOP-PCR products of genomic DNA also gave a smear with the majority of DNA fragments ranging from 400 bp to 1 kb (Fig. 3.2B).

Figure 3.4. Electrophoresis of all 24 CTA_4 DOP-PCR-amplified CSLs on a 1% agarose gel. The origin of each sample is indicated above each lane. CSLs made by microdissection are indicated in bold, the remainder were made by flow-sorting. DNA markers were *SPP*-1/*Eco*RI (M_1) and pUC19/*Hpa*II (M_2).

The scheme for the layout of the second batch of arrays is shown in Figure 3.5. Each array had 8 blocks consisting of 4 different pairs of blocks, each block being vertically duplicated. Within each block, 4 replicate dots of each sample were spotted next to each other in columns. Therefore, each array had eight replicate dots for each chromosome or control. One slide was tested for single-cell array CGH and the resultant JPEG ratio (Cy5/Cy3) image is shown (Fig. 3.5). Based on this image, as expected all dots were present and evenly shaped except that some dots had comet tails (smearing). The negative sample gave almost no hybridisation and the positive sample produced strong hybridisation. This batch of arrays was comprehensively tested for single-cell array CGH (Chapter 4).

Figure 3.5. Manufacture of the second batch of arrays. These arrays had 8 blocks arrayed in 4 duplicate blocks (blue, red, green, and purple). **A** and **B**): Within each block, 4 replicate dots of each sample were vertically spotted in a column. Samples were spotted in the orders of $CSL_{1, 5, 9, 13, 17, 21, (-)}$ (blue), $CSL_{2, 6, 10, 14, 18, 22, (+)}$ (red), $CSL_{3, 7, 11, 15, 19, X, (b)}$ (Green), $CSL_{4, 8, 12, 16, 20, Y, (b)}$ (purple). Negative control (-) contained spotting buffer and two blank controls (b) were spotted with empty printing pins. The image (**C**) is somewhat distorted; the real dimensions of the entire array were approximately 1 cm X 2 cm. The center-to-center spacing between two adjacent dots was 400 µm. The diameter of individual dots was around 100 µm. The large spacing between blocks was not deliberately designed but caused by mechanical restriction of array printing. **C**): The JPEG image of the Cy5 and Cy3 fluorescence intensity obtained from single-cell array CGH. The negative sample gave virtually no hybridisation signal. The positive sample produced strong hybridization signals.


A
1 5 9 13 17 21 (-)
1 5 9 13 17 21 (-)
1 5 9 13 17 21 (-)
1 5 9 13 17 21 (-)
2 6 10 14 18 22 (+)
2 6 10 14 18 22 (+)
2 6 10 14 18 22 (+)
2 6 10 14 18 22 (+)
3 7 11 15 19 X b
3 7 11 15 19 X b
3 7 11 15 19 X b
3 7 11 15 19 X b
4 8 12 16 20 Y b
4 8 12 16 20 Y b
4 8 12 16 20 Y b
4 8 12 16 20 Y b
B
C

3.4 Manufacture of the third batch of arrays

Differences in the normal ratio deviations were subsequently observed among different chromosomes in single-cell array CGH experiments using the second batch of arrays (Chapter 4). Unexpected deviations exceeding the chosen ratio threshold of 0.75 to 1.25 for diagnosis of aneuploidy could be seen for some chromosomes, especially those with a higher molecular smear in their CSLs, leading to misdiagnosis of aneuploidy for these chromosomes. In order to solve this problem, the third batch of arrays was manufactured using the eleven modified CSLs, which had the high molecular weight smear removed. All original CSLs were also spotted on this batch of arrays for comparative study. In addition, the third batch of arrays also had some dots containing individual PCR products (<1 kb) to test the sensitivity of single-cell array CGH analysis.

3.4.1 Array probes

45 different samples were spotted on the third batch of arrays, including:

(1) 11 modified CSLs for chromosomes 1, 2, 4, 5, 8, 9, 10, 11, 16, 21 and Y

(2) 6 individual PCR-amplified fragments of genes of Duchenne Muscular Dystrophy (DMD), Sex-determining Y region (SRY), and cystic fibrosis (CF)

(3) 2 probes (21_{vys} and X_{vys}) already tested on the first batch of arrays (Section 3.2.1)

(4) 24 original CSLs already tested on the second batch of arrays (Section 3.3.1)

(5) 1 negative control, containing H_2O

(6) 1 positive control sample already tested on the 2^{nd} array (Section 3.3.4)

3.4.2 Preparation of PCR-amplified fragments

Six individual DNA fragments were amplified from a normal male genomic DNA using conventional PCR. One fragment was amplified from the SRY gene and another from the gene responsible for Cystic Fibrosis. The other four fragments were from the DMD gene, which were amplified from the exons 17, 44, 45, and 48. Each fragment was separately amplified in a Minicycler (MJ, Research) in a volume of 50 µl, containing 100-300 ng of genomic DNA, 1 U of *Taq* DNA polymerase (Applied Biosystems), 10 pM each of the forward and reverse primers (Table 3.1), and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, and 0.25 mM of each of all the four dNTPs. The sample was denatured at 95°C for 4 min, and cycled for 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min followed by a final extension step of 72°C for 5 min. 5 µl of each PCR product was run on a 1% agarose gel (Section 2.2.2) and the remainder was purified (Section 2.2.6.4). Primers for amplification of the SRY gene and the exon 17 of the DMD gene were previously given in Table 2.1.

3.4.3 Small-scale preparations of modified CSLs for FISH

Small-scale preparation (Fig. 3.6) was used for FISH analysis. Briefly, a 1% agarose gel was prepared using 0.5 X TBE buffer. Ethidium bromide (Sigma) was incorporated into both the gel and electrophoresis buffer (0.5X TBE) at a concentration of 0.2 µg/ml. 5 µl of each DOP-PCR-amplified CSL (Section 3.3.2) was loaded onto the gel with 6X gel loading buffer [0.25% bromophenol blue and 40% (W/V) sucrose]. After electrophoresis at 80 volts for 30 min, the gel slice containing the desired part of smear (ranging from 200 bp to 1000 bp) was cut from the gel under UV radiation (254 nm). DNA was then purified from the gel slice (Section 2.4).

For FISH analysis (Fig. 3.7), the purified DNA was labeled by DOP-PCR. The total 50 μl of PCR reaction mixture contained the purified DNA, 5U of *Taq* polymerase and a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 160 μM for each of dGTP, dCTP, and dATP, 120 μM dTTP, 40 μM of either SpectrumRed-dUTP or SpectrumGreen-dUTP (Vysis, USA), and 2 μM DOP-PCR 6MW primer. Cycling conditions were the same as used previously (Section 3.2.2). The resulting labelled products were purified (Section 2.2.6.4) and then used immediately for FISH studies (Section 2.2.5) or stored at -20°C.

3.4.4 Large-scale preparations of modified CSLs for array spotting

Large-scale preparation of the modified CSLs was used for array manufacture. To avoid contamination by both ethidium bromide and bromophenol blue, special strategies were used to cut the gel slice from the gel and are therefore described in some detail (Fig. 3.8). Briefly, a 1% agarose gel was made with three small wells at the left for loading DNA markers and the control sample and large wells to the right of these small wells for loading 50-80 μl of DOP-PCR-amplified CSL sample (Fig. 3.8A1). Both the gel and electrophoresis buffer (0.5X TBE) was free of ethidium bromide. A modified 6X gel loading buffer containing 40% sucrose was applied to load samples onto the gel. After electrophoresis at 80 volts for 30 min, the left part of the gel containing DNA markers and the control sample was cut from the gel (Fig. 3.8A2), and then immersed in 0.5X TBE buffer containing ethidium bromide (0.5 μg/ml) for 30-45 minutes. The stained gel slice was photographed under UV radiation (Section 2.2.3) (Fig. 3.8A3) and then reunited with the unstained right part of the gel (Fig. 3.8A4). Under UV-radiation and the guidance of both the visible stained markers and the control sample at the left, the gel slice containing the smear (ranging from 200 bp to 1 kb) of each sample at the right was determined and cut from the gel (Fig. 3.8A5). The rest of the right part of gel was stained with ethidium

bromide, and photographed under UV radiation (Section 2.2.3) (Fig. 3.8A6), and then reunited with the previously stained left part of the gel (Fig. 3.8A7). In the meantime, DNA was purified from the gel slice and eluted into 50 μl of Utrapure H_2O (Section 2.2.4). 5 μl of each purified modified CSL was then run on a 1% agarose gel (Fig. 3.8B) (Section 2.2.3).

3.4.5 Array spotting

All samples were dissolved in 3X SSC with a concentration of around 250 ng/μl and then spotted on glass slides (Superfrost glass slides, Menzel-Glaser, Germany) by the Microarray facility of Molecular Biosciences, Adelaide University, Australia. 50 slides were spotted. However, evaporation of spotting solution nearly to drying-out occurred prior to the completion of the array spotting, therefore the microarrayer failed to spot many of the designed dots on the slides. Post-processing of arrays was conducted as previously described (Section 3.2.4).

3.4.6 Results from the third of batch of arrays

Modification of the eleven CSLs, CSL_1, CSL_2, CSL_4, CSL_5, CSL_8, CSL_9, CSL_{10}, CSL_{11}, CSL_{16}, CSL_{21}, and CSL_Y was achieved by cutting the desired parts (ranging from 200 bp to 1 kb) of their smears from a 1% agarose gel, and designated in this thesis as CSL_{1m}, CSL_{2m}, CSL_{4m}, CSL_{5m}, CSL_{8m}, CSL_{9m}, CSL_{10m}, CSL_{11m}, CSL_{16m}, CSL_{21m}, and CSL_{Ym}. Small-scale preparation (Fig. 3.6) generated tiny amounts of DNA probes just enough for FISH analysis (Fig. 3.7), which proved the specificity of all modified CSLs. Large-scale preparation (Fig. 3.8) was required for array manufacture, and a more complicated procedure was designed to avoid contamination of both ethidium bromide and bromophenol blue. Consequently, any potential adverse impact of these two reagents on array CGH could be avoided. In addition, six specific DNA fragments, E17, E44, E45, E48, SRY, and CF, were amplified by standard PCR (Table 3.1) and verified on a 1% agarose gel (Fig. 3.9).

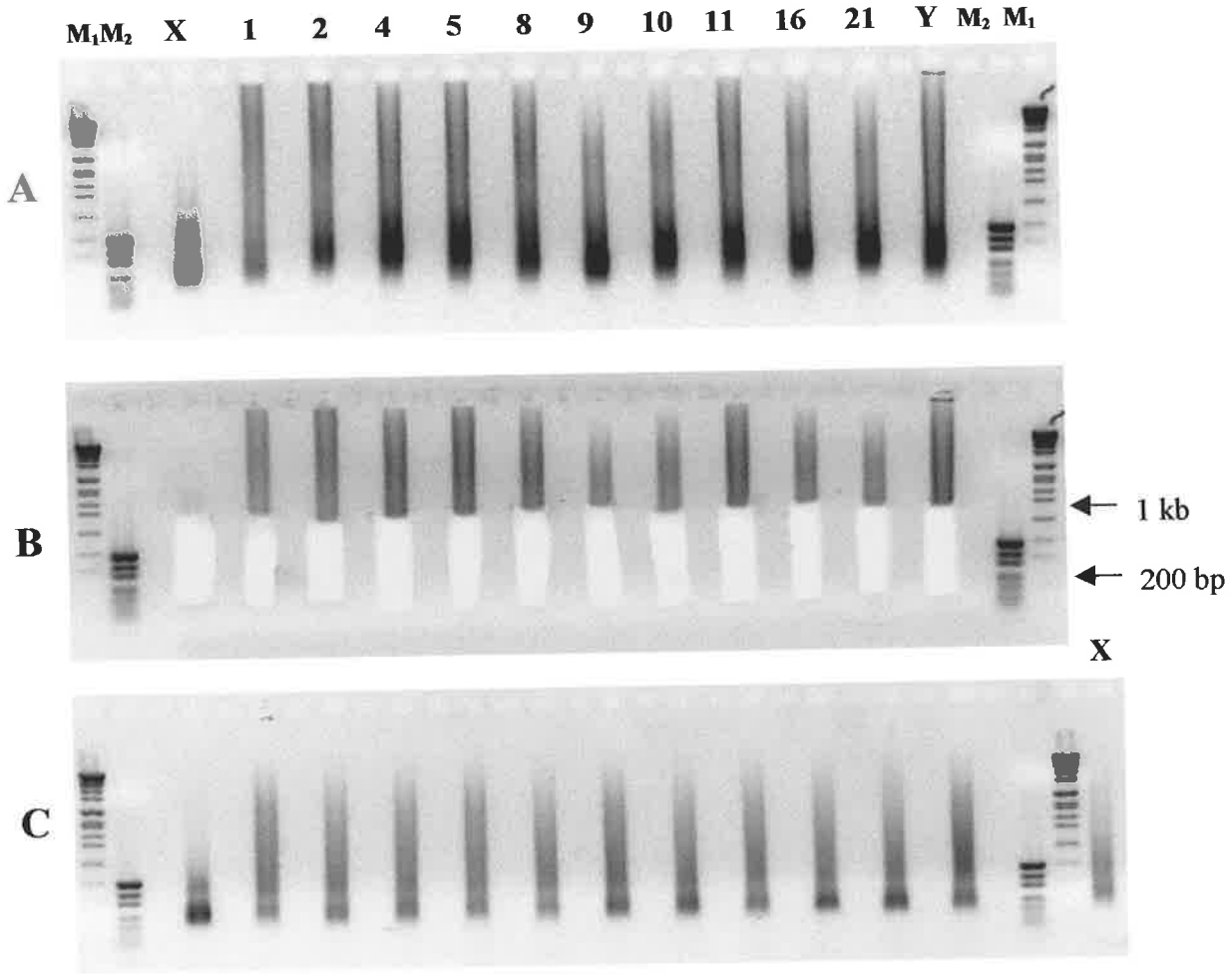


Figure 3.6. Small-scale preparation of modified CSLs for FISH analysis. Excision of the desired low molecular weight region of the smear (ranging from 200-1000bp) from a 1% agarose gel was conducted for eleven CSLs from 5µl of each DOP-PCR amplified CSL. The origin of each chromosome is indicated above each lane. Images shown were captured before (**A**) and after (**B**) the cutting procedure. DOP-PCR amplification of the recovered modified CSLs is shown in (**C**). The chromosome X-specific CSL was used as guide for the cutting procedure (**A**) and (**B**) and the positive control for DOP-PCR amplification (**C**). DNA markers were *SPP*-1/*Eco*RI (M_1) and pUC19/*Hpa*II (M_2).

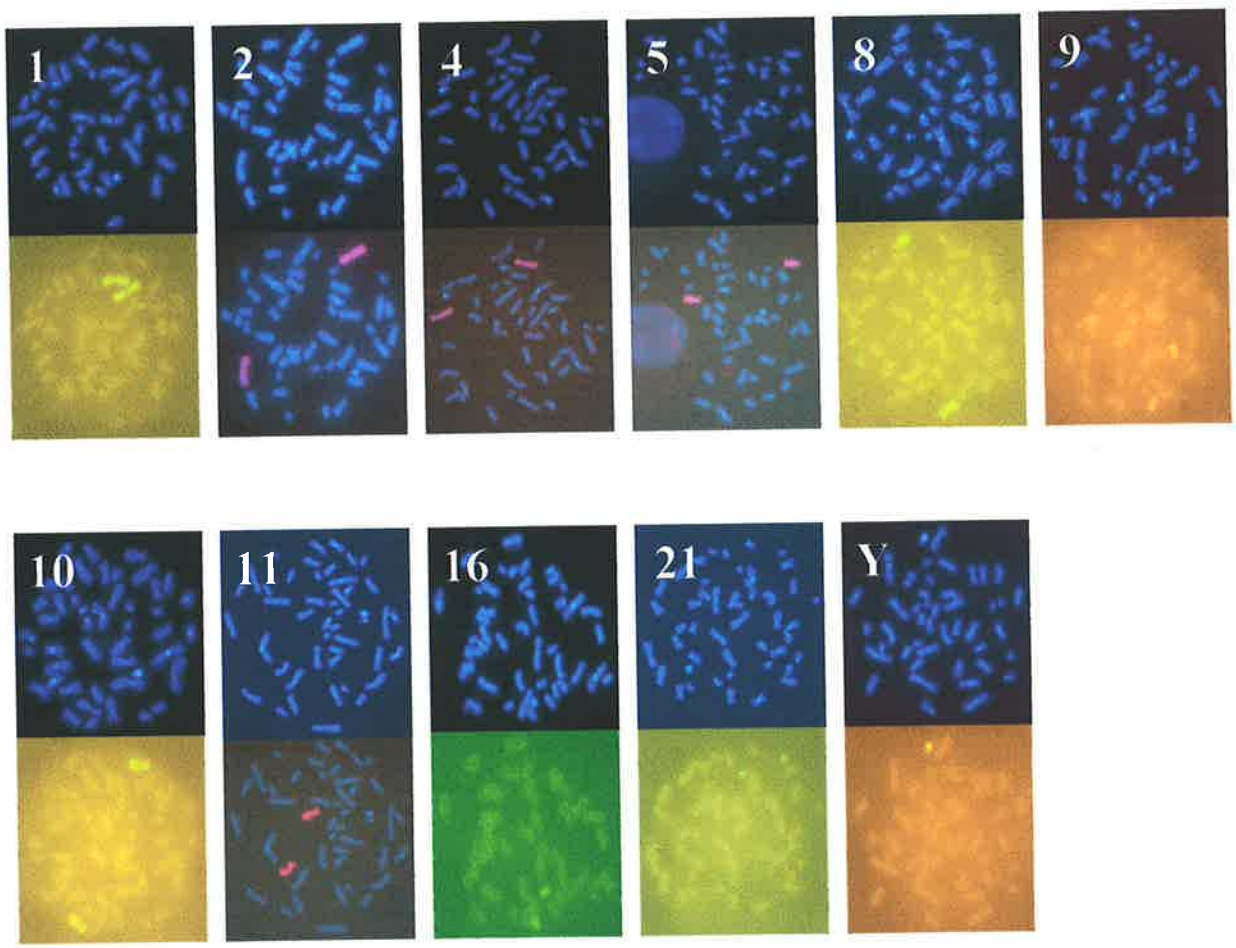


Figure 3.7. FISH images of eleven modified CSLs. CSLs labeled either by Spectrum Red-dUTP or Spectrum Green-dUTP hybridized to metaphase slides of a normal male (46,XY). One DAPI image (top) and another fluorescence image in red or green (bottom) were captured in each case. The origin of each modified CSL is indicated with a number within the image.

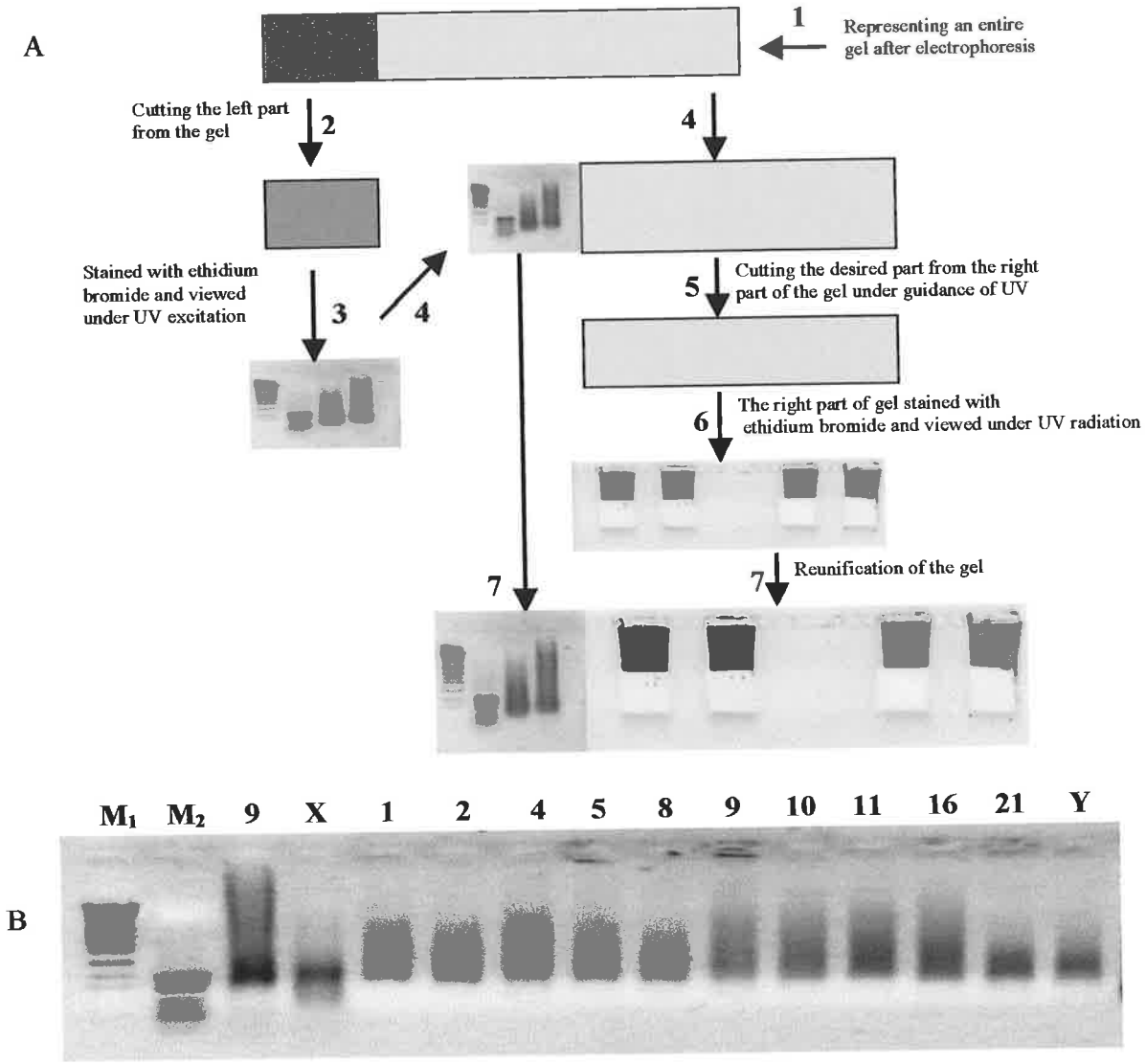


Figure 3.8. Large-scale preparation of the eleven modified CSLs for array spotting. A): Scheme for producing the modified probes from 50-80 µl DOP-PCR-amplified CSLs by excising the desired low molecular weight region (ranging from 200 bp to 1 kb) from a 1% agarose gel (3.4.4). **B)**: Electrophoresis of all eleven modified CSLs (1, 2, 4, 5, 8, 9, 10, 11, 16, 21, Y) on a 1% agarose gel by loading 5 µl (1/10 volume) of each recovered modified CSL. Original CSL_9 and CSL_X were run along with the modified CSLs on the gel. Compared to CSL_9, all modified CSLs produced a smear similar to that of CSL_X without the presence of higher molecular weight DNA fragments.

Table 3.1. PCR primers used for amplification of DNA fragments spotted on the third batch of arrays

Locus	Orientation	Primer sequences (5' → 3')	Products (bp)	Reference
DMD, Exon 44	F	GCAACCTTCCATTTAAAATCAGC	497	Hussey et al. (1999)
	R	GACAACAACAGTCAAAAGTAATTTCC		
DMD, Exon 45	F	AACATGGAACATCCTTGTGGGGAC	547	Chamberlain et al. (1990)
	R	CATTCCTATTAGATCTGTCGCCCTAC		
DMD, Exon 48	F	TTGAATACATTGGTTAAATCCCAACATG	506	Chamberlain et al. (1990)
	R	CCTGAATAAAGTCTTCCTTACCACAC		
CF, Exon 10	F	GCATAGCAGAACCTGAAACAGGA	550	Cui et al. (1995)
	R	GACGTTTGTCTCACTAATGAGTGAAC		

DMD: Duchenne Muscular Dystrophy
CF: Cystic Fibrosis
SRY: Sex-determining region Y
F: Forward primer for PCR
R: Reverse primer for PCR

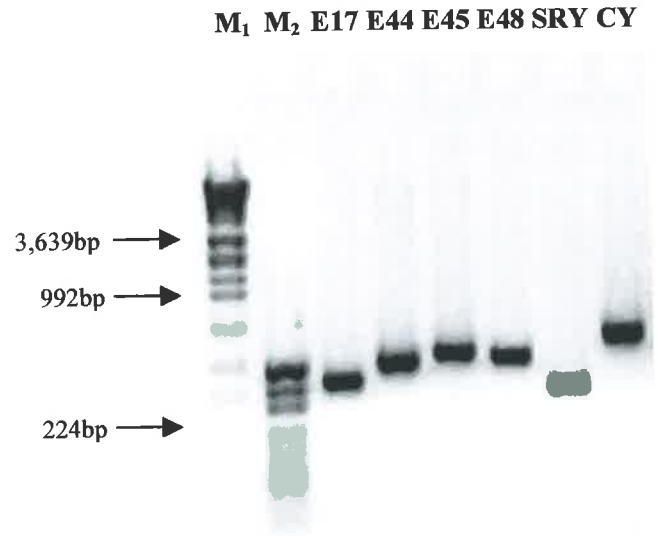
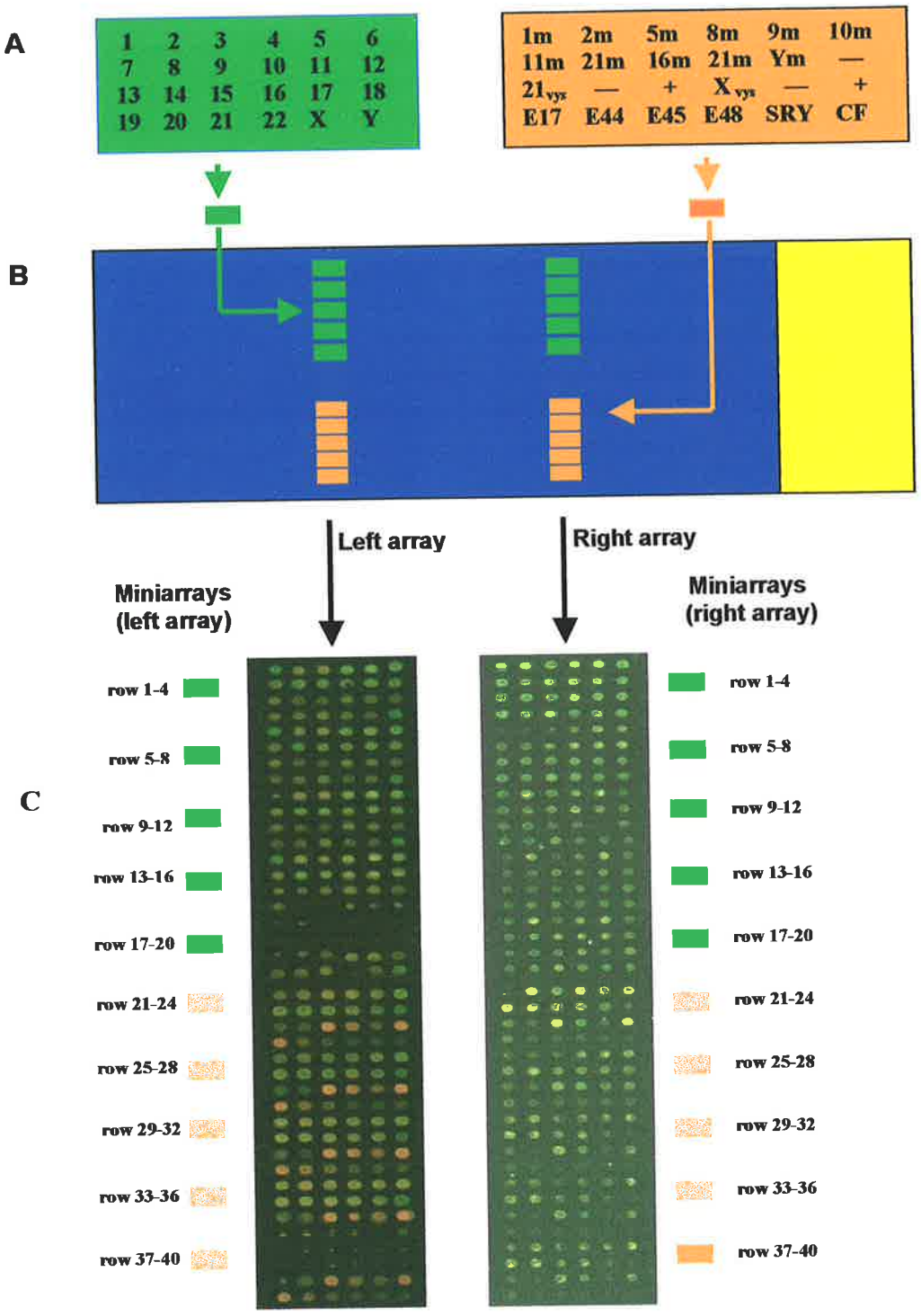


Figure 3.9. Electrophoresis of E17, E44, E45, E48, SRY, and CF. 10 µl of each PCR-amplified DNA fragment (Table 3.1) was run on a 2% agarose gel. The origin of each sample is indicated above each lane. DNA makers were *SPP*-1/*Eco*RI (M_1) and pUC19 /*Hpa*II (M_2).

The designed layout of the 3rd batch of arrays (Figs. 3.10A and 3.10B) contained two identical arrays (right and left), each of them had two blocks: the top block contained five identical miniarrays (Fig. 3.10A, left), each of them had all 24 original CSLs spotted in a configuration of 4 (row) X 6 (column); the bottom block also contained five identical miniarrays (Fig. 3.10A, right), each of them had eleven modified CSLs, 21_{vys}, X_{vys}, E17, E44, E45, E48, SRY, and CF, one positive sample spotted twice and another negative sample printed three times. Therefore, each array had 5 replicate dots of each sample, which were spotted away from each other to achieve a "random distribution" of all five replicates of the same sample. This format might improve the hybridization efficiency compared to that of the second batch of arrays with all replicates of the same sample spotted next to each other in a column (Fig. 3.5). Due to mechanical restriction of array spotting, the new format required a lengthy printing process of approximately 5 hours, almost 4 times longer than that used for the second batch of arrays. During such a lengthy duration, the spotting buffer (8 μl) evaporated so seriously that it dried out when the arrayer came to print the 4th row of the 4th miniarray of the left array. Without being noticed, the microarrayer continued to sample the dried wells and spotted another two rows. Once the error was noticed, spotting was paused and 8 μl of H_2O was added to the dried wells to resuspend the dried DNA. Spotting was then resumed as planned to spot the rest of the left array and then the entire right array.

Despite the evaporation problem, one slide was tested for single-cell array CGH in a normal male (Cy5)/female (Cy3) comparison. The resultant JPEG ratio images of both arrays (left and right) are shown (Fig. 3.10C). The dots on the left array shaped quite well but no dots could be seen on a few rows due to drying-out of the spotting buffer (Fig. 3.10C, left). By contrast, the dots on the right array were smaller and more variable (Fig. 3.10C, right). Consequently, both arrays were not good enough for any subsequent array CGH experiments.

