Molecular Genetic Diagnosis of Single Gene Disorders at the Single Cell Level

by

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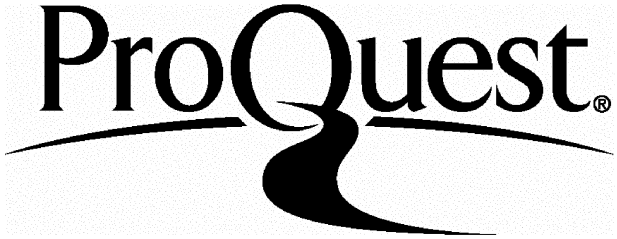

To my father

and the memory of my mother

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Abstract

Diagnosis of single gene disorders in isolated single cells can be accomplished after DNA amplification using the polymerase chain reaction (PCR). This has allowed preimplantation genetic diagnosis (PGD) to be performed for a range of inherited diseases. Essentially PGD is an early form of prenatal diagnosis, carried out on 1-2 cells biopsied from 8-10 cell embryos generated using *in vitro* fertilization (IVF) techniques. During the course of this study PGD protocols were designed for myotonic dystrophy (DM) and alpha and beta-thalassaemias.

The PGD protocols developed utilized multiplex-PCR amplification of a fragment encompassing the mutation site (within DM, β-globin, or α-globin) and a highly polymorphic marker. Microsatellite polymorphisms, deletions and insertions were analysed on a fluorescent sequencing apparatus. While nested-PCR and single stranded conformation polymorphism (SSCP) detected base-pair substitutions. Other analytical techniques investigated included long template amplification of trinucleotide repeat expansions, single cell sequencing and minisequencing.

The amplification of a hypervariable polymorphism, allows the generation of a very basic DNA fingerprint, assisting in the detection of contaminating DNA. Contamination is one of the most important problems facing single cell PCR. If the marker used is linked to the mutation site, it can also provide confirmatory diagnostic information, which is useful in cases of allele dropout (ADO). ADO is a phenomenon unique to single cell PCR in which one of the alleles in a heterozygous cell fails to amplify. Factors such as ADO were investigated using over 3,000 single cells, isolated by micromanipulation. This provided important information that might lead to the development of more reliable PGD protocols in the future.

Three cycles were carried out using the preimplantation diagnosis strategy for DM, resulting in two pregnancies; a singleton for one family and twins for the second. All three babies have been confirmed free of DM by CVS and were born healthy.

Table of Contents

Title 1

Dedication 2

Acknowledgements 3

Abstract 4

Table of contents 5

List of tables 11

List of figures 12

Abbreviations 14

Chapter 1 – Introduction 18

1.1 Genetic disease 19

1.1.1 Chromosome abnormalities 19

1.1.2 Single gene disorders 21

1.2 Diagnosis of genetic diseases 23

1.2.1 Prenatal diagnosis 24

1.2.1.1 Amniocentesis 24

1.2.1.2 Chorionic villus sampling (CVS) 26

1.2.1.3 Fetal blood sampling (FBS) 28

1.3 Preimplantation genetic diagnosis (PGD) 29

1.3.1 *In vitro* fertilisation (IVF) 31

1.3.2 Sampling techniques for PGD 34

1.3.2.1 Polar body biopsy 34

1.3.2.2 Cleavage stage embryo biopsy 35

1.3.2.3 Blastocyst biopsy 37

1.4 PGD analysis techniques 38

1.4.1 Sex determination 38

1.4.2 Diagnosis of chromosome abnormalities 40

1.4.3 Diagnosis of single gene disorders 42

1.5 Single cell PCR 43

1.5.1 Amplification failure (AF) 43

1.5.2 Allele drop out (ADO) 45

1.5.3 Contamination 49
1.5.4 Nested PCR 50
1.5.5 Fluorescent PCR (F-PCR) 51
1.5.6 Multiplex PCR 52
1.5.7 Mutation analysis 53
1.5.7.1 Restriction fragment length polymorphism (RFLP) 54
1.5.7.2 Amplification refractory mutation system (ARMS) 54
1.5.7.3 Heteroduplex analysis (HA) 57
1.5.7.4 Single strand conformational polymorphism (SSCP) 57
1.5.7.5 Denaturant gradient gel electrophoresis (DGGE) 60
1.6 Objectives **60**

Chapter 2 – Materials and methods **61**
2.1 Materials **62**
2.1.1 Chemicals 62
2.1.2 Enzymes 63
2.1.3 Nucleic acids 64
2.1.4 DNA samples 64
2.1.5 Solutions and buffers 65
2.1.6 Single cell manipulation 65
2.1.7 Apparatus 66
2.2 Methods **67**
2.2.1 DNA extraction from blood samples 67
2.2.2 Cell collection 68
2.2.2.1 Buccal cells 68
2.2.2.2 Human blastomeres and embryos 68
2.2.3 Single cell isolation 69
2.2.3.1 Single buccal cells 69
2.2.3.2 Single human blastomeres 69
2.2.4 DNA extraction from single cells and cell clumps 70
2.2.5 Polymerase chain reaction (PCR) 70
2.2.5.1 Oligonucleotides 70
2.2.5.2 Standard PCR 71
2.2.5.3 Multiplex PCR 72

2.2.5.4 Nested PCR 72
2.2.5.5 Fluorescent PCR 73
2.2.5.6 Fluorescent gap PCR 73
2.2.6 Precautions against contamination 74
2.2.7 Analysis of PCR products 74
2.2.7.1 Agarose gel electrophoresis 74
2.2.7.2 Fluorescent PCR analysis 75
2.2.7.2.1 F-PCR analysis using ALFExpress™ 76
2.2.7.2.2 F-PCR analysis using ABI Prism™310 76
2.2.7.3 Single stranded conformation polymorphism (SSCP) analysis 77
2.2.7.3.1 Silver stained SSCP using PhastSystem™ 77
2.2.7.3.2 Silver stained SSCP using GenePhor™ 77
2.2.7.3.3 Fluorescent SSCP using ALFExpress™ 78
2.2.7.4 Fluorescent DNA sequencing 79
2.2.7.5 Fluorescent DNA minisequencing (SNaPshot™) 80
2A. Appendix to materials and methods 81
2A.1 Solutions for DNA extraction 81
2A.2 DNA polymerase buffers for PCR 81
2A.3 Buffers and gels for electrophoresis 82
2A.4 Silver staining of PhastGel™ 83
2A.5 Silver staining of GeneGel® 83

Chapter 3 – Thalassaemias 86
3.1 Introduction 87
3.1.1 β-Thalassaemia 90
3.1.2 α-Thalassaemia 96
3.1.3 Aims 99
3.2 Materials and methods 100
3.2.1 Mutation detection of β-thalassaemia 100
3.2.2 PGD strategy for β-thalassaemia, IVSI-110 102
3.2.3 PGD strategy for β-thalassaemia, codon 41-42 103
3.2.4 PGD strategy for α-thalassaemia, $--^{SEA}$ 103
3.2.5 Single cell sequencing 105

3.2.6 Single cell mini-sequencing (SNaPshot™) 105

3.3 Results **106**

3.3.1 Mutation detection of β-thalassaemia 106

3.3.2 PGD strategy for β-thalassaemia, IVSI-110 111

3.3.3 PGD strategy for β-thalassaemia, codon 41-42 120

3.3.4 PGD strategy for α-thalassaemia, $--^{SEA}$ 122

3.3.5 Single cell sequencing 124

3.3.6 Single cell mini-sequencing (SNaPshot™) 128

3.4 Discussion **130**

3.4.1 Mutation detection of β-thalassaemia 130

3.4.2 PGD strategy for β-thalassaemia, IVSI-110 137

3.4.3 PGD strategy for β-thalassaemia, codon 41-42 143

3.4.4 PGD strategy for α-thalassaemia, $--^{SEA}$ 145

3.4.5 Single cell sequencing 146

3.4.6 Single cell minisequencing (SNaPshot™) 151

3.4.7 Conclusion 152

Chapter 4 – Myotonic dystrophy **155**

4.1 Introduction **156**

4.2 Materials and methods **161**

4.2.1 PGD strategy for DM 161

4.2.2 Work up for DM PGD cases 164

4.2.3 Clinical DM PGD cases 164

4.2.4 Hi-fidelity PCR for DM expansion detection 164

4.3 Results **166**

4.3.1 PGD strategy for DM 166

4.3.2 Work up for DM PGD cases 174

4.3.3 Clinical DM PGD cases 181

4.3.3.1 Cycle 1, family A 181

4.3.3.2 Cycle 2, family A 185

4.3.3.3 Cycle 1, family B 188

4.3.4 Hi-fidelity PCR for DM expansion detection 190

4.4 Discussion **193**
4.4.1 PGD strategy for DM 193
4.4.2 Work up for DM PGD cases 198
4.4.3 Clinical DM PGD cases 199
4.4.4 Hi-fidelity PCR for DM expansion detection 202
4.4.5 Conclusion 205

Chapter 5 – Investigation of factors affecting single cell PCR **206**
5.1 Introduction **207**
5.2 Materials and methods **209**
5.2.1 Freezing and thawing 211
5.2.2 Amount of DNA necessary to avoid ADO 211
5.2.3 Cell type/quality and the affect of DNA degradation 212
5.2.4 Examination of preferential amplification 212
5.2.5 Multiplex PCR 212
5.2.6 Deviation from expected amplification characteristics 213
5.2.7 PCR cycling conditions 213
5.2.8 DNA sequence composition, primers and chromatin structure 214
5.2.9 Fragment length 214
5.2.10 Definition of amplification characteristics 215
5.3 Results **216**
5.3.1 Freezing and thawing 216
5.3.2 Amount of DNA necessary to avoid ADO 216
5.3.3 Cell type/quality and the affect of DNA degradation 218
5.3.4 Examination of preferential amplification 219
5.3.5 Multiplex PCR 220
5.3.6 Deviation from expected amplification characteristics 223
5.3.7 PCR cycling conditions 224
5.3.8 DNA sequence composition, primers and chromatin structure 227
5.3.9 Fragment length 230
5.4 Discussion **232**
5.4.1 Freezing and thawing 232
5.4.2 Amount of DNA necessary to avoid ADO 233
5.4.3 Cell type/quality and the affect of DNA degradation 234

5.4.4 Examination of preferential amplification 235
5.4.5 Multiplex PCR 236
5.4.6 Deviation from expected amplification characteristics 237
5.4.7 PCR cycling conditions 238
5.4.8 DNA sequence composition, primers and chromatin structure 240
5.4.9 Fragment length 241
5.4.10 Conclusion 242

Chapter 6 – Discussion and conclusion 246
6.1 The future of PGD techniques 251
6.1.1 The future of techniques to analyse single gene defects 252
6.1.2 The future of techniques to analyse chromosomes 253
6.2 Laws and ethics 254

Bibliography 257
Publications arising from this thesis 287

List of Tables

2A.1	Automated silver staining process of the PhastGel™	84
2A.2	Manual silver staining process of the GeneGel™	85
3.1	Spectrum of β-thalassaemia mutations in at risk populations	94
3.2	Primer details for thalassaemias	101
3.3	Primer details for single cell mini-sequencing	105
3.4	Comparison study of lysis buffers	113
3.5	Control study of two polymerase enzymes	115
3.6	Comparison of HUMTH01 and β-globin genes amplification	118
4.1	Primer details for DM	162
4.2	Long template PCR systems	165
4.3	Amplification efficiencies and ADO rates of DM, APOC2 and D21S1414	173
4.4	Details of families 'A' and 'B'	175
4.5	Amplification efficiencies and ADO rates of DM and APOC2	178
4.6	PGD results of family 'A', cycle 1	182
4.7	PGD results of family 'A', cycle 2	186
4.8	PGD results of family 'B', cycle 1	189
5.1	Additional primer details for studying single cell PCR efficiency	210
5.2	Control studies for freezing and thawing effect and amount of DNA	217
5.3	Cell types study	218
5.4	Cell quality study	219
5.5	Preferential amplification study	221
5.6	Multiplex effect studies	222
5.7	ADO and AF relationship study	224
5.8	PCR cycling conditions studies	226
5.9	PCR cycling conditions studies (continued)	228
5.10	DNA sequence composition study	229
5.11	DNA sequence and fragment length study	229

List of Figures

1.1	Intracytoplasmic sperm injection (ICSI)	33
1.2	Cleavage stage embryo biopsy	37
1.3	Polymerase chain reaction (PCR)	44
1.4	Allele drop out (ADO)	46
1.5	Nested PCR	51
1.6	Restriction fragment length polymorphism (RFLP)	55
1.7	Amplification refractory mutation system (ARMS)	56
1.8	Heteroduplex analysis	58
1.9	Single strand conformational polymorphism (SSCP) analysis	59
3.1	Active globins during different periods of human life	88
3.2	A patient with β-thalassaemia major	90
3.3	The pathophysiology of β-thalassaemia	92
3.4	Major β-thalassaemia mutations	93
3.5	Various forms of α-thalassaemia	97
3.6	α-Globin gene complex and α-thalassaemia, $--^{SEA}$	104
3.7	SSCP analysis results of β-thalassaemia on PhastSystem™	107
3.8	SSCP analysis results of β-thalassaemia on GenePhor™	107
3.9	SSCP analysis results of β-thalassaemia, IVSI-110 on GenePhor™	108
3.10	F-SSCP results of β-thalassaemia, IVSI-110	109
3.11	F-SSCP results of β-thalassaemia mutations	110
3.12	PGD protocol β-thalassaemia, IVSI-110	111
3.13	HUMTH01 fragment analysis results from single and multiplex PCR	113
3.14	HUMTH01 fragment analysis results from two polymerase enzymes	114
3.15	Results from 2% agarose gel electrophoresis of two polymerase enzymes	116
3.16	HUMTH01 fragment analysis results from different PCR cycles	117
3.17	HUMTH01 fragment analysis results from multiplex PCR	119
3.18	SSCP analysis results of β-thalassaemia, IVSI-110 on GenePhor™	120
3.19	F-PCR detection of β-thalassaemia, codon 41-42	121
3.20	F-PCR analysis of β-thalassaemia, codon 41-42 from single cells	122
3.21	F-PCR detection of α-thalassaemia, $--^{SEA}$	123
3.22	Single cell sequencing result of β-thalassaemia, IVSI-110 with SA	124

3.23	Single cell sequencing result of β-thalassaemia, IVSI-110 with ADOM	125
3.24	Single cell sequencing result of β-thalassaemia, IVSI-110 with ADON	125
3.25	Single cell sequencing result of β-thalassaemia, IVSI-110 with PAN	125
3.26	Single cell sequencing result of β-thalassaemia, codon 41-42 with SA	126
3.27	Single cell sequencing result of β-thalassaemia, codon 41-42 with ADON	127
3.28	Single cell sequencing result of β-thalassaemia, codon 41-42 with PAM	127
3.29	Single cell minisequencing results of β-thalassaemia, IVSI-110	128
3.30	Single cell minisequencing results of β-thalassaemia, codon 41-42	129
3.31	Single cell minisequencing results of sickle disease	130
3.32	β-Globin gene sequences	134
3.33	β-Globin and δ-globin genes sequences	134
3.34	δ-Globin gene sequences	135
4.1	DM primers mapped to the DM CTG repeats	163
4.2	Inheritance of DM and APOC2	167
4.3	Amplification of DM and APOC2	168
4.4	Amplification of DM and D21S1414	168
4.5	F-PCR analysis results of APOC2	169
4.6	F-PCR analysis results of DM	170
4.7	F-PCR analysis results of DM and APOC2	171
4.8	Triplex amplification of DM, APOC2 and D21S1414	173
4.9	DM and APOC2 analysis results of 'A' family	176
4.10	Inheritance of DM and APOC2 in 'A' family	176
4.11	DM and APOC2 analysis results of 'B' family	177
4.12	Inheritance of DM and APOC2 in 'B' family	177
4.13	D21S1414 analysis results of 'A' family	180
4.14	D21S1414 analysis results of 'B' family	180
4.15	Morphology and PGD results of embryo A4	183
4.16	Morphology and PGD results of embryo A8	183
4.17	Morphology and PGD results of embryo A15	184
4.18	PGD results of blastomeres A4.1 and A6.1 and embryo AA6	187
4.19	Gel electrophoresis results of long template PCR systems	191
5.1	Relationship between fragment size and ADO rates	231
5.2	Relationship between fragment size and amplification efficiency	231
5.3	Relationship between amplification efficiency and ADO rates	238

Abbreviations

θ	recombination fraction
µl	microlitre
µM	micromolarity
A	ampere
AA	appropriate/adequate amplification of both alleles
ACU	Assisted Conception Unit
ADO	allele drop out
ADOL	allele drop out of the longer allele
ADOM	allele drop out of the mutant allele
ADON	allele drop out of the normal allele
ADOS	allele drop out of the shorter allele
AF	amplification failure
ALF	automated laser fluorescence
APS	ammonium persulfate
ARMS	amplification refractory mutation system
ART	assisted reproductive technology
ASO	allele-specific oligonucleotide
bp	base pair
BSA	bovine serum albumin
CCD	charged couple device
CGH	comparative genome hybridisation
CMC	chemical mismatch cleavage
CPM	confined placental mosaicism
CVS	chrorionic villus sampling
d	day
DGGE	denaturant gradient gel electrophoresis
DM	myotonic dystrophy
DMD	Duchenne muscular dystrophy
DMPK	myotonin-protein kinase gene
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

dNTP	deoxynucleotides triphosphate
DOP-PCR	degenerate oligonucleotide primed PCR
EDTA	ethylenediaminetetra-acetic acid disodium salt
Exo I	Exonucease I
FAPC	familial adenomatous polyposis coli
FBS	fetal blood sampling
FISH	fluorescent *in situ* hybridisation
F-PCR	fluorescent PCR
FS	frameshift
F-SSCP	fluorescent single stranded conformation polymorphism
g	gram
GIFT	gamete intrafallopian transfer
h	hour
HA	heteroduplex analysis
Hb	haemoglobin
hCG	human chorionic gonadotrophin
HD	Huntington's disease
HFEA	Human Fertilisation and Embryology Authority
HIV	human immunodeficiency virus
HLA	human lymphocyte antigen
Hm	homozygote
HPRT	hypoxanthine phosphoribosyl transferase
Ht	heterozygote
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilisation
IVSI-110	point mutation of intervening sequence I, nucleotide 110 in the beta-globin gene from G to A
IVSs	intervening sequences
Kb	kilo base pair
LCR	locus control region
M	molarity
MCAD	medium chain acyl CoA dehydrogenase
M-FISH	multi-fluorochrome FISH
mg	milligram

min	minute
ml	millilitre
mm	millimetre
mM	millimolarity
OMIM	Online Mendelian Inheritance in Man
PA	preferential amplification
PAL	preferential amplification of longer allele
PAM	preferential amplification of mutant allele
PAN	preferential amplification of normal allele
PAS	preferential amplification of shorter allele
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	primer extension preamplification
PESA	percutaneous epididymal aspiration
PGD	preimplantation genetic diagnosis
PGS	preimplantation genetic screening
PK	proteinase K
PND	prenatal diagnosis
POP-4™	Performance Optimised Polymer 4
PROST	pronucleate stage tubal transfer
PZD	partial zona dissection
QF-PCR	quantitative fluorescent PCR
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
SA	symmetrical/equal amplification of both alleles
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl (lauryl) sulphate
sec	second
SKY	spectral karyotyping
SSCP	single stranded conformation polymorphism
STR	short tandem repeat
SUZI	subzonal sperm insemination
TESA	testicular sperm aspiration
TOP	termination of pregnancy

v/v	volume/volume
Vh	volt hour
W	watt
w/v	weight/volume
w/w	weight/weight
WGA	whole genome amplification
Z	lod score
ZIFT	zygote intrafallopian transfer
ZP	zona pellucida

Chapter 1

Introduction

1.1 Genetic disease

The process of human development involves genetic and environmental elements. Disruption of any essential factors can lead to developmental abnormalities with various results from harmless to lethal. In general, around 3-5% of newborns possess some forms of congenital abnormalities (Shepard, 1986). The incidence of abnormalities is higher at older age, when the symptoms of late onset disorders are expressed. Congenital anomalies account for 25-30% of perinatal deaths (stillbirths after 28 weeks of pregnancy and neonatal deaths during the first week), whereas genetic causes are involved in 80% of cases. Gross structural abnormalities are detected in 80-85% of all spontaneous abortions. The causes include single gene defects and chromosome abnormalities, i.e. trisomy, monosomy or triploidy, which account for 50% of the causes. The understanding of genetic disease, together with modern technology, has helped in early detection. Consequently, the opportunity of early treatment or prophylactics and improving the quality of life and the survival of the patient, or avoiding new cases, is increasingly possible.