Figure 3.10. Manufacture of the third batch of arrays. A): This array was constructed with two basic miniarrays with a common configuration of 4 rows by 6 columns. The original 24 CSLs are indicated by the numbers of 1, 2, ··· 21, 22, X, and Y, and the eleven modified CSLs by 1m, 2m, 4m, 5m, 8m, 9m, 10m, 11m, 16m, 21m, and Ym. **B**): Two identical arrays (left and right) were spotted on every slide, with a spacing of approximately 1.5 cm away from each other so each of them can be used in a separate array CGH experiment. Each array had two blocks (top and bottom). Each block had five identical miniarrays. **C**): The JPEG image of the Cy5 and Cy3 fluorescence intensity obtained from both the left and right arrays of a single-cell array CGH experiment in a normal male (Cy5)/female comparison. Many dots are missing from the left hand side array due to evaporation of the spotting solution. By contrast, dots of the right hand array were present but varied with respect to amount of DNA spotted and evenness of shape.


A
1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
1m 2m 5m 8m 9m 10m
11m 21m 16m 21m Ym —
21vys — + Xvys — +
E17 E44 E45 E48 SRY CF
B
Left array
Right array
C
Miniarrays (left array)
Miniarrays (right array)
row 1-4
row 5-8
row 9-12
row 13-16
row 17-20
row 21-24
row 25-28
row 29-32
row 33-36
row 37-40

3.4.7 Discussion of the third batch of arrays

Evaporation of spotting solution is a seemingly inescapable phenomenon but normally it results in no serious consequence if an optimal relative humidity of around 45% and temperature of around 20°C is maintained (Hegde et al. 2000). However, it can turn into a disaster if a lengthy spotting duration is required, as clearly demonstrated in this study. The impacts of evaporation can be minimized by many strategies. The first and simplest is to increase the volume of initial spotting solution. 8 μl of initial spotting solution was used in this study, which was in the widely regarded normal range of 6 to 10 μl, but this was not enough for a spotting duration of about 5 hours. Drying-out of any spotting solution might not have occurred before the completion of spotting if 12-15 μl had been used in this study. The second strategy is to shorten the spotting duration by printing all replicates of the same probe next to each other in a row (or column). With an initial spotting solution of 8 μl, 30 slides had been successfully spotted for the second batch of arrays used in this study, each of which had eight replicates per sample spotted next to each in a column (Section 3.3). The third strategy is to use 20-50% dimethyl sulfoxide (DMSO) instead of high-salt, 1-5X SSC, spotting solution (Galbraith et al. 2001; Snijders et al. 2001; Tran et al. 2002) DMSO is hygroscopic and has a low vapour pressure, therefore permitting a lengthy spotting duration without significant evaporation. Moreover, it denatures DNA, allowing it to bind better to the slides and providing more single-stranded targets for hybridisation (Hegde et al. 2000).

The hybridization to the left and right hand sides was not analysed due to the disappointing morphology of the spots, the fact that many were missing and the known problem with evaporation of the spotting buffer. Nevertheless the intensity of the E17, E44, E45, E48 SRY and CF dots (4^{th} row of each miniarray in both bottom blocks, Fig. 3.10) decreased across the row.

This was suspicious of contamination from the previous dot spotted which was a strongly hybridizing positive control. It was difficult to explain how such contamination from sample carryover could have occurred. However, finding another explanation of how 6 probes, which should give very similar hybridization intensities gave a "serial dilution" type of effect is also not obvious. The questionable contamination issue increased our lack of confidence with the DNA printing procedure.

3.5 Manufacture of the fourth batch of arrays

The 4th array was manufactured with five replicate dots per sample on each array. In order to avoid a lengthy spotting duration, all replicates of the same sample were spotted next to each other in a row (or column), a format similar to that of the second batch of arrays (Section 3.3).

3.5.1 Array Probes

37 different samples were spotted on this batch of arrays, including:

(1) 24 original CSLs already tested on the second batch of arrays (Section 3.3.2)

(2) 11 modified CSLs for chromosomes 1, 2, 4, 5, 8, 9, 10, 11, 16, 21 and Y (Section 3.4.4)

(3) 1 negative control containing H_2O

(4) 1 positive sample of DOP-PCR products of male genomic DNA (Section 3.3.4)

3.5.2 Array spotting of the 4th batch of arrays

All samples were dissolved in 3X SSC with a concentration of around 250 ng/μl and then spotted in 5 replicates for each sample on glass slides (Polysine™ microscope glass slides, Menzel-Glaser, Germany) by the Microarray facility of Molecular Biosciences, Adelaide University, Australia. 40 slides were to be spotted but the printing pins ran out of spotting solution before they finished spotting all 40 slides. Post-processing of arrays was conducted as previously described (Section 3.2.4).

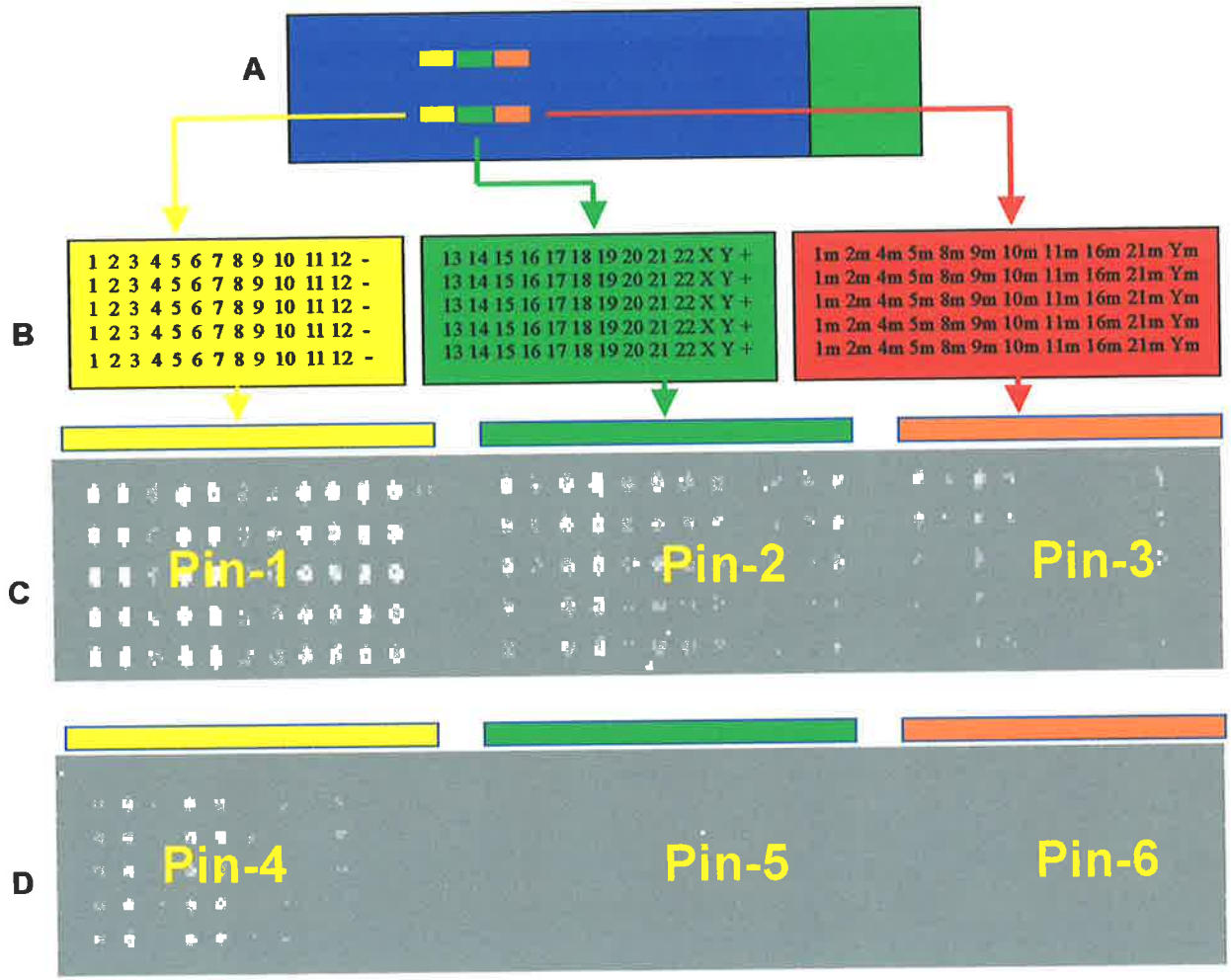
3.5.3 Results from the fourth batch of arrays

The scheme for the layout of this array is shown in Figure 3.11. Two identical arrays (top and bottom) were spotted on every slide (Fig. 3.11A). Each array had three blocks including the left, middle, and right blocks (Fig. 3.11B). Each block was spotted using a different printing pin, and

six pins were used to spot both the top and bottom arrays simultaneously (Fig. 3.11C and 3.11D). Within a block, five replicates per sample were spotted next to each other in a column. Samples were spotted in the orders (from left to right) of $CSL_{1\text{-}2\text{-}3\text{-}4\text{-}5\text{-}6\text{-}7\text{-}8\text{-}9\text{-}10\text{-}11\text{-}12}$-negative (left block), $CSL_{13\text{-}14\text{-}15\text{-}16\text{-}17\text{-}18\text{-}19\text{-}20\text{-}21\text{-}22\text{-}X\text{-}Y}$-positive (middle block), and $CSL_{1m\text{-}2m\text{-}4m\text{-}5m\text{-}8m\text{-}9m\text{-}10m\text{-}11m\text{-}16m\text{-}21m\text{-}Ym}$ (right block).

The 13th array slide was tested using single-cell array CGH (Section 2.2.6) and the resultant two TIFF ratio (Cy5/Cy3) images are shown as Fig. 3.11C and 3.11D, for the top and bottom arrays, respectively. Based on these two images, Pin-1 successfully spotted all expected dots on the left block of the top array. However, Pin-2, Pin-3, and Pin-4 failed to spot the majority of the expected dots, and both Pin-5 and Pin-6 failed to spot any of the all expected dots. The failure to spot dots on the 13th slide was due to the printing pins running out of spotting solution when they came to print this slide.

Figure 3.11. Manufacture of the fourth batch of arrays. A): Two identical arrays (top and bottom) were spotted on every slide. **B**): Each array had three blocks including the left, middle, and right blocks. **C** and **D**): Each block was spotted using a different printing pin. Six pins were used to spot both the left and right arrays simultaneously. Within a block, five replicates per sample were spotted next to each other in a column. Samples were spotted in the three blocks in the orders (from left to right) of $CSL_{1\text{-}2\text{-}3\text{-}4\text{-}5\text{-}6\text{-}7\text{-}8\text{-}9\text{-}10\text{-}11\text{-}12}$-negative (left), $CSL_{13\text{-}14\text{-}15\text{-}16\text{-}17\text{-}18\text{-}19\text{-}20\text{-}21\text{-}22\text{-}X\text{-}Y}$-positive (middle), and $CSL_{1m\text{-}2m\text{-}4m\text{-}5m\text{-}8m\text{-}9m\text{-}10m\text{-}11m\text{-}16m\text{-}21m\text{-}Ym}$ (right). One slide (13^{th} slide) was tested for single-cell array CGH and the resultant TIFF image of the Cy5 and Cy3 fluorescence intensity of both the top and bottom arrays are given in (**C**) and (**D**). Pin-1 spotted quite well but Pin-2, Pin-3, and Pin-4 failed to spot most of the expected dots; and even worse, Pin-5 and Pin-6 failed to spot any dots. Such failure to spot dots on the 13^{th} slide was due to the printing pins being worn and running out of spotting solution when they came to print this slide, out of a planed total of 40 slides.


A
B
1 2 3 4 5 6 7 8 9 10 11 12 -
1 2 3 4 5 6 7 8 9 10 11 12 -
1 2 3 4 5 6 7 8 9 10 11 12 -
1 2 3 4 5 6 7 8 9 10 11 12 -
1 2 3 4 5 6 7 8 9 10 11 12 -
13 14 15 16 17 18 19 20 21 22 X Y +
13 14 15 16 17 18 19 20 21 22 X Y +
13 14 15 16 17 18 19 20 21 22 X Y +
13 14 15 16 17 18 19 20 21 22 X Y +
13 14 15 16 17 18 19 20 21 22 X Y +
1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
C
Pin-1
Pin-2
Pin-3
D
Pin-4
Pin-5
Pin-6

3.5.4 Discussion of the fourth batch of arrays

Normally, one printing pin takes up a DNA sample volume of ~250 nl each time by capillary action and deposits less than 1 nl to print a spot. Therefore, 250 nl should be enough to spot 200 dots and therefore should have been sufficient for spotting 40 slides with 5 replicates per sample (200 dots). The same set of printing pins had successfully spotted the second batch of arrays, being required to print 120 dots from a single sampling volume of ~ 250 nl (Section 3.3). One year later, these pins ran out of spotting solution much earlier than their 120th printing, with Pin-5 and Pin-6 as early as their 30~40th printing. Microscopic examination of these pins found that all six pins were worn out to some extent, especially Pin-5 and Pin-6. These pins may take up less and/or deposit more on printing than good pins, thereby leading to running out of spotting solution earlier than expected. On being tested with 50% DMSO rather than 3X SSC as the spotting solution, these pins could spot more dots but still far less than the expected number of dots from a single sampling (data not shown). As a consequence, these pins (especially Pin-5 and Pin-6) could not be used for accurate printing.

3.6 Manufacture of the fifth batch of arrays

Since the Microarray Facility used to print both the 3rd and 4th batches of arrays failed to produce arrays of good quality twice (Sections 3.4 and 3.5), a new microarray facility was used to spot the fifth batch of arrays.

3.6.1 Array probes

All 37 samples spotted on this array were produced exactly the same as for the 4th batch of arrays (Section 3.5) except that the positive sample was derived from genomic DNA sample of Male Donor 2. Namely:

(1) 24 original CSLs already tested on the second batch of arrays (Section 3.3.2)

(2) 11 modified CSLs for chromosomes 1, 2, 4, 5, 8, 9, 10, 11, 16, 21 and Y (Section 3.4.4)

(3) 1 negative control containing H_2O

(4) 1 positive sample of DOP-PCR products of male genomic DNA (Section 3.3.4)

3.6.2 Array spotting

Arrays were spotted by Microarray facility of Clive & Vera Ramaciotti Center for Gene Function Analysis, School of Biotechnology & Biomolecular Sciences, The University of New South Wales, Australia. Briefly, all samples were dried in wells of a 384-well plate at room temperature and resuspended in 6 μl of 150 mM sodium phosphate to reach a final concentration of about 250ng/μl. Spotting from this plate on to SuperAmine slides (TeleChem, Sunnyvale, CA, USA) was carried out by a Chipwriter Pro (BioRad) arrayer. Two identical arrays were spotted per slide, each array had eight replicate spots per sample.

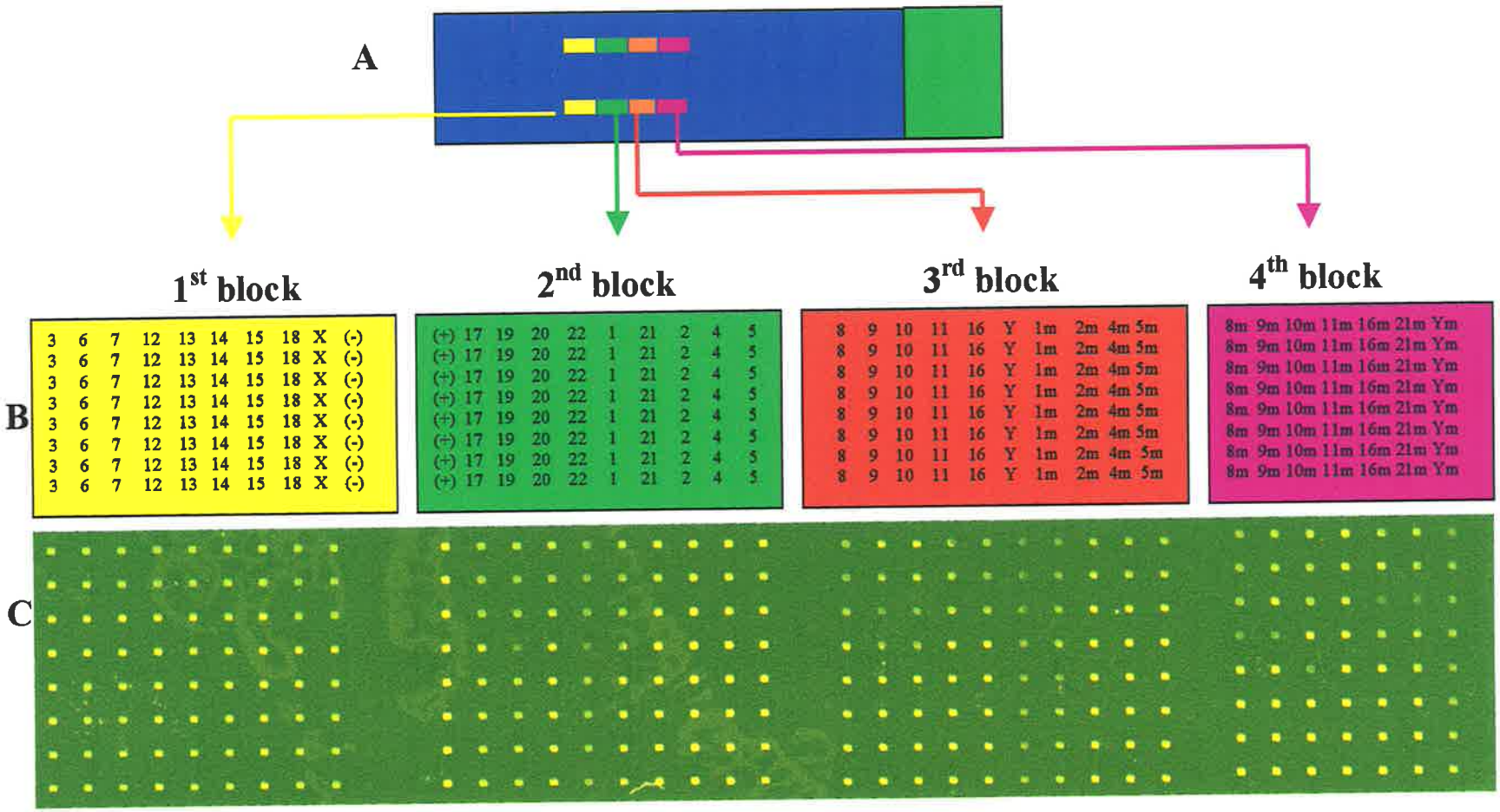
3.6.3 Post-processing of arrays

Post-processing of slides was different from that used for the previous four batches of arrays. The slides were allowed to age in a dust-free slide box for several days, then baked at 80°C for 80 min. After cooling to room temperature, the slides were washed with constant mixing in 0.2% SDS for 4 min followed by three times in MilliQ H_2O for 1 min each. Slides were plunged into nearly boiling MilliQ H_2O for 2-3 min to denature the DNA, dehydrated in cold 95% ethanol for 1 min, and finally dried either by spinning at 800 rpm for 5 min or by air drying in the dark. Slides were stored *in vacuo* at room temperature in the dark for up to 2 months for processed slides and up to 5 months if unprocessed. UV-crosslinking and chemical blocking were recommended to be not necessary for this batch of arrays by the Microarray Facility, and so these steps were not taken.

3.6.4 Results from the fifth batch of arrays

The scheme for the layout of this array is shown in Figure 3.12. Two identical arrays were vertically spotted on each slide with a spacing of around 1 cm between them, allowing each of them to be used independently for a separate array CGH experiment (Fig. 3.12A). Each array had four blocks, and eight replicates per sample, spotted next to each other in a column (Fig. 3.12B). One TIFF ratio image (Cy5/Cy3) of single-cell array CGH tested on one array is illustrated (Fig. 3.12C). All dots in this image appear to have been successfully spotted and they are evenly shaped. The negative and positive control samples gave appropriate signals. The results of single-cell array CGH analyses on this batch of arrays are reported later (Chapters 5 and 6).

Figure 3.12. Manufacture of the fifth batch of arrays. A): Two identical arrays (top and bottom) were spotted on every slide. **B)**: Each array had four blocks. Within each array, eight replicates per sample were spotted next to each other in a column. Samples were spotted in the orders (from left to right) of $CSL_{3\text{-}6\text{-}7\text{-}12\text{-}13\text{-}14\text{-}15\text{-}18\text{-}X}$-(-) ($1^{st}$ block), (+).$CSL_{17\text{-}19\text{-}20\text{-}22\text{-}1\text{-}21\text{-}2\text{-}4\text{-}5}$ (2^{nd} block), $CSL_{8\text{-}9\text{-}10\text{-}11\text{-}16\text{-}Y\text{-}1m\text{-}2m\text{-}4m\text{-}5m}$ (3^{rd} block), and $CSL_{8m\text{-}9m\text{-}10m\text{-}11m\text{-}16m\text{-}21m\text{-}Ym}$ (4^{th} block). The dimension of each array was approximately 0.5 cm X 2 cm and the center-to-center spacing between two adjacent dots was 400 μm with the diameter of dots around 100 μm. There was a (vertical) spacing of 1 cm between the top and bottom arrays. **C)**: A TIFF image of the Cy3/Cy5 fluorescence intensity of single-cell array CGH hybridized to one array. The evenness of all dots appeared to be very good. The negative sample gave no hybridization signal and the positive sample produced very strong hybridization signals.


A
1st block
2nd block
3rd block
4th block
B
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
3 6 7 12 13 14 15 18 X (-)
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
(+) 17 19 20 22 1 21 2 4 5
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8 9 10 11 16 Y 1m 2m 4m 5m
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
8m 9m 10m 11m 16m 21m Ym
C

3.7 Summary of array manufacture

Five different batches of arrays were manufactured for this study. Among them, the 2nd and 5th batches were properly spotted and tested comprehensively in single-cell array CGH (Chapters 4-6). However, similar experiments were not performed on the other three batches of arrays. Limited results from the 1st batch of arrays demonstrated that WCP probes of 21_{vys} and X_{vys} (Vysis, USA) were probably unsuitable for this project. Other fluorophore-labeled commercial CSLs, directly used for array CGH analysis, might also not be suitable because of their high autofluorescence. Both the 3rd and 4th batches of arrays were defective because of poor quality of array printing and were therefore not used for array CGH. The microarrayer failed to print many dots on the 3rd batch of arrays because the initial 8 μl of spotting solution evaporated to the point of drying-out before the completion of the spotting process. With some of the worn printing pins running out of spotting solution much earlier than expected, most of the dots failed to be printed on the 4th batch of arrays. The importance of optimizing the arraying conditions with any supplier prior to spotting any actual array slides is highlighted by the above errors.

Chapter 4

Detection of aneuploidy and gender determination in single cells by DNA microarray CGH using the 2nd batch of arrays

4.1 Aims

All experiments in the present study were carried out using the second batch of arrays (Section 3.3 and Fig. 3.5). Initial experiments were performed on single normal male and female lymphocytes from peripheral blood (Section 2.2.1.1), and aimed to optimise and eventually to develop an array CGH approach for analysing a single cell sample. A further aim was to test the feasibility of using this approach for gender determination using the results generated by CSL_X and CSL_Y and the correct diagnosis of the copy number of autosomes using results produced by autosomal CSLs. Experiments using 47,XX,+13, 47,XY,+18, and 47,XY,+21 single amniocytes (2.2.1.2) were finally carried out to test the feasibility of using this approach for detection of aneuploidy.

4.2 Results

4.2.1 Amplification and labelling of the single cells by DOP-PCR

Random amplification of the single genomes within single lymphocytes or amniocytes was performed using one round of DOP-PCR (Section 2.2.6.2). 5 μl of each amplified product was then labelled with either Cy3- or Cy5-dUTP by another round of DOP-PCR (Section 2.2.6.3). After amplification, 1/10 volume of each labelled product was run on a 1% agarose gel and

always gave a smear ranging from 300 bp to 2,500 bp containing two specific bands approximately at 450 bp and 600 bp (Fig. 4.1).

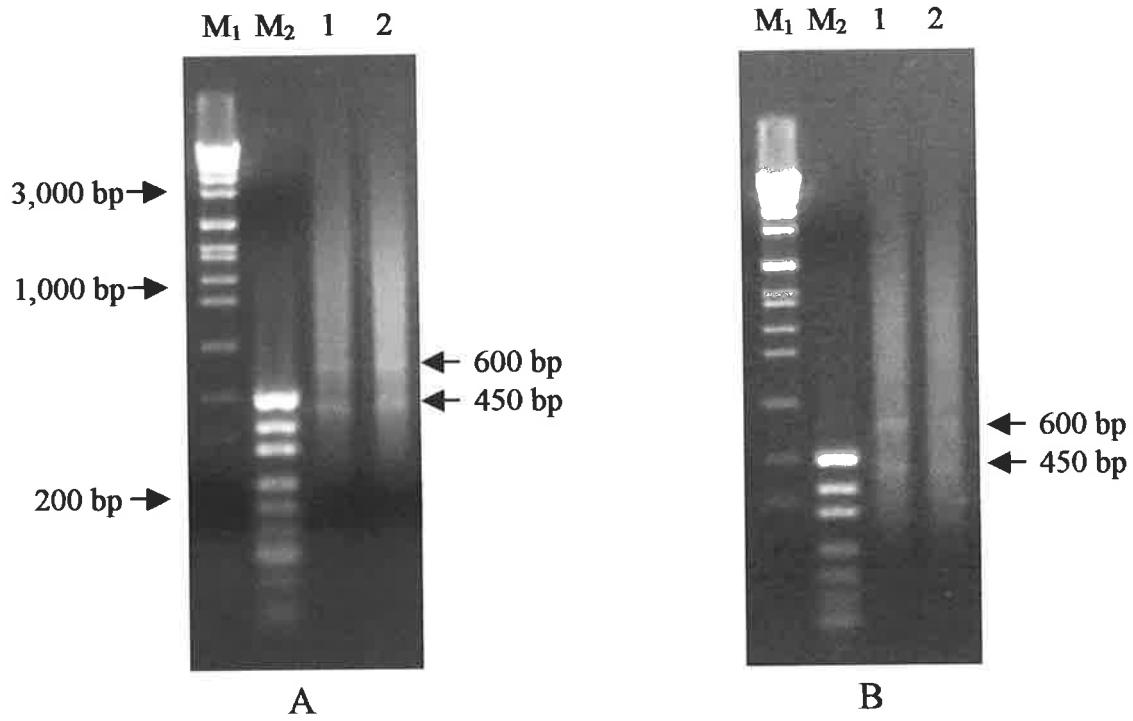


Figure 4.1. Electrophoresis of Cy3- or Cy5-labelled single-cell DOP-PCR products on a 1% agarose gel. **A)**: Labelled DOP-PCR products of a single normal male (Cy5, *lane 1*) and female (Cy3, *lane 2*) lymphocytes. **B)**: Cy3-labelled DOP-PCR products of a single female trisomy 13 (*lane 1*) and a single male trisomy 18 (*lane 2*) amniocyte. DNA markers were *SPP*-1/*Eco*RI (M_1) and pUC19 /*Hpa*II (M_2). Note that each labelled product gives a smear ranging from 300 bp to 2,500 bp containing two specific bands approximately at 450 bp and 600 bp.

4.2.2 Development of the single-cell array CGH approach

In order to develop/optimize the single-cell array CGH protocol in the present study, one single 46,XY male lymphocyte and another single 46,XX female lymphocyte was labelled with both Cy3 and Cy5 (Section 2.2.6.3). Two array CGH experiments of male(Cy3) versus female(Cy5) and female(Cy3) versus male(Cy5) were performed repeatedly using three different protocols (Table 4.1) by varying the amount of both labelled products and Cot-1 DNA as well as changing the washing buffer and temperature.

The first protocol (Table 4.1, Protocol A) failed to produce the expected ratios of > 1.25 and < 0.75 for the X-chromosomal DNA library (CSL_X) (results not shown). The ratios obtained for all 24 CSLs fell within the range 0.75-1.25 in both types of array CGH experiments (results not shown). In the JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from the array CGH in female(Cy3)/male(Cy5) comparison (Fig. 4.2), all the dots containing a DNA sample gave a bright signal and the negative sample containing H_2O produced almost no signals. These results indicated that sufficient hybridization was obtained but it was not specific enough to produce the expected ratio of > 1.25 for the CSL_X dots. To solve this problem, the array slides were rewashed with a washing buffer containing 50% formamide in 2X SSC instead of 2X SSC only, but this treatment failed to improve the results (data not shown). This indicated that increasing the stringency of washing buffer alone could not significantly reduce the non-specific signals.

Table 4.1. Single-cell array CGH protocols

	Protocol A	Protocol B	Protocol C (Section 2.2.6.5)
Cy_3-/Cy_5-labelled DOP-PCR products (µl)	10	2	5
Human Cot-1 DNA (µg)	40	70	70
Salmon sperm DNA (µg)	none	none	20
Preannealing of probe mixture at 37°C (min)	30	30	80
Hybridization at 37°C (hours)	15-20	15-20	15-20
Humidity during hybridization	50%	50%	95%
Post-hybridization washing	twice in 2X SSC at 60°C for 10 min twice in 0.1X SSC at 60°C for 5 min once in 1X SSC at RT for 10 min three times briefly in MilliQ H_2O	twice in 2X SSC at 60°C for 10 min twice in 0.1X SSC at 45°C for 5 min once in 1X SSC at RT for 10 min three times briefly in MilliQ H_2O	twice in 50% formamide and 2X SSC at 45°C for 10 min twice in 2X SSC at 45°C for 5 min once in 1X SSC at RT for 10 min three times briefly in MilliQ H_2O

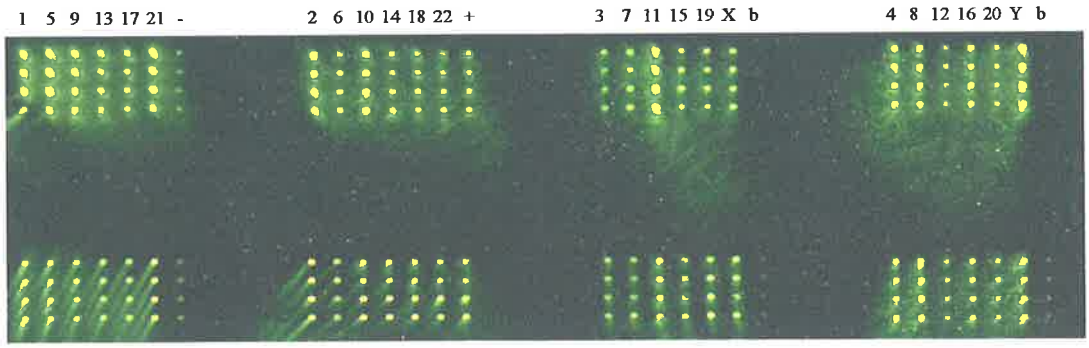


Figure 4.2. JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of normal female (Cy3) versus male (Cy5) single lymphocytes carried out using the 2nd batch of arrays (Section 3.3) and Protocol A (Table 4.1). Scanning was performed by a GenePix 4000B scanner: PMT volts (500/600, Cy3/Cy5) and laser ScanPower (33%/100%, Cy3/Cy5). Note that the blank control dots (b) give almost no signals but very faint signals can be seen on the negative dots. A brighter signal can be found on all dots containing either a chromosomal DNA library (CSL) or the positive sample. However, many dots have a comet tail.

Both array CGH experiments were then repeated using Protocol B (Table 4.1). Compared to Protocol A, this protocol used less labelled products and more Cot-1 DNA in an effort to enhance the suppression of repetitive sequences. Results showed that almost all dots gave extremely weak signals in both experiments (images not saved). These results suggested that the 2 μl of each labelled product applied in Protocol B, which was one fifth the amount of the 10 μl applied in Protocol A, might be too little to produce reliable signals.

Both of the above array CGH experiments were repeated again using Protocol C (Table 4.1), which is the standard protocol (Section 2.2.6.5). 5 μl of each of Cy3- and Cy5-labelled DOP-PCR products was used in this protocol, which was 2.5 times the amount applied in Protocol B but half the amount used in Protocol A. Following this change, the CSL_X dots gave the expected ratio, falling outside the range 0.75-1.25 in both array CGH experiments. These results suggested that Protocol C was optimized and able to determine the copy number difference of the X chromosome between the single male and female lymphocytes. Therefore, Protocol C was used for all further experiments in this study.

A constant humidity of 95% was required in Protocol C for the hybridization step. Without such a high humidity, the 10 μl of hybridization solution dried very quickly and the labelled products tended to bind non-specifically to the array area. In post-hybridization washing, it was hard or impossible to wash this contamination off the array area completely, thereby resulting in a high background. As a result, images obtained under these circumstances never qualified for accurate array CGH analysis (Fig. 4.3).

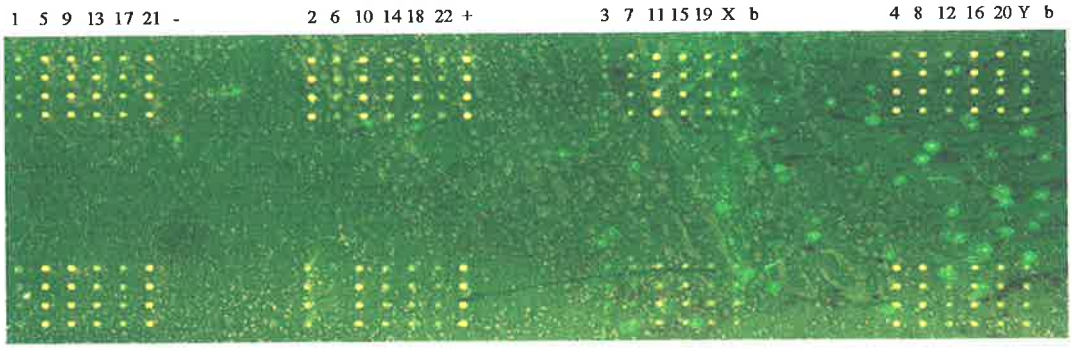


Figure 4.3. JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of normal female (Cy3) versus male (Cy5) single lymphocytes conducted using a relative humidity of approximately 45%, which is much lower than that of 95% applied in Protocol C (Table 4.1). Scanning was carried out by a GenePix 4000B scanner: PMT volts (550/600, Cy3/Cy5) and laser ScanPower (100%/100%, Cy3/Cy5). Note that the low relative humidity accidentally used in this experiment resulted in a high fluorescence background due to the difficulty to wash the labelled products non-specifically sticking to the array area off the slide.

4.2.3 Results of array CGH experiments using the second batch of arrays

4.2.3.1 Array CGH of 46,XX(Cy3) versus 46,XY(Cy5)

In this experiment, the test female lymphocyte was labelled with Cy3 and the reference male lymphocyte with Cy5. Of the total 192 (24 x 8) dots (Fig. 4.4A), 18 (9%) dots failed to pass the filtering criteria (Section 2.2.7.3) and therefore were excluded from analysis (Table 4.2, Experiment 1). The ratios obtained for all the 22 autosomal DNA libraries (CSLs) fit well within the range 0.75 to 1.25 (Table 4.3, Experiment 1). As expected, the X-chromosomal DNA library (CSL_X) gave a ratio of 1.63, which was less than its theoretical 2:1 ratio of 2.0, but greater than the higher threshold value of 1.25. Therefore, the two copies of the X chromosome present in the test female lymphocyte were correctly identified by such a ratio. However, a ratio of < 0.75 would be expected for the Y-chromosomal DNA library (CSL_Y) but a value of 1.02 was observed, thereby failing to indicate the absence of the Y chromosome in the test female lymphocyte.

Very surprisingly, the positive dots in this experiment showed in redder compared to all other dots and gave a very low ratio of 0.45. Both the positive control DNA sample spotted on the microarray and the reference single male lymphocyte used in this experiment were from the same normal Male Donor 1 (46,XY).

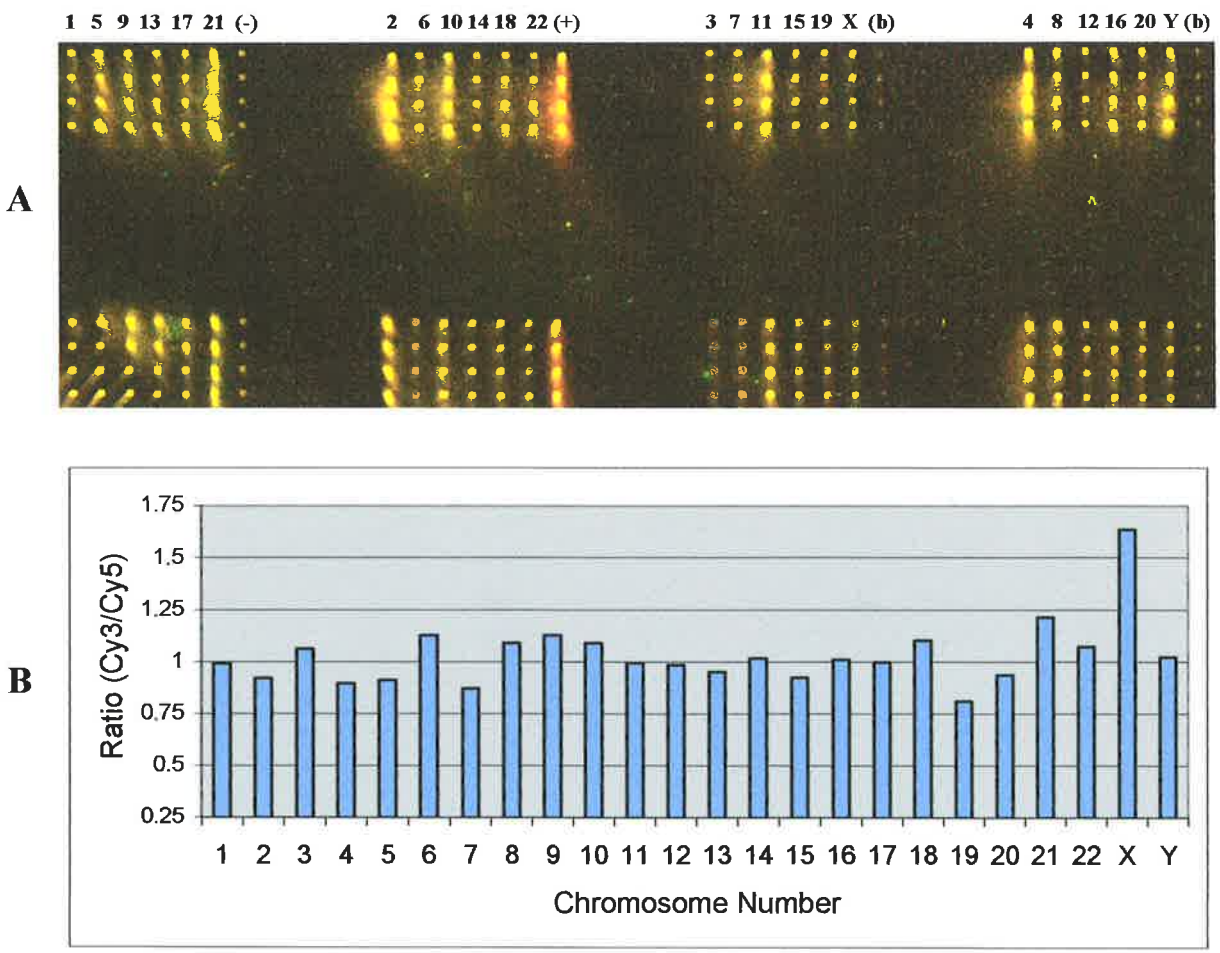


Figure 4.4. Results of an array CGH experiment of a normal female(Cy3) versus male(Cy5) lymphocyte (Section 4.2.3.1). **A**): JPEG image of the Cy3 and Cy5 fluorescence intensity obtained by a GenePix 4000B scanner: PMT volts (500/690, Cy3/Cy5) and laser ScanPower (100%/100%, Cy3/Cy5). **B**): Ratios of all of the 24 CSLs are shown in a graph (Table 4.3, Experiment 1). Note that the CSL_X ratio of 1.63 is > 1.25 as expected, but the ratio of 1.02 for CSL_Y is above the expected value of <0.75. As expected, ratios of all 22 autosomal CSLs are within the cutoff thresholds of 0.75 and 1.25. Both the positive control DNA sample spotted on the microarray and the reference single male lymphocyte used in this experiment were from the same normal Male Donor 1 (46,XY), and the positive dots fluoresce strongly in red.

Table 4.2 Number of dots failed to pass the filtering criteria and therefore were excluded from single-cell CGH analysis performed using the 2nd batch of arrays

Experiment	%>B635 + 2SD > 70 (1)	%>B532 + 2SD > 70 (2)	Rgn R^2 <0.60 (3)	(1) and (2)	(1) and (3)	(2) and (3)	(1), (2), and (3)	Maximal No. of dots excluded from a single CSL	Total No. of dots excluded
1. 46,XX_{Cy3}/46,XY_{Cy5}	0	3	6	2	0	5	2	3 (CSL_{18})	18(9 %)
2. 46,XY_{Cy3}/46,XX_{Cy5}	6	6	5	1	3	9	1	4 (CSL_{19})	31(16%)
3. 47,XX,+13_{Cy3}/46,XY_{Cy5}	2	0	7	3	5	3	1	4 (CSL_{22})	21(11%)
4. 47,XY,+18_{Cy3}/46,XX_{Cy5}	1	2	2	3	3	7	6	4 ($CSL_{3,6,Y}$)	24(12%)

Dots excluded from analysis by one single parameter only: (1), (2), and (3)
Dots excluded from analysis simultaneously by two parameters: (1) and (2), (1) and (3), and (2) and (3)
Dots excluded from analysis simultaneously by three parameters: (1), (2) and (3)
Rgn R^2 is the coefficient of determination for the least-squares regression fit of a given feature (Axon Instruments, Union City, CA, USA)

Table 4.3. Results of single-cell array CGH experiments performed on the 2nd batch of arrays

Source of test/reference	Normalized ratios (Cy3/Cy5) of CSLs																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
(1) 46,XX_{Cy3}/46,XY_{Cy5}	0.99	0.92	1.06	0.89	0.91	1.12	0.87	1.09	1.12	1.09	0.99	0.98	0.95	1.01	0.92	1.01	0.99	1.10	0.80	0.93	1.21	1.07	1.63	**1.02**
(2) 46,XY_{Cy3}/46,XX_{Cy5}	0.91	1.07	0.89	1.00	1.02	0.94	1.11	0.93	0.90	0.99	1.01	1.00	0.99	0.99	1.12	0.98	0.96	0.96	**1.28**	1.05	0.87	1.02	**0.62**	**0.94**
(3) 47,XX,+13_{Cy3}/46,XY_{Cy5}	0.86	0.87	0.83	0.79	0.93	1.15	1.04	1.18	0.99	1.09	0.78	1.17	**1.42**	1.07	1.02	0.91	1.19	1.00	0.99	**1.26**	**0.72**	0.94	1.28	0.58
(4) 47,XY,+18_{Cy3}/46,XX_{Cy5}	0.93	0.84	1.08	0.89	**0.70**	0.98	1.04	1.03	0.79	0.77	0.86	1.23	0.89	1.10	1.14	0.92	**1.40**	**1.40**	1.22	1.07	0.92	1.04	**0.67**	**1.39**

Ratios marked in green and red are expected ratios resulting in the correct diagnosis of aneuplodies or gender determination
Ratios marked in bold are unexpected ratios resulting in the misdiagnosis of autosomal aneuploidy or gender determination
All other ratios are all within the cutoff thresholds of 0.75 and 1.25
Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

4.2.3.2 Array CGH of 46,XY(Cy3) versus 46,XX(Cy5)

Compared to the experiment described above, this experiment swapped the dye labelling with the test male lymphocyte labelled with Cy3 and the reference female lymphocyte with Cy5. Of the total 192 dots (Fig. 4.5A), 31 (16%) dots couldn't pass the filtering criteria (Section 2.2.7.3) in this experiment and therefore were excluded from analysis (Table 4.2, Experiment 2). The ratios of all autosomal DNA libraries (CSLs) were within the range 0.75 to 1.25 with the exception of the chromosome 19-DNA library (CSL_{19}), which produced a ratio of 1.28 (Table 4.3, Experiment 2). As expected, the X-chromosomal DNA library (CSL_X) gave a ratio of 0.62, which was higher than its theoretical 1:2 ratio of 0.50, but less than the lower threshold value of 0.75. Therefore, the single copy of the X chromosome present in the test male lymphocyte was accurately predicted by this ratio. However, a ratio of >1.25 would be expected for the Y-chromosomal DNA library (CSL_Y) but a value of 0.94 was found instead. Therefore, the Y chromosome present in the test male lymphocyte was not correctly detected.

In this experiment, the positive dots showed in greener compared to all other dots and gave a very high ratio of 1.94. Both the positive control DNA sample spotted on the microarray and the test single male lymphocyte used in this experiment were also from the same normal Male Donor 1 (46,XY).

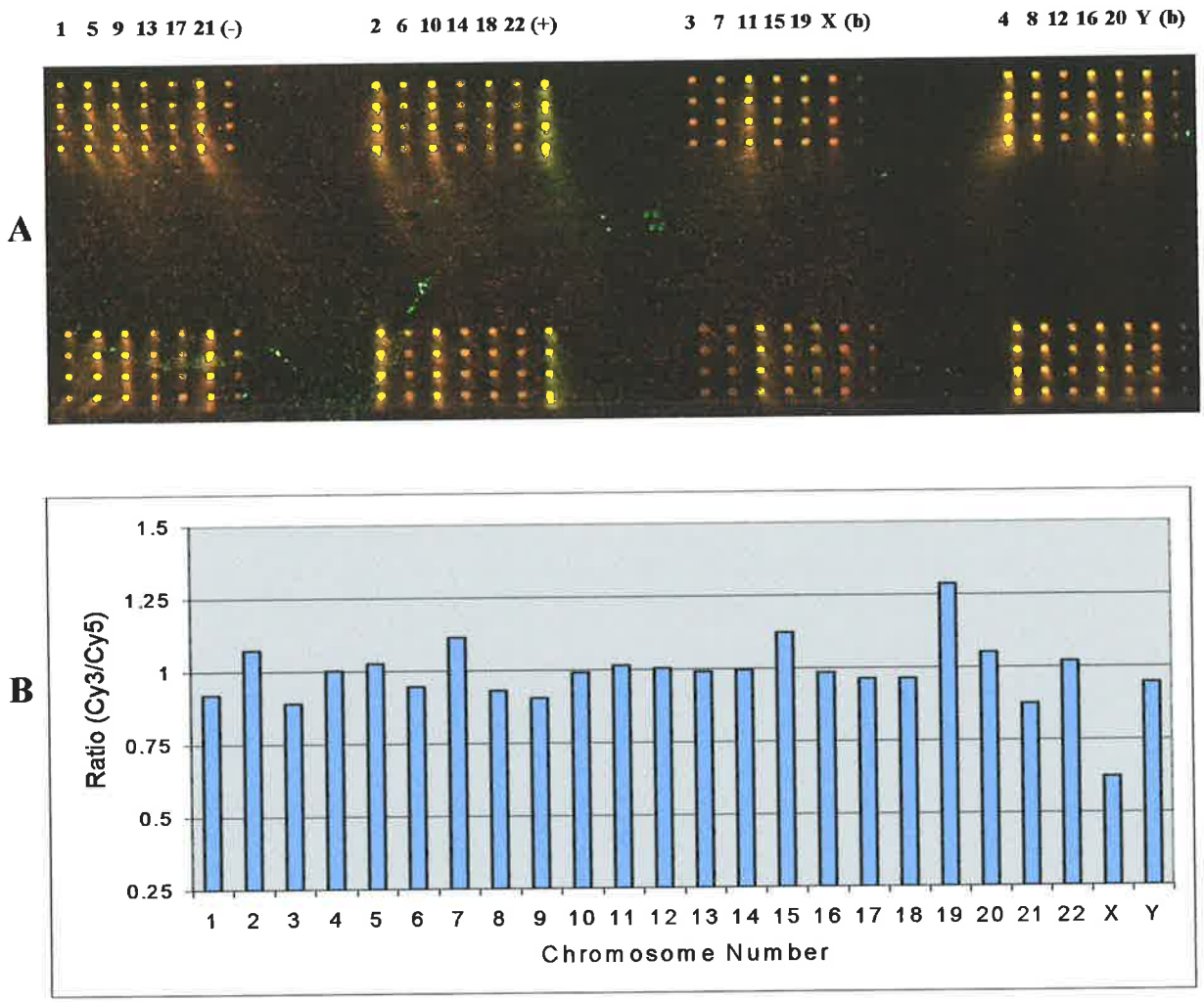


Figure 4.5. Results of an array CGH experiment of a normal male (Cy3) versus female(Cy5) lymphocyte (Section 4.2.3.2). **A**): JPEG image of the Cy3 and Cy5 fluorescence intensity scanned by a GenePix 4000B scanner: PMT volts (480/670, Cy3/Cy5) and laser ScanPower (100%/100%, Cy3/Cy5). **B**): Ratios of all of the 24 CSLs shown in a graph (Table 4.3, Experiment 2). Note that the CSL_X gives an expected ratio of 0.62, which is < 0.75 as expected. But an expected ratio of > 1.25 was not obtained for the CSL_Y. Ratios of all autosomal CSLs are within the cutoff thresholds of 0.75 and 1.25 except that the CSL_{19} gives an unexpected ratio of 1.28. Both the positive control DNA sample spotted on the microarray and the test single male lymphocyte used in this experiment were also from the same normal Male Donor 1 (46,XY) and the positive sample fluoresces strongly in green.

4.2.3.3 Array CGH of 47,XX,+13 (Cy3) versus 46,XY (Cy5)

In this experiment, the test female trisomy 13 amniocyte (47,XX,+13) was labelled with Cy3 and the reference male cell was a single lymphocyte (46,XY) labelled with Cy5. Out of the total of 192 dots, 21 (11%) were excluded from analysis (Fig. 4.6A) due to the failure to pass the filtering criteria (Table 4.2, Experiment 3). For the autosomes, the chromosome 13-DNA library (CSL_{13}) gave the highest ratio of 1.42, which was only a little lower than its theoretical 3:2 ratio of 1.50, but greater than the higher threshold value of 1.25 (Table 4.3, Experiment 3). Such a ratio demonstrates the presence of one additional copy of chromosome 13 in the test amniocyte. Unexpectedly, two autosomal DNA libraries including CSL_{20} and CSL_{21} produced ratios of 1.26 and 0.72, slightly falling outside the threshold range of 0.75 to 1.25. Ratios of the remaining 19 autosomal CSLs were all within the cutoff thresholds of 0.75 and 1.25. For the gonosomes, as expected, the X-chromosomal DNA library (CSL_X) gave a ratio of 1.28 (> 1.25). The Y-chromosomal DNA library (CSL_Y) produced a ratio of 0.58, in this case < 0.75, but considering the unreliability of the CSL_Y library, the result must be attributed to chance. The positive control DNA sample spotted on the microarray (Male Donor 1) is not from the same donor as the reference single male lymphocyte (Male Donor 2), and the positive dots gave a ratio of approximately 1:1 in this experiment.

In summary, this experiment produced the expected ratios for three critical DNA libraries, CSL_{13}, CSL_X, and CSL_Y, resulting in the correct diagnosis of trisomy 13 and the gender of female for the test amniocyte. However, both CSL_{20} and CSL_{21} gave deviant ratios, thereby incorrectly indicating that the test amniocyte also had trisomy 20 and monosomy 21.

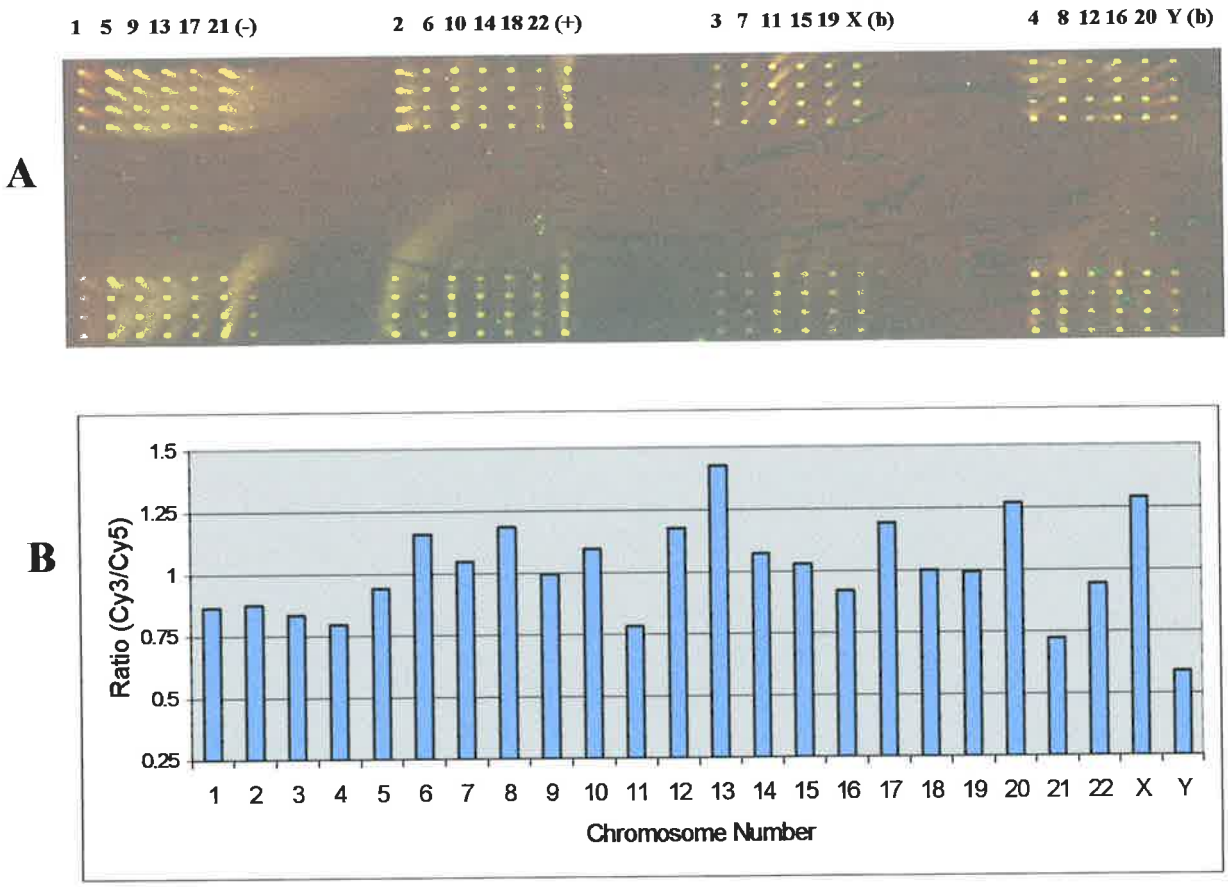


Figure 4.6. Results of an array CGH experiment of a single 47,XX,+13 (Cy3) amniocyte versus a single male 46,XY (Cy5) lymphocyte (Section 4.2.3.3). **A)**: JPEG image of the Cy3 and Cy5 fluorescence intensity scanned by a GenePix 4000B scanner: PMT volts (570/730, Cy3/Cy5) and laser ScanPower (100%/100%, Cy3/Cy5). **B)**: Ratios of all of the 24 CSLs shown in a graph (Table 4.3, Experiment 3). The CSL_{13}, CSL_X, and CSL_Y all give the expected ratio of 1.42, 1.28, and 0.58, respectively. However, the CSL_{20} and CSL_{21} show unexpected ratios of 1.26 and 0.72, respectively. The positive control DNA sample spotted on the microarray (Male Donor 1) is not from the same donor as the reference single male cell (Male Donor 2), and the positive dots fluoresce strongly in yellow and gave a ratio of approximately 1:1.

4.2.3.4 Array CGH of 47,XY,+18 (Cy3) versus 46,XX (Cy5)

In this experiment, the test male trisomy 18 amniocyte (47,XY,+18) was labelled by Cy3 and the reference single female lymphocyte (46,XX) by Cy5. Out of the total of 192 dots, 24 (12%) were excluded from analysis (Fig. 4.7A) after filtering the raw data (Table 4.2, Experiment 4). For the autosomes, the chromosome 18-specific DNA library (CSL_{18}) produced a ratio of 1.40, which was lower than its theoretical 3:2 ratio of 1.50, but greater than the higher threshold value of 1.25 (Table 4.3, Experiment 4). The presence of one additional copy of chromosome 18 in the test amniocyte was therefore determined. Two autosomal DNA libraries including CSL_5 and CSL_{17} gave ratios of 0.70 and 1.40, respectively, exceeding the cutoff thresholds of 0.75 and 1.25. Ratios of the remaining 19 autosomal CSLs all fell within the cutoff thresholds of 0.75 and 1.25. For the gonosomes, as expected, a ratio of 0.67 (< 0.75) was obtained for the X-chromosomal DNA library (CSL_X) and a ratio of 1.39 (> 1.25) for the Y-chromosomal DNA library (CSL_Y), but again the Y chromosomal result must be attributed to chance. In this experiment, the positive control DNA sample spotted on the microarray (Male Donor 1) is not from the same genome as the reference single female cell, and the positive dots gave a ratio of approximately 1:1.

In summary, in this experiment, the expected ratios were obtained for three DNA libraries including CSL_{18}, CSL_X, and CSL_Y leading to the correct diagnosis of trisomy 18 and the gender of male for the test amniocyte. However, both CSL_5 and CSL_{17} produced a deviant ratio, thereby misdiagnosing the test amniocyte as also carrying monosomy 5 and trisomy 17.

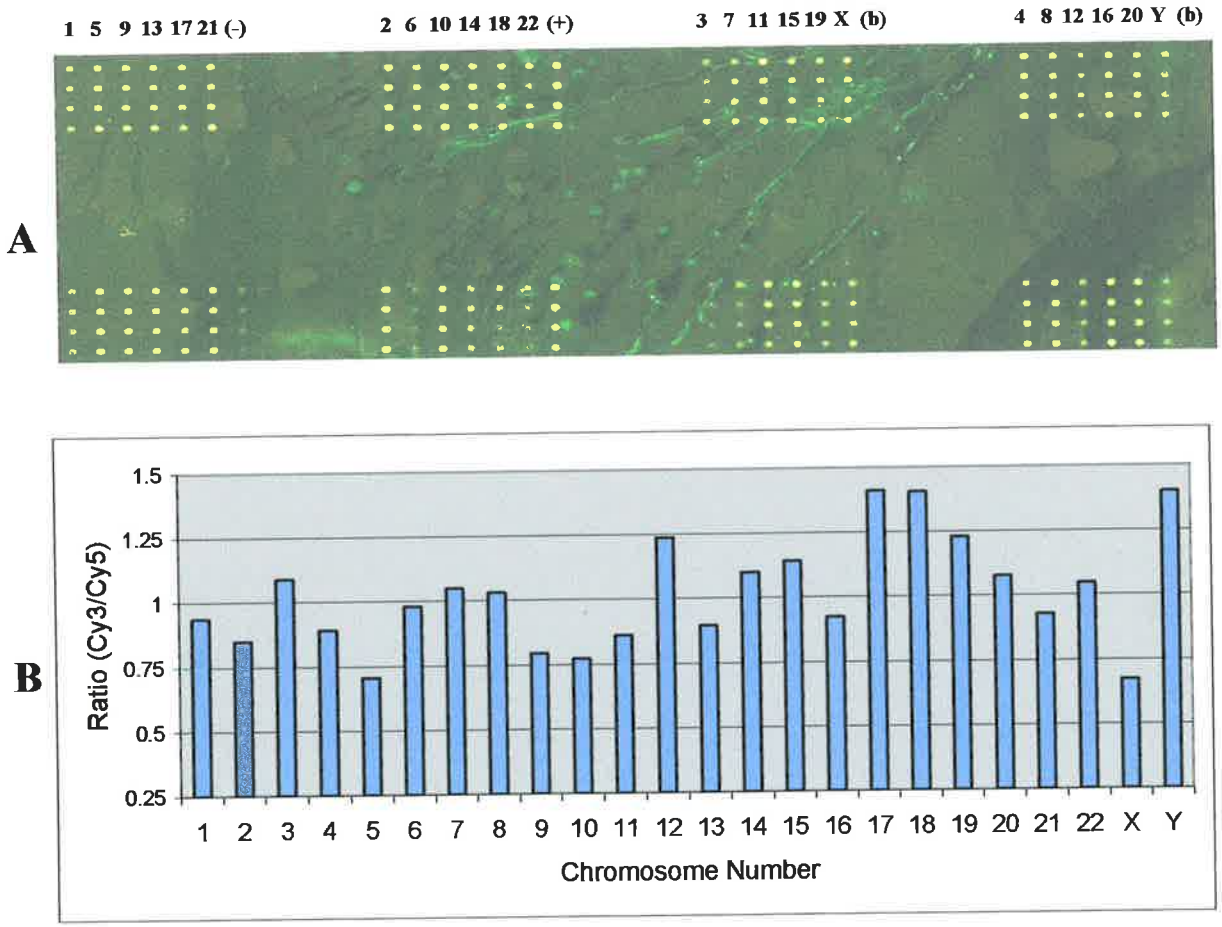


Figure 4.7. Results of an array CGH experiment of a single 47,XY,+18 (Cy3) amniocyte versus a single female 46,XX (Cy5) lymphocyte (Section 4.2.3.4). **A**): JPEG image of the Cy3 and Cy5 fluorescence intensity scanned by a GenePix 4000B scanner: PMT volts (580/670, Cy3/Cy5) and laser ScanPower (100%/100%, Cy3/Cy5). **B**): Ratios of all of the 24 CSLs shown in a graph (Table 4.3, Experiment 4). The CSL_{18}, CSL_X, and CSL_Y all give the expected ratio of 1.40, 0.67 and 1.39, respectively. However, the CSL_5 and CSL_{17} show unexpected ratios of 0.70 and 1.40, respectively. As with Figure 4.6, the positive control DNA sample spotted on the microarray (Male Donor 1) is not from the same genome as the reference single female cell resulting in a yellow fluorescence.

4.2.3.5 Array CGH of 47,XX,+21

Array CGH was also tested using single 47, XY,+21 amniocytes, but the results expected for trisomy 21 were not obtained after several attempts (data not shown).

4.2.4 Fluorescence in situ hybridization (FISH)

To rule out the possibility that poor specificity of some of the DNA libraries (CSLs) might contribute to the deviant ratios found in the above studies, FISH to metaphase 46,XY chromosome spreads was performed to test the specificity of each of all the 24 human CSLs used in this study.