1.1.1 Chromosome abnormalities

Chromosome abnormalities are the major cause of pregnancy loss and mental retardation in humans. Principal types of chromosomal anomalies found include aneuploidy and polyploidy. Aneuploidy is the abnormal chromosome number other than a multiple of the basic euploid number, which is 23 in humans, while the less common polyploidy involves one or more extra sets of chromosomes. The overall incidence of

aneuploidy in livebirths is 0.3%, while those in stillbirths and spontaneous abortions are higher; 4.3% and 34.7%, respectively (Griffin, 1996). Trisomy 21 (Down's syndrome) is the commonest chromosome abnormality at birth (0.13%), and trisomy 18 (Edwards' syndrome, 0.01%) and trisomy 13 (Patau's syndrome, 0.005%) are the second and third most common. Surprisingly, trisomy 16, which has never been found in livebirth due to its lethality, contributes to the most frequent trisomy as it is found in about one third of all trisomies in spontaneous abortions.

Sex chromosome aneuploidy has less severe phenotypes than autosomal aneuploidy and; therefore is more likely to survive until term. Common sex chromosome aneuploidies include XXX, XXY (Klinefelter's syndrome), XYY and monosomy X (Turner's syndrome). Monosomy X is the main aneuploidy in spontaneous abortions, but its incidence is much lower in livebirths indicating a high incidence of miscarriages during pregnancy (Griffin, 1996). The incidence of sex chromosome aneuploidy may be underestimated because some are asymptomatic, consequently, they are not diagnosed unless the patient has a problem, usually infertility.

The technology of *in vitro* fertilisation (IVF) (Steptoe and Edwards, 1978) has allowed the study of preimplantation embryos. Using karyotyping an incidence of aneuploidy in preimplantation embryos of approximately 20% was observed (Angell *et al.*, 1986). Further studies used Fluorescent *in situ* hybridisation (FISH) and revealed that approximately half of normally developing preimplantation embryos possess chromosome abnormalities and mosaicism (Delhanty *et al.*, 1997; Harper *et al.*, 1995), and up to 70% of abnormally developing embryos were chromosomally abnormal (Munné *et al.*, 1993). The possible chromosome patterns found in preimplantation

embryos include uniformly normal, uniformly abnormal, mosaic and chaotic or uncontrolled division. These findings were confirmed by studies using comparative genome hybridisation (CGH; **Section 6.1.2**), which allows every chromosome to be examined (Voullaire *et al.*, 2000; Wells and Delhanty, 2000).

The major cause of chromosome aneuploidy and polyploidy is nondisjunction which is due to abnormal spindle formation. Nondisjunction during meiosis I is the leading cause of aneuploidy in most chromosomes, except trisomy 18. The majority of autosomal trisomies (88%) originate from maternal nondisjunction; however, in monosomy X 80% of the lost sex chromosomes are paternal. Maternal nondisjunction during meiosis I is the commonest cause of trisomy 21. It was noted in 1933 that the increasing incidence of Down's syndrome is related to advanced maternal age (Penrose, 1933). The same relationship was also found for the other trisomies (Hassold *et al.*, 1996). The occurrence of chromosome abnormalities during early human development may be a crucial cause of infertility problems or recurrent pregnancy loss in some patients.

1.1.2 Single gene disorders

Mutations in single genes can lead to inherited disorders with Mendelian inheritance patterns. Individual single gene disorders are rare, but together affect around 1% of the general population. More than 6,800 different single gene disorders have been catalogued to date (http://www3.ncbi.nlm.nih.gov/omim/, 2001) and can be categorised by their inheritance patterns as autosomal dominant (over 2,900 disorders), autosomal recessive (over 2,800 disorders) and X-linked disorders (over 1,000 disorders).

Autosomal dominant disorders are those which are present in heterozygous subjects. The chance of transmitting the defective gene from an affected parent to their offspring is 50%. It is usually possible to trace the inherited gene back several generations. The disorders with autosomal recessive inheritance are only expressed in the homozygote state. Heterozygous individuals with one mutant and one normal gene are carriers and are clinically unaffected. If both parents are heterozygotes, the risk of having an homozygote affected offspring is 25% and the rest will be carriers (50%) and homozygote normal (25%).

X-linked diseases involve genes located on the X chromosome which can be recessive or dominant. X-linked recessive disorders are usually transmitted by healthy heterozygote female carriers to affected males. The affected males can produce carrier daughters (with 50% risk) who can give rise to affected sons (with 50% risk). However, the affected males cannot pass on the disease to their sons. The disorders with X-linked dominant inheritance are generally similar to autosomal dominant defects. An affected female has a 50% chance of having affected daughters and sons. However, all daughters of an affected male will be affected, while all sons will be normal.

The understanding of inheritance patterns of single gene diseases has helped in predicting the chance of transmitting a disease. This knowledge is useful for providing genetic counselling and the application of prenatal diagnosis in order to prevent new cases. However, unusual phenomena, i.e. mosaicism, anticipation, imprinting, uniparental disomy, variable expressivity and reduced (incomplete) penetrance may interfere with the study of patterns of inheritance in some disorders. Modern molecular technology has

allowed genetic diagnosis and the understanding of gene functions possible. At present, the role of disease control is very challenging.

1.2 Diagnosis of genetic diseases

Couples who are affected or carry a genetic defect possess a high risk of passing on the abnormality to their children. Prior to the era of prenatal diagnosis (PND), such subjects had to decide whether to be childless or risk having children who might inherit the genetic disorder. With the evolution of molecular genetic technologies, the testing of the growing number of inherited genetic diseases has become feasible. In addition to the correlation of the particular gene defect to each disorder, such tests are notably useful in identifying asymptomatic carriers who are at risk of transmitting the genetic abnormality to their offspring. This knowledge, when incorporated with genetic counselling and appropriate prenatal diagnosis techniques, helps in reducing the number of the births of new affected cases (Petrou and Modell, 1995; Tongsong *et al.*, 2000b). The members of the affected families whose tests show no causative mutation can abolish the anxiety and alter their life style and reproductive attitude. In presymptomatic patients of some diseases, i.e. myotonic dystrophy (DM) and Huntington's disease (HD), the genotypes of the mutant genes can approximately predict the age of onset and severity of the diseases. In addition, with early detection of some defective genes, the symptoms can be prevented or alleviated by diet control (Farrell *et al.*, 1997), drug administration (Charache *et al.*, 1995), blood transfusion (Piomelli *et al.*, 1969) or surgical removal (Vasen *et al.*, 1996).

Mutation analysis can be performed directly if the responsible genes in the family have been identified and cloned. However, in cases where the causative mutations are still

unspecified or direct analysis techniques are unavailable, the examination of polymorphic loci adjacent to the causative mutation can be employed for linkage analysis in order to track down the transmission of the mutant gene in the family. The polymorphic linked markers with point variation can be identified using restriction fragment length polymorphisms (RFLP), while those with hypervariable microsatellite loci which give more alleles with varied size are more useful.

1.2.1 Prenatal diagnosis

The combination of molecular techniques for diagnoses of genetic diseases and procedures to collect fetal cells in utero enables prenatal diagnosis of many genetic disorders at an early stage of pregnancy. Normal testing results reassure the parents that their fetus is free from the disease; while abnormal results give them a chance of deciding to discontinue the pregnancy, to have fetal therapy where possible, or to continue the pregnancy.

1.2.1.1 Amniocentesis

Amniocentesis is the commonest invasive procedure for prenatal diagnosis. At between 15 and 18 weeks of gestation when the total amniotic fluid volume is approximately 200ml, 15-20ml of the fluid containing fetal cells can be taken using a 20- or 22- gauge needle transabdominally under continuous ultrasound surveillance. The first 2ml of liquid is discarded to reduce the risk of maternal cell contamination. Amniocentesis was first performed in the treatment of polyhydramnios for amniotic fluid drainage (Prochownik, 1877). This technique was used for hypertonic saline infusion to

induce abortion in 1937 (Aburel, 1937). However, the first application of amniocentesis for prenatal diagnosis was documented in 1952 for the evaluation of Rhesus isoimmunisation (Bevis, 1953). After that the method of X-chromatin identification in amniocytes from second trimester amniocentesis became possible (Fuchs and Riis, 1956; Makowski *et al.*, 1956). Karyotyping from amniotic fluid cells was first performed in 1966 (Steele and Breg, 1966). The first fetal diagnosis of trisomy 21 and galactosaemia (an inborn error of metabolism) were reported in the same year (Nadler, 1968). Since then amniocentesis has been applied for various uses.

The complications to the mothers are very rare and almost negligible. However, common symptoms include spot bleeding and amniotic fluid leakage. The risk of abortion following amniocentesis is slightly higher than that of a control group, 2.4% vs 1.2%, (Working Party on Amniocentesis, 1978). Other fetal complications, including skin dimples, cardiac tamponade (Berner *et al.*, 1972), pneumothorax (Hyman *et al.*, 1973), arteriovenous fistula (Gottdiener *et al.*, 1975), gangrene of the leg (Lamb, 1975) and eye injury (Cross and Maumenee, 1972), have been documented. However, a larger study found no difference in the risk of birth defects among the amniocentesis group when compared to a control group (Baird *et al.*, 1994; Simpson *et al.*, 1976; Working Party on Amniocentesis, 1978). Early amniocentesis during 10 and 14 weeks of gestation as an alternative to chorionic villus sampling (CVS) has been carried out (Hanson *et al.*, 1987) in order to obtain early diagnostic results and consequently reduce the risk caused by late termination of pregnancy. However, a randomised controlled trial demonstrated an increased fetal loss rate and a raised incidence of talipes in the early amniocentesis group compared to the second trimester amniocentesis group (The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group, 1998). The relationship between early

amniocentesis and orthopedic malformations were also observed in other reports (Stripparo *et al.*, 1990; Sundberg *et al.*, 1997). Therefore, amniocentesis is usually not performed until 15 weeks of pregnancy. Modern analysis methods, including FISH (Klinger *et al.*, 1992) and polymerase chain reaction (PCR) (Pertl *et al.*, 1994) have been successfully applied to uncultured amniocytes in order to accelerate the diagnostic reports. However, the test results are seldom completed before 16 weeks of pregnancy.

1.2.1.2 Chorionic villus sampling (CVS)

Chorionic villus sampling (CVS) is a technique to retrieve fetal tissue for analysis in the first trimester. The major advantage of this technique includes the opportunity to obtain sufficient fetal cells for the tests and early fetal diagnosis results which enable first trimester abortion in cases of affected fetuses. Moreover, direct DNA analysis, fast karyotyping following a rapid culture technique and biochemical analysis from the biopsied chorionic villus are also possible. Three large studies have revealed no difference (Lippman *et al.*, 1992), slightly increased (Jackson *et al.*, 1992) and significantly higher (MRC working party on the evaluation of chorion villus sampling, 1991) fetal loss rate following CVS when compared with amniocentesis. Therefore, CVS is categorised as a relatively safe procedure and the method of choice for identifying single gene disorders which possess high risks of having affected offspring (25% with recessive and 50% with dominant disorders). Limb-reduction defects were found to be associated with the CVS procedure (Brambati *et al.*, 1992; Burton *et al.*, 1992; Firth *et al.*, 1991). However, a larger study, which did not include pregnancies at 6-8 weeks of gestation, showed no difference in the incidence of limb defects following CVS from the

normal population (Froster and Jackson, 1996). Therefore, it is advised not to perform CVS earlier than 10 weeks.

CVS was first performed transcervically (Mohr, 1968), but was unpopular due to the more acceptable amniocentesis. The development of modern high resolution ultrasound has helped CVS in ensuring its safety and it is consequently gaining in its popularity. At present there are two sampling approaches, transcervical and transabdominal, which are usually carried out during 10-12 weeks of pregnancy. For transcervical CVS, a catheter (Ward *et al.*, 1983) or biopsy forceps (Vaughan and Rodeck, 1992) are gently inserted though the cervix and directed to take the chorion samples under continuous ultrasound observation. The transabdominal technique was first published in 1984 (Smidt-Jensen and Hahnemann, 1984). The chorionic villus samples can be obtained by introducing an 18- or 20-gauge needle transabdominally into the chorionic bed under continuous ultrasound guidance. The transcervical approach is preferable when the placenta is posterior, while the transabdominal technique is easier when the placenta is anterior. The fetal loss rates and the efficiency of transcervical and transabdominal techniques were not significantly different in 2 randomised clinical studies (Brambati *et al.*, 1991; Jackson *et al.*, 1992). Recently, the minimal invasive transcervical fetal cell retrieval technique was proposed to be another possible prenatal diagnosis procedure which is safe and effective (Rodeck *et al.*, 1995).

The diagnostic results from CVS are based on the analysis of the extraembryonic tissues which may not always be identical to the fetus (Kalousek and Dill, 1983). Mitotic nondisjunction during the early stage of embryonic development can give rise to an extra cell line residing in the extraembryonic structures. This event is called confined placental

mosaicism (CPM), can be found in approximately 1% of pregnancies and can lead to discordant results between the CVS and the fetus. The occurrence of uniparental disomy in the fetuses following the diagnosis of CPM supports the trisomic zygote rescue theory (Kalousek *et al.*, 1993; Purvis-Smith *et al.*, 1992; Roberts *et al.*, 1997). Therefore, confirmatory amniocentesis or fetal blood sampling are required in some cases to confirm CVS results.

1.2.1.3 Fetal blood sampling (FBS)

Fetal blood sampling from the umbilical cord or cordocentesis was performed using a fetoscopic technique (Rodeck and Campbell, 1979). However, this technique has been superseded by the percutaneous ultrasound guided approach (Daffos *et al.*, 1983) when high resolution ultrasound was developed. Using a 22- or 23-gauge needle, the umbilical cord can be punctured at the cord insertion to the placenta or free loop depending on the location of the placenta. Fetal blood samples are useful for the diagnosis of fetal isoimmunisation, hypoxia, infection, haemoglobinopathy, metabolic disorders, single gene defects and quick karyotyping. Transient bleeding at the puncture site and temporary fetal bradycardia are common but unserious complications. The procedure related fetal loss rate is around 0.2-2.3% (Buscaglia *et al.*, 1996; Daffos *et al.*, 1985; Tongsong *et al.*, 2000a; Weiner and Okamura, 1996). Therefore, cordocentesis is another choice of fetal diagnosis which is relatively safe and effective.

1.3 Preimplantation genetic diagnosis (PGD)

At present the strategy of population screening, genetic screening and offering appropriate prenatal diagnosis procedures is the best tool to control the incidence of new cases with genetic disorders in many countries. However, amniocentesis, CVS and FBS are invasive operations which possess some risks of miscarriage and malformation to the fetuses. Additionally, in the case of a positive result, termination of pregnancy is the only option if the couple want a healthy family. Preimplantation genetic diagnosis (PGD) is the genetic testing of preimplantation stage embryos for inheritable chromosome abnormalities or single gene defects. This allows the selection of unaffected embryos prior to the establishment of a pregnancy, and therefore gives the couple the chance to start a pregnancy with a disease free baby. Consequently, the need for a termination of an affected pregnancy can be eliminated. This approach is found to be more acceptable than prenatal diagnosis in some groups of patients, in particular those with moral or religious objections to pregnancy termination. Subfertile couples carrying a chromosomal translocation or gonadal mosaicism may benefit by increasing the chance of starting a pregnancy with a normal zygote. PGD can help carriers of an inherited disease avoid repeated pregnancy termination. Moreover, some individuals at risk of having offspring with late onset or non-life-threatening diseases consider this approach more acceptable than PND. Therefore, PGD is the alternative approach to avoid a pregnancy affected with a genetic disease.

The concept of PGD was first established by sexing rabbit blastocysts (Edwards and Gardner, 1967). However, none of the embryos survived after the procedure. The biopsy technique was employed to improve the survival rates (Gardner and Edwards,

1968). Karyotyping was applied for sex determination in bovine embryos (Hare *et al.*, 1976). *In vitro* fertilisation of human oocytes was successful in 1969 (Edwards *et al.*, 1969) and *in vitro* culture conditions up to the blastocyst stage of human embryos were established in 1971 (Steptoe *et al.*, 1971). This knowledge has made it possible to study preimplantation human embryos.

The preliminary model of preimplantation diagnosis was carried out using biochemical testing for hypoxanthine phosphoribosyl transferase (HPRT) enzyme deficiency, which is the underlying cause of Lesch-Nyhan syndrome, in mouse embryos (Monk *et al.*, 1987). β-Globin test was the first model of biopsy and DNA based analysis for PGD in mouse embryos (Holding and Monk, 1989). In human, it was shown that the development of the 8-cell stage preimplantation embryo was not compromised after the biopsy (Hardy *et al.*, 1990). The first PGD pregnancies were from sex determination using PCR to avoid X-linked diseases (Handyside *et al.*, 1990). The embryos were generated by IVF procedures and single blastomeres were biopsied from cleavage stage embryos (Handyside *et al.*, 1989). Since then (data until May 2000), over 1,000 PGD cycles have been carried out worldwide resulting in more than 160 healthy children (ESHRE PGD Consortium Steering Committee, 2000). Approximately 40% of PGD cycles were for the diagnosis of single gene disorders, a third were for chromosomal abnormalities and a quarter were for sexing in patients at risk of X-linked diseases. Almost half of the patients decided to have PGD due to moral or religious objection to pregnancy termination, a third possessed infertility problems that needed IVF treatment and carried a genetic disorder, while a quarter had experienced termination of pregnancy and wanted to avoid another.