FISH signals of uniform painting were obtained for all target chromosomes and specifically only the q arms of the five acrocentric chromosomes (13-15, 21 and 22) (Fig. 4.8A and 4.8B). No cross-hybridization was found in any experiments. As expected, the centromeres were weakly painted and a clear gap could be seen at the centromere for many chromosomes, especially chromosomes, 1, 2, 3, 4, 6, 7, 8, 9, 10, and 12 (Fig. 4.8A). These results demonstrate that all the 24 CSLs still maintained high specificity after one additional round of DOP-PCR amplification in our laboratory. However, the addition of human Cot-1 DNA and probe preannealing prior to hybridization was required for each of the reamplified CSLs to paint on the target chromosome without any cross-hybridization. Therefore, all the 24 reamplified CSLs may still contain some repetitive sequences, which might have been amplified with high efficiency in the repeated DOP-PCR amplifications following the repeat-depletion procedure.

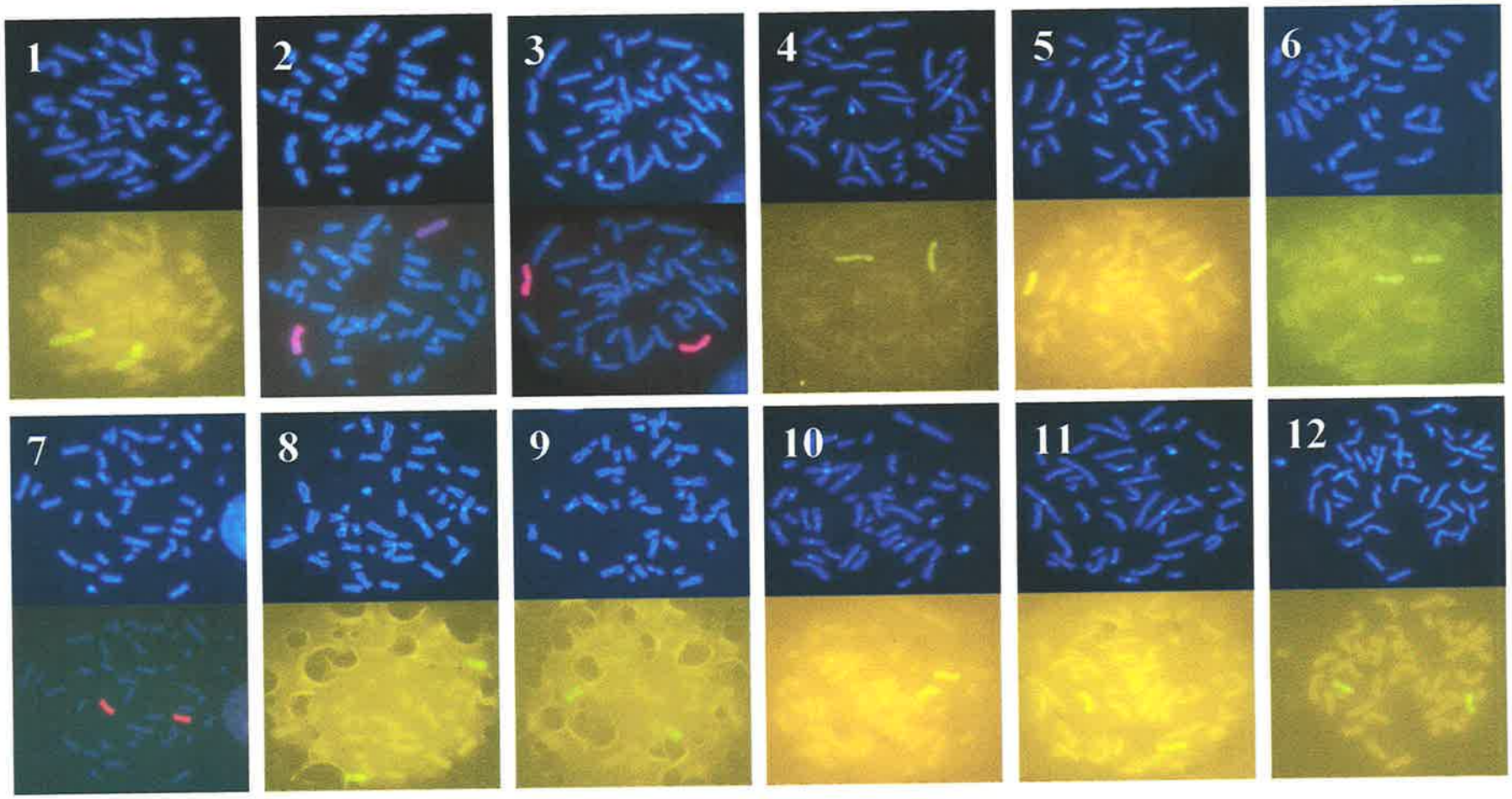


Figure 4.8A. FISH images of whole chromosome specific DNA libraries (CSL_{1-12}) spotted as probes on the 2nd batch of arrays. CSLs (FISH probes) were labelled either by SpectrumRed-dUTP or SpectrumGreen-dUTP and hybridized to normal male (46,XY) metaphase chromosome spreads. The DAPI image (top) and its respective fluorescent image in red or green (bottom) are shown in each case. The origin of each CSL is marked with a number within the image.

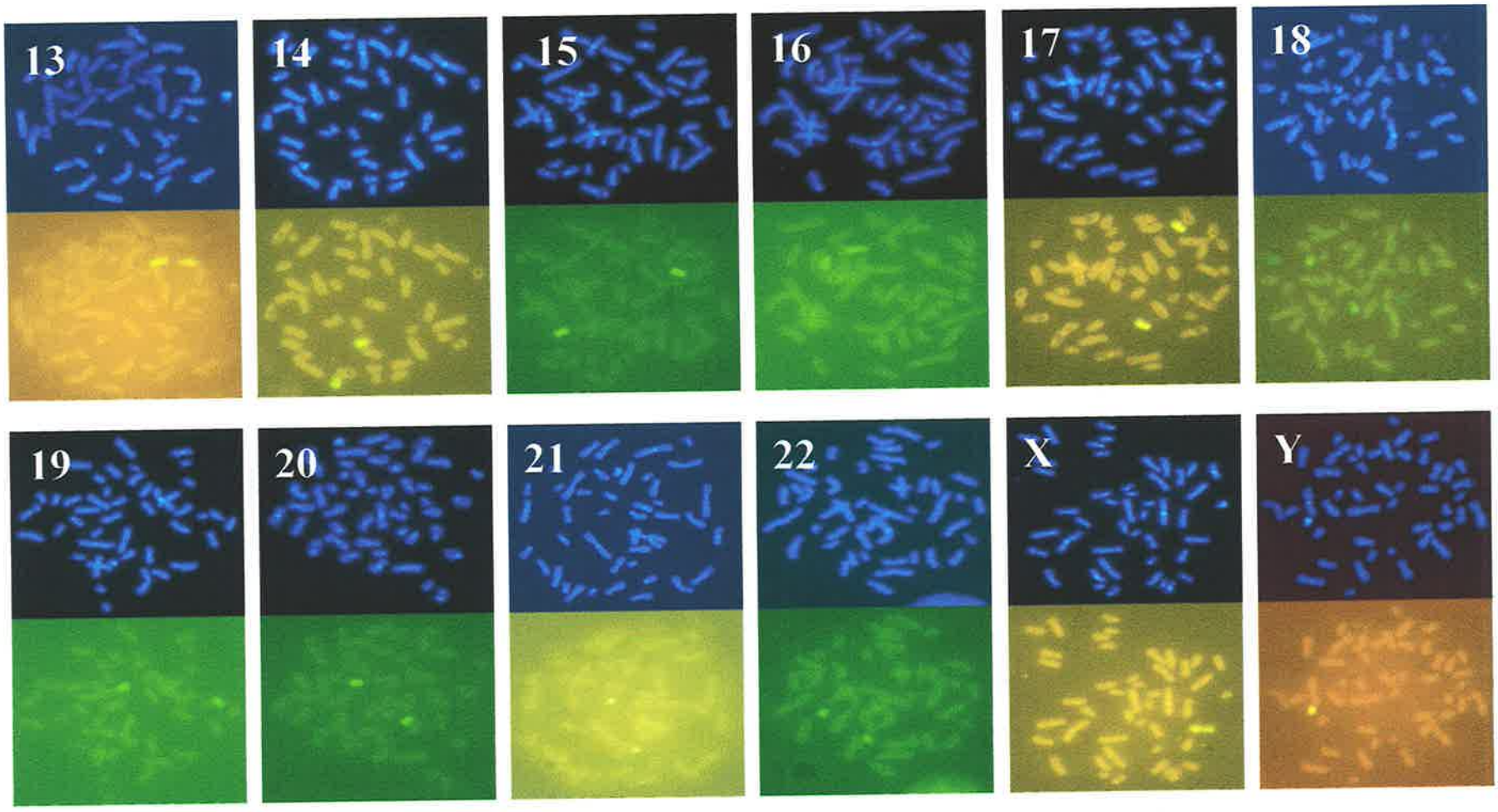


Figure 4.8B. FISH images of whole chromosome specific DNA libraries ($CSL_{13\text{-}Y}$) spotted as probes on the 2nd batch of arrays. CSLs (FISH probes) were labelled either by SpectrumRed-dUTP or SpectrumGreen-dUTP and hybridized to normal male (46,XY) metaphase chromosome spreads. The DAPI image (top) and its respective fluorescent image in red or green (bottom) are shown in each case. The origin of each CSL is marked with a number within the image.

4.3 Discussion

In a recent study (Voullaire et al. 2000), DOP-PCR products amplified from single blastomeres removed from human preimplantation embryos showed a smear containing bands at 450 and 600 bp and sometimes two further bands, at approximately 1250 and 1650 bp. They sequenced the 600-bp band showed it to have 99% homology with mitochondrial DNA. From the presence of the same 600 bp band (Section 4.2.1 and Fig. 4.1), it can be inferred that mitochondrial DNA was also amplified in the present study.

The novelty of this study is that a set of all human 24 whole chromosome-specific DNA libraries (CSLs) were spotted on glass slides for CGH analysis. In order to obtain a reliable signal from a single-cell array CGH analysis, DOP-PCR amplified DNA libraries reputedly free of repetitive sequences were specifically selected for this project. In all four experiments (Section 4.2), an overall 94% (81/86) of the ratios obtained from the autosomal DNA libraries (CSLs) was found to be within the selected cutoff thresholds of 0.75 and 1.25 (Table 4.3). The copy-number of the X chromosome could be correctly predicted in all 4 trials. In the cases of trisomic amniocytes 47,XX,+13 and 47,XY,+18 tested, the additional copy of the aneuploid chromosomes were correctly identified. These results demonstrated the feasibility of detecting aneuploidy and gender determination in a single cell using array CGH technology.

However, six chromosome-specific DNA libraries (CSLs) including CSL_5, CSL_{17}, CSL_{19}, CSL_{20}, CSL_{21}, and CSL_Y produced at least one unexpectedly deviant ratio in this study, thereby resulting in misdiagnosis of aneuploidy for the five relevant autosomes and gender determination merely relying on the results generated by the CSL_X. All of these six CSLs could specifically paint their target chromosomes in FISH to metaphase chromosome spreads

(Fig. 4.8A and 4.8B), thereby indicating that such deviant ratios were unlikely to be caused by poor specificity of the respective DNA libraries. In metaphase CGH, deviant profiles of ratios are frequently observed for the four chromosomes, 17, 19, 20, and 22, the first three of which were also found to be problematic in the current array CGH study. For accurate diagnosis, these chromosomes are always excluded from metaphase CGH analysis (Larramendy et al. 1998; Voullaire et al. 2000; Malmgren et al. 2002) and should perhaps also be excluded from array CGH analysis as presently developed.

Out of three other CSLs giving unexpectedly deviant results in the present study, CSL_5 and CSL_Y were initially derived from flow-sorted chromosomes and CSL_{21} from microdissected chromosomes (Bolzer et al. 1999). These three CSLs (Fig. 3.2) produced the longer smear extending up to more than 3 kb in size after being run on a 1% agarose gel. Similar smears were also found for eight other CSLs including CSL_1, CSL_2, CSL_4, CSL_8, CSL_9, CSL_{10}, CSL_{11}, and CSL_{16}. In the present study, the ratios obtained from these eight CSLs were found to be within the cutoff thresholds of 0.75 and 1.25 but gave a wider range (Table 4.3) compared to those CSLs with a typical smear ranging from 200bp to 800bp (Fig. 3.2). Another consistent finding among these eleven CSLs was that they tended to produce a brighter signal in array CGH analysis (Fig. 4.4) conducted in the present study even if all dots on the array containing the same amount of sample DNA. In addition, many dots of these eleven CSLs showed smearing, which might lead to inaccurate background subtraction and therefore had adverse impact on the ratios of the respective dots (Figs. 4.4-4.7). Optimization of DOP-PCR conditions failed to produce the typical smear for these eleven CSLs (Section 3.3.6 and Fig. 3.3). These results raised a possibility that the high molecular weight part of the longer smear may pose an adverse impact on the ratios, particularly for CSL_5, CSL_{21}, and CSL_Y, resulting in the deviant ratios for these three chromosomes found in this study.

In one recent study of Veltman *et al.* (2002), dots giving a value of any of (1) % > B532 + 2 SD < 30, (2) % > B635 + 2 SD < 30, and Rgn R^2 < 0.5 were excluded from array CGH analysis. Using such filtering criteria, almost all dots in the present study would have been included in the analysis. In order to enhance the accuracy of analysis, the filtering criteria used in the present study were much more stringent than that used in Veltman *et al.* (2002). Any dots which failed to pass all of the criteria: (1) % > B532 + 2SD > 70, (2) % > B635 + 2 SD > 70, and R^2 < 0.6, were excluded from analysis in the present study. Furthermore, three additional parameters of (1) Dia. > 50 μm, (2) F635% = 0, and (3) F532% = 0 were also included in data filtering to ensure that saturated or imporperly printed dots (size too small in this case) would not have been included in the analysis in the present study. Using these filtering criteria, 9-16% of dots per hybridization were excluded from analysis in the present array CGH study (Table 4.2). For some CSLs, up to 4 out of the total 8 replicate dots of the same CSL per hybridization were unqualified for analysis (Table 4.2). Therefore, the use of 8 replicates for each CSL in the present study was very important to ensure that a diagnosis could be made on every chromosome even if many replicate dots of a single CSL had to be excluded from analysis.

The positive control dots in Figures 4.4 and 4.5 have very skewed fluorescence deviating from the 1:1 green to red fluorescence. In these experiments, the positive control DNA sample was DOP-PCR products from Male Donor 1 genomic DNA and the single male reference (Section 4.2.3.1) or test (Section 4.2.3.2) cell was also from the same donor. When a male cell from Male Donor 2 (Fig. 4.6) or a female cell (Fig. 4.7) was used as the single cell reference this very skewed fluorescence disappeared and the positive control dots gave a ratio of approximately 1:1. It is possible that genomic DNA repetitive sequence present in the positive

control dots hybridises more efficiently and completely to the Male Donor 1 reference cell labelled DNA because it is a "self to self" hybridisation.

4.4 Conclusions

In conclusion, this study provided the first evidence to show the feasibility of detecting aneuploidy, including trisomy 13 and 18, and gender determination in single cells using array CGH technology. Requiring just 30 hours, this array CGH approach may be more suitable than metaphase CGH for PGD aneuploidy screening if it could be successfully performed using single blastomeres or polar bodies. However, further improvement of this approach was obviously required. Particular attention needed to be given to modifying or replacing the six problematic chromosomal DNA libraries (CSLs). Until this problem is solved, the six relevant chromosomes need to be excluded from array CGH analysis. Lastly and most importantly, the reliability of the array CGH approach awaited to be further tested in larger series of experiments using DNA arrays or CGH protocols more robust than those used in this Chapter.

Chapter 5

Detection of aneuploidy and gender determination in single cells by DNA microarray CGH using the 5th batch of arrays

5.1 Introduction and aims

Single-cell array CGH analyses described in the previous Chapter demonstrated the feasibility of detection of aneuploidy and gender determination using DNA arrays consisting of whole chromosomal DNA libraries (CSLs), and also produced unexpectedly deviant ratios for six different CSLs, thereby resulting in misdiagnosis for those chromosomes.

The specific aims of the present study were to test the reliability of single-cell array CGH analysis in larger series of experiments, and to improve the results for the problematic CSLs by use of modified CSLs (Section 3.4.4) or modified reference material, containing a pooled mixture of multiple single-cell DOP-PCR reactions. One hypothesis tested was that the modified CSLs would produce better ratio profiles in single-cell array CGH analysis than their original counterparts. Another hypothesis tested was that deviant ratios might be derived from extreme variations in DOP-PCR amplification of the single genome of the samples. The use of modified reference material might eliminate, or at least minimise such deviant ratios originating from variation of the results of PCR when using reference material derived only from single cells.

5.2 Results

5.2.1 Array CGH on normal male and female single lymphocytes

Four array CGH experiments (Table 5.1, Section A) were initially performed including (1) female(Cy3) versus female(Cy5) cell, (2) male(Cy3) versus female(Cy5) cell, (3) male(Cy3) versus male(Cy5) cell, and a (4) female(Cy3) versus male(Cy5) cell. In all four experiments, the ratios of all autosomal CSLs were found to be within the cutoff thresholds of 0.75 and 1.25 with the exception that a deviant ratio was observed once for CSL_2 and twice for CSL_9 The CSL_X dots gave the expected results in all four trials, with a ratio of 0.97 in the female(Cy3)/female(Cy5), 0.63 in the male(Cy3)/female(Cy5), 0.94 in the male(Cy3)/male(Cy5), and 1.58 in the female(Cy3)/male(Cy5) comparisons. By contrast, the CSL_Y dots failed to produce the expected ratio of >1.25 and <0.75 in the male(Cy3)/female(Cy5) and female(Cy3)/male(Cy5) comparisons, respectively. The results of (4), comparison of a female(Cy3) versus a male(Cy5) cell, are presented as an example (Fig. 5.1).

To test further the reliability of single-cell array CGH analysis, 10 more experiments of female(Cy3)/male(Cy5) comparisons were carried out using 9 single male cells and 10 single female cells (Table 5.1, Section B). Results of these 10 experiments were combined with the 4 previous experiments (Table 5.1, Section A) for statistical analysis. Overall, up to 8 of the total 192 (24 x 8) dots per experiment failed to pass the filtering criteria (Section 2.2.7.3) and were then excluded from analysis (Table 5.2). The mean ratios of all 22 autosomal CSLs were close to the theoretical 2:2 ratio of 1.0 (Table 5.1, Section C). Of the total 308 (22 x 14) autosomal ratios in all 14 experiments, 300 (97%) were found to be within the range 0.75-1.25. Sixteen different autosomal CSLs ($CSL_{1,\ 3,\ 5\text{-}8,\ 10,\ 12\text{-}16,\ \text{and}\ 18\text{-}21}$) always gave a ratio in the

Table 5.1. Results of 14 separate single-cell array CGH experiments performed using normal male and female lymphocytes on the 5th batch of arrays after analysis of the whole set of all 24 original CSLs only

Experiments of Array CGH	Ratios (Cy3/Cy5) of chromosomal DNA libraries (CSLs)																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A. Possibiliy of array CGH in single cells																								
(1) 46,XX_{Cy3}/46,XX_{Cy5}	0.94	0.98	1.04	0.99	0.98	0.97	1.00	0.95	1.02	1.01	0.99	0.97	0.99	1.00	1.01	0.98	1.05	1.01	0.99	1.00	1.04	0.98	0.97	0.89
(2) 46,XY_{Cy3}/46,XX_{Cy5}	0.82	**1.25**	0.85	1.19	1.12	0.84	0.86	0.93	**1.32**	1.23	1.18	0.88	0.94	0.88	0.87	1.23	0.85	0.99	0.90	0.85	1.04	1.14	0.63	**1.02**
(3) 46,XY_{Cy3}/46,XY_{Cy5}	0.88	0.97	0.95	0.95	0.97	0.94	0.96	1.10	**1.45**	1.18	1.02	0.95	0.95	0.95	0.99	1.16	0.93	0.96	0.93	0.93	0.98	0.93	0.94	0.98
(4) 46,XX_{Cy3}/46,XY_{Cy5}	0.96	0.85	1.08	0.89	0.86	1.16	1.16	1.02	0.90	0.99	0.87	1.12	1.12	1.06	1.17	0.88	1.03	0.90	0.98	1.16	0.91	0.95	1.58	**0.90**
B. Reliability of array CGH in single cells																								
(1) 46,XX_{Cy3}/46,XY_{Cy5}	0.89	1.12	0.94	1.04	0.88	1.07	1.07	0.87	0.96	1.08	0.78	0.98	0.95	0.98	1.07	0.97	1.18	1.08	0.95	1.10	1.04	0.97	1.54	0.57
(2) 46,XX_{Cy3}/46,XY_{Cy5}	0.93	1.01	1.16	1.12	0.99	1.06	1.12	0.98	1.22	1.07	1.02	1.07	1.15	1.16	1.01	0.94	**0.71**	0.96	0.88	0.81	0.93	0.79	1.39	0.64
(3) 46,XX_{Cy3}/46,XY_{Cy5}	1.03	0.97	0.97	1.03	1.00	1.00	1.07	1.08	1.00	0.97	1.00	1.01	0.99	0.99	1.09	1.01	0.91	0.97	0.88	0.90	1.04	1.00	1.35	**0.92**
(4) 46,XX_{Cy3}/46,XY_{Cy5}	0.94	1.18	0.88	1.02	1.10	0.92	0.90	1.08	1.19	1.08	0.85	0.98	0.99	0.97	1.00	0.90	0.93	1.00	0.92	1.03	1.17	0.96	1.33	**1.09**
(5) 46,XX_{Cy3}/46,XY_{Cy5}	0.89	1.15	0.94	1.12	0.96	0.96	0.94	0.79	1.04	1.20	1.20	0.99	0.88	0.96	0.86	1.20	0.97	1.16	0.92	1.02	1.20	**0.74**	1.31	**0.77**
(6) 46,XX_{Cy3}/46,XY_{Cy5}	1.03	0.94	1.08	0.78	0.88	1.03	0.96	0.93	0.91	0.93	0.93	1.09	1.15	1.06	1.16	0.85	**1.25**	0.95	1.15	1.02	0.92	1.01	1.52	**0.81**
(7) 46,XX_{Cy3}/46,XY_{Cy5}	0.96	1.04	0.95	0.97	1.02	1.01	0.83	0.94	0.97	0.96	0.96	1.04	1.02	0.99	1.04	1.09	0.88	0.98	1.06	1.11	1.06	1.05	1.38	**0.94**
(8) 46,XX_{Cy3}/46,XY_{Cy5}	1.07	1.07	0.89	1.11	1.07	0.89	0.90	1.05	1.13	1.09	1.23	0.91	0.95	0.89	0.92	1.04	0.91	0.95	0.95	0.95	1.08	0.95	**1.07**	**1.21**
(9) 46,XX_{Cy3}/46,XY_{Cy5}	0.87	1.05	1.03	1.03	1.01	0.97	1.01	0.88	1.05	0.99	1.15	0.99	1.01	1.00	1.15	1.04	0.97	1.05	0.89	0.90	1.01	0.90	1.31	**0.87**
(10)46,XX_{Cy3}/46,XY_{Cy5}	1.04	0.93	1.18	**0.70**	0.79	1.15	0.92	0.93	1.01	1.03	**0.69**	1.16	1.13	1.11	1.10	1.00	0.98	1.03	0.95	1.15	0.93	1.21	1.44	**0.98**
C. Statistical analysis																								
(1) Mean	0.95	1.04	1.00	1.00	0.97	1.00	0.98	0.97	1.08	1.06	0.99	1.01	1.02	1.00	1.03	1.02	0.97	1.00	0.95	1.00	1.03	0.97	1.38[a]	0.88[b]
(2) Standard deviation	0.07	0.10	0.10	0.13	0.09	0.09	0.09	0.09	0.15	0.09	0.16	0.07	0.08	0.07	0.10	0.11	0.13	0.06	0.07	0.11	0.08	0.12	0.14	0.18
(3) Number of experiments	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
(4) Number of mean within threshold of 0.75 to 1.25	14	13	14	13	14	14	14	14	12	14	13	14	14	14	14	14	12	14	14	14	14	13	13	4

Ratios marked in green and red are expected ratios resulting in the correct diagnosis of gender determination
Ratios marked in bold are unexpected ratios resulting in the misdiagnosis of autosomal aneuploidy or gender determination
All other ratios are all within the cutoff thresholds of 0.75 and 1.25 as expected
[m]a pooled mixture of at least 5 (but up to 10) single cell DOP-PCR products labeled by either Cy3- or Cy5-dUTP
[a]and[b]Means averaged from 11 female(Cy3)/male(Cy5) array CGH comparisons only
Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

Figure 5.1. Analysis of all 24 original CSLs and re-analysis using the eleven modified CSLs carried out for a single-cell array CGH experiment performed on the 5th batch of arrays. A): JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from a single-cell array CGH experiment of 46,XX(Cy3)/46,XY(Cy5). The origin of each sample is indicated above each lane. The 11 modified CSLs are indicated in blue and their 11 respective original CSLs in green. The remaining 13 original CSLs are indicated in red. All of the original and modified CSLs were simultaneously hybridized in each array CGH experiment. As expected, the dots of CSL_X show greener, and the dots of CSL_{Ym} are redder compared to all other dots. However, in most array CGH experiments conducted in this study, it is not possible to determine the gender and to detect autosomal aneuploidies by visible colour changes, and any diagnosis should be made based on the analysis of the ratios of the Cy3 and Cy5 fluorescence intensity. **B**): All of the 24 original CSLs marked with red and green were first analysed together, and **C**): Re-analysis was then carried out using the 13 original CSLs marked in red and the 11 modified CSLs marked in blue. **D**): Ratios obtained from the analysis and re-analysis of the same array CGH experiment compared graphically. As expected, the ratios of all autosomal CSLs are within the range 0.75 to 1.25 and the CSL_X gives a ratio of >1.25 in both cases. An expected ratio of <0.75 was not obtained for the original Y-chromosomal DNA library (CSL_Y). However, the modified Y-chromosomal DNA library (CSL_{Ym}) produced an expected ratio of 0.72, slightly less than the lower threshold value of 0.75.

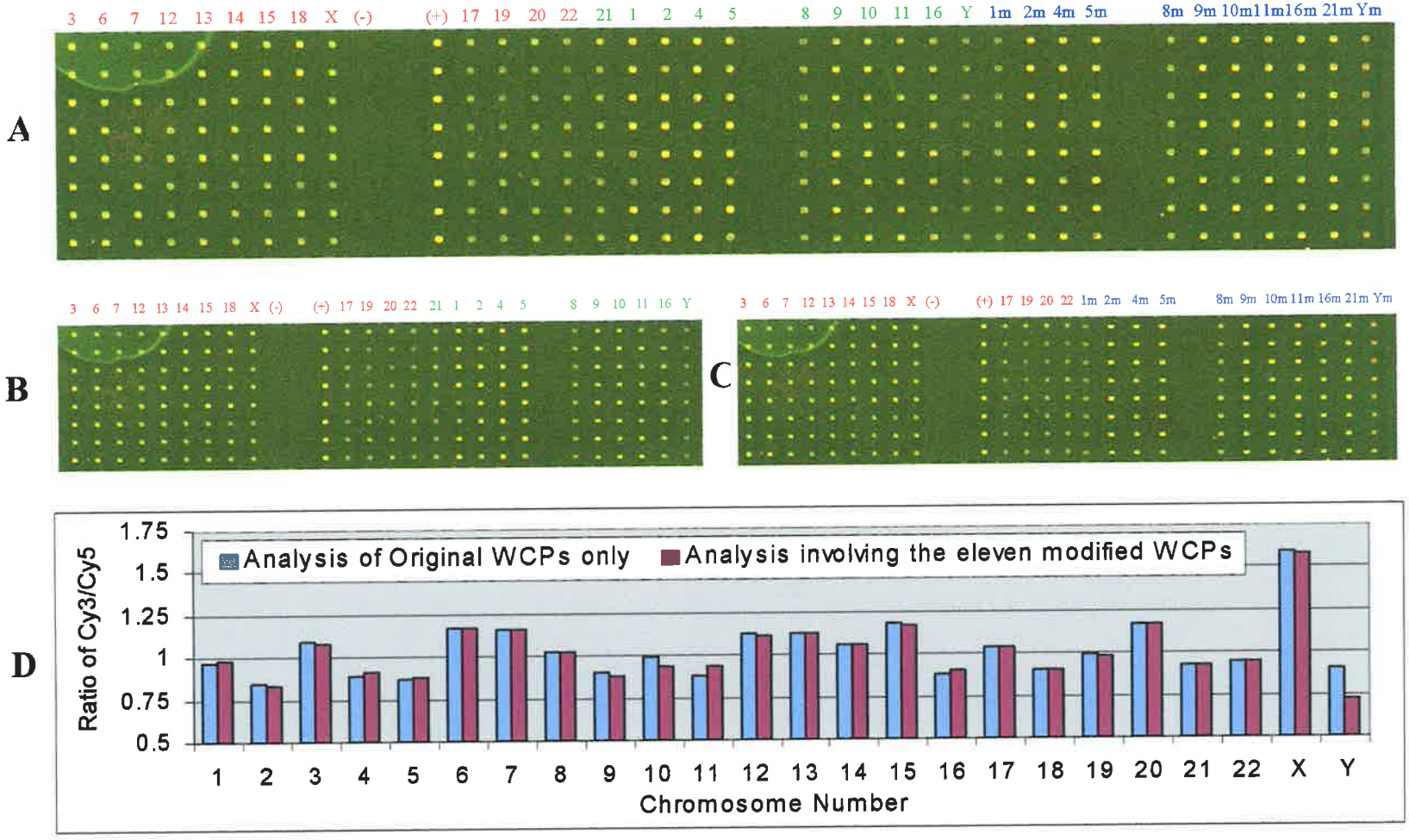

A
3 6 7 12 13 14 15 18 X (-) (+) 17 19 20 22 21 1 2 4 5 8 9 10 11 16 Y 1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
B
3 6 7 12 13 14 15 18 X (-) (+) 17 19 20 22 21 1 2 4 5 8 9 10 11 16 Y
C
3 6 7 12 13 14 15 18 X (-) (+) 17 19 20 22 1m 2m 4m 5m 8m 9m 10m 11m 16m 21m Ym
D
Analysis of Original WCPs only
Analysis involving the eleven modified WCPs
Ratio of Cy3/Cy5
1.75
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

Table 5.2. Number of dots failed to pass the filtering criteria and then excluded from analyses of 14 separate single-cell array CGH experiments performed using single normal lymphocytes on the 5th batch of arrays

Experiments		% > B635 + 2 SD > 70	% > B532 + 2 SD > 70	SNR635 > 3.0	SNR 532 > 3.0	Maximal No. of replicates excluded from a single CSL[a]	Total No. of dots excluded[b]
A: Possibility of array CGH in single cells	(1) $46,XX_{Cy3}/46,XX_{Cy5}$	1	3	2	5	2 (CSL_7)	5 (2.6%)
	(2) $46,XY_{Cy3}/46,XX_{Cy5}$	1	3	2	3	1 ($CSL_{7,17,19}$)	3 (1.5%)
	(3) $46,XY_{Cy3}/46,XY_{Cy5}$	4	8	7	8	2 ($CSL_{4,14}$)	8 (4.1%)
	(4) $46,XX_{Cy3}/46,XY_{Cy5}$	1	1			1 (CSL_7)	1 (0.5%)
B: Reliability of array CGH in single cells	(1) $46,XX_{Cy3}/46,XY_{Cy5}$	1	5	2	5	2 (CSL_{16})	7 (3.6%)
	(2) $46,XX_{Cy3}/46,XY_{Cy5}$	1	1			1 ($CSL_{2,16}$)	2 (1.0%)
	(3) $46,XX_{Cy3}/46,XY_{Cy5}$	2	4	4	4	2 (CSL_{17})	4 (2%)
	(4) $46,XX_{Cy3}/46,XY_{Cy5}$					0	0 %
	(5) $46,XX_{Cy3}/46,XY_{Cy5}$	1	3		3	1 ($CSL_{3,7,18}$)	3 (1.5%)
	(6) $46,XX_{Cy3}/46,XY_{Cy5}$	7	8	7	8	2 (CSL_{18})	8 (4.1%)
	(7) $46,XX_{Cy3}/46,XY_{Cy5}$					0	0 %
	(8) $46,XX_{Cy3}/46,XY_{Cy5}$		4		6	1 ($CSL_{1,2,3,4,11,17}$)	6 (3.1%)
	(9) $46,XX_{Cy3}/46,XY_{Cy5}$	2	5	2	5	1 ($CSL_{1,6,11,14,19}$)	5 (2.6%)
	(10)$46,XX_{Cy3}/46,XY_{Cy5}$	1	2		1	1 ($CSL_{1,20}$)	2 (1%)

Definitions for % >B635 + 2SD >70, % > B532 + 2SD > 70, SNR635, and SNR532 were previously described (2.2.7.3)
[a]Number of replicate dots of the same CSLs failed to the pass filtering criteria and were then excluded from analysis
[b]Total number of dots of all of the 24 CSLs per array CGH experiment failed to pass filtering criteria and were excluded from analysis
Some dots were excluded from analysis by more than one filtering parameter (details not shown)

range 0.75-1.25. However, one deviant ratio was observed once for each of four $CSL_{2, 4, 11, and\ 22}$, and twice for CSL_9 and CSL_{17}. The CSL_X gave a mean ratio of 1.38 ± 0.14 (mean ± s.d.) in 11 independent female(Cy3)/male(Cy5) comparisons, and it was possible to deduce the correct copy number of the X chromosome by these ratios in 13 out of 14 separate array CGH experiments (Table 5.1, Section C). By contrast, the expected ratio of <0.75 was obtained for the CSL_Y dots in only 2 out of 11 female(Cy3)/male(Cy5) comparisons (mean = 0.88 ± 0.18) (Table 5.1, Section C).

5.2.2 Improvement of the ratio profiles with modified CSLs

To try to improve the ratio profiles, all the 14 experiments (Section 5.2.1) were reanalysed with the 11 original CSLs containing the longer smear replaced by their respective modified CSLs (Table 5.3, Sections A and B, Fig. 5.1). Overall, the mean ratios of all autosomal CSLs were all close to the theoretical 2:2 ratio of 1.0 (Table 5.3, Section C). Of the total 308 (22 x 14) autosomal ratios, 296 (96%) were within the range 0.75-1.25. Sixteen different autosomal CSLs ($CSL_{3, 5\text{-}10, 12\text{-}16, and\ 18\text{-}21}$) always produced an expected ratio falling within the cutoff threshold range of 0.75 and 1.25. One deviant ratio was observed once for CSL_{11m} and CSL_{22}, twice for CSL_{2m} and CSL_{4m}, and three times for CSL_{1m} and CSL_{17}, respectively. The CSL_X dots gave a mean ratio of 1.40 ± 0.15 (mean ± s.d.) in 11 independent female(Cy3)/male(Cy5) comparisons, and the copy-number of the X chromosome was correctly predicted by these ratios in 13 out of 14 array CGH experiments (Table 5.3, Section C). An expected ratio of <0.75 was obtained for the CSL_{Ym} in 6 out of 11 female(Cy3)/male(Cy5) comparisons (mean =0.79 ± 0.20) (Table 5.3, Section C). These results indicate that the replacement of the original CSLs with modified CSLs are unable to eliminate the unexpectedly deviant ratios previously observed (Section 5.2.1).

Table 5.3. Results of 14 separate single-cell array CGH experiments on the 5th batch of arrays after re-analysis with the respective original CSLs replaced by the eleven modified CSLs

Experiments of Array CGH	Ratios (Cy3/Cy5) of chromosomal DNA libraries (CSLs)																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A. Possibiliy of array CGH in single cells																								
(1) 46,XX_{Cy3}/46,XX_{Cy5}	0.93	1.00	1.00	1.01	0.98	0.98	1.00	0.97	0.99	0.98	0.99	0.97	0.99	1.01	1.01	0.99	1.05	1.01	0.99	1.00	1.02	0.98	0.97	0.90
(2) 46,XY_{Cy3}/46,XX_{Cy5}	0.86	**1.31**	0.84	**1.26**	1.19	0.83	0.85	0.92	1.23	1.10	1.22	0.87	0.93	0.88	0.86	1.16	0.84	0.98	0.89	0.84	1.21	1.13	0.62	**1.04**
(3) 46,XY_{Cy3}/46,XY_{Cy5}	0.96	0.92	0.95	0.79	0.87	0.94	0.96	0.83	0.89	0.88	0.78	0.95	0.95	0.95	0.99	0.86	0.93	0.96	0.93	0.93	0.76	0.93	0.94	**0.61**
(4) 46,XX_{Cy3}/46,XY_{Cy5}	0.98	0.83	1.08	0.91	0.88	1.16	1.15	1.02	0.88	0.93	0.93	1.11	1.12	1.05	1.17	0.90	1.03	0.89	0.98	1.16	0.92	0.94	1.58	0.72
B. Reliability of array CGH in single cells																								
(1) 46,XX_{Cy3}/46,XY_{Cy5}	**0.61**	1.07	1.01	0.98	0.87	1.15	1.15	0.80	0.90	1.06	0.82	1.06	1.03	1.06	1.15	0.91	**1.27**	1.16	1.02	1.18	0.87	1.04	1.66	0.53
(2) 46,XX_{Cy3}/46,XY_{Cy5}	**0.68**	1.18	1.15	1.23	1.10	1.05	1.10	0.95	0.98	1.02	1.16	1.05	1.13	1.15	0.99	1.07	**0.70**	0.94	0.87	0.80	1.10	0.78	1.37	0.69
(3) 46,XX_{Cy3}/46,XY_{Cy5}	0.97	0.97	0.99	1.02	1.02	1.02	1.09	1.01	1.00	0.96	0.96	1.03	1.01	1.01	1.11	0.98	0.93	0.99	0.90	0.92	0.99	1.02	1.38	**0.83**
(4) 46,XX_{Cy3}/46,XY_{Cy5}	**0.72**	**1.25**	0.94	1.09	1.03	0.99	0.97	0.78	0.88	0.91	0.95	1.06	1.07	1.05	1.08	0.99	1.00	1.00	0.99	1.11	1.04	1.03	1.43	0.56
(5) 46,XX_{Cy3}/46,XY_{Cy5}	0.90	1.20	0.93	1.11	1.05	0.95	0.93	0.81	1.13	1.17	1.08	0.98	0.87	0.95	0.85	1.00	0.96	1.14	0.91	1.01	1.11	**0.73**	1.29	0.65
(6) 46,XX_{Cy3}/46,XY_{Cy5}	0.96	0.91	1.10	0.78	0.86	1.05	0.98	0.96	0.92	0.97	0.99	1.10	0.85	1.07	1.18	0.87	**1.27**	0.96	1.17	1.04	0.94	1.02	1.55	**0.91**
(7) 46,XX_{Cy3}/46,XY_{Cy5}	0.88	1.08	0.96	1.03	1.07	1.01	0.84	0.96	0.96	0.96	0.99	1.04	1.02	0.99	1.04	1.07	0.88	0.98	1.06	1.11	0.96	1.06	1.38	0.73
(8) 46,XX_{Cy3}/46,XY_{Cy5}	1.08	1.15	0.89	1.13	0.98	0.89	0.90	1.03	1.22	1.09	1.22	0.91	0.96	0.89	0.92	0.97	0.91	0.96	0.95	0.95	1.06	0.95	**1.07**	**1.22**
(9) 46,XX_{Cy3}/46,XY_{Cy5}	0.85	1.04	1.02	1.00	0.99	0.97	1.00	0.93	1.11	1.00	1.10	0.99	1.00	0.99	1.14	1.06	0.97	1.04	0.88	0.89	1.08	0.89	1.30	**0.92**
(10)46,XX_{Cy3}/46,XY_{Cy5}	0.92	0.92	1.19	**0.70**	0.78	1.15	0.92	0.95	1.05	1.00	**0.68**	1.16	1.14	1.11	1.11	0.97	0.98	1.03	0.96	1.16	0.92	1.20	1.40	**0.98**
C. Statistical analysis																								
(1) Mean	0.88	1.06	1.01	1.00	0.98	1.01	0.99	0.92	1.01	1.00	0.99	1.02	1.00	1.01	1.04	0.99	0.98	1.01	0.96	1.01	1.00	0.98	1.40[a]	0.79[b]
(2) Standard deviation	0.12	0.14	0.09	0.16	0.11	0.09	0.10	0.08	0.12	0.07	0.15	0.08	0.08	0.07	0.10	0.08	0.15	0.07	0.07	0.12	0.11	0.12	0.15	0.20
(3) Number of experiments	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
(4) Number of mean within threshold of 0.75 to 1.25	11	12	14	12	14	14	14	14	14	14	13	14	14	14	14	14	11	14	14	14	14	13	13	6

Ratios marked in green and red are expected ratios resulting in the correct diagnosis of gender determination
Ratios marked in bold are unexpected ratios resulting in the misdiagnosis of autosomal aneuploidy or gender determination
All other ratios are within the cutoff thresholds of 0.75 and 1.25 as expected
[a]and[b]Means averaged from 11 separate female(Cy3)/male(Cy5) array CGH comparisons only
Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

5.2.3 Improvement of the ratio profile with pooled normal male reference material

To improve the ratio profiles, another strategy tried in this study was that the normal male reference material was changed from a single cell to a pooled mixture of at least 5 (but up to 10) single cell DOP PCR reactions. One array CGH experiment of mixed female (Cy3)/mixed male single-cell DOP-PCR products (Cy5) (Table. 5.4, Experiment 1) was initially conducted and the resulting ratios for all the CSLs except the CSL_Y were found as expected. One experiment with no deviant results (Table 5.1, Section B, Experiment 1) was then repeated using the pooled normal male reference, and no deviant ratios were observed following the change in the reference material. In this repeated trial, much improvement of the ratio was obtained for CSL_{11} (Fig. 5.2A, Table 5.4, Experiments 2).

Four other experiments that previously produced deviant result(s) were further repeated and three of these (Table 5.4, Experiments 3-5) showed an improvement of the deviant results. For one (Table 5.1, Section A, Experiments 2), the ratio of 1.25 for CSL_2 decreased to 1.16 and for CSL_9 the ratio decreased from 1.32 to 1.23, falling within the threshold of 0.75-1.25 (Fig. 5.2B). For another (Table 5.1, Section B, Experiment 2), the ratio of 0.71 was corrected to 1.02 for CSL_{17} (Fig. 5.2C). For a third (Table 5.1, Section B, Experiment 6), the ratio of 1.25 for CSL_{17} was decreased to 1.13 (Fig. 5.2D). For the fourth (Table 5.1, Section B, Experiment 5), a previous deviant ratio of 0.74 for CSL_{22} increased to 1.09, however five other CSLs now produced deviant ratios from 1.25 to 1.31 (Fig. 5.2E). Despite these last results (Fig. 5.2E), there is an indication that the profiles of ratios can be improved using pooled normal male or female single cell DOP-PCR products as the reference material. Therefore for all further experiments in this study, the reference materials were all generated by this method.

Table 5.4. Results of 6 separate array CGH performed on the 5th batch of arrays using a pooled reference material versus a single normal lymphocyte

Experiments of Array CGH	Ratios (Cy3/Cy5) of chromosomal DNA libraries (CSLs)																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
(1) $^{m}46,XX_{Cy3}/^{m}46,XY_{Cy5}$	0.97	0.96	1.05	0.94	1.00	1.06	1.05	0.96	0.89	0.86	0.95	1.11	1.14	1.11	1.08	0.87	1.04	0.97	1.06	1.00	0.95	0.94	1.69	**0.88**
(2) $46,XX_{Cy3}/^{m}46,XY_{Cy5}$	0.99	1.00	1.02	0.95	0.93	0.99	0.98	0.94	1.07	0.97	1.05	1.00	1.04	0.99	0.95	0.94	1.09	0.98	1.10	0.92	0.99	1.06	1.31	**0.88**
(3) $46,XY_{Cy3}/^{m}46,XY_{Cy5}$	0.90	1.16	0.96	1.00	1.03	0.87	0.88	0.93	1.23	1.03	1.12	0.89	0.95	1.03	0.97	1.05	0.93	1.00	1.00	0.86	1.12	1.10	0.86	0.96
(4) $^{m}46,XX_{Cy3}/46,XY_{Cy5}$	0.90	1.01	0.98	1.00	0.95	1.03	1.07	1.01	0.98	1.03	1.09	1.01	1.03	1.00	1.09	0.97	1.02	0.91	0.89	1.11	0.96	0.89	1.39	**0.91**
(5) $46,XX_{Cy3}/^{m}46,XY_{Cy5}$	1.09	0.83	1.11	0.80	0.81	1.10	0.99	0.99	0.90	0.89	0.90	1.07	1.13	1.15	1.12	0.87	1.13	1.02	1.15	1.09	0.94	0.98	1.48	**0.91**
(6) $46,XX_{Cy3}/^{m}46,XY_{Cy5}$	0.83	**1.27**	0.84	**1.25**	1.19	0.78	0.79	0.86	**1.25**	1.14	**1.28**	0.83	0.86	0.88	0.87	1.09	0.95	1.06	0.95	0.86	**1.31**	1.09	1.42	**0.92**

Ratios marked in green are expected ratios resulting in the correct diagnosis of gender determination
Ratios marked in bold are unexpected ratios resulting in the misdiagnosis of autosomal aneuploidy or gender determination
All other ratios are within the cutoff thresholds of 0.75 and 1.25 as expected
^{m}a pooled mixture of at least 5 (but up to 10) single cell DOP-PCR products labeled by either Cy3- or Cy5-dUTP
Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

Figure 5.2. Comparison of the profiles of ratios for single-cell array CGH analyses after the normal male or female reference material changed from a single cell to a pooled mixture of 5 to 10 single cell DOP-PCR reactions.

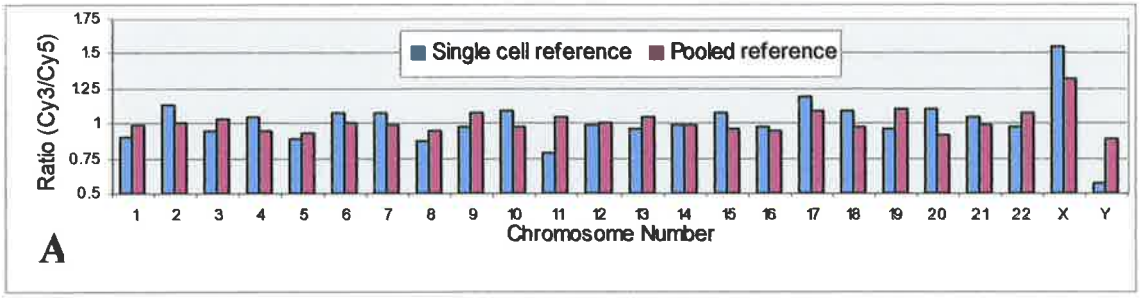
A): Repeat of a single-cell array CGH experiment of 46,XX (Cy3)/46,XY (Cy5), which previously gave no deviant results, using pooled normal male reference DNA labelled with Cy5. No deviant ratios were observed following the change in the reference material.

B): Repeat of a single-cell array CGH of 46,XY(Cy3)/46,XX(Cy5) following a change of reference DNA from a single-cell female to pooled normal male reference DNA labelled with Cy5. The ratio of 1.25 for CSL_2, and 1.32 for CSL_9 was decreased to 1.16 and 1.23, respectively. As expected, the ratio of <0.75 found for CSL_X in the original male/female comparison was then changed to a value of 0.86, falling within the range 0.75-1.25 in the repeated male/male comparison.

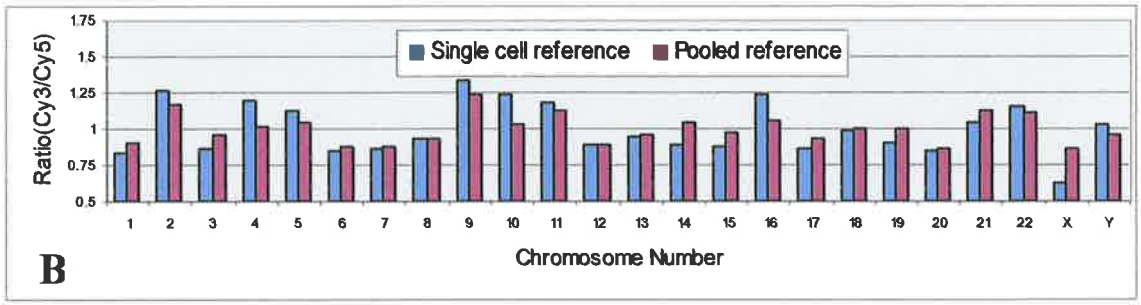
C): In a single-cell array CGH of 46,XX(Cy3)/46,XY(Cy5), the ratio of 0.71 was corrected to 1.02 for CSL_{17} after the single male cell reference DNA was replaced by the pooled normal male reference DNA labelled with Cy5.

D): In a single-cell array CGH of 46,XX(Cy3)/46,XY(Cy5), the ratio of 1.25 was decreased to 1.13 for CSL_{17} after using the pooled normal male reference DNA labelled with Cy5.

E): Following the change from single-cell to pooled reference DNA, a previously deviant ratio of 0.74 for CSL_{22} found in a single-cell array CGH of 46,XX(Cy3)/46,XY(Cy5) was increased to 1.09. However, five other CSLs, including CSL_2, CSL_4, CSL_9, CSL_{11}, and CSL_{21}, then showed deviant ratios ranging from 1.25 to 1.31.


Single cell reference
Pooled reference
Ratio (Cy3/Cy5)
1.75
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

A


Single cell reference
Pooled reference
Ratio(Cy3/Cy5)
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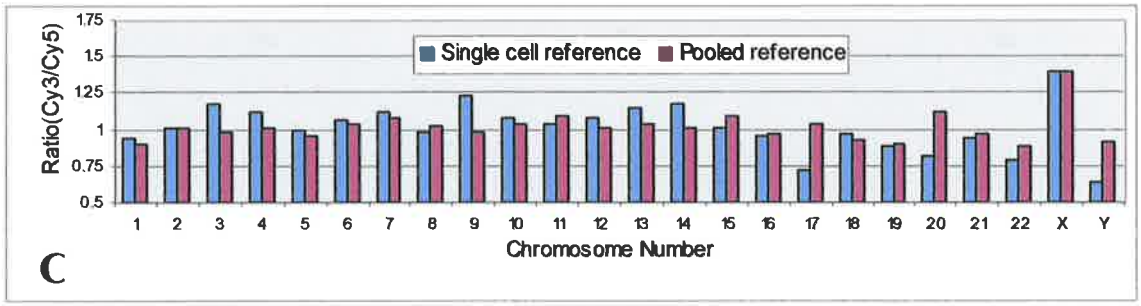
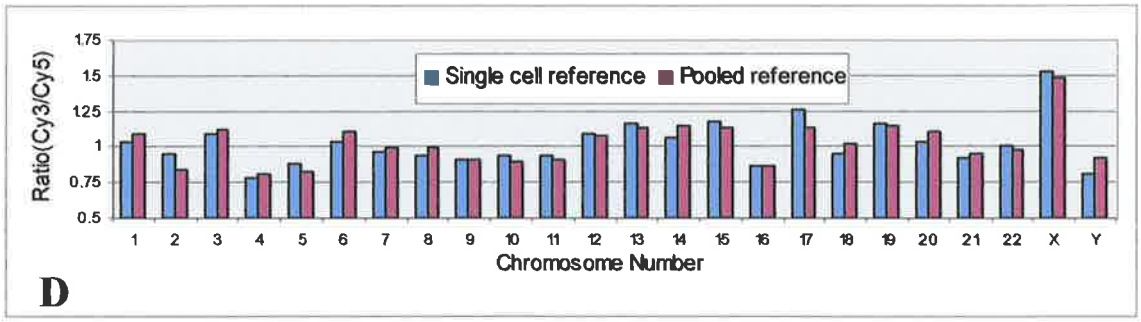
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

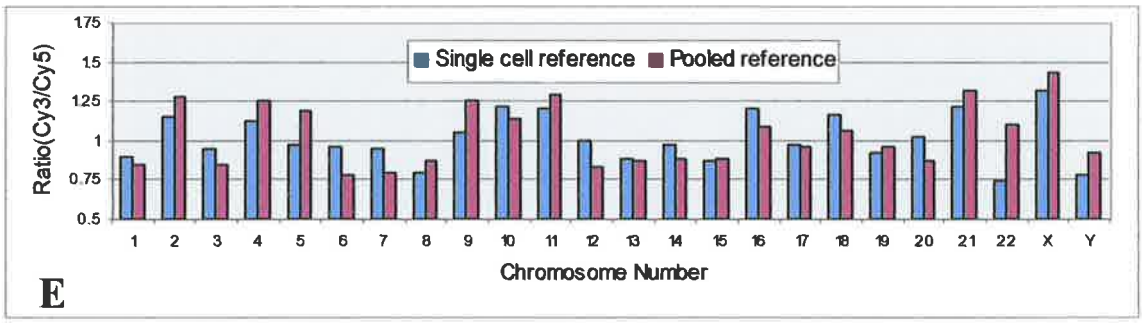
B


Single cell reference
Pooled reference
Ratio(Cy3/Cy5)
1.75
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

C


Single cell reference
Pooled reference
Ratio(Cy3/Cy5)
1.75
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

D


Single cell reference
Pooled reference
Ratio(Cy3/Cy5)
1.75
1.5
1.25
1
0.75
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Chromosome Number

E

5.2.4 Array CGH analysis on three trisomic cell lines

Single-cell array CGH analysis was carried out to test the possibility of diagnosis of three fibroblast cell lines including GM02948A (47,XY,+13), GM07189 (47,XY,+15), and GM01359 (47,XY,+18). In each case, single fibroblast cells were isolated from these cell cultures, amplified and labelled with Cy3 by DOP-PCR, and then compared to pooled normal male reference material labelled with Cy5.

When a single cell isolated from GM02948A cell line was tested, the CSL_{13} dots gave a ratio of 1.32, well above the upper cut-off threshold of 1.25. The ratios for all 23 other CSLs fell well within the 0.75-1.25 range, ranging from 0.85-1.20 (Table 5.5, Section A, Experiment 1; Fig. 5.3). These results indicate the correct diagnosis of the extra copy of chromosome 13 contained in the 47,XY,+13 karyotype of the GM02948A cell line.

Similarly, a single cell from GM01359 cell line was tested and it produced a ratio of 1.27 for CSL_{18}, which was above the upper threshold of 1.25. The ratios for all 23 other CSLs varied from 0.87 to 1.09, falling well within the range 0.75-1.25 (Table 5.5, Section A, Experiment 2; Fig. 5.4). Therefore, the presence of one additional copy of the chromosome 18 in the GM01359 cell line (47,XY,+18) was correctly determined.

When the GM07189 cell line was tested, one single cell gave the highest ratio of 1.27 for the CSL_{15}, which was above the upper threshold of 1.25. The ratios of all 23 other CSLs varied only from 0.94 to 1.08 and fell well within the 0.75-1.25 range (Table 5.5, Section A, Experiment 3; and Fig. 5.5). These results indicate that this test cell had an extra copy of chromosome 15. However, another single cell tested failed to produce a ratio of >1.25 for the CSL_{15}. In this experiment, a ratio within the range 0.75-1.25 was observed for all 24 CSLs

(Fig. 5.6), indicating that this test cell was effectively chromosomally normal. These discordant results between the two cells tested for the GM07189 cell line suggested that this cell line might be mosaic, although a non-mosaic 47,XY,+15 cell line had been specified by Coriell Cell Repositories (Camden, NJ, USA). This suspicion was subsequently confirmed by G-banding analysis, which revealed that the GM07189 cell line was a mosaic: 47,XY,+15 (27 metaphases, 68%)/47,XY,t(3;16)(q22;p11.2),+der(16p) (13 metaphases, 32%) (Figs. 5.5A and 5.6A). Therefore, it is quite possible that the test cell had a 47,XY,t(3;16)(q22;p11.2),+der(16p) karyotype. We would not expect any positive deviant ratios because this unbalanced abnormality involved imbalances of only a small region of chromsome 16. Further G-banding analyses of the two other cell lines confirmed the claimed karyotypes of 47,XY,+13 for GM02948A (Fig. 5.3A) and 47,XY,+18 for GM01359 (Fig. 5.4A).

The unexpected clone of 47,XY,t(3;16)(q22;p11.2),+der(16p) cells found in the culture of GM07189 is most likely to be a culture-induced abnormality due to the extended time of culture (approx. two months) after receipt of these cells; there was no evidence of trisomy in this clone. It is probable the trisomy 15 cell line was the initial cell line and 47 centromeres have been retained by the extra chromosome 15 centromere being somehow incorporated into the rearrangement involving chromosomes 3 and 16 (G.C. Webb, personal communication).

Another two cell lines: GM0111145 (47,XY,+9) and GM04435 (48,XY,+16,+21) were also imported from Coriell Cell Repository (Camden, NJ, USA). But the GM0111145 cell was found to be chromosomally normal (46,XY) by G-banding analysis (data not shown) and the GM04435 cell line failed to proliferate since no vital cells were available upon receipt. Therefore, no single-cell array CGH experiments were performed on these two cell lines.

Table 5.5. Results of single-cell array CGH experiments performed on the 5th batch of arrays from
A): Diagnoses of three trisomic fibroblast cell lines
B): An array CGH experiment scanned using six different settings of PMT gains
C): An array CGH experiment scanned for six times over a period of 73 days after hybridization

Experiments of	Ratios (Cy3/Cy5) of chromosomal DNA libraries (CSLs)																							
Array CGH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A. Diagnosis of trisomy																								
(1) 46,XY,+13$_{Cy3}$/^{m}46,XY$_{Cy5}$	1.20	0.85	1.00	0.85	0.92	1.07	0.98	1.09	0.91	0.86	0.89	1.05	1.32	1.08	1.07	0.88	1.01	1.00	1.10	0.94	0.99	0.95	0.91	1.19
(2) 46,XY,+18$_{Cy3}$/^{m}46,XY$_{Cy5}$	0.93	1.06	0.92	1.04	1.04	0.87	0.87	1.00	1.09	1.00	1.01	0.97	0.99	0.97	1.03	0.97	0.93	1.27	1.07	0.95	1.09	0.91	0.88	0.89
(3) 46,XY,+15$_{Cy3}$/^{m}46,XY$_{Cy5}$	1.00	1.01	0.94	1.01	0.99	0.97	0.99	0.88	1.08	0.99	1.04	1.08	0.96	1.03	1.27	0.95	0.95	0.93	0.96	0.90	1.04	0.98	0.92	0.95
B. An array CGH of 46,XX$_{Cy3}$/46,XY$_{Cy5}$ scanned using six different settings of PMT gains (Cy3/Cy5)																								
(1) 400/500	0.97	1.01	1.03	0.95	0.93	1.00	0.95	1.00	0.93	1.04	1.04	1.06	1.01	0.98	0.91	1.06	1.00	1.10	0.94	0.98	1.02	1.02	1.35	0.85
(2) 500/550	0.98	0.99	1.03	0.93	0.92	0.98	0.99	0.91	1.04	0.93	1.03	1.02	1.05	1.01	0.96	0.93	1.10	0.96	1.12	0.96	0.99	1.10	1.32	0.90
(3) 600/670	0.99	1.00	1.02	0.95	0.93	0.99	0.98	0.94	1.07	0.97	1.05	1.00	1.04	0.99	0.95	0.94	1.09	0.98	1.10	0.92	0.99	1.06	1.31	0.88
(4) 650)/750	1.00	0.98	1.05	0.95	0.91	0.99	0.99	0.92	1.04	0.96	1.02	1.02	1.07	1.00	0.96	0.91	1.09	0.96	1.10	0.92	0.99	1.08	1.34	0.87
(5) 700/Cy5	0.99	0.98	1.04	0.94	0.93	0.98	0.98	0.92	1.06	0.94	1.02	1.01	1.06	1.00	0.99	0.90	1.11	0.99	1.12	0.93	0.98	1.06	1.31	0.84
(6) 800/900	1.01	1.00	1.07	0.92	0.91	0.99	0.98	0.92	1.04	0.94	1.00	1.04	1.06	0.99	0.97	0.91	1.10	1.00	1.13	0.95	0.94	1.06	1.32	0.85
C. An array CGH of 47,XY,+18$_{Cy3}$/^{m}46,XY$_{Cy5}$ scanned for six times over a period of 73 days after hybridization																								
(1) day 1	0.93	1.06	0.92	1.04	1.04	0.87	0.87	1.00	1.09	1.00	1.01	0.97	0.99	0.97	1.03	0.97	0.93	1.27	1.07	0.95	1.09	0.91	0.88	0.89
(2) day 8	0.93	1.06	0.90	1.05	1.07	0.86	0.86	1.02	1.11	1.00	1.03	0.95	0.96	0.96	1.01	0.99	0.92	1.25	1.07	0.94	1.12	0.92	0.88	0.89
(3) day 21	0.96	1.04	0.94	1.03	1.05	0.90	0.87	0.99	1.06	0.96	1.00	0.99	0.99	0.99	1.04	0.94	0.91	1.29	1.08	0.93	1.09	0.92	0.89	0.89
(4) day 31	0.93	1.04	0.95	1.05	1.06	0.88	0.90	0.99	1.08	0.99	0.99	0.97	0.97	0.99	1.02	0.95	0.90	1.32	1.06	0.93	1.09	0.92	0.89	0.85
(5) day 55	0.96	1.03	0.94	1.04	1.05	0.89	0.90	1.00	1.06	0.97	0.98	0.98	0.98	0.99	1.04	0.94	0.95	1.27	1.08	0.93	1.10	0.91	0.88	0.88
(6) day 73	0.95	1.05	0.98	1.07	1.06	0.90	0.88	0.96	1.05	0.96	0.95	0.98	1.00	1.03	1.02	0.90	0.90	1.30	1.07	0.95	1.07	0.92	0.91	0.85

Ratios marked in green are expected ratios resulting in the correct diagnoses of aneuploid chromosomes in A), genders in B), and trisomy 18 in C)
All other ratios are all within the cutoff thresholds of 0.75 and 1.25 as expected
^{m}a pooled mixture of at least 5 (but up to 10) single cell DOP-PCR products labeled with Cy5-dUTP
Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

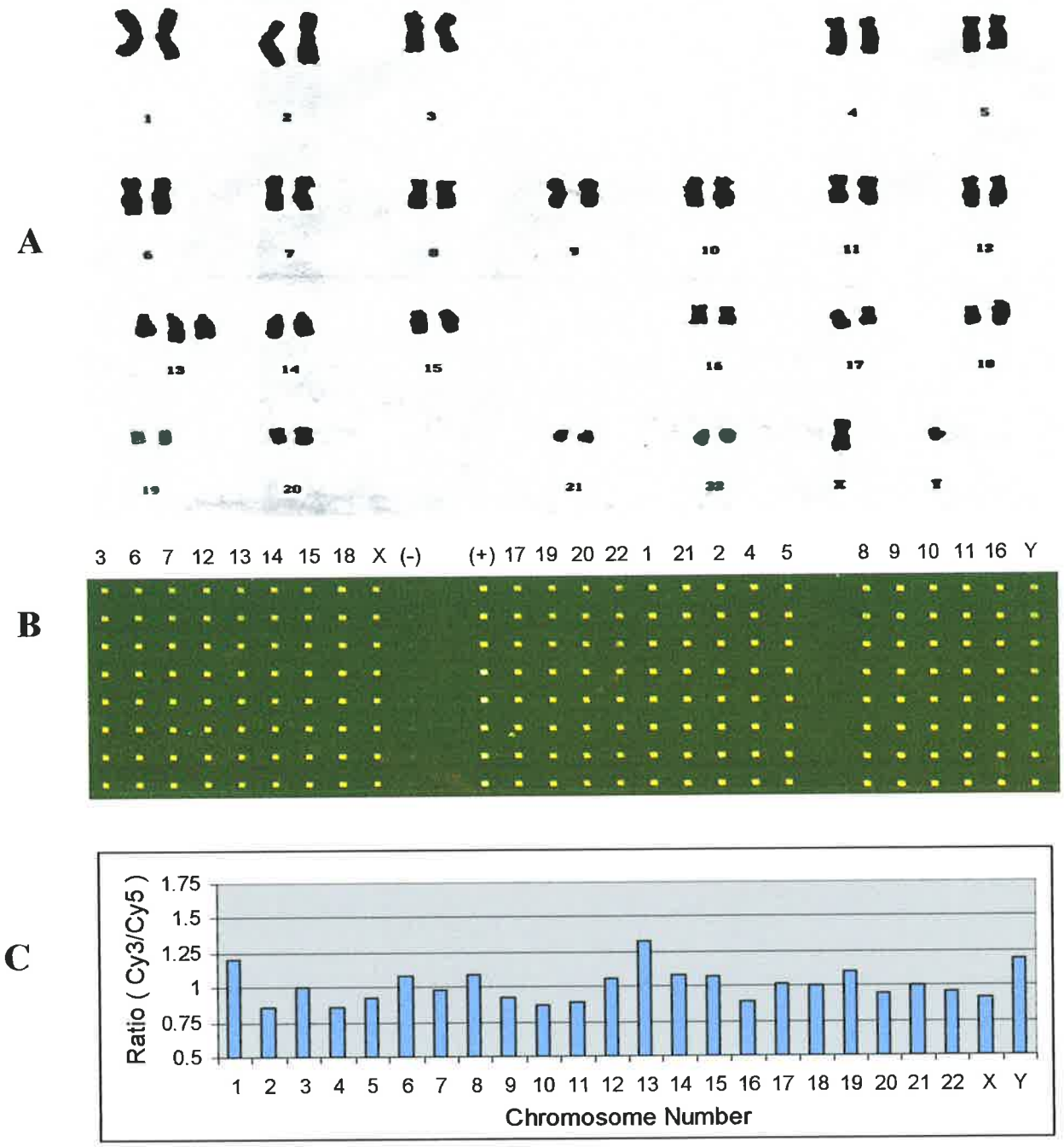


Figure 5.3. Diagnosis of the GM02948A (47,XY,+13) cell line using single-cell array CGH analysis. A): Results of G-banding analysis showing a 47,XY,+13 karyotype. **B)**: JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of a single cell isolated from the GM02948A cell culture (Cy3) versus a pooled mixture of 5 to 10 normal male single-cell DOP-PCR products (Cy5) captured using a GenePix 4000B scanner. **C)**: Analysis of the image shown in B) by GenePix Pro 4.0.1.12 showing the ratios of Cy3/Cy5 for all of the 24 original CSLs. As expected, the CSL_{13} gives the highest ratio of 1.32 (> 1.25), with the ratios of all other CSLs falling well within the range of 0.75 and 1.25.