FISH has been used for sexing (Griffin *et al.*, 1994), and detecting numerical chromosomal abnormalities and chromosome translocations (Conn *et al.*, 1998 and 1999; Iwarsson *et al.*, 2000). PCR is used for identifying single gene disorders, including Duchenne muscular dystrophy (Liu *et al.*, 1995), Fragile X syndrome (Dreesen *et al.*, 1995; Sermon *et al.*, 1999b), Tay Sachs disease (Gibbons *et al.*, 1995), Marfan's syndrome (Blaszczyk *et al.*, 1998; Harton *et al.*, 1996; Sermon *et al.*, 1999a), DM (Sermon *et al.*, 1997; Sermon *et al.*, 1998a) (see also **Section 4.3.3**), Charcot Marie Tooth type 1A (De Vos *et al.*, 1998), familial adenomatous polyposis coli (FAPC) (Ao *et al.*, 1998), Huntington's chorea (Sermon *et al.*, 1998b), severe inherited skin diseases (McGrath and Handyside, 1998), sickle cell anaemia (Rechitsky *et al.*, 1998; Xu *et al.*, 1999), spinal muscular atrophy (Dreesen *et al.*, 1998), β-thalassaemia (Kanavakis *et al.*, 1999; Kuliev *et al.*, 1999), congenital adrenal hyperplasia (Van de Velde *et al.*, 1999), Lesch Nyhan syndrome (Ray *et al.*, 1999), and medium chain acyl CoA dehydrogenase (MCAD) deficiency (Ioulianos *et al.*, 2000; Sermon *et al.*, 2000). Current data shows no significant difference in pregnancy rates between those from PGD and routine IVF cycles (ESHRE PGD Consortium Steering Committee, 2000).

1.3.1 In vitro fertilisation (IVF)

Initially the oocytes were collected laparoscopically, cultured and fertilised *in vitro* and the embryos transferred into the uterus, namely *in vitro* fertilisation and embryo transfer (IVF-ET) (Steptoe and Edwards, 1978). The main indication was for patients with fallopian tube obstruction. However, the procedure has become very useful for a growing number of reproduction problems, including male infertility and PGD. The

techniques of drug induced ovarian superovulation and transvaginal ultrasound-guided oocyte collection were later employed. Other modifications, i.e. gamete intrafallopian transfer (GIFT) (Guastella *et al.*, 1985), zygote intrafallopian transfer (ZIFT) (Hamori *et al.*, 1988) and pronucleate stage tubal transfer (PROST) (Yovich *et al.*, 1987), were also developed. The introduction of micromanipulation has allowed methods assisting male infertility, i.e. subzonal sperm insemination (SUZI) to inject sperm inside the zona pellucida (Laws-King *et al.*, 1987) and partial zona dissection (PZD) to facilitate sperm penetrating the oocyte (Cohen *et al.*, 1989), become possible.

The greatest breakthrough for solving sperm problems has been intracytoplasmic sperm injection (ICSI; **Figure 1.1**) developed when a sperm was accidentally injected into the ooplasm (Palermo *et al.*, 1992). ICSI has become a popular assisted reproduction procedure and given rise to over 1,000 babies worldwide without an increased risk of congenital anomalies (Bonduelle *et al.*, 1996; Loft *et al.*, 1999; Tarlatzis and Bili, 1998). However, genetic defects associated with infertility, i.e. cystic fibrosis (Schlegel *et al.*, 1995), Y chromosome deletions (Pryor *et al.*, 1997) and chromosomal abnormalities (Wilkins-Haug *et al.*, 1997), can be passed on and cause the same problem in the offspring (Chandley and Hargreave, 1996). In addition to mature sperm, surgical removal of immature sperm from the testis (testicular sperm aspiration, TESA) and epididymis (percutaneous epididymal aspiration, PESA) have been employed in azoospermic patients (Shrivastav *et al.*, 1994; Silber *et al.*, 1994).

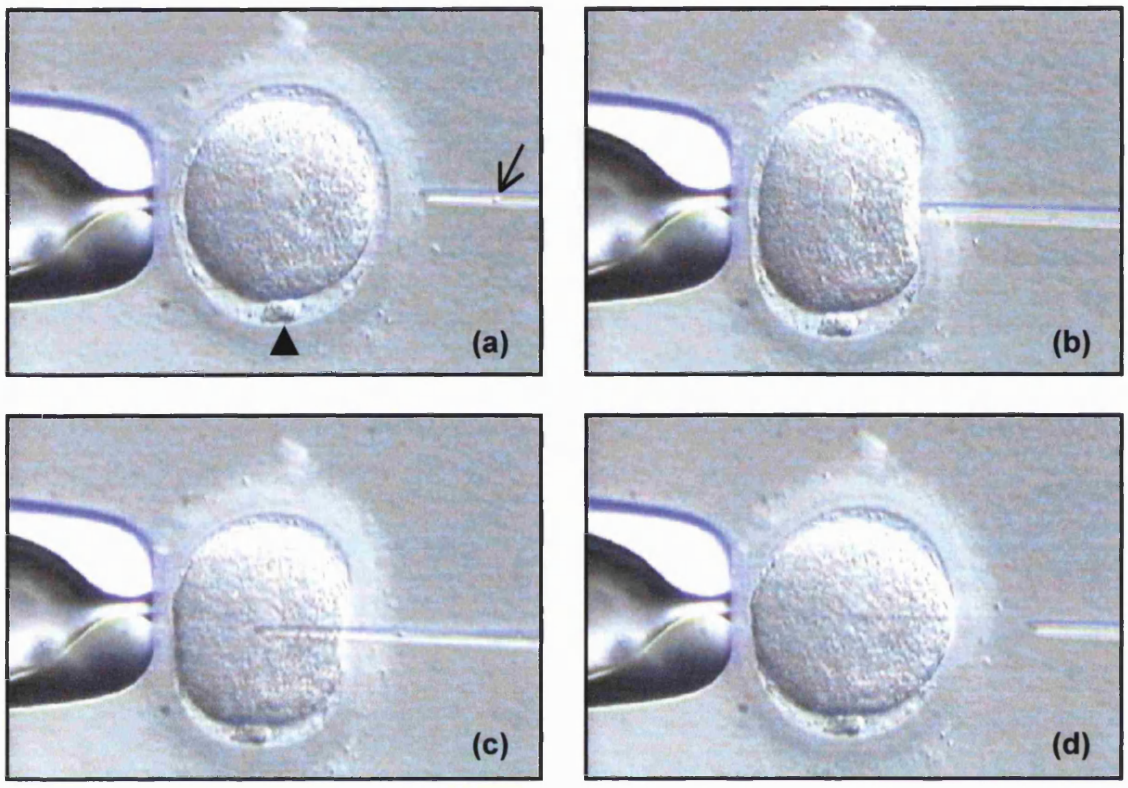


Figure 1.1 Intracytoplasmic sperm injection (ICSI): **(a)** and **(b)** the oocyte is stabilised by a holding pipette (left) with negative pressure, a hole is created in the zona pellucida using an injection needle (right) containing a sperm (arrow), **(c)** and **(d)** the injection needle is advanced into the ooplasm and the sperm is expelled into the ooplasm before the injection needle is withdrawn. Arrow = sperm, arrow head = first polar body. Procedure performed by Alpesh Doshi, ACU (Assisted Conception Unit), UCL.

With all the modern assisted reproduction techniques (ART), the pregnancy rates from IVF procedures are still relatively low. In order to obtain as many oocytes as possible, the ovarian superovulation protocol is employed. However, the drug regimen used in the stimulation protocol is unique for the individual patient due to the different hormonal status and response. The serum oestradiol level and follicular growth are monitored during the drug administration. When at least 3 follicles with a diameter of

18mm are observed by ultrasound scanning, ovulation induction is carried out using a single dose of human chorionic gonadotrophin (hCG). Thirty six hours after hCG administration, oocytes are collected using transvaginal needle aspiration under ultrasound guidance (Oehninger and Hodgen, 1990). The oocytes are incubated with washed sperm or ICSI is performed (Palermo *et al.*, 1992). Fertilisation is assessed 18 h later; two pronuclei indicating normal fertilisation. At 48 h post-oocyte retrieval, embryo development and morphology are evaluated and the 2 or 3 best quality embryos are selected for transfer. In IVF cycles, surplus embryos can be cryopreserved for use in future cycles, donated for research, donated to another couple or discarded.

1.3.2 Sampling techniques for PGD

For PGD, some of the oocyte or embryo has to be removed for the genetic analysis. This could be by polar body, cleavage stage or blastocyst biopsy. The samples are used for testing of the particular disease and normal embryos chosen for transfer.

1.3.2.1 Polar body biopsy

The first polar body is extruded from the ovulated oocyte after the first meiotic division is complete and has been used for genetic analysis (Munné *et al.*, 1995; Verlinsky *et al.*, 1996). The analysis results of the first polar body are complementary to that of the oocyte. In a heterozygote patient, if the genetic defect is found in the first polar body, it can be assumed that the genetic material of the same locus in the oocyte is normal and the oocyte can be chosen for fertilisation.

The concept of preconception diagnosis without an invasive procedure to the embryo is more acceptable to some couples and a longer time is available for the analysis. However, the risk of recombination (crossing-over) during meiotic division can make the predicted results uncertain. The chance of recombination varies from 50% for the telomeric genes to less than 1% for the centromeric genes. Therefore, both the first and second polar bodies are needed for the completion of the predictive results (Verlinsky *et al.*, 1996), but the second polar body is not extruded until after fertilisation. The analyses of both the first and second polar bodies make this technique somewhat labour-intensive. Moreover, male factors can not be analysed by this method, consequently, sexing for X-linked diseases and the prediction of mutations, in particular autosomal dominant disorders, carried by male partners are not possible. For autosomal recessive diseases in which both parents are carriers, oocytes with a mutant gene will be discarded, even though they may produce a heterozygote embryo when fertilised with a sperm with the normal gene. Finally, post-zygotic events, i.e. mosaicism, are not disclosed by this method.

1.3.2.2 Cleavage stage embryo biopsy

After fertilisation, the zygote undergoes cleavage cell division every 24 h. On day 3 when the embryo is at the 6-8 cells stage, two blastomeres can be taken without affecting embryonic metabolism or development and more than 90% of the embryos survive the biopsy procedure (Hardy *et al.*, 1990). Blastomeres at this stage are thought to be totipotent, not being assigned to be any specific tissues. However, embryo biopsy at the 4-cell stage may compromise the development of embryos (Tarin *et al.*, 1992).

Therefore, embryo biopsy on day 3 is the most popular technique for PGD worldwide (ESHRE PGD Consortium Steering Committee, 2000).

The procedure involves zona drilling and blastomere aspiration. On a micromanipulator, the embryo is held by suction on a flame-polished holding pipette. A hole is drilled in the zona pellucida by applying acid Tyrodes solution from a narrow pipette (Hardy *et al.*, 1990). The size of the hole generated from this technique is difficult to control. Other zona drilling techniques include partial zona dissection (PZD) by cutting the zona in a V shape mechanically (Malter and Cohen, 1989) and a laser which gives more control of the hole size (Boada *et al.*, 1998; Montag *et al.*, 1998; Veiga *et al.*, 1997). Drilling a hole in the zona pellucida may help in raising IVF success rates due to assisting embryo hatching (Gordon *et al.*, 1988). The blastomere is gently aspirated through the hole using a 30 micron pipette (**Figure 1.2**) (Handyside *et al.*, 1990). In the case of compacted embryos, incubation in calcium magnesium free medium at 37°C for 10 min, to temporally disrupt the cells junctions, can help in blastomere aspiration (Dumoulin *et al.*, 1998).

The disadvantages of cleavage stage biopsy are that only 1-2 cells can be obtained and only 24 h is available for the analysis in order to transfer the embryos on day 4. Therefore, the diagnostic techniques need to be quick, sensitive and accurate. Due to the risk of chromosomal mosaicism in preimplantation embryos (Delhanty *et al.*, 1997; Harper *et al.*, 1995; Munné *et al.*, 1995) and allele specific amplification failure (ADO) from PCR assays, the analysis of two blastomeres from each embryo is recommended to reduce the chance of misdiagnosis (**Section 1.5.2**).

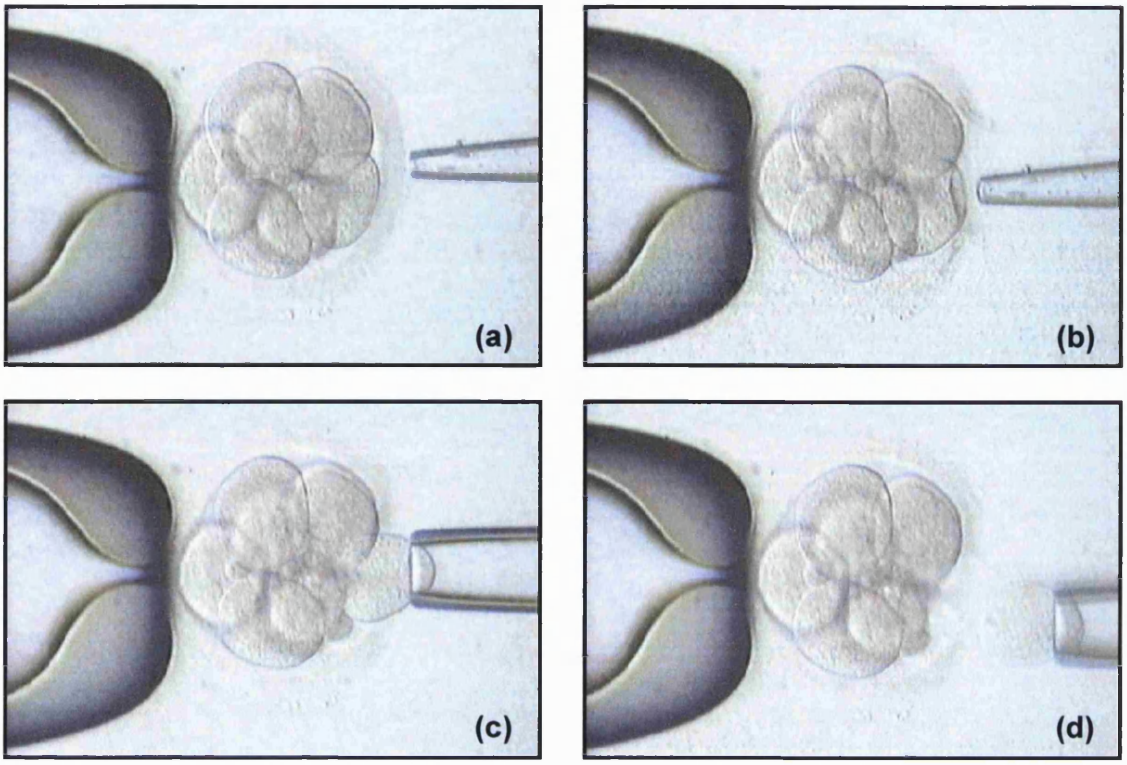


Figure 1.2 Cleavage stage embryo biopsy: **(a)** the embryo is stabilised by a holding pipette (left) with negative pressure, **(b)** a hole is created in the zona pellucida (zona drilling) using acid Tyrodes applied from a fine pipette (right) with a concentrated flow, **(c)** and **(d)** a blastomere is gently pulled away from the embryo using a third pipette (right) with negative pressure through the hole in the zona pellucida. Procedure performed by Alpesh Doshi, ACU, UCL.

1.3.2.3 Blastocyst biopsy

Human blastocysts can be obtained from uterine lavage of *in vivo* fertilised embryos (Buster and Carson, 1989; Formigli *et al.*, 1990) or extended culture of IVF embryos. However, the first mentioned technique is ineffective. Blastocyst biopsy can be performed on day 6 to day 7 post-fertilisation when the blastocyst contains approximately

120 cells. A blastocyst is made up of two cell types; inner cell mass which will give rise to the embryo and trophectoderm which will give rise to the placental membranes. A hole is drilled in the zona pellucida and the embryo is kept in the culture medium until the trophectoderm cells herniate from the embryo (Dokras *et al.*, 1990). 10-30 trophectoderm cells can be taken for the analysis (Muggleton Harris *et al.*, 1995). The restrictions for clinical application of this technique involve the analysis of extraembryonic cells which may not represent that of the inner cell mass and the poor *in vitro* embryonic development after day 3 to day 4. Only approximately half of IVF embryos develop to the blastocyst stage *in vitro* (Gardner *et al.*, 1998; Jones *et al.*, 1998). For these reasons, this method has never been applied clinically.

1.4 PGD Analysis techniques

1.4.1 Sex determination

For X-linked diseases where the causative genes have not been cloned or direct mutation analysis is not possible, sex determination is an alternative for avoiding affected embryos. Male embryos possess a 50% risk of developing the X-linked disease; therefore, only female embryos are selected for transfer. There are over 1,000 X-linked diseases, including Duchenne muscular dystrophy (DMD) and haemophilia. The first clinical PGD report was sex determination to avoid X-linked disorders using PCR to amplify a specific repetitive sequence on the Y chromosome (Handyside *et al.*, 1989). However, in the subsequent report of the same series a misdiagnosis was documented, probably due to amplification failure of the Y chromosome sequence (Handyside and Delhanty, 1993). This was supported by a later study (Kontogianni *et al.*, 1996). Attempts were made in

order to improve the accuracy of this method, i.e. amplifying a fragment from the homologous X and Y-linked locus, ZFX and ZFY (Chong *et al.*, 1993), multiplex amplification of the repetitive sequences of both X and Y chromosomes (Grifo *et al.*, 1992), amplifying the amelogenin gene (Nakahori *et al.*, 1991) and the steroid sulphatase gene (Liu *et al.*, 1994). For these techniques, the amplified fragments of the X and Y chromosomes are from the homologous locus, but different in size or restriction endonuclease site which enables differentiation between both fragments using the same primer set.