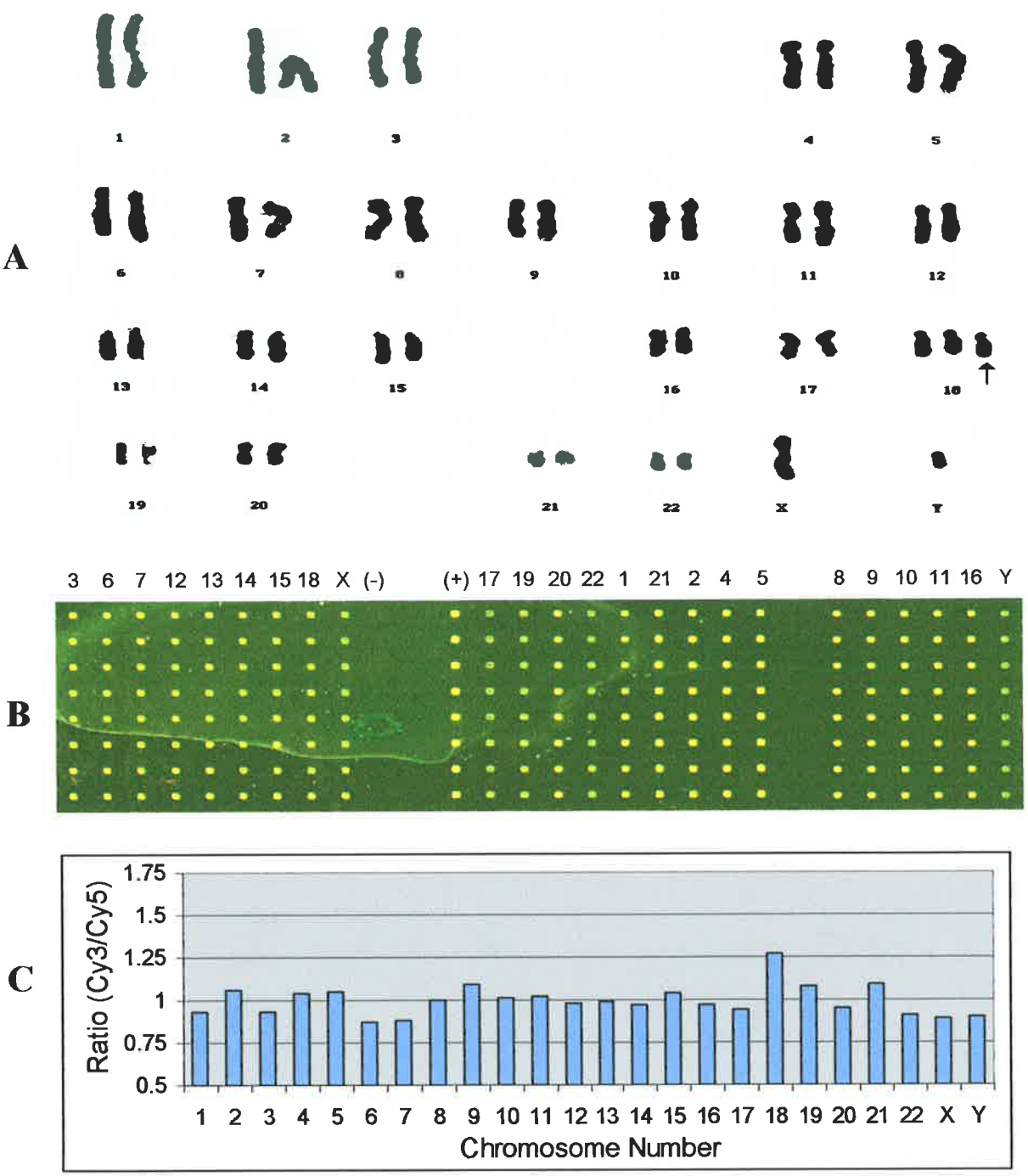


Figure 5.4. Diagnosis of the GM01359 (47,XY,+18) cell line using single-cell array CGH analysis. A): Results of G-banding analysis showing a 47,XY,+18 karyotype. **B)**: JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of a single cell isolated from the GM01359 cell culture (Cy3) versus a pooled mixture of 5 to 10 normal male single-cell DOP-PCR products (Cy5) captured using a GenePix 4000B scanner. **C)**: Analysis of the image shown in B) by GenePix Pro 4.0.1.12 showing the ratios of Cy3/Cy5 for all of the 24 original CSLs. As expected, the CSL_{18} gives the highest ratio of 1.27 (> 1.25), with the ratios of all other CSLs falling well within the range of 0.75 and 1.25.

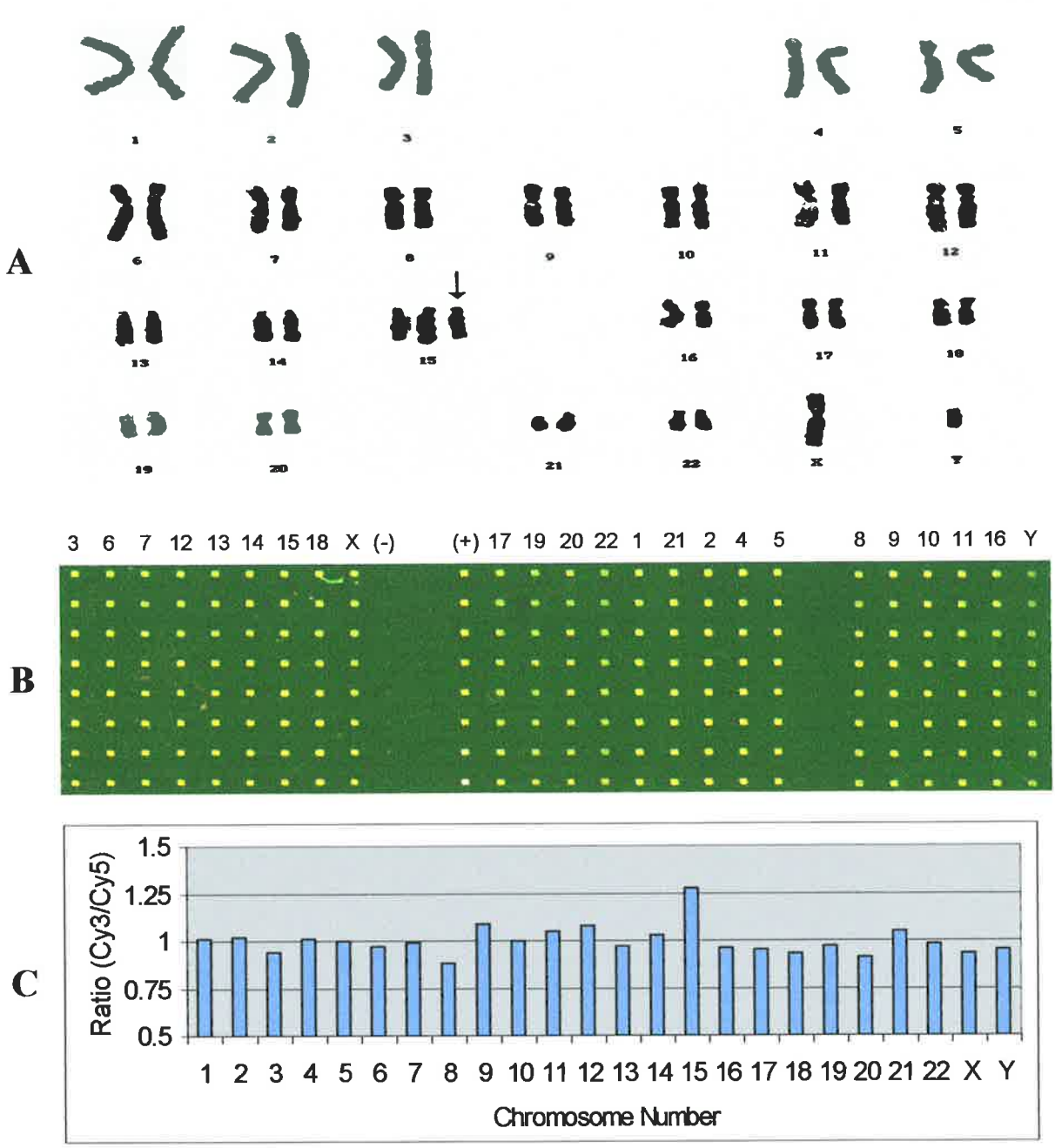


Figure 5.5. Diagnosis of the GM07189 cell line (47,XY,+15/47,XY,t(3;16)(q22;p11.2),+der(16p) using single-cell array CGH analysis. A): Results of G-banding analysis for one cell isolated from the GM07189 cell culture showing a 47,XY,+15 karyotype. **B)**: JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of a single cell isolated from the GM07189 cell culture (Cy3) versus a pooled mixture of 5 to 10 normal male single-cell DOP-PCR products (Cy5) captured using a GenePix 4000B scanner. **C)**: Analysis of the image shown in B) by GenePix Pro 4.0.1.12 showing the ratios of Cy3/Cy5 for all of the 24 original CSLs. The CSL_{15} gives the highest ratio of 1.27 (> 1.25) along with the ratios of all other 23 CSLs ranging from 0.94 to 1.08, falling well within the range of 0.75 and 1.25. These results indicate that the test cell was 47,XY,+15.

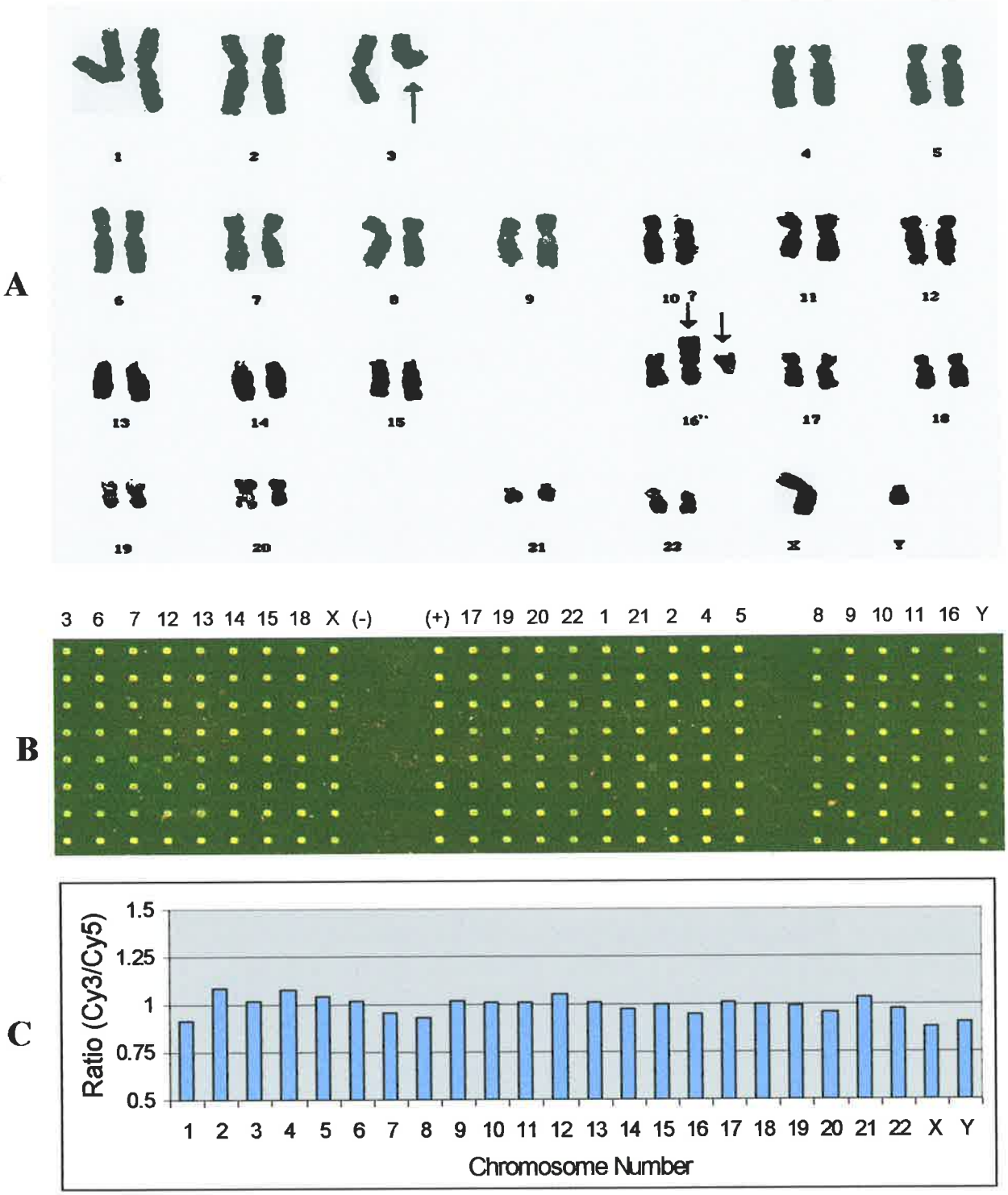


Figure 5.6. Diagnosis of the GM07189 cell line (47,XY,+15/47,XY,t(3;16)(q22;p11.2),+der(16p) using single-cell array CGH analysis. A): Results of G-banding analysis for one cell isolated from the GM07189 cell culture showing a 47,XY,t(3;16)(q22;p11.2),+der(16p) karyotype. **B)**: JPEG image of the Cy3 and Cy5 fluorescence intensity obtained from an array CGH experiment of a single cell isolated from the GM07189 cell culture (Cy3) versus a pooled mixture of 5 to 10 normal male single-cell DOP-PCR products (Cy5) captured using a GenePix 4000B scanner. **C)**: Analysis of the image shown in B) by GenePix Pro 4.0.1.12 showing the ratios of Cy3/Cy5 for all of the 24 original CSLs, which are plotted in a graph. Ratios of all of the 24 CSLs range from 0.88 to 1.08, falling well within the range of 0.75 and 1.25. These results indicate that the test cell was most likely a cell with the 47,XY,t(3;16)(q22;p11.2),+der(16p) karyotype.

5.2.5 Effects of photomultiplier tube gains on ratio profiles

To test the effect of photomultiplier tube (PMT) gains, one slide of a single female (Cy3)/pooled male(Cy5) comparison was scanned using six different PMT gain settings (Fig. 5.7). An expected ratio of ≥1.25 for the CSL_X dots was obtained in all cases along with ratios for all 23 other CSLs falling within the range 0.75 to 1.25 (Table 5.5, section B; Fig. 5.7). The ratio of CSL_Y also fell within this range, but as found previously this was an inadmissible result. Ratios of CSLs obtained from the scanning of six different PMT gain settings were not significantly different (single-factor analysis of variance, F = 0.0032, $P = 0.99$).

However, in the cases of extreme PMT gains of less than 400, a large number of dots tended to give a very low signal to background ratio. These dots always failed to pass the filtering parameter of SNR635 and/or SNR532 and therefore had to be excluded from analysis (data not shown). In the cases of PMT gains higher than 800, up to 44 of the total 192 dots (24 x 8) per array CGH experiment produced a strong signal that saturated the scanner. Therefore these dots failed to pass the filtering parameters of 635 % = 0 and/or 532 % = 0 (Table 5.6). Because of this reason, only 1 out of the total 8 replicates of CSL_2 could be included in the analysis when PMT gains of 800/900 were employed for Cy3/Cy5 scanning (Table 5.6). For accurate diagnosis, PMT gains were always set in this study in the range 500-700 for both Cy3 and Cy5 channels.

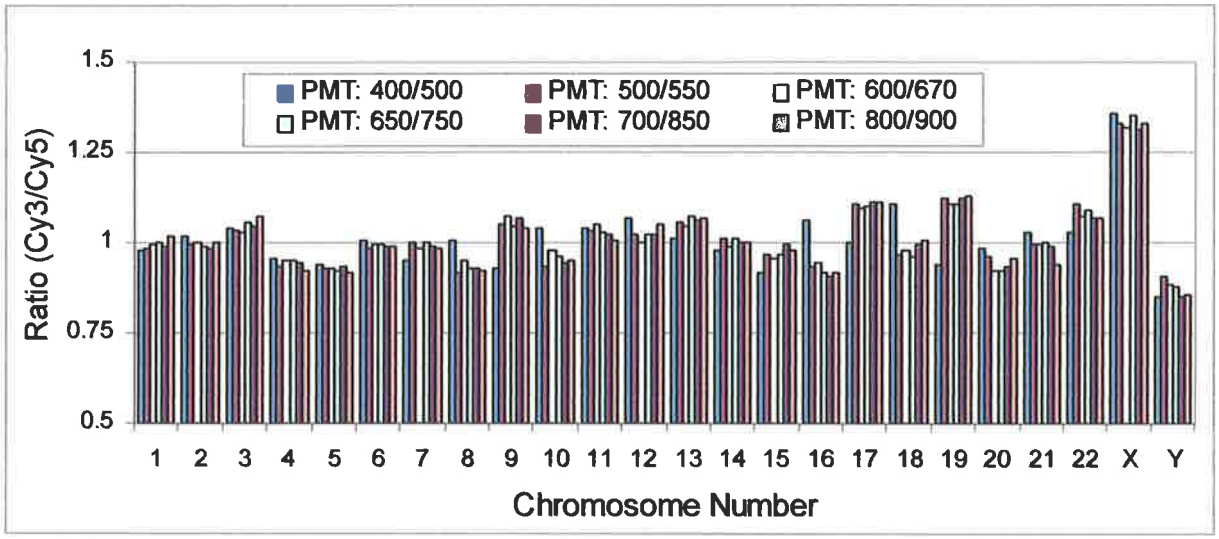


Figure 5.7. Impact of photomultiplier tube (PMT) gains on the reliability of the diagnosis obtained from single-cell array CGH analysis. A hybridized array slide using PCR product from a normal female cell (Cy3) versus pooled normal male (Cy5) DOP-PCR products was scanned using six different settings of PMT gains (voltages) ranging from 400 to 800 for the Cy3 and 500 to 900 for the Cy5 channels. The diagnosis of the test cell as 46,XX was possible for all PMT settings used. The ratios for the X-chromosomal DNA library (CSL_X) ranged from 1.31 to 1.35 and all ratios for the autosomes fell within the range 0.75-1.25 (Table 5.5B). As found previously, the expected ratio of < 0.75 for the Y chromosome was not observed.

Table 5.6. Number of dots which failed to pass the filtering criteria and were then excluded from analyses of an array CGH experiment of 46,XX(Cy3)/46,XY(Cy5) scanned using six different photomultiplier tube (PMT) gains (Cy3/Cy5)

PMT gain settings (volts)	F635 % = 0	F532 % = 0	% > B635 + 2 SD > 70	% > B532 + 2 SD > 70	SNR635 > 3.0	SNR 532 > 3.0	Maximal No. of replicates excluded from a single CSL[a]	Total No. of dots excluded[b]
400/500			1	3	1	4	1 ($CSL_{6, 7, 21, and Y}$)	4 (2%)
500/550			1	4	1	4	1 ($CSL_{6, 7, 21, and Y}$)	4 (2%)
600/670			1	3	1	3	1 ($CSL_{6, 7, and Y}$)	3 (1.5%)
650/750	1	1	1	2	1	4	1 ($CSL_{6, 7, 21, and Y}$)	4 (2%)
700/850	6	6			1	2	4 (CSL_2)	4 (2%)
800/900	22	41	1	1	1	1	7 (CSL_2)	44 (22.9%)

Definitions for F635 %=0, F532%=0, % >B635 + 2SD >70, % > B532 + 2SD > 70, SNR635, and SNR532 were previously described (2.2.7.3)
[a]Number of replicate dots of the same CSLs failed to pass the filtering criteria and were then excluded from analysis
[b]Total number of dots of all of the 24 CSLs per array CGH experiment failed to pass the filtering criteria and were then excluded from analysis
Some dots were excluded from analysis by more than one filtering parameter (details not shown)

5.2.6 Effects of rescanning on ratio profiles

To explore how long the integrity and stability of array CGH results are maintained after hybridization, one slide of single 47,XY,+18 (Cy3)/pooled 46,XY (Cy5) was scanned six times over a period of 73 days. In all cases, an expected ratio of ≥1.25 was obtained for CSL_{18} with all ratios for the other CSLs falling within the range 0.75 to 1.25 (Table 5.4, section C, and Fig. 5.8). Ratios of CSLs obtained from rescanning six times were not significantly different (single-factor analysis of variance, F = 0.0064, P = 0.99). These results suggest that the hybridised slides could be stored at room temperature in the dark for up to 73 days with no change in the resulting diagnosis.

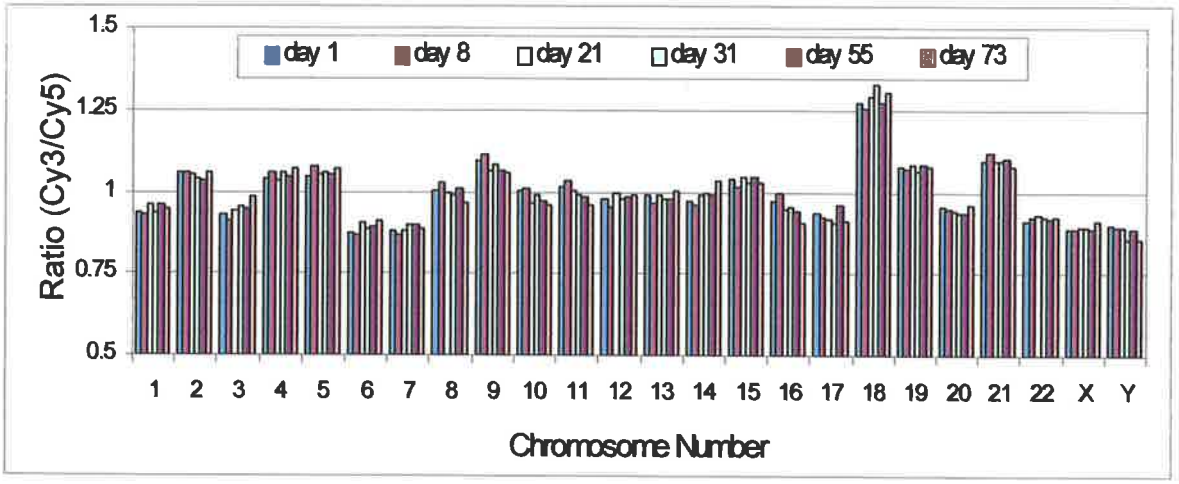


Figure 5.8. Effect of repeated scanning over an extended period of time on the reliability of the diagnosis obtained from single-cell array CGH analysis. One hybridized array slide of a single trisomy 18 (46,XY,+18) cell (Cy3) versus pooled normal male labelled (Cy5) DOP-PCR products was scanned six times over a period of 73 days. The diagnosis of the test cell as a male trisomy 18 cell was possible each time. The ratios obtained for CSL_{18} ranged from 1.25-1.32, and all ratios for the 23 other CSLs fell within the expected range of 0.75-1.25. (Table 5.5C) However, the results obtained for the Y chromosome are inadmissible, although they are all in the expected 0.75-1.25 range.

5.3 Discussion

5.3.1 Reliability of single-cell array CGH analysis

5.3.1.1 Reliability of array CGH for diagnosis for the X chromosome

To test the reliability of diagnosis for the X chromosome, a single male and single female cells were compared in all four possible ways (Table 5.1, Section A). The CSL_X dots gave the expected ratios in all four cases, indicating that single-cell array CGH analysis is able correctly to predict the copy-number of the X chromosome in four different comparisons including 2X/2X, 1X/2X, 1X/1X, and 2X/1X. Further experiments (Table 5.1, Section B) demonstrate that the copy number of the X chromosome was correctly determined in 13 out of 14 (92.9%) separate array CGH experiments. The only failure was a ratio of 1.07 for CSL_X found in a female(Cy3)/male(Cy5) comparison, which could lead to misdiagnosis of a female cell as a male cell (Table 5.1, Section B, Experiment 8). This error would not result in serious clinical consequences but will reduce the number of embryos available for transfer because this embryo would not be transferred in a PGD clinical case where only female embryos are transferred to a woman carrier for a X-linked genetic disorder.

5.3.1.2 Reliability of array CGH for diagnosis for the autosomes compared with FISH

An overall diagnostic accuracy of 97% (300/308) for all 22 autosomes was obtained in this study in a total of 14 separate single-cell array CGH experiments. This can be favourably compared to that of single-cell FISH analysis which had an accuracy of 91-96% per probe per cell for euploid samples and even lower for trisomic samples (Ruangvutilert et al. 2000a). This equates to an overall accuracy of 55-80% for a five chromosome FISH test. In other

FISH analyses, Munne *et al.* (1998) reported a total misdiagnosis rate of 15% when 9 different chromosomes (X, Y, 13, 14, 15, 16, 18, 21, and 22) were analysed per blastomere using two consecutive rounds of FISH hybridisations (Munne et al. 1998a). And Liu *et al.* (1998) analysed six different chromosomes (11, 13, 18, 21, X, and Y) per cell and the rate of nuclear loss and absence of signals was found to be 6% and 13% respectively after the third round of FISH analysis (Liu et al. 1998a). Clearly, the single-cell array CGH approach developed in this study not only allows all 22 autosomes to be analysed per cell but this method also give a more accurate diagnosis than any FISH method.

5.3.1.3 Reliability of array CGH for diagnosis for the Y chromosome

The CSL_Y dots gave the expected ratio in only 4 out of 14 separate single-cell array experiments (Table 5.1, Section C). Without a correct diagnosis on the Y chromosome, gender determination would merely rely on the results produced by the X chromosome. Therefore, it is impossible to differentiate normal male 46,XY from Turner syndrome 45,X and normal female 46,XX from Kleinfelter male syndrome 47,XXY.

In single-cell metaphase CGH, only the euchromatic region of the Y chromosome gives a reliable ratio for the determination of the Y chromosome and not the heterochromatic regions (especially the distal part of its q-arm) (Voullaire et al. 2000; Wilton et al. 2001). In order to avoid misdiagnosis for the Y chromosome, its heterochromatic regions (Speicher et al. 1995) or even the entire Y chromosome (Franke et al. 2001) are always excluded from metaphase CGH analysis. As a consequence, determination of the copy number of the Y chromosome relies on the ratios generated by the very small euchromatic regions (Kallioniemi et al. 1992; Speicher et al. 1995; Voullaire et al. 1999).

The Y chromosomal DNA library (CSL_Y) used in this study was initially made by flow-sorted Y chromosomes (Bolzer et al. 1999), and therefore it contained the DNA sequences of the entire Y chromosome including both euchromatic and heterochromatic regions. This might be the major reason for the failure in this study to determine this chromosome in 10 out of 14 array CGH experiments. If that is the case, microdissection of the p arm only might result in a much better probe for the Y chromosome as it avoids the presence of repetitive sequences of the heterochromatic region in the long arm. Experiments to correct the Y chromosome problem are currently underway in our laboratory.

5.3.2 Improvement of the ratio profiles for single-cell array CGH analysis

5.3.2.1 Deviant ratios found in single-cell array CGH analysis

Deviant ratios were found in the present study for six autosomal DNA libraries including CSL_2, CSL_4, CSL_9, CSL_{11}, CSL_{17}, and CSL_{22}. Deviant ratios were also previously observed for five autosomes including CSL_5, CSL_{17}, CSL_{19}, CSL_{20}, and CSL_{21} (Section 4.3). Overall, 10 different autosomal CSLs have produced unexpectedly deviant results in single-cell array CGH experiments conducted so far in this study. Four out of these 10 chromosomes, 17, 19, 20, and 22, have been also found to be problematic in metaphase CGH, and sometimes they had to be excluded from CGH analysis (Speicher et al. 1993; Kallioniemi et al. 1994; Larramendy et al. 1998; Voullaire et al. 2000; Malmgren et al. 2002).

The remaining six problematic CSLs, CSL_2, CSL_4, CSL_5, CSL_9, CSL_{11}, and CSL_{21}, all had showed a longer smear on a gel (Fig. 3.2). As previously discussed (Section 4.3), the high molecular weight part of this wide smear might be associated with the deviant ratios found in single-cell array CGH analysis. To test this hypothesis, the high molecular part of the wide

smear was removed from these six and five other original CSLs (Section 3.4.4) and the resultant eleven modified CSLs were spotted along with their original CSLs on the 5th batch of arrays (Fig. 5.1A).

5.3.2.2 Effects on the ratio profiles using modified CSLs

No significant difference (single-factor analysis of variance, F = 0.06236, P = 0.80; Fig. 5.9) was found between the mean ratios obtained from the analyses using all the original CSLs only (Table 5.1, Section C) and those generated after the reanalyses involving the eleven modified CSLs (Table 5.3, Section C). The analysis of all the original CSLs only gave an overall misdiagnosis rate of 2.6% (8/308) for the autosomal CSLs and a similar rate of 3.9% (12/308) was obtained after the reanalysis with the eleven modified CSLs included (Table 5.7). Deviant ratios were found for eight different CSLs in both cases, seven of which, CSL_2, CSL_4, CSL_{11}, CSL_{17}, CSL_{22}, CSL_X and CSL_Y, were the same in both original and modified form (Table 5.7). These results indicate that the ratio profiles could not be improved by use of the modified autosomal CSLs. However, the modified Y chromosomal DNA library (CSL_{Ym}) might be the only exception. The CSL_{Ym} dots gave an expected ratio of < 0.75 in 6 out of 11 separate female/male array CGH experiments compared to 2 out of 11 produced by the original Y chromosomal DNA library (CSL_Y) (Table 5.7). Despite this improvement the CSL_{Ym} is still not reliable for single-cell array CGH analysis because it produced incorrect results in almost half number of experiments (5/11). Therefore, only the original CSLs were included in the analyses of all other array CGH experiments conducted in this study.

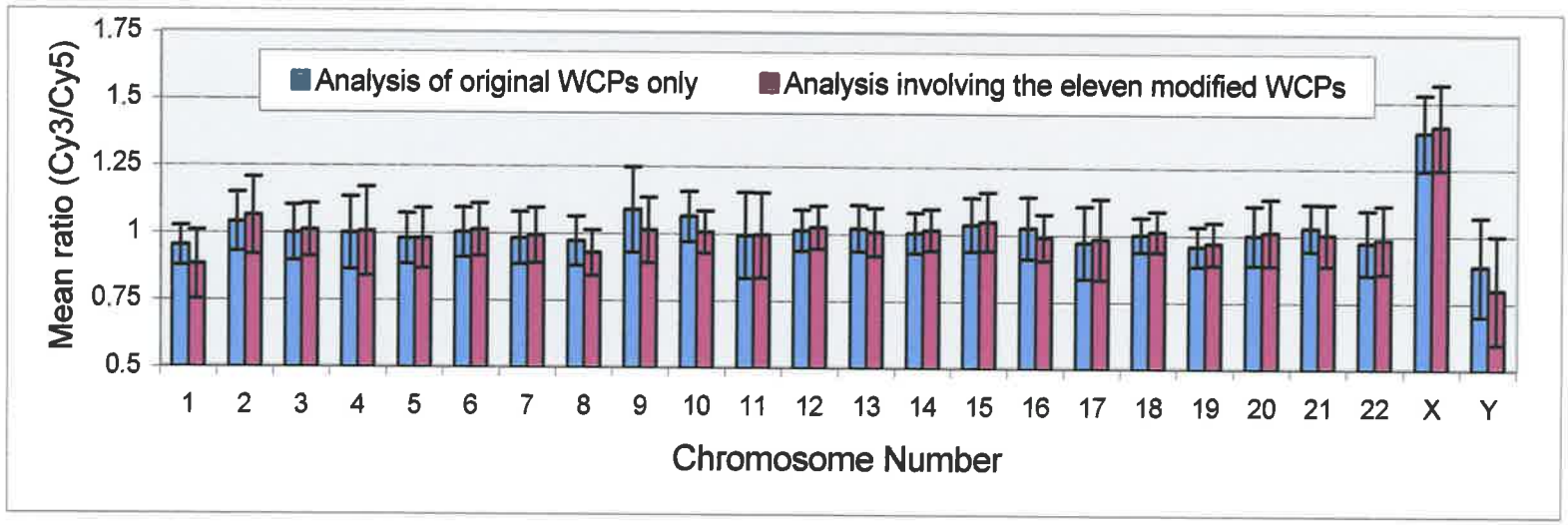


Figure 5.9. Comparative analysis between the mean ratios (mean ± 1 SD) obtained after the analysis of all the original CSLs only and the mean ratios (mean ± 1 SD) generated after re-analysis using the eleven modified CSLs in a total of 14 separate single-cell array CGH experiments. All ratios of the autosomal CSLs in both cases are close to the theoretical 2:2 ratio of 1, and the CSL_X gives an expected mean ratio of >1.25 in both cases. However, both CSL_Y and CSL_{Ym} failed to produce an expected mean ratio of <0.75. The difference found in the mean ratios in both cases was not significantly different (single-factor analysis of variance, F = 0.06236, $P = 0.80$).

Table 5.7. Deviant ratios found in a total of 14 single-cell array CGH experiments after analysing all of 24 original CSLs and re-analysing using the eleven modified CSLs

Type of CSLs analysed	No. of deviant ratios found for chromosomal DNA libraries (CSLs)									
	CSL_1	CSL_2	CSL_4	CSL_9	CSL_{11}	CSL_{17}	CSL_{22}	CSL_X	CSL_Y	Total No. for all autosomal CSLs[a]
Original CSLs only	0	1	1	2	1	2	1	1	6[b]	8 (2.6%)
Modified CSLs involved	3	2	2	0	1	3	1	1	2[b]	12 (3.9%)

[a]Total number of autosomal deviant ratios found in 14 separate female/male array CGH experiments.
[b]No. of deviant ratios found in 11 separate female/male array CGH experiments.

5.3.2.3 Improvement of the ratio profiles with pooled normal male reference material

The use of pooled normal male or female reference material improved the ratio profiles in 3 out of 4 single-cell array CGH experiments, which previously gave at least one deviant ratio (Section 5.2.3). These results support the obvious expectation of variable DOP-PCR amplification of the DNA of a single genome from a single cell as the starting material. Thus perhaps extremely biased amplification of a particular chromosome can result in a deviant ratio for the respective chromosomal DNA library (CSL). Using a reference of pooled multiple single-cell DOP-PCR products has the obvious advantage of averaging out the signals from the reference material and eliminating extreme variations in the single-cell DOP-PCR products of the reference material.

Obviously, variable DOP-PCR amplification might also occur for the DNA of single cells under test, rather than used for reference. However, due to the high level of mosaicism within human preimplantation embryos, it is not permissible to pool more than one test blastomere for PGD aneuploidy screening using CGH analysis. For example, if two blastomeres, removed from a mosaic embryo, are normal and trisomy 21, and the amplified DNA is pooled, then array CGH analysis is most unlikely to show the trisomy 21 because the ratio for the CSL_{21} would be the average of normal and trisomy 21 results. Therefore, some extreme variations in test samples cannot be avoided by pooling more than one cell. Obviously, if more than one blastomere can be available from an embryo, they should be analysed independently rather than pooled; this would give the classic scientific advantage of repetition.

DOP-PCR biased amplification was one of the primary concerns at the beginning of this study. Except for pooling the reference material, two other strategies had already been taken

in this study to minimise the impact of such biased amplification on the ratio profiles. The first was to spot DNA from a library derived from the whole of the chromosome or the long arm in the case of the acrocentric chromosomes as probes on the arrays. The principle underlying this strategy is that the quantity of amplified products from single cells may be more for some DNA fragments or less for some other fragments but such fluctuations should be averaged out when the signals are averaged over numerous DNA fragments contained in the CSLs. The second strategy adopted in this study was the use of only 26 cycles of first round DOP-PCR for random amplification of the DNA contained in the single cell (Section 2.2.6.2) along with 25 cycles of second round DOP-PCR for labelling (Section 2.6.3). This approach may introduce less variation in the DOP-PCR amplification compared to the 35 cycles (Voullaire et al., 1999) or the 50 cycles (Wells et al., 1999) of DOP-PCR used for single-cell amplification, because exponential amplification no longer exists after 23-25 cycles of PCR amplification (Prior et al. 1990).

Despite all these strategies the pooled reference material did not eliminate all of the aberrant results. Therefore, further refinements in the probes spotted onto the glass slide as well as changes in the amplification technique should be taken into consideration to yield more accurate results in the future.

5.3.3 Sensitivity of single-cell array CGH analysis

5.3.3.1 Ratio thresholds for detecting single-copy changes of chromosome

In this study, 14 separate single-cell array CGH experiments in normal versus normal comparisons served to assess the normal ratio deviations of the CSLs arrayed on the slides. This number can be favourably compared to the 5 to 8 independent array CGH experiments

conducted for the same purpose in many published array CGH studies (Pinkel et al. 1998; Hui et al. 2001; Veltman et al. 2002; Wilhelm et al. 2002). Generally, ratio thresholds for detecting genomic imbalances should be broader than the normal deviations observed in the normal versus normal array CGH experiments, and therefore they may be variable in different studies and even different array CGH protocols. The most frequently used thresholds include a range of 0.85 to 1.15 (Cai et al. 2002), 0.82 to 1.18 (Hui et al. 2001), 0.80 to 1.20 (Veltman et al. 2002), or 0.75 to 1.25 (Wessendorf et al. 2002). And it is a general rule that probes with ratios which frequently fall outside of the selected range in the control trials should be replaced or excluded from array CGH analysis.

The range of 0.75 to 1.25 was selected in the present study as the ratio thresholds indicative of equal ploidy because 97% (300/308) of the autosomal ratios fit well within this range. However, an overall 2.6% of the autosomal ratios were found to be outside of this range in normal versus normal array CGH comparisons (Table 5.7). As a consequence, the use of the threshold values of 0.75 and 1.25 could result in false positive diagnoses of aneuploidy, observed once in 14 hybridisations for four autosomes 2, 4, 11, and 22, and observed twice in 14 hybridisations for autosomes 9 and 17 (Table 5.7). It is expected that misdiagnoses might be greatly reduced following the change to pooled reference material (Section 5.2.3). The exclusion of these six problematic CSLs from analysis avoids such misdiagnoses, but this approach would result in no diagnosis for the respective six chromosomes. One compromise may be that all of the six problematic CSLs be included in analyses but cautious interpretation should be given for any diagnosis of aneuploidy for any of the relevant six chromosomes.

5.3.3.2 Ratios of CSL_X for gender determination

The mean ratio of CSL_X obtained in this study from 11 separate single-cell array CGH experiments in normal female/male comparisons was 1.38 ± 0.14 (Table 5.1, Section C), which is much lower than the theoretical 2:1 ratio of 2. Similar results have been reported even in array CGH studies using much more starting DNA, such as genomic DNA samples. For example, ratios of X chromosomal probes were found to be from 1.49 to 1.52 in female/male comparisons (Hui et al. 2001), and 0.69 ± 0.05 (Pinkel et al. 1998) or 0.59 ± 0.004 (Bruder et al. 2001) in male/female comparisons. These reported ratios are slightly better than those generated by CSL_X found in the present study and this might be the result of variations in single-cell DOP-PCR amplification of the samples. Nevertheless, in this study the CSL_X produced an expected ratio of >1.25 in 10 out of 11 independent female/male single-cell array CGH experiments even without the use of pooled normal male reference material.

The underrepresentation of the true copy number of X chromosome probes found in array CGH analysis can be caused by many different reasons (Pinkel et al. 1998; Pollack et al. 1999; Fiegler et al. 2003). The most important are thought to be incomplete suppression of the repetitive sequences and inaccuracy in background substruction. Other reasons may include probe autofluorescence, crosshybridization due to homology of X-Y or X-autosome sequences, and the effects of inactivation of one female X chromosome. Despite these deviant findings, a linear correlation between the copy number of the X chromosome and the ratio has been observed (Fiegler et al. 2003) when normal female DNA was compared to DNA samples of cell lines containing 1 to 5 copies of the X chromosome. However, the observed slope of the relation was 0.37, much lower than the expected value of 0.5, and somewhat in agreement with the results described above.

5.3.3.3 Ratios for diagnosis of anueploidy

In a recent report, DOP-PCR-based array CGH was used to test samples of about 500 cells from a cell line containing a trisomy 7 aneuploidy (Daigo et al. 2001). The mean ratio for the trisomic chromosome probes was 1.3, ranging from 1.22 to 1.45. In another study a ratio of 1.28 was used for the diagnosis of trisomic duplications (Veltman et al. 2002). In the present study, a ratio of 1.32 for CSL_{13}, 1.27 for CSL_{15}, and 1.27 for CSL_{18} was used to diagnose the three relevant trisomic single cells. These ratios fall within the range reported in the published array CGH studies.

In array CGH, a ratio of 0.59 ± 0.05 (Bruder et al. 2001) or 0.67 (Veltman et al. 2002) were observed for heterozygous deletions of autosomal loci. A similar ratio of 0.63 was found in this study for one deleted copy of the X chromosome in an array CGH experiment in a normal male/female comparison (Table 5.1, Section A, Experiment 2), thereby suggesting that autosomal monosomies are most likely detectable by single-cell array CGH using a ratio of < 0.75, as applied in the present study. A ratio of 0.26 was found in array CGH for homozygous deletions of autosomal loci (Bruder et al. 2001), suggesting that a similar ratio may be required for single-cell array CGH to make a diagnosis of autosomal nullisomy.

The results of single-cell array CGH of the present study and all other array CGH studies cited above once again indicate that under-estimation of the true copy number of array probes is a common phenomenon in array CGH analysis. Such under-estimation is most likely caused by reasons similar to those for the X chromosome (Section 5.3.3.2).

5.4 Summary of Chapter 5

Single-cell array CGH analyses conducted in the present study showed an overall accuracy of 97.4% for the diagnosis of all autosomes, and most of the misdiagnoses of aneuploidy for autosomes could be avoided by use of a pooled normal male (or female) reference material. These results indicate that this method is more reliable than interphase FISH methods, which are currently used in almost all PGD cycles for aneuploidy screening.

The ratios obtained in the present study for gender determination and diagnosis of trisomy fell within the range reported in the majority of array CGH studies published to date. These results indicate that the method applied in the present study is capable of detecting autosomal aneuploidy and X chromosomal variation using ratio thresholds such as 0.75-1.25 widely adopted in current array CGH studies. However, for more accurate gender determination, the CSL_Y used in this study will have to be replaced by other Y chromosome-specific DNA probes, which will give a correct ratio for this chromosome.

Chapter 6

Chromosomal and molecular analyses of single blastomeres of preimplantation embryos by single-cell array CGH and locus-specific PCR

6.1 Introduction and aims

Many studies have revealed that at least 50% of all IVF-created human cleavage-stage embryos are aneuploid or mosaic (Delhanty et al. 1993; Delhanty et al. 1997; Munne et al. 1998a; Wells and Delhanty 2000). This raises the possibility that embryos transferred in PGD cycles for single-gene defects may have chromosomal abnormalities. Therefore, there is a need to screen for aneuploidy in the case of PGD for single-gene defects in order to transfer embryos unaffected with either single-gene defects or aneuploidies. To achieve this goal, the primary priority is to develop PGD protocols capable of analysing both chromosomal and single-gene defects using the same biopsied polar bodies or blastomeres. Such PGD protocols would be particularly beneficial to couples in which maternal ages are > 35 years undergoing PGD for single-gene defects because these patients are not only carriers of single-gene defects but also at increased risk for aneuploid pregnancy (Boue et al. 1975; Hassold 1980; Boue et al. 1985; Hassold and Chiu 1985). Currently, PGD tests are separately conducted, either for chromosomal analysis using FISH, or for specific gene analysis using PCR.

During the last few years, significant progress has been made with PGD methodologies resulting in a few PGD protocols theoretically suitable for simultaneous analysis of

chromosomal and single-gene defects being published. Three promising methods include: sequential FISH and PCR analysis (Rechitsky et al. 1996; He et al. 1999), metaphase CGH combined with PCR-based single-gene analysis (Wells et al. 1999), and quantitative fluorescence PCR analysis (QL-PCR) (Blake et al. 1999). However, none of these three methods have been reportedly used for clinical PGD application.

The aims of this phase of present study were to test the feasibility of using array CGH for aneuploidy screening on single blastomeres of preimplantation embryos, and further to explore the possibility of using DOP-PCR products, randomly preamplified from single blastomeres, for both array CGH analysis and gene-specific PCR analysis.

6.2 Results

6.2.1 Random amplification and labelling of single blastomeres by DOP-PCR

After DOP-PCR preamplification and labelling with Cy3 (Fig. 6.1, left), all of the 12 blastomeres (Section 2.2.8), which were obtained from the three frozen IVF-created cleavage-stage embryos donated to research, produced a satisfactory Cy3-labelled product ranging from 300 bp to 2500 bp containing two specific bands, of approximately 450 bp and 600 bp, after being size fractionated on a 1% agarose gel stained with ethidium bromide (Fig. 6.2).

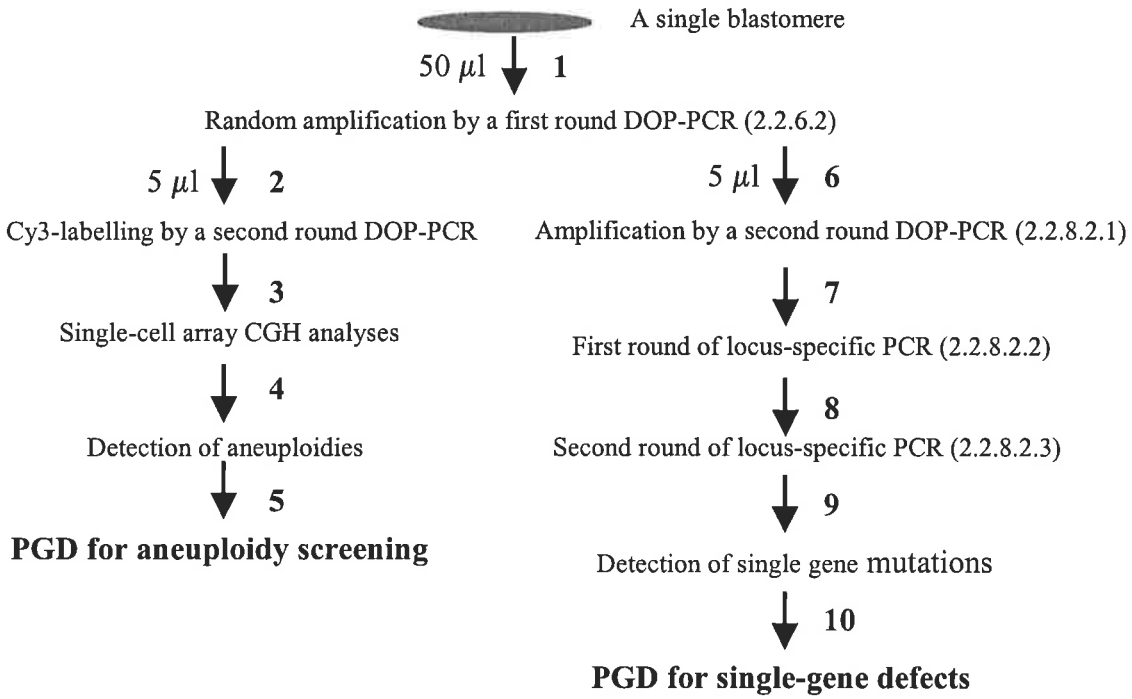


Figure 6.1. Protocol for chromosomal and molecular analyses of single blastomeres of human IVF-created cleavage stage embryos

Step 1): Each blastomere was preamplified by a first round of DOP-PCR.

Steps 2-5): 5 μl of each first round DOP-PCR product was labelled with Cy3 by another round of DOP-PCR for single-cell array CGH analysis to detect aneuploidies for PGD for aneuploidy screening.

Steps 6-10): 5 μl of each first round DOP-PCR was used to seed another round of DOP-PCR followed by two rounds of locus-specific PCR, from which specific genes were amplified. Mutation analyses of the products for PGD for single-gene defects was then followed.

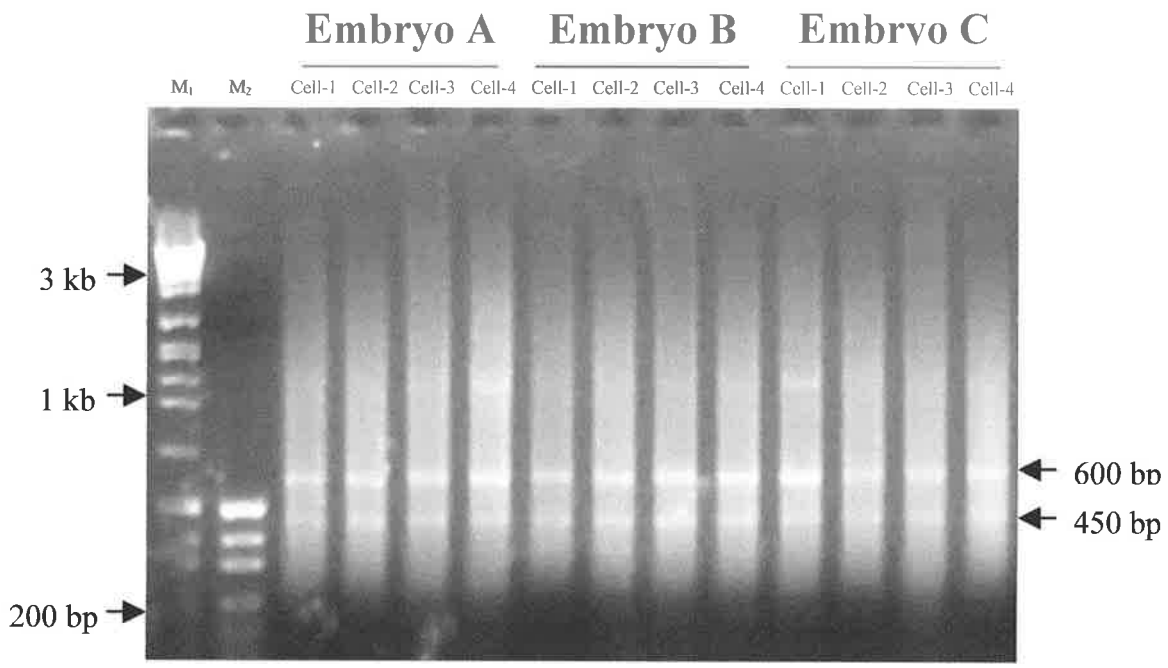


Figure 6.2. Agarose gel electrophoresis of Cy3-labelled DOP-PCR products generated from single blastomeres of IVF-created cleavage-stage embryos. Four blastomeres were present in each of three frozen embryos donated to research and all four blastomeres were dissociated resulting in a total of 12 separate single blastomeres. The DNA of each cell was preamplified and then labeled with Cy3 by DOP-PCR. The origin of each sample is indicated above each lane. DNA markers were *SPP*-1/*Eco*RI (M_1) and pUC19 /*Hpa*II (M_2). Note that each labelled product gives a smear on a 1% agarose gel ranging from 300 bp to 2,500 bp and containing two specific bands approximately at 450 bp and 600 bp.

6.2.2 Chromosomal analyses of single blastomeres using Array CGH

Array CGH analysis was performed on the 5th batch of arrays (Section 3.6) using a pooled mixture of 5 to 10 normal male single-cell DOP-PCR products labelled with Cy5 as the reference material. Only the 24 original CSLs (Fig. 5.1B) were included in the data analysis, which was conducted by GenePix Pro 4.0.1.12 (Section 2.2.7). Only 10 out of the 12 available blastomeres could be analysed because of limited availability of arrays. Of the 10 cells analysed using array CGH, 2 failed to produce analysable results due to a high fluorescence background, probably the result of the relative humidity adopted for the hybridization step accidentally being much lower than the standard 95%.

Of the 8 blastomeres producing analysable array CGH results (Table 6.1), three were found to be normal with an apparently female karyotype (46,XX) (embryo A blastomeres 1 and 4, embryo C blastomere 2). Four cells were aneuploid, two of which had trisomy 21 and apparently female karyotypes (embryo A blastomere 2, embryo B blastomere 1). Two other cells were aneuploid for chromosome 21 (embryo B blastomere 2) and 18 (embryo C blastomere 3) with possible monosomies for chromosomes 1 and 12 respectively. In these latter two cells the ratios for CSL_1 and CSL_{12} respectively fell just below the 0.75 threshold and therefore the results of these two CSLs need to be interpreted with caution. Finally, one blastomere (embryo A blastomere 3) gave an apparently chaotic karyotype with a ratio of <0.75 for six different CSLs including $CSL_{1, 7, 8, 14, 17, \text{and } 20}$, and a ratio of >1.25 for seven other CSLs containing $CSL_{2, 5, 10, 12, 13, 18, \text{and } 21}$. This result suggests that this blastomere had monosomy for six chromosomes,1, 7, 8, 14, 17, and 20, and trisomy for seven other chromosomes, 2, 5, 10, 12, 13, 18, and 21. Such extensive aneuploidy is termed a chaotic karyotype.

Table 6.1. Results of array CGH analyses performed on the 5th batch of arrays for eight single blastomeres removed from three human IVF-created cleavage-stage embryos

Experiments of Array CGH	Ratios (Cy3/Cy5) of chromosomal DNA libraries (CSLs)																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A: Array CGH trials tested for embryo A																								
(1) blastomere-1	1.05	0.92	1.13	0.96	0.87	1.08	1.02	0.92	1.06	0.91	0.86	1.07	1.07	1.14	1.19	0.94	1.19	0.86	0.92	0.83	1.09	0.93	1.54	0.89
(2) blastomere-2	0.85	1.04	0.94	1.06	1.09	0.89	0.91	0.96	1.19	1.01	1.00	1.04	1.00	0.93	0.93	1.04	0.97	1.02	1.02	0.91	1.26	0.91	1.43	0.85
(3) blastomere-3	0.72	1.62	0.81	1.16	1.85	1.20	0.60	0.65	0.75	1.42	1.08	1.32	1.36	0.60	1.22	1.02	0.63	1.96	0.75	0.60	1.30	0.85	0.90	1.35
(4) blastomere-4	0.98	1.01	1.09	1.22	0.79	0.92	0.95	1.03	1.22	1.08	1.20	0.98	0.97	0.86	0.84	1.18	0.77	1.13	0.87	0.88	1.24	0.90	1.42	0.98
B: Array CGH trials tested for embryo B																								
(1) blastomere-1	0.90	1.14	0.99	1.17	1.05	0.90	0.94	0.98	1.10	1.12	1.15	0.94	0.91	0.99	0.88	1.16	0.92	0.92	0.96	0.85	1.27	0.79	1.39	0.96
(2) blastomere-2	0.73	1.23	1.08	1.08	1.14	0.91	0.96	0.88	0.98	0.98	1.11	0.86	0.96	0.96	0.95	1.14	0.81	1.20	0.93	0.94	1.48	0.84	1.34	0.91
C: Array CGH trials tested for embryo C																								
(1) blastomere-2	0.96	0.89	1.07	0.92	0.94	0.89	0.92	0.86	1.01	0.94	0.90	1.07	1.00	1.03	1.05	0.95	1.08	1.01	1.17	1.03	1.14	1.16	1.44	0.87
(2) blastomere-3	1.03	1.06	1.08	1.24	1.03	0.95	0.94	1.12	1.17	0.94	1.15	0.73	0.90	0.86	0.79	0.95	1.19	1.28	0.86	0.78	1.24	0.86	1.32	1.21

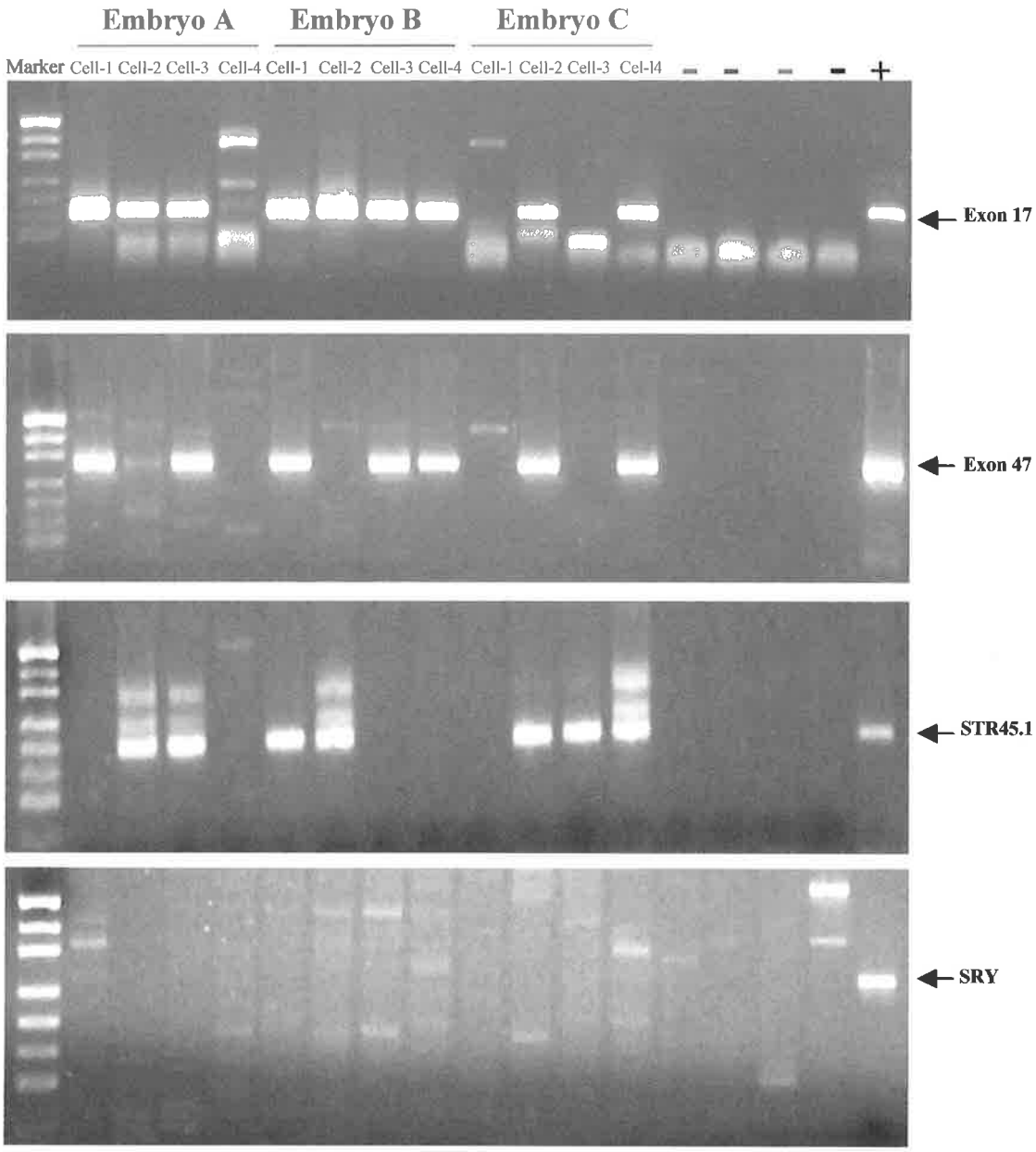
(1) For all array CGH analyses, a single blastomere labelled with Cy3 was compared to a pooled mixture of 5 to 10 independent single-cell (46,XY) DOP-PCR products labeled with Cy5.
(2) Ratios marked in green and red are respectively indicative of trisomies and monosomies in the test blastomere.
(3) Ratios marked in blue indicate that the test cell was female
(4) Ratios just below the 0.75 threshold need to be interpreted with caution and are stated here as evidence for a possible monosomy only.
(5) Ratios of CSL_Y were inadmissible.
(6) Raw data of Array CGH reported in this Table can be found in the CD attached to the back cover of this thesis

All of the three embryos analysed were observed to be mosaic. Of the four cells analysed for embryo A, two were normal, one was trisomy 21, and the other had extensive aneuploidy (chaotic). Both cells analysed for embryo B had trisomy 21, and one of them had a possible monosomy 1. Of the two cells analysed for embryo C, one was normal and the other was trisomy 18 with a possible monosomy 12. Gender determination revealed that all three embryos had an apparently female karyotpye and this was consistent for all cells from each embryo except the chaotic blastomere (embryo A blastomere 3) (Table 6.1) for which no weight can be given to the observed ratio of 0.90 for CSL_X for the purposes of gender assignment.