Despite the improvement of the PCR technique in identifying embryo sex, FISH has become the method of choice (Griffin *et al.*, 1994). The major advantage of FISH over PCR involves the information of copy number of the chromosomes analysed, consequently providing the chance to distinguish normal female embryos (XX) from an embryo with triploidy or Turner's syndrome (XO) (Griffin *et al.*, 1992). A 45,XO embryo possesses a high risk of being affected by the X-linked disorder as the absent sex chromosome is more likely to be paternal (Hassold *et al.*, 1988). In addition to the chromosome specific probes with different labels for the X and Y chromosomes, the incorporation of a third probe for an autosome can reveal the occurrence of mosaicism and ploidy abnormalities (Delhanty *et al.*, 1997). The diagnosis of female embryos is based on the presence of two X signals in the absence of a Y. The risk of misdiagnosis from contamination is minimal for FISH, compared with PCR where any contamination is significant. Therefore, FISH has become the preferred method for embryo sexing (ESHRE PGD Consortium Steering Committee, 2000).

1.4.2 Diagnosis of chromosome abnormalities

Chromosome abnormalities, mostly aneuploidies, are involved in half of spontaneously aborted pregnancies. Couples carrying a balanced translocation and those with gonadal mosaicism possess an even higher risk of having chromosomally abnormal fetuses. These patients are likely to have a history of recurrent spontaneous miscarriages or repeated induced abortions following positive prenatal diagnosis. Therefore, PGD would give them a better chance to start with a normal pregnancy. The test can be carried out using polar bodies (Munné *et al.*, 1998e) or blastomeres from cleavage stage embryos (Conn *et al.*, 1998) using FISH. Testing for chromosome abnormalities is the major indication for PGD (ESHRE PGD Consortium Steering Committee, 2000).

To examine every chromosome at the same time, to detect aneuploidy and polyploidy, traditional karyotyping using G-banding is the ideal technique (Angell *et al.*, 1986). However, most blastomeres in embryos are in interphase and are difficult to arrest in metaphase. Therefore, karyotyping is not an effective method for PGD. FISH uses a fluorescent-labelled probe which is a fragment of single-stranded DNA which specifically hybridises to its complementary target sequence in the genome. The probe is visualised by ultraviolet light. This provides the advantage of a quick interphase analysis. The disadvantage of FISH is that only a limited number of chromosomes can be analysed at one time. In addition to the growing number of fluorochromes used, newly developed techniques including interphase chromosome conversion (Evsikov and Verlinsky, 1999; Willadsen *et al.*, 1999) and comparative genome hybridisation (CGH) (Voullaire *et al.*, 1999; Wells *et al.*, 1999) will allow more chromosomes to be analysed (**Section 6.1.2**).

Preimplantation genetic screening (PGS) for age-related aneuploidy using FISH on polar bodies and blastomeres has been performed (Munné *et al.*, 1998b; Verlinsky *et al.*, 1998). The use of FISH probes for chromosomes X, Y, 13, 18 and 21 can cover 95% of congenital chromosome abnormalities. However, applying PGS is labour-intensive. Moreover, older age women are more likely to produce fewer good quality oocytes and PGS would reduce the number of embryos for transfer while the false positive rate is approximately 15% (Munné *et al.*, 1998b), which is higher than the chance of a monosomic embryo surviving to term. Also the chance of mosaicism in normally developing preimplantation embryos is high (Delhanty *et al.*, 1997; Harper *et al.*, 1995; Laverge *et al.*, 1997; Munné *et al.*, 1994). The use of 6 probes for chromosomes X, Y, 13, 16, 18 and 21 labelled with 3 fluorochromes in 6 different ratios has been reported (Munné *et al.*, 1998c). Additional probes for chromosomes 14, 15 and 22 were used in a re-probing method; however, a misdiagnosis of trisomy 21 has occurred (Munné *et al.*, 1998b), possibly due to overlapping signals, a reduced hybridisation efficiency or disomy/trisomy mosaicism.

Recent studies have shown that interphase FISH is not as efficient as metaphase FISH (Ruangvutilert *et al.*, 2000). Moreover, the additional number of probes used and re-probing technique leads to reduced hybridisation efficiency. The efficiency of the combination of the probes for PGD should be tested on lymphocytes and spare embryos before clinical application. For this reason, a scoring criteria for interpreting FISH signals has been introduced (Hopman *et al.*, 1988; Munné *et al.*, 1998c). The presence of mosaicism and chaotic embryos can lead to misdiagnosis as the analysis results of the biopsied blastomeres may not represent the whole embryo. In the case of a mosaic embryo, if a normal blastomere is biopsied from an otherwise abnormal embryo, the

embryo will be diagnosed as normal when it is affected. Therefore, the diagnosis of a normal embryo should be based on the results of two normal nuclei in order to reduce the risk of misdiagnosis.

For structural chromosome abnormalities, PGD for Robertsonian translocations using chromosome paints on polar bodies (Munné *et al.*, 1998e) and locus specific probes for the acrocentric chromosomes on cleavage stage biopsied blastomeres (Conn *et al.*, 1998; Iwarsson *et al.*, 2000) have been reported. Clinically reports of PGD for reciprocal translocation have been described using chromosome painting on first polar bodies (Munné *et al.*, 1998d) and 3 colour FISH, two probes flanking the breakpoint of one chromosome and the third for the other chromosome on blastomeres (Conn *et al.*, 1999; Iwarsson *et al.*, 2000). The use of flanking probes cannot differentiate balanced from normal embryos; however, the development of spanning probes can (Munné *et al.*, 1998a).

1.4.3 Diagnosis of single gene disorders

PGD protocols for more than 20 single gene defects have been reported (Wells and Delhanty, 2001). Almost 400 PGD cycles using PCR have been carried out, resulting in nearly 100 pregnancies (ESHRE PGD Consortium Steering Committee, 2000).

1.5 Single cell PCR

PCR (**Figure 1.3**) is a powerful molecular technique for quickly amplifying a particular DNA fragment to a stage that can be further analysed by other methods (Saiki *et al.*, 1985). A variety of modified techniques have been developed for different purposes, including forensic analysis, evolutionary biology, genetic screening, mutation analysis, PND and PGD of single gene disorders. More sophisticated and modern techniques have been employed. However, amplification failure, ADO and contamination are still major problems encountered during PCR at the single cell level. Due to the wide variety of mutations within the same and among different genes, specific analysis techniques are needed for individual types of mutations.

1.5.1 Amplification failure (AF)

The problem regarding amplification failure of single cell PCR emerged when a misdiagnosis from the first series of PGD for X-linked disorders was reported (Hardy and Handyside, 1992). In order to avoid transfer of male embryos, Y-chromosome specific sequences were amplified from the biopsied blastomeres. The absence of amplification is indicative of a female embryo. However, by this strategy the failure of amplification leads to the same diagnostic result. Amplification failure is not an unusual phenomenon encountered during PCR at the single cell level and can be found in around 10% of samples (Kontogianni *et al.*, 1996).

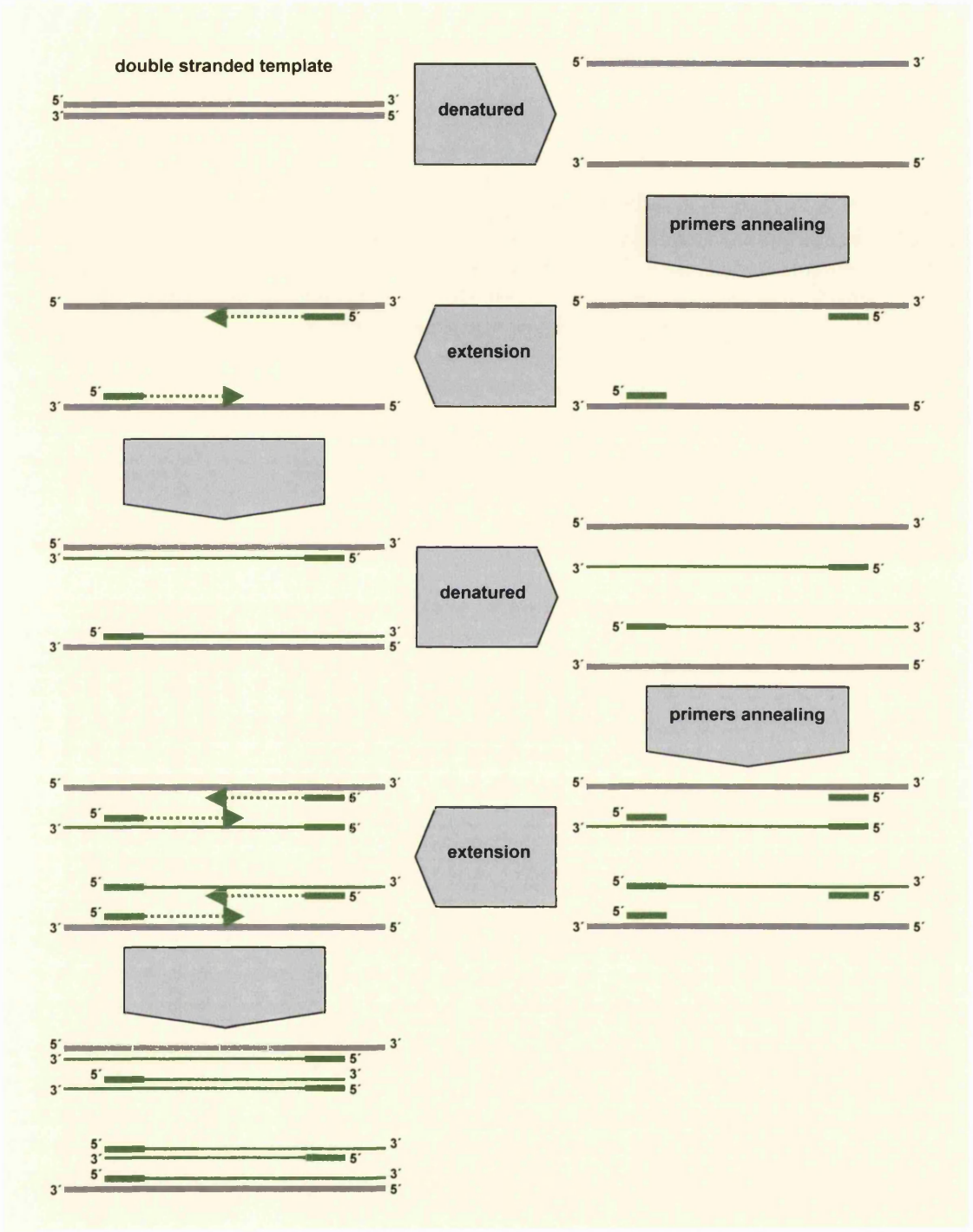


Figure 1.3 Polymerase chain reaction (PCR). The double stranded DNA templates are separated by denaturation at 94-97°C. The temperature is then reduced allowing annealing of primers to complementary genomic sequence. Optimal annealing temperature is determined experimentally. The temperature is then raised to 68-72°C to maximise the efficiency of the *Taq* polymerase enzyme, which initiates replication from the 3′ end of each primer. The amplification reaction is repeated using both the original DNA sample and newly synthesised fragments as templates. Amplification of the both sense and anti-sense strands generated gives rise to an exponential increase of PCR products.

It is difficult to empirically determine the cause of amplification failure although there are usually several likely candidates: the isolated cell can be lost during transfer to the PCR tube, or the cell could be anucleate or in the process of degeneration. For this reason, current protocols are assigned not to interpret a missing result as normal, but as an affected genotype. This design will not cause serious misdiagnosis in cases of amplification failure, but may reduce the number of normal embryos available for transfer. Amplification failure may be caused by the suboptimal conditions of the lysis protocol, primers combinations, PCR protocol or poor cell quality. Therefore, the protocols should be carefully optimised before clinical application.

1.5.2 Allele drop out (ADO)

Another common problem of single cell PCR is ADO or allele specific amplification failure when one of the two alleles in a heterozygote sample randomly fails to amplify (Findlay *et al.*, 1995a). Consequently only a single allele is detected after PCR, giving the appearance of homozygosity (**Figure 1.4**). This event is specific to PCR with low copy number of the templates and can lead to misinterpretation. Misdiagnoses that might have resulted from ADO have been documented (Grifo *et al.*, 1994; Harper and Handyside, 1994; Verlinsky, 1996). This problem is particularly crucial in the analysis of dominant disorders or recessive disorders with two different mutations (compound heterozygotes). For PGD of a recessive disease the occurrence of ADO would not normally lead to the transfer of an affected embryo, but could reduce the number of heterozygous (unaffected carrier) embryos detected.

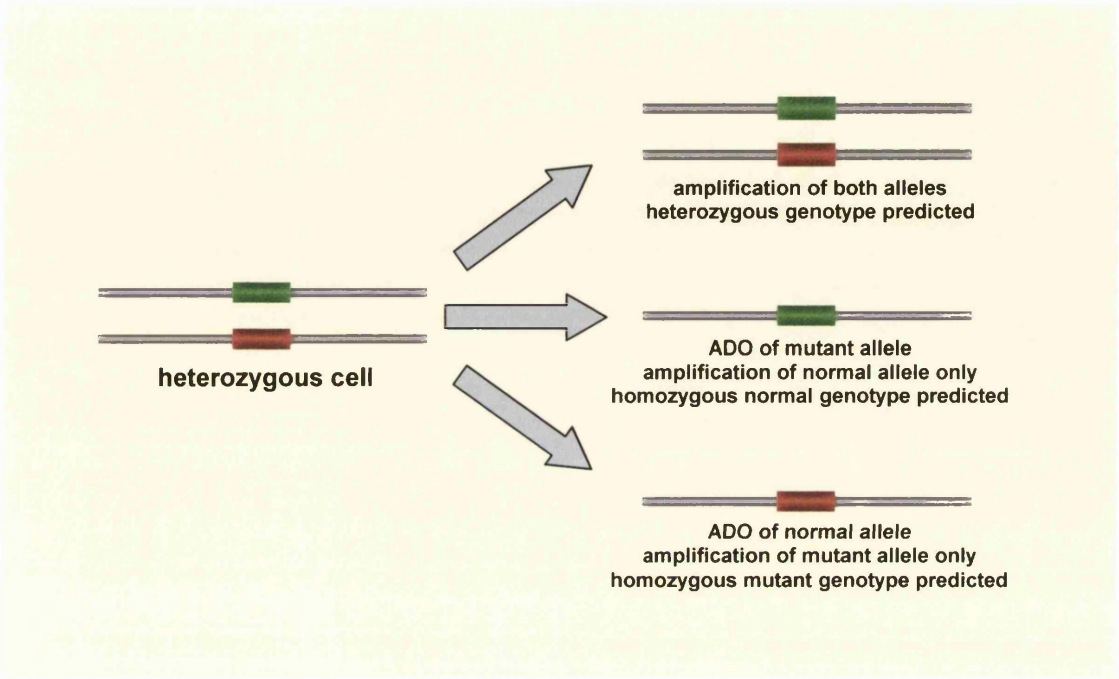


Figure 1.4 ADO in PGD of a heterozygous cell. ADO of the mutant allele leads to a homozygous normal diagnosis, while ADO of the normal allele leads to a homozygous mutant diagnosis.

In the case of a recessive disease with a single mutation involved, a heterozygous cell with ADO of the mutant allele would lead to a normal result and embryo transfer of an embryo carrying the mutation which is considered acceptable. ADO of the normal allele in the same case would lead to a homozygote affected result and this embryo would not be considered for transfer. Therefore, this could lower the chance of obtaining a pregnancy if significant numbers of embryos were mistakenly excluded from transfer, but it would not lead to a serious misdiagnosis. However, in the case of a recessive disease caused by two different mutations within the same gene (i.e. a compound heterozygous genotype) then it is possible for ADO to cause a misdiagnosis and lead to transfer of affected embryos. There are two instances in which this can occur. The first, which has

caused several reported misdiagnoses, occurs when only one of the two mutations has been characterized, or only one mutation can be visualized by the techniques employed. In this case ADO affecting the one detectable mutation causes the embryo to appear normal. However, 50% of such embryos will actually be affected. The second problem arises in cases where both mutations are detectable, but a distance too great for amplification in a single PCR fragment separates them. In this case a multiplex PCR may be performed, with each mutation amplified and analysed separately. Thus, an affected embryo will give a heterozygous result at each mutation site, but the presence of both mutations indicates that the embryo is actually affected. In cases such as this ADO affecting either of the mutation sites in an affected cell will produce a heterozygous, unaffected carrier, result. On rare occasions ADO could independently affect the mutant alleles at each of the two sites, thus giving a homozygous normal result when the embryo is in fact affected. In the diagnosis of a dominant disorder, ADO of the normal allele would give rise to an affected result. However, failure to amplify the mutant allele can lead to the transfer of affected embryos, as only the normal allele is amplified and subsequently detected.

On average, ADO occurs in around 2-20% of single cell PCR amplifications (Ray and Handyside, 1996). There are several theories as to the cause of ADO, the foremost of which are: DNA degradation leading to PCR-refractory breaks in both DNA strands; and inaccessibility of the DNA template due to imperfect PCR conditions or incomplete cell lysis. Possible means to improve amplification efficiency and minimize ADO include using highly sensitive fluorescent PCR (F-PCR) (Findlay *et al.*, 1995a), increasing the PCR denaturation temperature (Ray and Handyside, 1996), and the use of different cell lysis buffers (El-Hashemite and Delhanty, 1997). However, none of these measures seems

to consistently eliminate ADO. The most experienced PGD labs are generally able to reduce ADO rates to 5-10% (i.e. one of the two alleles affected in 5-10% of amplifications). However, ADO rates higher than this are not uncommon and in rare cases they may exceed 40%, significantly compromising the diagnosis (Wells and Sherlock, 1998). Methodology to avoid misdiagnosis caused from ADO involves the incorporation of a polymorphic linked marker analysis with the mutation analysis reaction as a multiplex PCR (Kuliev *et al.*, 1998) or in separate reactions after whole genome amplification (WGA) (Ao *et al.*, 1998) for the back up linkage analysis result. However, due to the timing and restricted genome coverage (Zhang *et al.*, 1992), multiplex PCR is still the preferred technique used for PGD (Harper and Wells, 1999). It is recommended to obtain the results from two cells of each embryo to reduce the risk of misdiagnosis from ADO (Kontogianni *et al.*, 1996).

In cases where both alleles are present, but one allele has been amplified more than its partner, the heterozygous locus is said to display preferential amplification (PA). This is distinguished from ADO in that both alleles are above the threshold of detection. Sometimes PA is a consequence of an allele difference in DNA sequence or fragment length, in which case the effect is not random like ADO, but affects one allele more often than the other. PA is not of great diagnostic significance except in its most extreme forms. However, it is PA along with ADO that prevents the use of quantitative PCR for aneuploidy detection at the single cell level (Sherlock *et al.*, 1998).