6.2.3 Molecular analyses of single blastomeres using locus-specific PCR

Four separate nested locus-specific PCR amplifications were performed for each of the total 12 blastomeres after being preamplified by two rounds of DOP-PCR (Fig. 6.1, right). The four DNA fragments amplified in this study were: one from the SRY gene (Sex-determining region Y) and three the DMD gene (Duchenne Muscular Dystrophy). The DMD fragments were from the exons 17 and 47 and a (CA)n-bearing locus in the intron 45 (STR45.1). Positive control tubes containing 100 ng of a normal male genomic DNA and negative control tubes containing 1-3 μl of 1x PCR buffer from the last wash droplet were always included. The sequences of primers for locus-specific PCR amplification were previously given in Tables 2.1 and 2.2. As expected, no products (Fig. 6.3) were seen for all 16 (4 x 4) negative controls, and all 4 positive controls gave the correct products. The successful amplification rate was 9/12 for DMD exon 17, 8/12 for DMD exon 47, and 7/12 for DMD STR45.1. By contrast, all 12 blastomeres tested gave no products for the SRY gene, supporting the finding that all three embryos analysed in this study were female.

Figure 6.3. Agarose gel electrophoresis of locus-specific PCR products using DOP-PCR products preamplified from single blastomeres of three human IVF created cleavage-stage embryos. Four blastomeres were removed from each of three frozen embryos resulting in a total of 12 separate single blastomeres, each of which was preamplified by two rounds of DOP-PCR followed by nested locus-specific PCR. The four DNA fragments amplified include the SRY gene (Sex-determining region Y) and three DNA fragments of the DMD gene (Duchenne Muscular Dystrophy), including DMD exon 17, DMD exon 47, and one short tandem repeat (STR) in the intron 45 (DMD STR45.1). Four negative control tubes containing 1-3 μl of 1x PCR buffer from the last wash droplet and one positive control tube containing 100 ng of a normal male genomic DNA are also shown. The origin of each sample is indicated above each lane. DNA marker was pUC19 /*Hpa*II. The size of the exon 17 product is 154 bp, the exon 47 product is 309 bp, the SRT45.1 product is around 160 bp, and the SRY gene product is 254 bp in length. As expected, no products are observed for all 16 (4 x 4) negative controls but all 4 (1 x 4) positive controls give the correct products. No products are found for the SRY gene in all 12 blastomeres, indicating that the three embryos tested were all female. The successful amplification rate is 9/12 for exon 17, 8/12 for exon 47, and 7/12 for SRT45.1.


Embryo A
Embryo B
Embryo C
Marker Cell-1 Cell-2 Cell-3 Cell-4 Cell-1 Cell-2 Cell-3 Cell-4 Cell-1 Cell-2 Cell-3 Cel-l4 - - - - +
Exon 17
Exon 47
STR45.1
SRY

Genomic DNA samples of the patients producing the embryos analysed in this study were not available for genetic analysis of DMD STR45.1. However, polyacrylamide gel electrophoresis (Fig. 6.4) of DMD STR45.1 PCR products tagged by the HEX-labelled primers indicated that allele dropout (ADO) occurred in two blastomeres (embryo B blastomere 2 and embryo C blastomere 2) out of the seven blastomeres, which produced the STR45.1 PCR products.

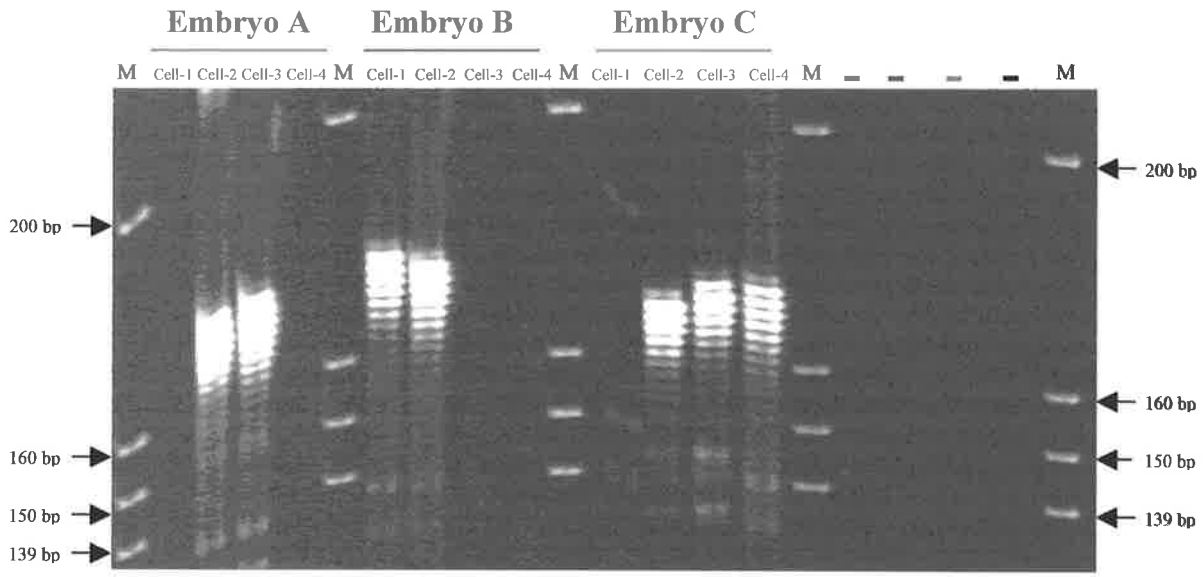


Figure 6.4. Electrophoresis of HEX-tagged DMD STR45.1 PCR products on a 5% denaturing polyacrylamide gel containing 42% Urea using GEL-SCAN 2000 DNA Fragment Analyzer. The origin of each sample is indicated above each lane. Details of the samples are the same as described in the legend to Fig. 6.3 except that only HEX-tagged DMD STR45.1 PCR products were loaded for electrophoresis. DNA marker was Genescan-350 TAMRA (Perkin Elmer, USA). As expected, no products are seen for all four negative controls. The successful amplification rate for DMD SRT45.1 is 7/12. The genotypes of the parents of each of the three embryos are not known. Precise assignment of allele types is not possible nevertheless the results of the gel indicate that two cells show allele drop-out (ADO), and the genotypes of the seven blastomeres can be described as follows:

Embryo A	Cell 2 alleles 1/2
	Cell 3 alleles 1/2
Embryo B	Cell 1 alleles 1/2
	Cell 2 allele 2 (presumed ADO of allele type 1)
Embryo C	Cell 2 allele 2 (presumed ADO of allele type 1)
	Cell 3 allele 1/2
	Cell 4 allele 1/2

6.3 Discussion

6.3.1 Chromosomal analyses of embryos using array CGH and metaphase CGH

Metaphase CGH analyses of single blastomeres of frozen (Wells and Delhanty 2000) or fresh (Voullaire et al. 2000) preimplantation embryos donated to research have shown that only one quarter (3/12) of embryos were chromosomally normal, with most of the remaining embryos being mosaic (6-7/9) or chaotic (1-2/9). However, the majority (8-9/12) of the embryos analysed in these previously published studies had at least one normal cell. These studies also revealed that all cells from each embryo were consistent for gender determination except for the cells carrying extensive aneuploidy or derived from chaotic embryos in which each cell showed a different array of chromosome abnormalities (Voullaire et al. 2000). Chaotic embryos found in these studies may result from the absence of some cell cycle checkpoints during meiosis (LeMaire-Adkins et al. 1997; Wells and Delhanty 2000) or a low mitochondrial membrane potential in oocytes (Wilding et al. 2003). In one recent study of Malmgren *et al.* (2002), 28 embryos were analysed using single-cell metaphase CGH and all were found to be mosaic; most of these embryos (22/28) were donated from patients undergoing PGD for chromosomal translocations. These patients are expected to have high rates of meiotically derived abnormality but they are not expected to be more prone to mitotic abnormalities. These authors conclude that patients undergoing PGD for chromosome translocations would benefit from general aneuploidy screening also.

Similar results to those previously published were found in the present array CGH study, where all of the three embryos analysed were found to be mosaic, and two of which had at least one normal cell. Inconsistency of gender determination was only found for blastomere-3

of embryo A giving a ratio of 0.90 for CSL_X suggesting that this cell was male, which was different from female found for all of the three other cells tested for the same embryo. This blastomere had extensive aneuploidies for 13 different autosomes, and the existence of the Y chromosome in this cell was reduced by the failure of PCR amplification by the SRY gene primers (Fig. 6.3). These results demonstrate that the determination of the copy number of the X chromosome for blastomeres with extensive aneuploidies is not accurate for both metaphase CGH and array CGH technologies, thereby giving a misleading result for gender for such cells.

6.3.2 Application of metaphase CGH and array CGH for PGD aneuploidy screening

The use of metaphase CGH to screen all human chromosomes for aneuploidy in preimplantation embryos is hindered by the time of 5 to 6 days required to perform the analysis. Wilton *et al.* (2001) froze the embryos after blastomere biopsy, and this strategy allowed them to obtain the first healthy baby following PGD aneuploidy screening using metaphase CGH analysis. One disadvantage of this approach is that the freezing-thawing process may reduce the developmental capacity of the embryo (Joris et al. 1999; Magli et al. 1999). In order to avoid this problem, Wells *et al.* (2002) used first polar body biopsy instead of blastomere biopsy, thereby obtaining at least two more days for metaphase CGH analysis. This strategy has been performed in one PGD cycle for a 40-year-old IVF patient. Of the total 11 first polar bodies analysed in this study, only one was found to be chromosomally normal and the corresponding normal embryo was subsequently transferred but there was no resulting pregnancy. The major limitation of this method is the inability to detect chromosomal aberrations of paternal and postzygotic origins.

Obviously, these metaphase CGH-based protocols for PGD aneuploidy screening require further modification before being widely accepted in clinical application. In the present array CGH study, both chromosomally normal and abnormal blastomeres were identified, and testing all blastomeres of the same embryos resulted in the diagnosis of mosaic embryos. These results demonstrate the feasibility of the use of array CGH for PGD aneuploidy screening. Taking about 30 hours, this array CGH approach allows embryos to be transferred in the same IVF cycle that created them, making it more useful for PGD aneuploidy screening than metaphase CGH.

6.3.3 Molecular analyses of single blastomeres using DOP-PCR followed by locus-specific PCR

Whole genome amplification (WGA) of single cells using DOP-PCR followed by locus-specific PCR in order to analyse numerous genes from a single cell was initially reported by Wells and colleagues (1999). The authors tried to amplify 10 different loci from each of a total of 25 single cells, including single blastomeres of human preimplantation embryos, single fibroblasts, buccal cells, and amniocytes, and they found that the average amplification rate varied among different loci from 73% to 100% with an average of 85%. A similar protocol was employed in the present study but a much lower average amplification rate of 67% (8/12) was obtained for the three DNA fragments of the DMD gene. This difference might be due to the different types of cells studied. DMD STR45.1 is a (CA)n variable locus with an overall heterozygosity frequency of 88.7% (Clemens et al. 1991), and therefore it can be used for linkage analysis. However, only 7 out of the 12 blastomeres analysed in the study (Fig. 6.4) gave a product for DMD STR45.1 locus, and two of which are presumed to have displayed ADO. These results indicate that further improvement of locus-specific PCR

amplification is required for the current protocol before it can be accepted for PGD of single-gene defects. However, the results of gender determination for all three embryos by PCR amplification of the SRY gene were consistent with those obtained from array CGH analysis, suggesting that sexing by both methods is reliable at least on a technical rather than perhaps a biological basis. The rate of failure of amplification of the SRY gene has been reported to be 0-3.6% in conventional single-cell PCR (Cui et al. 1994; Hussey et al. 1999).

In the present study, two blastomeres yielded no PCR products for all four different loci. One was blastomere-1 of embryo C, which gave a satisfactory Cy3-labelled DOP-PCR for array CGH analysis (Fig. 6.2). This cell might contain only anuclear fragments, and the DOP-PCR products amplified from this cell might be derived from mitochondrial DNA only. If that is the case, no signals of the test DNA would be expected for dots of all CSLs in array CGH analysis. Unfortunately, array CGH analysis was not performed on this cell. The other was blastomere-4 of embryo A (Fig. 6.3), which gave no PCR products for all three DMD loci, although it was diagnosed to be chromosomally normal using array CGH analysis (Table 6.1). These results indicate that somewhat biased amplification of the genome occurs in single-cell DOP-PCR but this may have no impact on the accuracy of single-cell array CGH analysis. The reason underlying this result might be that biased amplification of individual loci is unlikely to affect the final results of array CGH analysis derived from a whole chromosomal DNA library (CSL) containing DNA fragments from numerous loci, but it is obviously detrimental to a specific locus analysis. Finally and interestingly, blastomere-3 of embryo A had extensive aneuploidies (Table 6.1), but it gave satisfactory results in all of the locus-specific PCR (Fig. 6.3).

6.3.4 PGD for both chromosomal and single-gene defects using the same biopsied blastomeres

Although the array CGH approach used in the present study facilitates PGD aneuploidy screening allowing embryos to be transferred in the same IVF cycles that created them, it gave a very low PCR amplification efficiency of 58% to 75% for specific loci (Fig. 6.3). Therefore, the PCR amplification efficiency of individual genes requires further improvement before this approach can be reliably performed for single-gene disorders coincident with chromosomal abnormalities. One promising possible solution to this problem is the addition of locus-specific primers in the single-cell DOP-PCR random amplification reaction. This method is termed "Selectively-Enhanced Primer-extension-preamplification (SEP)" (Hussey et al. 1997). By this method, locus-specifically amplified DNA products should be able to be obtained along with the randomly amplified products from the whole genome of a single cell, thus probably increasing the PCR amplification efficiency of specific genes as required for PGD of single gene defects. However, it remains to be trialed whether the excess of the specific primers of the targeted gene, or the highly-amplified DNA produced by them, interferences with the results of CGH.

Multiplex quantitative fluorescence PCR (QF-PCR) is another method reportedly capable of detection of both single gene mutations and aneuploidy in a single cell (Findlay et al. 1999; Blake et al. 2001; Katz et al. 2002b). In this approach, aneuploidy can be diagnosed after fingerprinting analysis using short tandem repeats (STRs) such as di-, tri-, and tetranucleotide microsatellite markers. A normal diploid cell gives an allelic ratio of 1:1 at a heterozygous locus, whereas a trisomic cell shows an allelic ratio of 1:1:1 if heterozygosity is available for all three alleles or an allelic ratio of 2:1 when two of the three alleles are the same. This

quantitative analysis of ratio profiles is not accurate in the case of the presence of allele dropout (ADO) and preferential allele amplification, both of which are very common especially at STR loci in single-cell PCR analysis. In one recent study (Katz et al. 2002a), a pentaplex chromosome 21 single-cell fingerprinting system was tested using both diploid and trisomy 21 buccal cells, and showed 10% preferential allele amplification rate, and approximately 8% ADO rate which would give a false result for aneuploidy screening. The authors report the use of this multiplex QF-PCR approach for aneuploidy screening for chromosome 21 along with analysis of the cystic fibrosis Δ F508 mutation at single cell level. These results are encouraging but this single-cell QF-PCR approach can analyse only a limited number of STR loci per cell, and therefore it's impossible to use this method for comprehensive PGD aneuploidy screening. For example, if a pentaplex is required for screening trisomy 21 only, a multiplex single-cell QF-PCR capable of analysing 15 different STR loci is needed for simultaneous screening of only three different chromosomes per cell. The availability of informative STR loci is another restriction of this approach, and this means that in most cases patient-specific multiplex fingerprinting analysis has to be developed. Additionally, it is clear that allele-dropout (ADO) and preferential amplification (PA) occur frequently at STR loci, and obviously ADO and PA can lead to misinterpretation of the genotypes.

Finally, sequential PCR and FISH analysis of a single blastomere fixed on a microscopic slide may also have the potential to be used for PGD to analyse both aneuploidy and singe gene mutations. The problem of this method is the decrease of amplification efficiency of specific genes as each round of PCR. The successful amplification rate could be as low as 40% at the fifth round of PCR (He et al. 1999). As a result, not much genetic information can be obtained from a single cell by this method.

6.4 Summary of Chapter 6

The present study is the first to demonstrate the feasibility of the use of array CGH to screen aneuploidy on single blastomeres of human IVF-created preimplantation embryos. With an experimental duration of around 30 hours, this approach can be applied to PGD for aneuploidy screening allowing the transfer of embryos in the same IVF cycle that created them. However, this approach will need to be modified before a concomitant diagnosis for single gene disorders is as good as the current technology for single cell analysis namely multiplex single cell PCR alone.

Chapter 7

Final discussion and future directions

7.1 Major achievements of this study

At the time this study commenced in July 2000, there were only a few published array CGH studies (Solinas-Toldo et al. 1997; Geschwind et al. 1998; Pinkel et al. 1998; Albertson et al. 2000). All of these studies required DNA samples of 0.5-1.0 μg far in excess of the ~ 6 pg contained in a single cell (Morton 1991) to obtain a hybridisation signal reliable for array CGH analysis. Since the commencement of this project, array CGH analysis has been successfully performed in one study to analyse laser-microdissected tumour samples with as few as 500 cells (Daigo et al. 2001). The present study is the only one to date to demonstrate the feasibility of detecting aneuploidy and gender determination in a single cell using array CGH technology. Compared to single-cell metaphase CGH requiring 3-5 days to perform (Wells and Delhanty 2000; Wilton et al. 2001), the array CGH approach developed in the present study takes only 30 hours, making it more suitable for PGD aneuploidy screening with allowance for embryo transfer to occur in the fresh IVF cycle. Furthermore, this array CGH approach has been used to analyze all of the entire 24 human chromosomes except for the Y chromosome, and this compared favorably to the FISH method, which can analyse only 5-9 chromosomes per cell in the same time period.

7.2 Novelty of this study

Although a few human chromosome-specific DNA libraries were once spotted on glass slides for array CGH analysis (Solinas-Toldo et al. 1997) the present study is the first to perform array CGH analysis using DNA microarrays consisting of a full set of 24 human whole-chromosome DNA libraries. The major reason for using whole-chromosome DNA libraries instead of a single PAC or BAC clone is that the whole chromosome is combined into one spot and therefore it is more likely to produce a reliable signal than a spot only consisting of a single PAC or BAC clone in array CGH analysis performed using single cells. The DNA libraries used in Solinas-Toldo *et al* (1997) were derived from flow-sorted chromosomes and subcloned in the vector pBS. To avoid any potential contamination from the vector genome, all of the 24 whole-chromosome DNA libraries selected for the present study were not established by such cloning technology but developed by DOP-PCR amplification of either flow-sorted or microdissected chromosomes (Bolzer et al. 1999). Furthermore, in the present study, the Cy3- and Cy5-labelled target DNA was also obtained by DOP-PCR amplification of the test and reference single cells. Therefore, the use of DOP-PCR-amplified DNA libraries on the microarray allowed DOP-PCR products from the target genome to hybridize to likewise generated DOP-PCR products on the glass slides (probes). This "self hybridising to self" strategy used in the present study aimed to enhance the hybridization efficiency of the array CGH analysis. It does this by presumably increasing the chance that the limited amount of DNA products generated from a single cell will easily find a matching partner on the array.

The complexity of chromosome-specific DNA libraries (CSLs) is several orders of magnitude higher than individual PAC and BAC clones. Therefore, repetitive DNA sequences contained in

the DNA libraries spotted on the microarrays could result in non-specific signals if the suppression of repetitive sequences from the labelled products using Cot-1 DNA is insufficient. One solution to this problem is to prehybridize the microarrays with Cot-1 DNA (Geschwind et al. 1998), and another, initially proposed by Solinas-Toldon *et al.* (1997) but never tried up to date in the literature, is to remove the repetitive sequences from the DNA libraries prior to array printing. With the concern that an additional prehybridization step in the array CGH protocol could add to the background level (Wessendorf et al. 2002) as well as the length of time to perform the procedure, repeat-depleted chromosome-specific DNA libraries were therefore selected for this project. Depletion of repetitive sequences from these 24 DNA libraries had been achieved using affinity chromatography in combination with negative subtraction hybridization using human Cot-1 DNA and some other repetitive DNA sequences as subtractors (Section 3.3.1). However, despite the use of DNA libraries apparently free of repetitive sequences, 70 μg of human Cot-1 DNA per array CGH hybridisation was still needed in the present study to suppress the non-specific signals from repetitive sequences, which were possibly still residual in the DNA libraries and more likely produced in large number by DOP-PCR of the test and reference samples. In addition, results (Table 4.1) were further improved by extending the length of time for preannealing (37°C) from 30 min to 80 min and increasing the stringency of washing buffer using solution of 50% formamide, 2X SSC (45°C) instead of 2X SSC (60°C) only.

7.3 Significance of using multiple dots of each probe in array CGH analysis

In array CGH, it is a general practice to exclude dots from analysis due to mechanical problems such as printing artefacts including misplacement, irregular morphology, overlying debris, and the failure to print the dots. Dots may also be disqualified for analysis because of

problems produced by hybridization such as signal intensity too low, approaching the background level, or signal intensity too high, saturating the detectors, and poor signal/background ratios (Section 1.6.7). In the present study, generally zero, one or two dots (Table 5.2) were excluded from analysis except for the more extreme PMT values where more dots needed to be excluded (up to 7 out of the total 8 replicate dots of a single CSL) (Table 5.6). These results highlight the significance of spotting multiple dots for each probe on the microarray and in fact this has become a general rule in array CGH studies. Triplicates of dots per sample are most frequently applied (Daigo et al. 2001; Snijders et al. 2001; Veltman et al. 2002). Others used up to date include duplicates (Fiegler et al. 2003), quadruplicates (Pinkel et al. 1998), five replicates (Bruder et al. 2001), six replicates (Cai et al. 2002), eight replicates (Solinas-Toldo et al. 1997), and even 10 replicates (Wessendorf et al. 2002). This practice of using replicas allows dots of good hybridization quality to be selected for analysis. This strategy is similar to that of analysing metaphases of good hybridization quality only in metaphase CGH analysis (Kallioniemi et al. 1992; Karhu et al. 1997).

7.4 Applications and limitations of single-cell array CGH analysis

The single-cell array CGH approach developed in this study overcomes a major limitation of single-cell metaphase CGH, which is the length of time required to perform the analysis. This new approach takes about 30 hours, making it more acceptable for PGD aneuploidy screening than metaphase CGH by obviating the need to freeze embryos until a second cycle is reached in the mother. Although this study focuses on developing aneuploidy screening for PGD, this technology may also be used for analysing a limited number of uncultured amniocytes and chorionic villus cells for prenatal diagnosis, limited amounts of material in dissected tumour

samples, and perhaps most importantly, single foetal cells isolated non-invasively from peripheral blood of pregnant women.

The method used in this study was developed specifically to screen aneuploidy across the whole karyotype. As a result, the biggest disadvantage is the inability to detect deletions and duplications involving specific regions of chromosomes, which may be detected by metaphase CGH on genomic DNA (Kallioniemi et al. 1992). Using more array spots each from DNA libraries pertaining to smaller regions of the chromosome might solve this problem. Like all CGH analyses (Kallioniemi et al. 1994), single-cell array CGH cannot detect balanced aberrations (such as balanced translocations or inversions, haploidy and polyploidy), and of course, point mutations. The chromosomal abnormality of 47,XY,t(3;16)(q22;p11.2),+der(16p), probably contained in one of the test cells, was not detected (Section 5.2.4), and this highlights the inability of the method applied in this study for the diagnosis of unbalanced abnormalities with minor change of the chromosomal complement. However, this method may be useful for detecting unbalanced Robertsonian translocations since the chromosomal DNA libraries (CSLs) of the five acrocentric autosomes 13, 14, 15, 21, and 22 used in the present study only contained the DNA sequences of the respective q arms (Guan et al. 1994).

DOP-PCR products preamplified from single blastomeres (Chapter 6) can be successfully used for aneuploidy screening using array CGH analysis, however, they gave a relative low amplification efficiency of 58% to 75% for specific loci, which is unacceptable for PGD conducted for single-gene disorders. These results indicate that this approach cannot be reliably applied to PGD for analysing both aneuploidy and single-gene mutations using the same single blastomeres or polar bodies until the efficiency of PCR amplification of specific loci is further increased.

7.5 Technical considerations

7.5.1 Array spotting conditions

One problem of array spotting found in this study was the extreme evaporation of spotting buffer (Section 3.4), and another was worn-out spotting pins (Section 3.5), both of which resulted in a similar consequence: the failure to print some dots on the arrays. These results highlight the significance of optimising the spotting conditions prior to spotting any actual array slides, especially if a lengthy duration of spotting from a single loading plate is required. Obviously, regular examination of the spotting pins is necessary and any extremely worn spotting pins found in such regular check-ups should be replaced. Other general conditions of array spotting which should also be optimised include: the relative humidity, temperature, and the volumes of the starting spotting buffer. Generally, UV-cross-linking and chemical blocking after array spotting was required (Sections 3.2-3.5) but this process was not necessary for the 5th batch of array slides made in this study (Section 3.6).

7.5.2 Labelling efficiency and handling the Cy3 or Cy5-dUTP products

Because it involves uniform DNA labelling, nick-translation is widely used for labelling the target DNA samples in array CGH studies. In this study, DOP-PCR was used to label the randomly amplified single-cell DOP-PCR products and appeared to give a satisfactory labelling efficiency and yield. A 1/10 volume (5 μl, approximately 0.5-1.0μg) of each labelled product was sufficient for a complete array CGH analysis. Direct labeling such as that reported here is quicker and easier to perform than nick translation.

The failure to label the test and/or reference sample was a frequent technical problem at the beginning of this study. This was probably due to repeated rounds of freezing and thawing the

Cy3- and Cy5-dUTPs as this treatment could break the phosphate bonds resulting in mono-phosphates, which are not polymerase substrates (Amersham Phamacia Biotech). Labelling efficiency was much improved by sub-aliquoting all newly received Cy-dUTPs into single-use portions to minimise freeze-thaw cycles.

Cy3-and Cy5-labelled dUTP are readily photobleached by exposure to light but this was not a problem in this study as all solutions containing fluorophores and the hybridized slides were handled in reduced light. Nevertheless, a chemical coating is commercially available called "Dyesaver" which prevents the photobleaching especially for the Cy5 fluorochrome, which is very sensitive to ozone (Genisphere Inc. Hatfield, PA, USA).

7.5.3 Significance of the use of freshly prepared single cells

It is a common practice in our laboratory to store isolated single cells at -20°C for up to 2-3 years. These cells were successfully used to amplify specific DNA fragments from the single genomes by conventional single-cell PCR (Hussey et al. 1999). However, a large number of array CGH experiments involving DOP-PCR failed to produce the expected results when such frozen lymphocytes, amniocytes, fibroblasts, and blastomeres were used (data not shown). This might be due to degradation of the whole single genome in the freezing process. Obviously, such degradation could lead to false ratio deviations and therefore freshly prepared single cells are necessary for single-cell array CGH analysis reported in the present study.

7.6 Conclusions and future directions

This thesis describes the development of a DNA microarray approach for comparative genomic hybridization of a single cell sample using a full set of human repeat-depleted chromosome-specific DNA libraries (CSLs). The feasibility and reliability of using this approach for detection of aneuploidy and gender determination was tested using various types of single cells including normal lymphocytes, trisomic amniocytes and fibroblasts, and blastomeres removed from human IVF cleavage-stage preimplantation embryos. Results showed that aneuploidy was be reliably detected by this method using a single cell sample. This method is capable of analyzing all of the human chromosomes except for Y chromosome and produce results within 30 hours, and therefore it may be more suitable for PGD aneuploidy screening than either FISH analysis or metaphase CGH approach.

Due to the limited availability of both arrays and embryos, only three embryos were analysed in this study, and the normal and abnormal karyotypes found by array CGH analysis were not independently verified by FISH or some other methods (Chapter 6). Although chromosomal analyses of single blastomeres is sometimes difficult to be verified because of the high level of mosaicism in human embryos, the reliability of the current array CGH needs to be further validated in larger series of experiments, ideally using embryos with an aneuploidy detected by FISH for comparison but ultimately the proof that microarray CGH increases the implantation rate and decreases the miscarriage rate will require a clinical trial with enough power to overcome the enormous variation expected in patient treatment. After this type of trial it may be possible to say which patients benefit from the technique and by how much.

The CSL_Y probe used in this study frequently gave inadmissible results, therefore for accurate gender determination this probe will have to be replaced by some other probes, such as a probe directed at the euchromatic regions of the Y chromosome only (instead of the whole chromosome). Such a probe can be developed by microdissecting the short arm and the proximal long arm of the Y chromosome followed by DOP-PCR amplification of the micro-dissected genetic materials. Any new probes for the Y chromosome would need to undergo extensive validation studies before determining that they are truly reliable.

Finally, it can be visualized that the methodology might be expanded to include high-density arrays of many dots specific for sub-chromosomal regions, and perhaps even single or small numbers of contiguous chromosomal bands to increase the resolution of single-cell array CGH analysis.

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