1.5.3 Contamination

In single cell PCR, contamination is another critical problem. ICSI has been a preferable method of fertilisation in order to reduce the risk of paternal (sperm) DNA contamination. All maternal cumulus cells should be removed prior to insemination to reduce the risk of maternal contamination. The biopsied blastomeres should be washed several times in clean medium to remove any remaining cumulus cells or DNA that may remain in the culture medium before transferring into the microcentrifuge tubes for PCR. PCR set up in a DNA-free environment away from the analysis area can help in reducing the chance of getting 'carry over' PCR products from previous amplifications. All media and reagents should be tested before use. The use of nested PCR by aliquoting the first amplified products for use as the target template in a second amplification reaction using another set of primers situated internally to those used in the first reaction was suggested to prevent carry over contamination (Wells and Sherlock, 1998). The application of a restriction enzyme to digest the extraneous DNA in the PCR mixture before adding the target DNA has also been carried out (Sermon *et al.*, 1997).

Despite all efforts, contamination can still occasionally take place and cause misdiagnosis. Misdiagnosis from maternal DNA has been reported (Sermon *et al.*, 1998b). For a dominant disease, if the mother carries the causative gene, maternal DNA contamination would not lead to misdiagnosis, but would reduce the embryo transfer number. However, in case of a recessive disease, the contamination of maternal heterozygote DNA in a homozygote affected sample would lead to a heterozygote result, and the transfer of affected embryos. In order to reduce the risk of misdiagnosis caused by contamination, the concept of DNA fingerprinting has been introduced to track down the

presence of contamination by co-amplifying a highly polymorphic marker together with the test gene as a multiplex reaction. The genotype in an embryo that deviates from the 4 possible combinations of parental alleles indicates the possibility of contamination.

1.5.4 Nested PCR

The templates with a very low copy number, i.e. a single copy, usually need more PCR cycles than those from genomic DNA, i.e. 50 *vs* 30 cycles, in order to have sufficient DNA fragments for further analysis using traditional gel electrophoresis. However, the very last thermal cycles are less efficient due to the reduced amount of substrates, i.e. primers, dNTPs, polymerase enzyme, etc. For this reason, a technique including a second amplification with a fresh identical PCR mixture was introduced for single cell PCR to increase the amplified fragment to a detectable level (Handyside *et al.*, 1992). Nested PCR (**Figure 1.5**) is an improved technique for single cell amplification which is popularly used in PGD (Wells and Sherlock, 1998). The technique involves two successive amplification reactions. In the first amplification reaction, a pair of locus specific primers is used to produce a DNA fragment that includes the mutation; however, the amplified products from this amplification is insufficient for the analysis. A small aliquot of the PCR products from the first reaction is used as the template for the second amplification. The second reaction tube contains fresh PCR mixture with a different set of primers. The second set of primers is situated within the first amplicon and generates a smaller DNA fragment. The resulting amplified products possess sufficient amplicons for visualisation and further analysis. Moreover, this technique is also useful in increasing the specificity and reducing the risk of contamination.

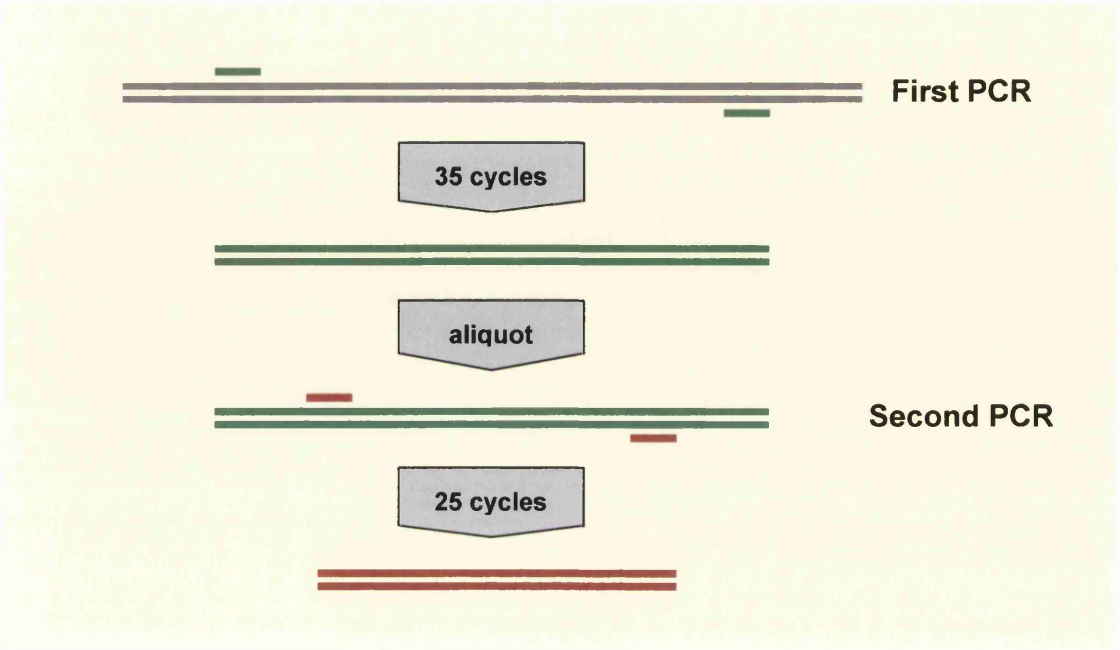


Figure 1.5 Nested PCR. An aliquot of the amplified products from the first reaction is used as to provide templates in a second amplification reaction using another set of primers (red) situated internally to those used in the first reaction (green).

1.5.5 Fluorescent PCR (F-PCR)

The traditional methods of visualising the PCR products following electrophoresis include ethidium bromide or silver staining or the use of radioactive labelled primers. The first two techniques need nested PCR to have sufficient amplified fragments for visualisation. The latter is a more sensitive means than the first two, but time consuming; therefore, it is not suitable for PGD. The introduction of F-PCR (Hattori *et al.*, 1992) has been useful for single cell PCR, increasing the sensitivity and specificity. The application of oligonucleotide primers attached to fluorescent molecules gives rise to amplified products labelled with fluorescent dye. When these F-PCR products migrate under electrophoresis to the position where the laser bisects the gel, the fluorescent molecules are activated by the laser and give a signal with a specific wavelength which can be

detected by a CCD (charged couple device) detector and analysed by computer software. By this strategy, the F-PCR products from a single cell can be detected after only 35 cycles of amplification. This eliminates the need for nested PCR and quickens the diagnosis. This technique allows the size standards to run in the same lane, consequently, the size analysis is as precise as a single base pair difference. In addition to fragment analysis, the application of F-PCR with various mutation analysis methods, i.e. SSCP (Ellison *et al.*, 1993), chemical mismatch cleavage (CMC) (Rowley *et al.*, 1995) and ARMS (Sherlock *et al.*, 1998), have been reported.

1.5.6 Multiplex PCR

One limitation of the molecular analysis from a single cell for PGD is that the amplification can be performed only once. Multiplex PCR (Findlay *et al.*, 1995b; Sherlock *et al.*, 1998) is a technique allowing more than one locus to be amplified by using a combination of unrelated sets of primers in a PCR tube. One concern of this technique is the interaction between unrelated primers or PCR products. Therefore, each combination of primer sets used in multiplex PCR needs to be optimised for the relative primer concentrations, annealing temperatures and reaction buffers. The PCR products of different loci can be distinguished by designing the primers to generate amplified fragments with different sizes. The analysis of multiplex PCR products has become more straightforward with the introduction of F-PCR. By labelling the primers with different dyes, the fragments generated from different sets of primers can be simply differentiated, although they possess the same fragment size. This strategy benefits the analysis of multiple loci from a single cell, i.e. the detection of more than one disease or the combination of mutation detection and a linked or unlinked polymorphic locus. The

additional information of an informative polymorphic marker is useful for a back up linkage analysis result (for a linked marker only) and contamination identification (Wells and Sherlock, 1998).

1.5.7 Mutation analysis

Following DNA amplification from a single cell to a detectable level, selection of an appropriate analysis method is another challenge for single cell PCR. For PGD, difficulties involve a single copy of the starting template, large number of samples to be analysed (including positive and negative control samples) and time limitation. Therefore, the analysis techniques need to be simple, quick, sensitive and accurate. The amplified products with size differences can be analysed using traditional agarose gel, polyacrylamide gel electrophoresis or a modern automated laser fluorescence sequencer for F-PCR. However, base pair substitutions without size alteration, or small deletions or insertions are more difficult to detect. For substitution mutations, major detection systems include those for a particular mutation and those that can identify several mutations at a time.

Scanning methods are helpful for searching for uncharacterised mutations and for diseases which are caused by a variety of mutations, i.e. β-thalassaemia. Popular techniques of this group include heteroduplex analysis (HA), single strand conformational polymorphism (SSCP) and denaturant gradient gel electrophoresis (DGGE). Mutation specific analysis techniques are useful for detecting common mutations and provides definite results. These techniques include restriction fragment length polymorphism (RFLP) and amplification refractory mutation system (ARMS)

1.5.7.1 Restriction fragment length polymorphism (RFLP)

RFLP is a mutation specific analysis technique. The analysis is based on the identification of the difference of the DNA sequence, i.e. mutation, by digestion of the DNA using restriction endonucleases. The bacterial enzymes can recognise a particular DNA sequence and cleave the DNA strand at the recognition site. By knowing the DNA sequence, a specific restriction enzyme can be used to digest either the normal or mutant sites giving a size difference between both alleles (**Figure 1.6**). The application of RFLP for PGD of thalassaemias has been successfully achieved (Kuliev *et al.*, 1999). An artificial restriction site can be created using a primer of modified sequence as site-specific mutagenesis (SSM) method if there is no natural one (Strom *et al.*, 1998).

1.5.7.2 Amplification refractory mutation system (ARMS)

ARMS is another mutation specific analysis method. This technique involves the annealing of allele-specific oligonucleotides and the use of three oligonucleotides: one for the common upstream sequences and two for the normal and mutant sequences (**Figure 1.7**). The allele-specific oligonucleotides in this method function as primers for PCR. Successful amplification of the DNA being tested indicates that the specific normal/mutant primer has annealed, and thus confirms the presence of the specific normal/mutant alleles. Primers for the normal and mutant alleles can be included in the same PCR tube and designed to have size difference or be labelled with different fluorescent dyes in order to differentiate both alleles (Sherlock *et al.*, 1998).

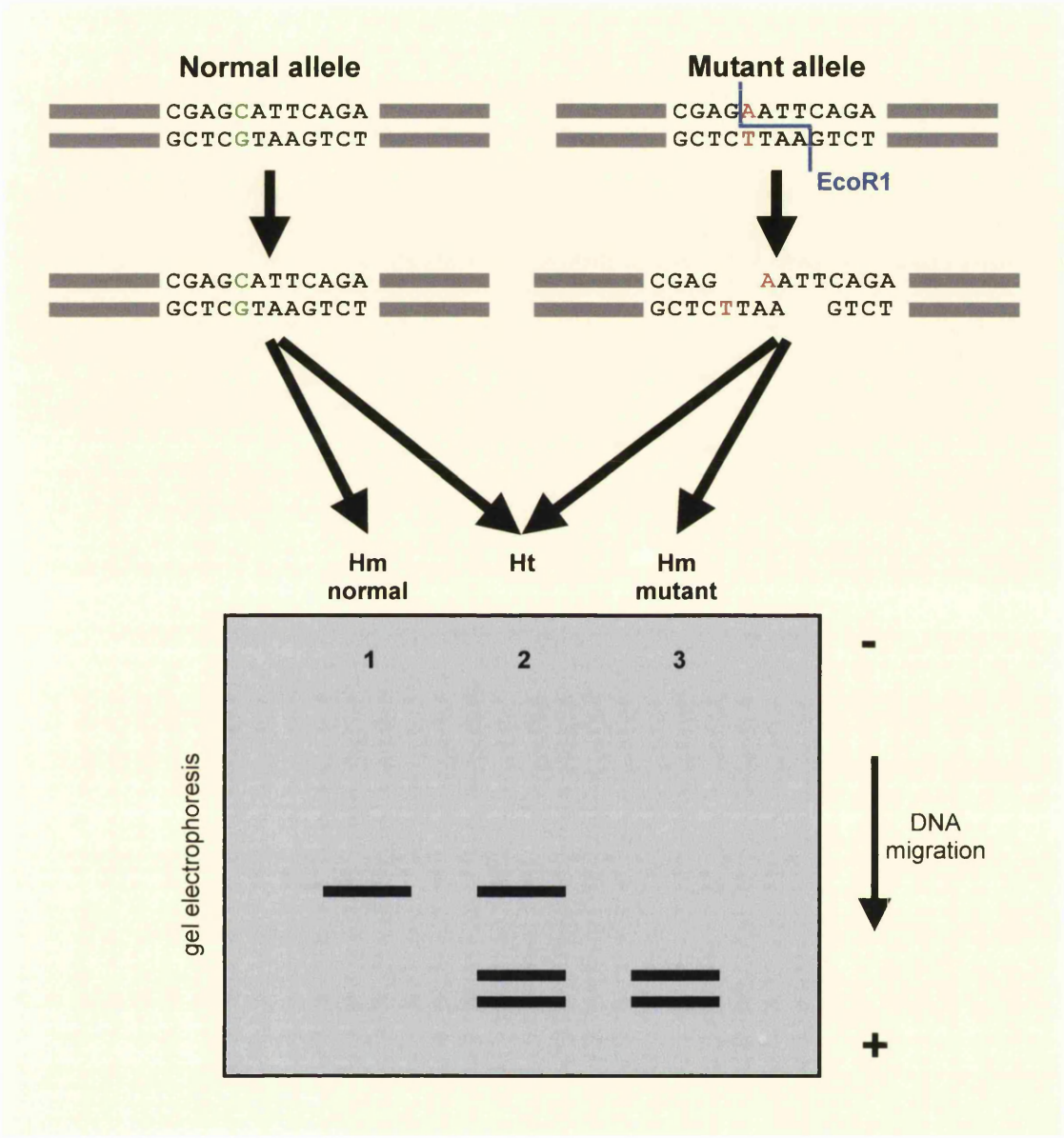


Figure 1.6 Restriction fragment length polymorphism (RFLP) analysis following PCR. The mutant allele with a cleavage site, GAATTC / CTTAAG, can be recognised by the EcoR1 restriction endonuclease, and is digested into 2 smaller fragments during gel electrophoresis (Lane 3). The homozygous (Hm) normal genotype without the cleavage site shows only one large fragment (Lane 1), while the heterozygous (Ht) sample gives rise to one large and two small fragments (Lane 2).

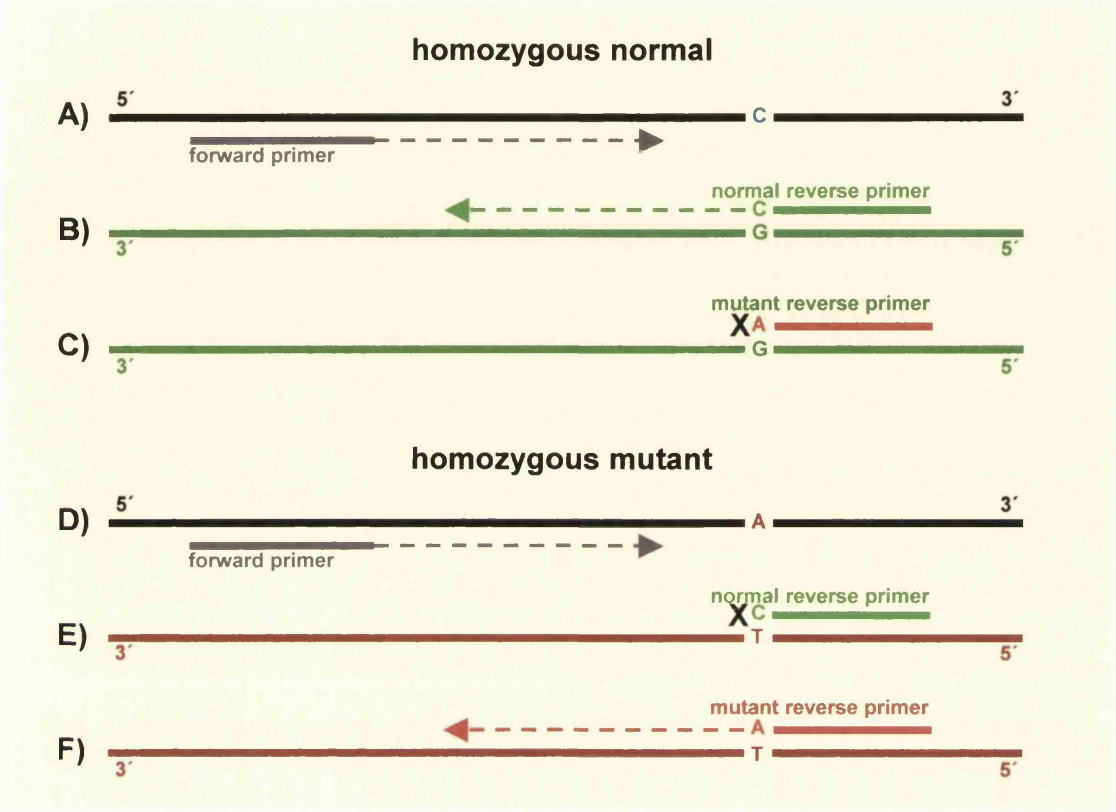


Figure 1.7 Amplification refractory mutation system (ARMS). For a homozygote normal sample, the combination of the 'forward primer' and the 'normal reverse primer' (A and B) gives rise to the amplification of the normal allele. The mismatch of the 3′ **A** terminal of the 'mutant reverse primer' with the **G** nucleotide of the normal allele (C) prevents the amplification by the 'mutant reverse primer'. For a homozygote mutant sample, the combination of the 'forward primer' and the 'mutant reverse primer' (D and F) gives rise to the amplification of the mutant allele. The mismatch of 3′ **C** terminal of the 'normal reverse primer' with the **T** nucleotide of the mutant allele prevents the amplification by the 'mutant reverse primer' (E). The amplification of both 'normal reverse primer' and 'mutant reverse primer' are successful in a heterozygote sample due to the presence of both normal and mutant alleles.

1.5.7.3 Heteroduplex analysis (HA)

When denatured and allowed to reanneal, the complementary strands of the normal and mutant alleles of a heterozygote sample will form hybrid molecules (heteroduplex) with an area of mismatch of the different sequence (**Figure 1.8**). The heteroduplex molecules will give extra bands during the electrophoresis due to their different migration pattern from the homoduplex molecules. Samples with homozygous genotypes do not generate an extra heteroduplex band, unless the PCR product of the different homozygous genotype is added. This technique has been successfully employed for PGD of cystic fibrosis (Handyside *et al.*, 1992).

1.5.7.4 Single strand conformational polymorphism (SSCP)

SSCP analysis is a scanning technique relying on the electrophoretic resolution of the sequence specific conformations of single stranded DNA fragments (Orita *et al.*, 1989). This strategy is useful in detecting small deletions and insertions and single base pair substitutions of the fragment size from 100 to 500bp. A single base pair mutation can give rise to a markedly different single strand conformation, which results in a different migration rate during the electrophoresis, and therefore the different alleles can be identified (**Figure 1.9**). By this method, multiple mutations that lie within the same amplified fragment can be identified using a single protocol. This can benefit the diagnosis of compound heterozygous genotypes. This technique has been carried out for PGD of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (Ioulianos *et al.*, 2000) and familial adenomatous polyposis coli (FAPC) (Ao *et al.*, 1998).

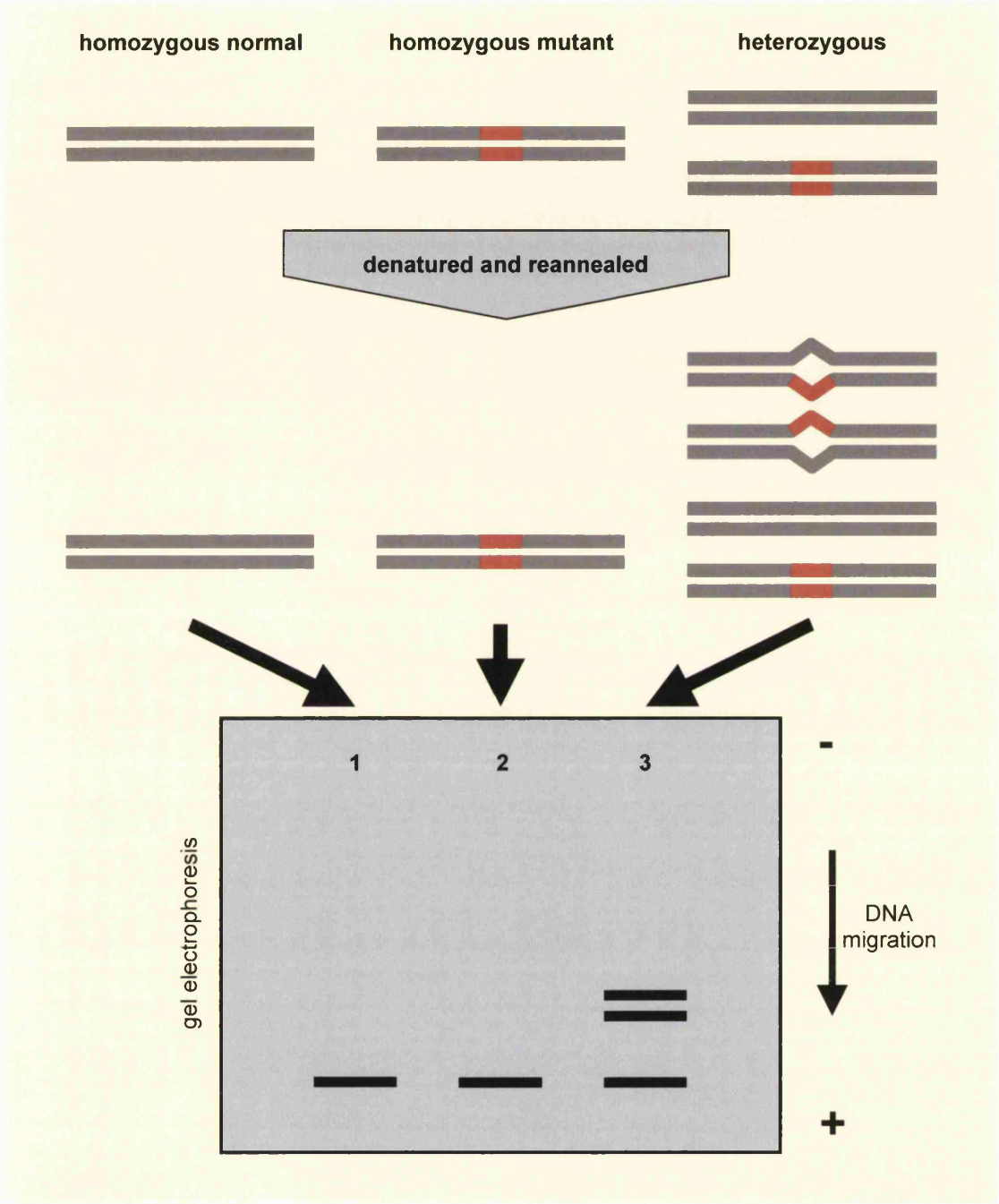


Figure 1.8 Heteroduplex analysis. A sample with a heterozygous genotype gives rise to hybrid molecules (heteroduplex) in addition to the homoduplex molecules. Heteroduplexes can be resolved by polyacrylamide gel electrohporesis, as they migrate slower than homoduplexes (lane 3).

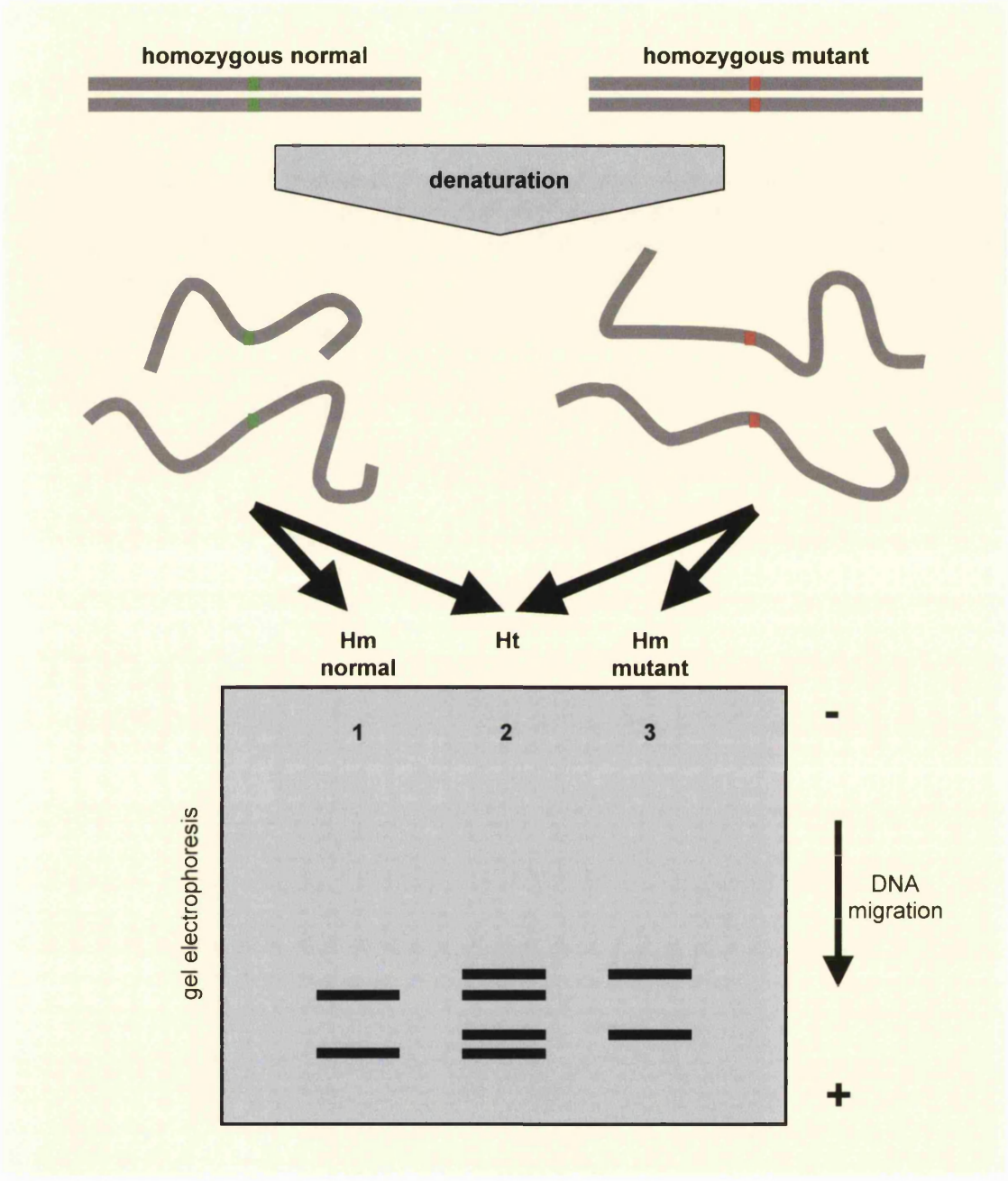


Figure 1.9 SSCP analysis. Single stranded fragments of DNA take on highly sequence dependent three dimensional conformations. A single base pair mutation can give rise to a markedly different conformation. The specific shapes of the sense and antisense strands of the mutant allele (Lane 3) can result in a different migration rate from those of the normal allele (Lane 1) during the electrophoresis on a non-denaturing gel. A heterozygous sample (Lane 2) theoretically possesses a combination of the normal and mutant band patterns.

1.5.7.5 Denaturant gradient gel electrophoresis (DGGE)

The related strategy to SSCP, DGGE is based on the altered melting characteristics due to sequence difference which influence migration rates during the electrophoresis through a polyacrylamide gel with an increasing concentration of denaturant. The test fragments in this method are usually amplified using specially designed primers with a stretch of approximately 40 guanine or cytosine residues (GC-clamp), which may affect the efficiency of single cell PCR. However, this technique has been successfully used in PGD of β-thalassaemia (Kanavakis *et al.*, 1999).

1.6 Objectives

The first objective of this study was to develop an appropriate method for the versatile mutation detection of β-thalassaemia for different mutations and to develop optimal PGD protocols for thalassaemias with different types of mutation, including β-thalassaemia IVSI-110 (single basepair substitution) and codon 41-42 (frameshift) mutations, α-thalassaemia, $--^{SEA}$ (large deletion) mutation, which entail a variety of PCR applications. This includes the development of a widely applicable PGD protocol using fluorescent sequencing and minisequencing. The second objective was to develop an optimal PGD protocol of DM (triplet repeat expansion) and to work up clinical PGD for DM for 2 families. The work up for the clinical diagnosis provided the opportunity to access the patients' samples and spare human embryos in order to test the protocols. Three clinical PGD cycles of DM were carried out, resulting in two pregnancies with three normal babies. The third aim was to study the causes of amplification failure and ADO and develop more efficient single cell PCR methods

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals utilized in this study were all AnalaR grade and mostly provided by **British Drug House (BDH), Poole, Dorset**. These included all laboratory reagents and chemicals used for silver staining and buffer preparation. The following companies supplied other chemicals:

Applied Biosystems, Warrington, England: Performance Optimised Polymer 4 (POP-4™) for ABI Prism™310, microcapillaries 5-47cm×50μm, sample application tubes without lids and septa for ABI Prism™310.

Bio-rad Laboratories, Hempstead, England: TEMED® (*N,N,N',N'*-Tetra-methyl ethylenediamine).

Flowgen, Lionfield, Staffordshire: 2×MDE® acrylamide gel for the ALF Express™.

Gibco BRL, Paisley, Scotland: Dulbecco's phosphate buffered saline (1×PBS) without Ca^{2+} and Mg^{2+}.

National Diagnostics, Hull: Sequagel-6® (6% polyacrylamide solution), Sequagel® complete buffer reagent for the ALF Express™.

Pharmacia Biotech, Herts, England: GeneGel® Excel 12.5/24 kit (12.5% non-denaturing polyacrylamide gels) for the GenePhor™, GeneGel® Clean 15/24 kit (15% dehydrated polyacrylamide gels) for the GenePhor™, PhastGel® Homogeneous 20 (ultra-thin pre-cast 20% non-denaturing polyacrylamide gels), solid Native and DNA Buffer Strips and sample applicators for the PhastSystem™. Ultrapure dNTP set (2′deoxynucleoside 5′-triphosphate, 100mM solutions).

Sigma® Chemical Company, Poole, Dorset: agarose (standard grade), ammonium persulfate (APS), bovine serum albumin (BSA, molecular biology grade), blue dextran sulphate, ethidium bromide, deionised formamide (molecular biology grade), IGEPAL CA-630 [tert-Octylphenoxy poly(oxyethylene) ethanol], lauryl sulfate (SDS) 10% solution (0.2μm filtered, DNase and RNase free), light weight mineral oil for PCR.

Qiagen Ltd, Crawley, England: QIAquick™ PCR Purification Kit.

2.1.2 Enzymes

Proteinase K (PK) (*Arthobacter leteus*) PCR-Grade, solution form was supplied by **Roche Diagnostics Ltd, East Sussex, England**. DNA polymerase was purchased from various sources. SuperTaq® (5u/μl), thermostable *Thermus aquaticus* (*Taq*) DNA polymerase and 10× SuperTaq Buffer were obtained from **HT Biotechnology LTD, Cambridge, England**. AmpliTaq Gold™ (5u/μl) DNA polymerase and 10× GeneAmp® Buffer, BigDye® Terminator Reaction Kit and SNaPshot™ Ready Reaction Mix were purchased from **Applied Biosystems, Warrington, England**. *Pfu* DNA Polymerase was obtained from **Promega Corporation, Southampton, England**, Expand Long Template (ELT) PCR System® and GC-RICH PCR System® from **Roche, East Essex, England** and Extensor Hi-Fidelity PCR Kit® from **ABgene®, Surrey, England**. Shrimp alkaline phosphatase (SAP) and Exonucease I (Exo I) were supplied by **Pharmacia Biotech, Herts, England**.

2.1.3 Nucleic acids

The DNA size standard 1KbPlus DNA Ladder™ (100bp-12kb ladder) was supplied by **GIBCOBRL®, Paisley, England**. Sizers with Cy5® fluorescent dye labelled for the ALF Express™ (50-300bp ladder) were obtained from **Pharmacia Biotech, Herts, England**. Genescan®-500 [TAMRA] size standard (1-500bp ladder) with TAMRA fluorochrome labelled for the ABI Prism™310 was purchased from **Applied Biosystems, Warrington, England**.

Oligonucleotide primers for PCR, nested PCR and those with Cy5® fluorescent dye labelled for fluorescent PCR analysed on the ALF Express™ were supplied by **Pharmacia Biotech, Herts, England**. Oligos with 6'FAM®, TET® and HEX® fluorochrome labelled for florescent PCR analysed on the ABI Prism™310 were obtained from **Applied Biosystems, Warrington, England**. All oligos were diluted to a working concentration at 10pmol/μl before used.

2.1.4 DNA samples

DNA samples and buccal cells samples of control normal genotypes were supplied by the staff in the Preimplantation Genetic Diagnosis group, Department of Obstetrics & Gynaecology, University College London. DNA samples of the FAP82 family with three generations of the members were provided by the Polyposis Registry, St. Marks Hospital, London. DNA samples of various α- and β-thalassaemia mutations were provided by the Perinatal Centre, University College London. Buccal cell samples of β-thalassaemia, IVSI-110 mutation, were obtained from the same unit. Buccal cell

samples of α-thalassaemia, $--^{SEA}$ mutation and β-thalassaemia, codon 41-42 mutation were collected from patients attending the Antenatal Clinic, Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiangmai University, Thailand and sent by air mail.

Blood samples and buccal cell samples of the couples coming for preimplantation genetic diagnosis of DM were taken after the consultation. Buccal cells of the affected members of the families were sent by post. Human embryos were obtained from the Assisted Conception Unit (ACU), University College London Hospital (UCLH). These were surplus to the IVF treatment and were unsuitable for transfer or freezing, and therefore were donated for research after informed written consent from the patients. The use of these spare human embryos was approved by UCL ethics committee and the Human Fertilisation and Embryology Authority (HFEA).

2.1.5 Solutions and buffers

All solutions and buffers were prepared as described in **Section 2A** using distilled deionised water. All were sterilized by autoclaving at 15llbs psi 121°C for 30 min. Storage was at room temperature unless otherwise stated.

2.1.6 Single cell manipulation

Nunc™ Nuncleon 50×9mm petri dishes were used for single cell isolation and purchased from **Gibco BRL, Paisley, Scotland**. Glass microcapillaries, 50, 75 and 100μl in size, used for handling the single cells were manufactured by **Drummond, USA** and

supplied by **Laser, Southampton**. Sterile cotton swabs and collection tubes were obtained from **Greiner Labortechnik LTD, Stonehouse**.

2.1.7 Apparatus

Four models of thermal cyclers were used for PCR amplification. Hybaid® Omnigene and Hybaid® Touchdown were manufactured by **Hybaid®, Middlesex**. GeneAmp2400 PCR System was made and serviced by **Applied Biosystems, Warrington, England** and Mastercycler Gradient® was from **Eppendorf-Netheler-Hinz GmbH, Cambridge**. Minigel mould (8mmx100mm) with a 20-well gel-slot-former was purchased from **Cambridge Electrophoresis, Cambridge**. GenePhor™ Electrophoresis Unit with EPS 600 Power Supply and PhastSystem™ with automated development chamber were employed for single stranded conformation polymorphism (SSCP) analysis and silver staining. Both machines were obtained from **Pharmacia Biotech, Herts, England**. Fluorescent PCR and fluorescent SSCP with Cy5® fluorescent dye was analysed on the Pharmacia Automated Laser Fluorescence Express (ALF Express™) DNA sequencer with DNA Fragment Analyser© software which was manufactured and serviced by **Pharmacia Biotech, Herts, England**. The external gel temperature controlling system, Multi-Temp® for the ALF Express™, was obtained from the same company. The analysis of fluorescent PCR with 6'FAM, TET and HEX fluorescent dyes was carried out on the ABI Prism™310 using GeneScan™ analysis software and automated laser fluorescence sequencing was performed on the ABI Prism™377 using Sequencing Analysis software. Both ABI Prism™310 and ABI Prism™377 were made and serviced by **Applied Biosystems, Warrington, England**. The dissecting microscope was the product of **Nikon™, Tokyo, Japan**.

2.2 Methods

2.2.1 DNA extraction from blood samples

The method described by Lahiri and Nurnberger (1991) was employed for DNA extraction from blood. Blood samples of 4.5ml were collected in tubes containing 400mM EDTA. Whole blood was transferred into centrifuge tubes, and 5 ml of low salt buffer, TKM1 (**Section 2A.1**) and 125µl of IGEPAL CA-630 (Sigma®) were added to lyse the red blood cells. The mixture was mixed well by inversion and shaking and centrifuged at 1,000g for 10 min. The supernatant was slowly decanted off and the small pellet was washed in 5ml of TKM1 and 125µl of IGEPAL CA-630 as previously. The washing and spinning were repeated until the redness of the pellet was diminished. The pellet was re-suspended in 100µl of TKM1 to which 800µl of TKM2 and 50µl of 10% (w/v) sodium dodecyl sulphate (SDS) were added to lyse the white blood cells. The solution was mixed thoroughly by pipetting up and down and the tube incubated at 55 °C for at least 20 min or until the lumps disappeared. 300µl of 6M NaCl were added to make certain that all cells were lysed, and the tube centrifuged at 10,000g for 5 min. The supernatant was transferred to a new centrifuge tube and the precipitated protein pellets were discarded. Two volumes of 100% ice-cold ethanol were added to the supernatant, and the tube inverted until the DNA strands precipitated. The tube was spun at 10,000g for 5 min, and the supernatant poured off. The saved precipitated DNA was re-suspended in 1ml of ice-cold 70% ethanol and spun at 10,000g for 5 min. The supernatant was discarded and DNA pellets were left to dry for approximately 5 min in a freeze-dryer. The DNA pellets were dissolved in 300µl of 1×TE (**Section 2A.1**) and stored at -20°C until further use.

2.2.2 Cell collection

2.2.2.1 Buccal cells

Buccal cell samples of the subjects of known genotype were collected by rinsing out the mouth with tap water in order to remove dead cells and food debris, and then swabbing the inside of the mouth with a cotton swab. The swab was rinsed in 5ml of sterile phosphate buffered saline (PBS) solution to create a cell suspension.

2.2.2.2 Human blastomeres and embryos

The patients underwent routine superovulation and oocytes were fertilised using intracytoplasmic sperm injection (ICSI). ICSI was used as a precaution to reduce the risk of sperm DNA contamination in subsequent PCR amplifications. Day-3 embryos were either biopsied by an embryologist or disaggregated to achieve single blastomeres. For disaggregation, each embryo was briefly transferred to a droplet of acid Tyrodes solution to dissolve the zona pellucida (ZP). Embryos were washed in a droplet of PBS containing 4% bovine serum albumin (BSA) and pipetted up and down until all blastomeres were disaggregated. For embryo biopsy, on day-3 post fertilisation (4-9 cell stage) zona drilling with acid Tyrodes solution was performed, removing 2 blastomeres from embryos consisting of 6 cells or more and 1 blastomere from embryos with 4-5 cells. Cleavage stage embryos were graded 1, 1^{-}, 2^{+}, 2, 2^{-} and 3 where grade 1 had the best morphology and grade 3 was a highly fragmented, poor quality embryo (Staessen *et al.*, 1992).

2.2.3 Single cell isolation

2.2.3.1 Single buccal cells

50μl of the concentrated cell suspension was transferred to a 5-cm petridish in a laminar flow cabinet. Approximately ten 30-μl drops of sterile PBS containing 4% BSA were spotted onto the petridish. About 0.5μl of cell suspension was transferred to an adjacent drop of PBS using a pulled glass micropipette, while visualising under a dissecting microscope. The transfer of cells was repeated 2-3 times to dilute the cell concentration until the isolation of a single cell was possible. Single cells were transferred in and out of at least four fresh PBS drops to wash away any contaminants and were transferred into individual thin-wall microcentrifuge tubes containing lysis buffer. A clump of 30-50 cells was taken as a positive control for each PCR amplification. 2μl of the last wash drop was taken as a blank for each single cell.

2.2.3.2 Single human blastomeres

Each single blastomere from biopsy or disaggregation was washed in fresh PBS drops four times or more and transferred into an individual thin-wall microcentrifuge tube containing lysis buffer. 2μl of the last wash drop was taken as a blank for each single blastomere.

2.2.4 DNA extraction from single cells and cell clumps

The lysis buffer used consisted of 2µl of 125µg/ml proteinase K (PK) and 1µl of 17µM sodium dodecyl sulphate (SDS) (El-Hashemite and Delhanty, 1997). These were pipetted into each of the microcentrifuge tubes before adding the single cells or cell clumps. The mixture was covered with a drop of light weight mineral oil to prevent contamination and evaporation before closing the lid. The lysis buffers were activated at 37°C for 1 h, and inactivated by incubating at 99°C for 15 min. After lysis the DNA from the single cells or cell clumps was ready for PCR or storage at -80°C.

2.2.5 Polymerase chain reaction (PCR)

2.2.5.1 Oligonucleotides

Full details of the oligonucleotides used as primers for PCR are provided in **Table 3.2, 4.1** and **5.1**. The approximate annealing (melting) temperatures for the oligonucleotide primers were estimated by the following the rule-of-thumb calculation:

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

Where A, T, G and C are the nucleotides adenine, thymine, guanine and cytosine. The working optimal annealing temperatures were determined empirically by experiments using the Mastercycler Gradient® thermal cycler. A temperature gradient of ±5°C from the calculated temperature across the block was applied. The temperatures

giving the most intense amplified products were chosen as the working annealing temperatures.

2.2.5.2 Standard PCR

The PCR mixture consisted of 0.2μM of each primer, 200μM deoxynucleoside triphosphates (dNTPs: dATP, dGTP, dCTP, dTTP) and 1 unit of SuperTaq® DNA polymerase with 1× SuperTaq Buffer and was made up to a total volume of 25μl with sterile distilled deionised water. The reaction mix was added to the genomic DNA or extracted DNA from single cells; one extra tube was taken as a PCR-mix-only negative control for each reaction. 25μl of lightweight mineral oil was added to prevent contamination and evaporation. This was prepared on ice in a laminar flow cabinet using dedicated pipettes and sterile pipette tips. The amplifications were performed on a thermal cycler with the conditions: denaturation 94°C 30 sec, annealing at 60°C 30 sec and extension at 72°C 45 sec for 35 cycles when genomic DNA was used as the template and the PCR product was analysed by gel electrophoresis. This was followed by a final extension at 72°C for 10 min to complete the extension step of the annealed primers and preceded with the primary denaturation step at 94°C for 4 min. For single cell PCR, 1.5 units of the polymerase enzyme were used in the reaction mixture. When AmpliTaq Gold™ polymerase with 1× GeneAmp® Buffer were employed instead of SuperTaq®, the primary denaturation step was set to 12 min to activate the enzyme. Other thermal programs (denaturation/annealing/extension) of 15 sec/15 sec/25 sec and 45 sec/45 sec/1 min were also carried out.

2.2.5.3 Multiplex PCR

The reaction mixture and conditions were similar to those of standard PCR (**Section 2.2.5.2**), but more than one set of primers were added in the PCR mixture. The additional primers were for the detection of a polymorphic linked or unlinked marker. The concentration of the primers was preliminary used at 0.2μM and the amplification efficiency was evaluated. If one of the primers gave poor results, the concentration of the primers and annealing temperatures were modified until the optimal PCR condition was achieved.

2.2.5.4 Nested PCR

In order to increase the amount of the test gene fragments from the low copy number of the templates, i.e. single cells or cell clumps, to be enough for further non-radioactive analysis, using agarose gel electrophoresis or silver stained SSCP, nested PCR was applied. The extracted DNAs from single cells or cell clumps were amplified as the standard PCR protocol (**Section 2.2.5.2**) using the outer set of primers (**Section 2.2.5.1**). Two μl of the outer amplification products were added to the inner nested amplification reaction, which comprised the same mixture as the outer amplification, except using 0.2μM of the inner set of primers (**Section 2A.3**) and amplifying for 25 cycles. This resulted in the production of a smaller fragment than that produced by the outer set of primers.

2.2.5.5 Fluorescent PCR

The technique of labelling the primers used in the amplification reaction on the 5′ end with the fluorescent dyes allowed detection of PCR products on an automated laser fluorescence sequencer. Either forward, reverse or both primers were tagged with Cy5® fluorochrome for the analysis on the ALF Express™ (**Section 2.2.7.2.1**) and 6'FAM®, TET® or HEX® fluorochromes for the ABI Prism™310 (**Section 2.2.7.2.2**). The PCR mixture preparation was the same as the standard PCR protocol (**Section 2.2.5.2**). Thermal cycler program was performed at 25 cycles when the template was from genomic DNA, 35 cycles when the template was from single cells and the amplified product was subjected to further nested PCR and 40 cycles when the template was from single cells and only fluorescent PCR analysis was carried out.

2.2.5.6 Fluorescent gap PCR

The reaction mixture and conditions were similar to fluorescent PCR analysis (**Section 2.2.5.5**), except 0.2μM of forward 'S1', reverse normal 'S2' and reverse mutant 'S3' primers (Ko *et al.*, 1992) were employed. The S1 and the S3 primers flank the α-thalassaemia, $--^{SEA}$ deletion, while the S2 primer anneals within the deleted area. In normal samples the S1 and S2 primers produce a product of 287 bp. S1 and S3 primers do not produce a product, as the large distance separating them (approximately 20kb) is refractory to PCR. However, in a mutant sample these primers are brought into close proximity by deletion of the intervening sequence and are thus able to create a product of 194bp. The annealing site of the S2 primer is deleted from mutant alleles, thereby

preventing amplification from this site. S2 and S3 primers were labelled with TET® and HEX® fluorescent dyes, respectively, for F-PCR analysis on an ABI Prism™310.

2.2.6 Precautions against contamination

PCR protocols at the single cell level were similar to standard PCR (**Section 2.2.5.2**); however, because the risk of contamination was acute the strategy for contamination prevention was crucial. All procedures, prior to amplification, including single cell isolation and PCR mixture preparation, were performed in a devoted room with restricted access and continuous positive pressure. While working in this room, clean gowns, gloves and overshoes were worn at all times. The PCR mixture was prepared in a laminar flow cabinet using separate pipettes and sterile filter pipette tips. A dedicated freezer for storing solutions, enzymes and primers and microcentrifuger were kept in this room. All working solutions, enzymes and primers were aliquoted into several tubes after diluting from the stock tubes so that each tube could be discarded after a few uses, reducing the chance of contamination and the risk that reagent efficiency would be impaired by freeze and thawing several times. Testing for contamination and efficiency was carried out before use.

2.2.7 Analysis of PCR products

2.2.7.1 Agarose gel electrophoresis

Electrophoresis on 2% (w/v) agarose gels was conducted to check the efficiency and specificity of the amplification reaction. 2% agarose gels were prepared by heating 1g

of agarose in 50ml of 1×TBE until all the agarose was dissolved. Five μl of 500μg/ml ethidium bromide was added into the melted agarose, which was poured into a minigel mould (8mmx100mm) with a 20-well gel-slot-former and left to set at room temperature. Five μl of each PCR product mixed with 2μl of loading buffer was loaded into well-slots. One Kb Plus DNA Ladder™ was loaded alongside the PCR products to allow evaluation of the DNA fragment size. Electrophoresis was performed at 100 volts for 30 min and the gels were examined under ultra-violet trans-illumination to visualise the PCR products. 500mg of agarose was used, instead of 1g, to make 1% agarose gel for the analysis of large amplified fragments.

2.2.7.2 Fluorescent PCR analysis

The analysis of PCR products using primers labelled with fluorescent dyes was carried out on two different automated laser fluorescence sequencers. The ALF Express™ used a gel-based electrophoresis technology with a polyacrylamide gel, while the ABI Prism™310 employed a capillary electrophoresis system with a special polymer (Performance Optimised Polymer 4, POP-4™). For both machines, the amplified products were loaded on to the mediator and electrophoresed through it. During electrophoresis, a laser stream passed through the mediator. When the fluorescent PCR fragments pass through the laser, the fluorochromes attached to the fragments were excited and emitted photons of specific wavelength. The signal was picked up by the CCD (charged couple device) detector and interpreted by the computer software.

2.2.7.2.1 Fluorescent PCR analysis using ALF Express™

In order to obtain the optimal analysis, the glass plates of the ALF Express™ were carefully cleaned using distilled deionised water and 100% ethanol. Detergents were disallowed due to their fluorescence component. Bind-silane (**Section 2A.4**) was applied to the top of the plate. 25ml of the 6% Sequagel-6® polyacrylamide gel (**Section 2A.4**) was poured between the 0.35mm glass plates and left to set at room temperature for 1.5 h. A mixture of 1µl fluorescent PCR products Cy5® labelled, 1µl each of 100bp and 300bp size standard and 3µl of loading buffer was prepared and denatured at 95°C for 5 min before loading into the gel lanes. The electrophoresis was run in 1× TBE for 120 min with the conditions: 1,500V, 38W, 25mA, 45°C. The data was analysed by DNA Fragment Analyser© software.

2.2.7.2.2 Fluorescent PCR analysis using ABI Prism™310

A mixture of 1µl fluorescent PCR products, 12µl of deionised formamide and 0.5µl of Genescan®-500 [TAMRA] size standard was prepared in a 0.5ml sample tube without a lid. The tube was capped with rubber septa, denatured at 95°C for 5 min and loaded on to a 48-tube sample rack in the ABI Prism™310 sequencer. The denatured sample was subjected to capillary electrophoresis using Performance Optimised Polymer 4 (POP-4™; 5 sec injection time, 15,000V, 60 °C, 24 min). The data was analysed by GeneScan™ analysis software.

2.2.7.3 Single stranded conformation polymorphism (SSCP) analysis

2.2.7.3.1 Silver stained SSCP using PhastSystem™

Two μl of formamide was added into 1.5μl of each amplified product, which were denatured at 95°C for 10 min. The denatured samples were kept on ice until they were loaded on to a 20% polyacrylamide gel (PhastGel™ Homogeneous 20) using the 12-tooth sample applicator, which allowed the transfer of approximately 0.3 μl of each of the 12 samples to the gel surface at the same time. The gel was pre-run at 400V, 20mA, 2W, 20°C for 9Vh prior to loading the samples. Automated gel electrophoresis was performed on the PhastSystem™ using Native Buffer Strips at 400V, 20mA, 2W for 350Vh. Different conditions, by changing the temperature (5°C, 10°C and 15°C), duration of pre-run (49Vh) and total running time of separation (200Vh, 250Vh, 300Vh and 400Vh), to find the optimal condition for the detection of the amplified products from various mutations were also carried out. DNA bands were located by silver staining, which was performed in the automated development chamber of the PhastSystem™. The staining process includes 16 steps using nine distinct solutions, which were newly prepared on the day of the procedure (**Section 2A.4**).

2.2.7.3.2 Silver stained SSCP using GenePhor™

The denatured amplified products, treated as those for SSCP using the PhastSystem™ (**Section 2.2.7.3.1**), were transferred on to the pre-formed wells on a pre-cast 112×110×0.5mm GeneGel® Excel 12.5% non-denaturing polyacrylamide gel or a GeneGel® Clean 15% dehydrated polyacrylamide gel. The first-mentioned was the

ready-to-use type, but the later needed to be re-hydrated using gel rehydration buffer provided with the gel on a rocking platform for 1 h at room temperature. The gel electrophoresis was performed on the GenePhor™ Electrophoresis Unit with EPS 600 Power at 600V, 15W, 25mA, 5°C for 1-1.5 h. Different conditions by changing the temperature, and total running time of separation to find the optimal condition for the detection of the amplified product of each mutation were also carried out. DNA bands were visualised by silver staining, which was performed manually on a rocking platform including 10 steps using 5 different solutions (**Section 2A.5**) which were freshly prepared on the day of the procedure (Harvey *et al.*, 1995).

2.2.7.3.3 Fluorescent SSCP using ALF Express™

A mixture of 1µl of the fluorescent PCR products and 5µl of loading buffer was prepared and denatured at 95°C for 10 min. The denatured samples were kept on ice until they were loaded into the gel lanes. The gel electrophoresis was run in 1× TBE at 1,500V, 25W, 60mA, 15°C for 120 min using 0.35 mm 0.5× nondenaturing polyacrylamide gel (**Section 2A.3**) on the ALF Express™. Several temperature conditions, including 4°C, 10°C and 20°C, were compared using external gel temperature controlling system, Multi-Temp®. Additionally, a number of different gel compositions were tested by changing the concentration of MDE® acrylamide gel (0.5× or 0.3×) and/or adding 10% glycerol in order to obtain the best conditions for versatile mutation analysis. The amplified products from labelled forward primer only and those from both labelled forward and labelled reverse primers were also studied. The data was analysed by DNA Fragment Analyser© software.

2.2.7.4 Fluorescent DNA sequencing

The PCR products were purified by the spin column technique using QIAquick™ PCR Purification Kit as per the manufacturer's instructions. Five volumes of Buffer PB were mixed with 1 volume of the amplified product in a spin column. The flow-through was discarded after centrifugation at 10,000g for 1 min. 750µl of Buffer PE was added to the spin column and this was spun at 10,000g for 1 min. The processed PCR products were collected in a fresh tube by adding 50µl of Elution Buffer EB and spinning at 10,000g for 1 min. The resulting DNA concentration was evaluated by comparing with the 1Kb Plus DNA Ladder using agarose gel electrophoresis. BigDye® Terminator sequencing mixture comprised 8µl of Terminator Ready Reaction Mix (BigDye® Terminator Reaction Kit), 3-10ng of DNA template, 3.2pmol of primer and was made up to a total volume of 20µl with sterile distilled deionised water. The mixture was covered with 1 drop of light weight mineral oil and kept on ice until the labelling reaction was ready. The reaction was performed with the following conditions: denaturation at 96°C 30 sec, annealing at 50°C 15 sec and extension at 60°C 4 min for 25 cycles. A temperature ramp of 1°C per sec was programmed in each step.

The sequencing products were transferred into 0.5ml centrifuge tubes containing 2µl of 3M sodium acetate, pH 4.6 and 50µl of 95% ethanol, mixed well and left at room temperature for 15 min to precipitate. The tube was spun at 10,000g for 30 min, the supernatant was carefully aspirated and discarded. The pellets were washed in 250 µl of 70% ethanol, mixed well and centrifuged at 10,000g for 5 min. After the supernatant was aspirated, the pellet was washed in 250 µl of 70% ethanol, dried and resuspended in 5µl of deionised formamide and 1µl of 25mM EDTA (pH8.0) with 25M blue dextran. One µl

of the sequencing product was loaded onto a polyacrylamide sequencing gel electrophoresis by the automated fluorescence sequencer ABI Prism™377 by Dr Henry Houlden, the Institute of Neurology, University College London. The data was analysed by Sequencing Analysis software.

2.2.7.5 Fluorescent DNA minisequencing (SNaPshot™)

The amplified products from single buccal cells were treated with shrimp alkaline phosphatase (SAP) and Exonucease I (Exo I) to remove primers and unincorporated dNTPs prior to SNaPshot™ primer extension. Four μl of the PCR products were added into 0.5ml centrifuge tubes containing 2μl of SAP (1 unit/μl), 0.2μl of Exo I (10 units/μl) and 6μl of sterile distilled deionised water. The mixture was covered with 1 drop of lightweight mineral oil. The mixture was incubated at 37°C for 1 h to activate the enzymes and 72°C for 15 min to inactivate the enzymes. SNaPshot™ reaction mixture was set up on ice and comprised 5μl of SNaPshot™ Ready Reaction Premix, 1μl of the minisequencing primer (0.15pmol/μl) (**Table 3.3**), 1μl of the purified template (0.15pmol/μl) and was made up to a total volume of 10μl with sterile distilled deionised water. The mixture was covered with 1 drop of lightweight mineral oil. The thermal cycling was performed with the conditions 96°C 30 sec, 50°C 15 sec and 60°C 1 min 30 sec for 25 cycles. The minisequencing products were kept on ice until the post-extension treatment step. The unincorporated [F]ddNTPs in the minisequencing products were removed by incubating with 1 unit of SAP at 37°C for 1 h and the enzyme was deactivated at 72°C for 15 min. The purified minisequencing products were stored on ice until the analysis on the ABI Prism™310. A mixture of 1μl purified minisequencing product and 12μl of deionised formamide was prepared and denatured at 95°C for 5 min.

The denatured sample was subjected to capillary electrophoresis using Performance Optimised Polymer 4 (POP-4™; Module E, 10 sec injection time, 15,000V, 60 °C, 24 min). The data was analysed by GeneScan™ analysis software.

2A. Appendix to Materials and Methods

2A.1 Solutions for DNA extraction

TKM1 (low concentration salt buffer): 10mM Tris-HCl, pH 8.0, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA

TKM2 (high concentration salt buffer): 10mM Tris-HCl, pH 8.0, 10mM KCl, 2mM EDTA, 10mM $MgCl_2$, 0.4M NaCl

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

2A.2 DNA polymerase buffers for PCR

All DNA polymerase buffers were provided with the particular enzymes by the manufacturers. The components of each buffer were described below:

10× SuperTaq Buffer: 100mM Tris-HCl, pH 9.0, 15mM $MgCl_2$, 500mM KCl, 1% Triton X-100, 0.1% (w/v) stabilizer

10× GeneAmp® Buffer: 100mM Tris-HCl, pH 8.3, 15mM $MgCl_2$, 500mM KCl, 0.01% (w/v) gelatin

***Pfu* DNA Polymerase 10× Reaction Buffer with $MgSO_4$:** 200mM Tris-HCl, pH 8.8, 20mM $MgSO_4$, 100mM KCl, 100mM $(NH_4)_2SO_4$, 1.0% Triton® X-100, 1mg/ml nuclease-free BSA

10× PCR Buffer for Expand Long Template (ELT) PCR System®: 20mM Tris-HCl, pH 7.5, 22.5mM $MgCl_2$, 100mM KCl, 1mM dithiothreitol (DTT), 0.1mM EDTA, 0.5% (v/v) Tween20, 0.5% (v/v) Nonidet P40, 50% (v/v) glycerol, detergents

5× GC-RICH PCR Reaction Buffer: 20mM Tris-HCl, pH 8.0, 15mM $MgCl_2$, 100mM KCl, 1mM DTT, 0.1mM EDTA, 0.5% (v/v) Tween20, 0.5% (v/v) Nonidet P40, 50% (v/v) glycerol, 5M GC-RICH resolution solution.

2A.3 Buffers and gels for electrophoresis

10× TBE Buffer: 0.9mM Tris-HCl, pH 8.0, 0.9mM Boric acid, 10mM EDTA

Loading buffer for agarose gel electrophoresis: 40% (w/v) sucrose, 4mM bromophenol blue, 4mM xylene cyanol

Loading dye for polyacrylamide gel electrophoresis on ALF Express™: 100% (v/v) deionised formamide, 5mg/ml Dextran Blue 2000

Bind silane: 80% (v/v) ethanol, 2% (v/v) glacial acetic acid, 0.3% (v/v) bind silane stock

6% polyacrylamide gels for F-PCR on ALF Express™: 20ml Sequagel-6®, 5ml Sequagel® complete buffer reagent, 200µl 10% (w/v) APS

0.5× MDE® acrylamide gel for F-SSCP on ALF Express™: 0.5× MDE® acrylamide gel, 1× TBE, 200µl 10% (w/v) APS, 10µl TEMED®

0.3× MDE® acrylamide gel for F-SSCP on ALF Express™: 0.3× MDE® acrylamide gel, 1× TBE, 200µl 10% (w/v) APS, 10µl TEMED®

2A.4 Silver staining of PhastGel™

Nine different solutions used for 16 steps of the automated silver staining process of the PhastGel™ are described in **Table 2A.1**.

2A.5 Silver staining of GeneGel®

Table 2A.2 shows the manual silver staining procedure of the GeneGel®.

Table 2A.1 Automated silver staining process of the PhastGel™ in the developing chamber of the PhastSystem™ included 16 steps using nine different solutions which were freshly prepared on the day of the procedure.

Step	Solution	Temperature (°C)	Time (min)	Purpose
1	distilled deionised water	20	0.5	Wash
2	50% (v/v) ethanol, 10% (v/v) acetic acid	50	2	Fixative
3	10% (v/v) ethanol, 5% (v/v) acetic acid	50	2	Fixative
4	10% (v/v) ethanol, 5% (v/v) acetic acid	50	4	Fixative
5	10% (v/v) gluteraldehyde	50	6	Sensitisation
6	10% (v/v) ethanol, 5% (v/v) acetic acid	50	3	Fixative
7	10% (v/v) ethanol, 5% (v/v) acetic acid	50	5	Fixative
8	distilled deionised water	50	2	Wash
9	distilled deionised water	50	2	Wash
10	0.4% (w/v) silver nitrate	40	10	Staining
11	distilled deionised water	30	0.5	Wash
12	distilled deionised water	30	0.5	Wash
13	2.5% (w/v) sodium carbonate, 0.03% (v/v) formaldehyde	30	1	Developer
14	2.5% (w/v) sodium carbonate, 0.03% (v/v) formaldehyde	30	10	Developer
15	3.7% (w/v) Tris-HCl, 2.5% (w/v) sodium thiosulphate	30	2	Background reduction
16	10% (v/v) glycerol	50	5	Preservative

Table 2A.2 Manual silver staining process of the GeneGel™ on a rocking platform involved 10 steps using five different solutions at room temperature (Harvey *et al.*, 1995).

Step	Solution	Time (min)	Purpose
1	10% (v/v) ethanol, 0.5% (v/v) acetic acid	2	Fixative
2	10% (v/v) ethanol, 0.5% (v/v) acetic acid	2	Fixative
3	0.1% (w/v) silver nitrate	7.5	Staining
4	0.1% (w/v) silver nitrate	7.5	Staining
5	distilled deionised water	0.2	Wash
6	1.5% (w/v) sodium hydroxide, 0.01% (w/v) sodium borohydride 0.4% (v/v) formaldehyde	0.2	Developer
7	1.5% (w/v) sodium hydroxide, 0.01% (w/v) sodium borohydride 0.4% (v/v) formaldehyde	20	Developer
8	distilled deionised water	0.2	Wash
9	distilled deionised water	0.2	Wash
10	10% (v/v) glycerol	30	Preservative

Chapter 3

Thalassaemias

3.1 Introduction

The thalassaemias are the world's commonest single group of hereditary disorders. They were first characterised by Thomas B. Cooley in 1925 in infants with marked anaemia and splenomegaly. Early nomenclatures of this disorder include von Jaksch's anaemia, splenic anaemia, erythroblastosis, Mediterranean anaemia and Cooley's anaemia. In 1936, Whipple and Bradford described the pathological mechanism of these conditions and 'thalassaemia' was named after the Greek word 'θαλασσα' which means 'the sea' as most patients in his series came from the Mediterranean region. However, later reports revealed that these disorders are also prevalent in other parts of the world, including the Middle East, Indian subcontinent, Far East and Southeast Asia. Clinical characteristics of the thalassaemias are extremely heterogeneous according to the level of defective globin chain production. The pathogenesis is caused by the reduced synthesis of the globin chains of haemoglobin, i.e. α, β, δβ or γδβ, which leads to an imbalance of globin chain production and the accumulation of free globin chains. The insoluble excess globin chains precipitate and give rise to haemolysis anaemia which results in compensatory bone marrow hyperplasia (Weatherall, 1998).

Human haemoglobin (Hb), which is responsible for oxygen transport, is a protein with a tetrameric structure composing of two α-like (α or ζ) and two β-like (ε, γ, δ or β) globin chains (Weatherall, 2001). The expression of the globin gene complex is varied during human life. Hb Gower2 ($\alpha_2\varepsilon_2$), Hb Gower1 ($\zeta_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$) are active during the embryonic period, while the major fetal haemoglobin is Hb F ($\alpha_2\gamma_2$). The principal adult haemoglobins are Hb A ($\alpha_2\beta_2$) and Hb A_2 ($\alpha_2\delta_2$) (**Figure 3.1**).

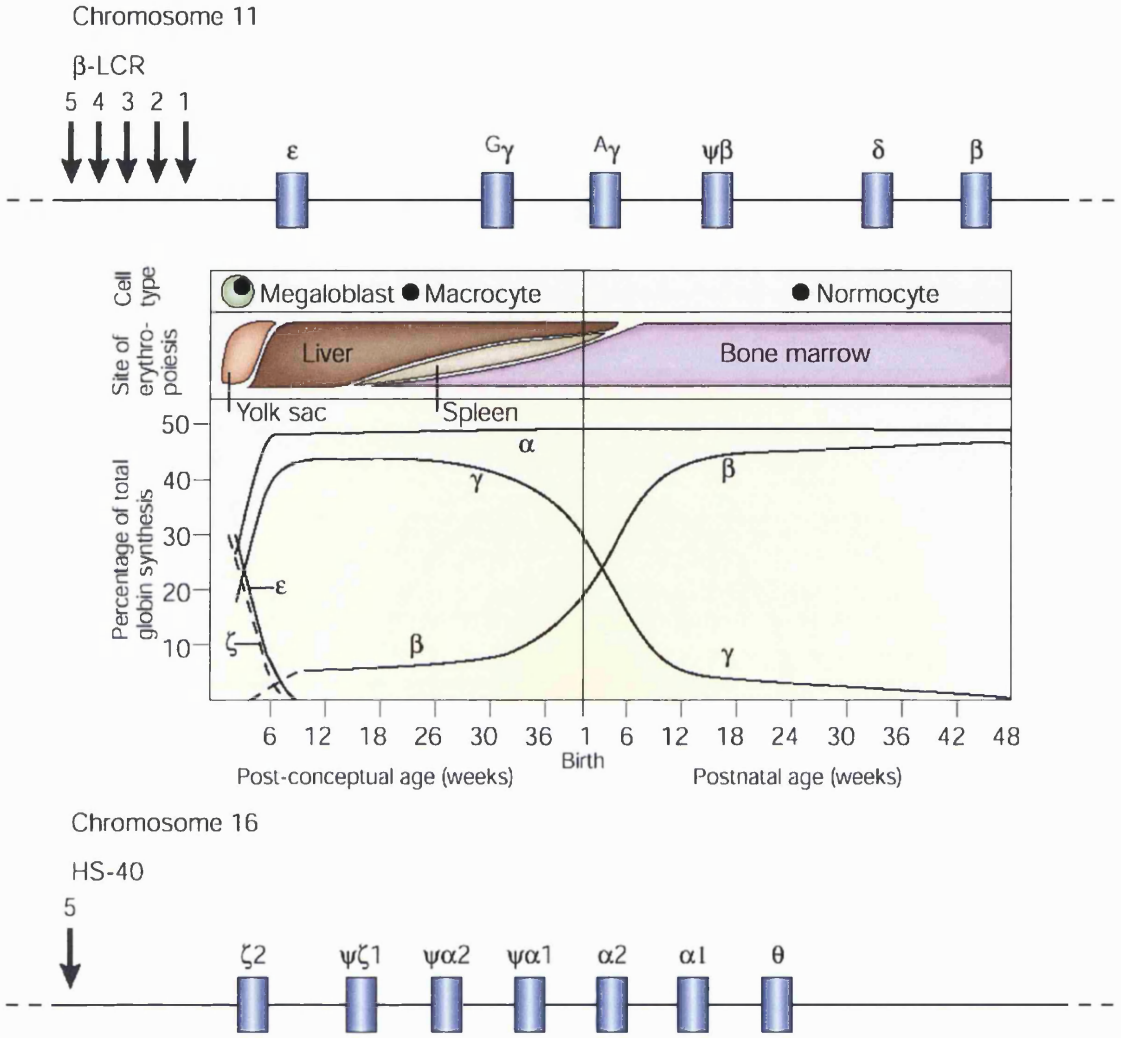


Figure 3.1 Diagram showing active globins, i.e. embryonic, fetal and adult globins during different periods of human life. Structural maps of α-globin and β-globin gene clusters on chromosomes 16 and 11 are also exhibited (Weatherall, 2001). (LCR = locus control region.)

The synthesis of each globin protein is controlled by each particular gene. The α-like globin cluster includes an embryonic gene (ζ2), fetal/adult genes (α1 and α2), pseudogenes (ψζ1, ψα1 and ψα2) and undetermined function gene (θ1) on chromosome 16 (16p13.3) in the order 5′-ζ2-ψζ1-ψα2-ψα1-α2-α1-θ-3′ (Higgs *et al.*, 1989). The β-globin

gene clusters are located on chromosome 11 (11p15.5) and consist of an embryonic gene (ε), fetal genes (Gγ and Aγ), adult genes (β and δ) and a pseudogene (ψβ) as the series 5′-ε-Gγ-Aγ-ψβ-δ-β-3′ (Thein, 1998). These two globin gene clusters might have come from the same fundamental Hb gene following duplication and modification in the evolutionary process as some globin chains share an identical sequence of amino acids.

Thalassaemias are clinically categorised as major, intermediate and minor depending on their symptoms (Thein, 1998). The patients with severe transfusion dependent anaemia are described as thalassaemia major. Those with anaemia and splenomegaly without the need of regular blood transfusion are thalassaemia intermedia. Thalassaemia minor are a silent carrier. This classification benefits clinical use, but does not provide all the genetic details. The diagnosis can be drawn by traditional haematological analysis. Transmission of thalassaemias is in a Mendelian autosomal recessive manner. Heterozygotes usually possess some haematological changes, but are asymptomatic. The more severe forms include patients that are homozygotes or compound heterozygotes. Modern molecular analysis shows that the nature of molecular defects are very heterogeneous and specific to each population.

One of the heterozygote advantages of thalassaemia is malarial resistance which was first postulated by Haldane (Haldane, 1948). One explanation for the protection effect against the malarial parasite *Plasmodium falciparum* is that when being infected by the parasites the abnormal red blood cells of the heterozygotes are destroyed rapidly. In the areas where malaria is endemic, the heterozygotes who possess a biological advantage over the normal homozygotes will have a better chance to survive. Therefore, malarial selection may be the major reason for the relationship of thalassaemia gene frequency and the malaria endemic regions (Flint *et al.*, 1998).

3.1.1 β-Thalassaemia

The term β-thalassaemia (http://www.ncbi.nlm.nih.gov/entrez/; 141900) is used to describe the conditions caused by deficient production of β-globin. The cases with a reduced β-globin level are called β^{+}-thalassaemia, while those with absent β-globin synthesis are β^{0}-thalassaemia. The patients with homozygote β^{0}-thalassaemia mutations are the most severe form. This group of patients possess a severe transfusion dependent anaemia and are clinically categorised as β-thalassaemia major. β-Thalassaemia major patients are born healthy. However, when the β-globin is needed to take over from the γ-globin (**Figure 3.1**), the patients will present with severe anaemia during the first year of life. Other characteristics include spleen enlargement and bone marrow expansion with skeleton deformities (**Figure 3.2**).

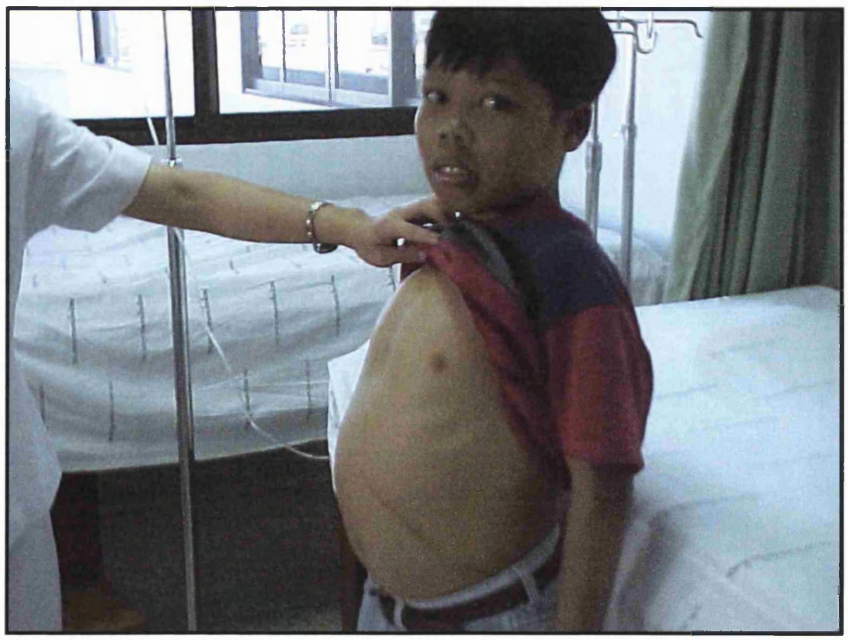


Figure 3.2 A patient with β-thalassaemia major showing abnormal skull shape due to bone marrow expansion and splenomegaly.

The defective β-globin production gives rise to an imbalanced globin chain condition, i.e. excess of α-globin. The excess α-globin precipitates in the red cell precursors and leads to ineffective erythropoiesis (**Figure 3.3**). Despite adequate blood treatment, the patients usually die from the complications of iron overload with an average age of twenty years, unless parenteral iron chelators are provided regularly (Hoffbrand and Wonke, 1997). When a heterozygote individual marries another carrier, they will have a 25% risk of having a severe transfusion dependent homozygous child, i.e. β-thalassaemia major. Co-inheritance of the β- and α-thalassaemias gives rise to a milder symptom due to the co-existent deficiency of α-globin chains. This more balanced globin chain synthesis results in a lesser degree of α-globin precipitation, subsequently a more effective erythropoiesis (Weatherall, 1995a). The disease is particularly common in Mediterranean, Middle Eastern and Asian populations (Old, 1996).

The β-globin gene sequence encodes for 146 amino acids over 1,600 bases. This involves 3 coding regions (exons) and 2 non-coding intervening sequences (IVSs or introns) (**Figure 3.4**). Over 200 different mutations in the β-globin gene have been documented (Huisman *et al.*, 1997). Most are point mutations or one or two base deletions/insertions, while large deletions are not common. The resulting interruption of gene functions can be at the transcriptional, translational or post-translational levels. Nonsense and frameshift types of mutations or the mutations affecting the initiation codon or the junction of a splice site usually give rise to β^0-thalassaemia, while most mutations located in the β-globin gene promoter region which affect the transcription or translation process are likely to cause β^+-thalassaemia (Old, 1996).

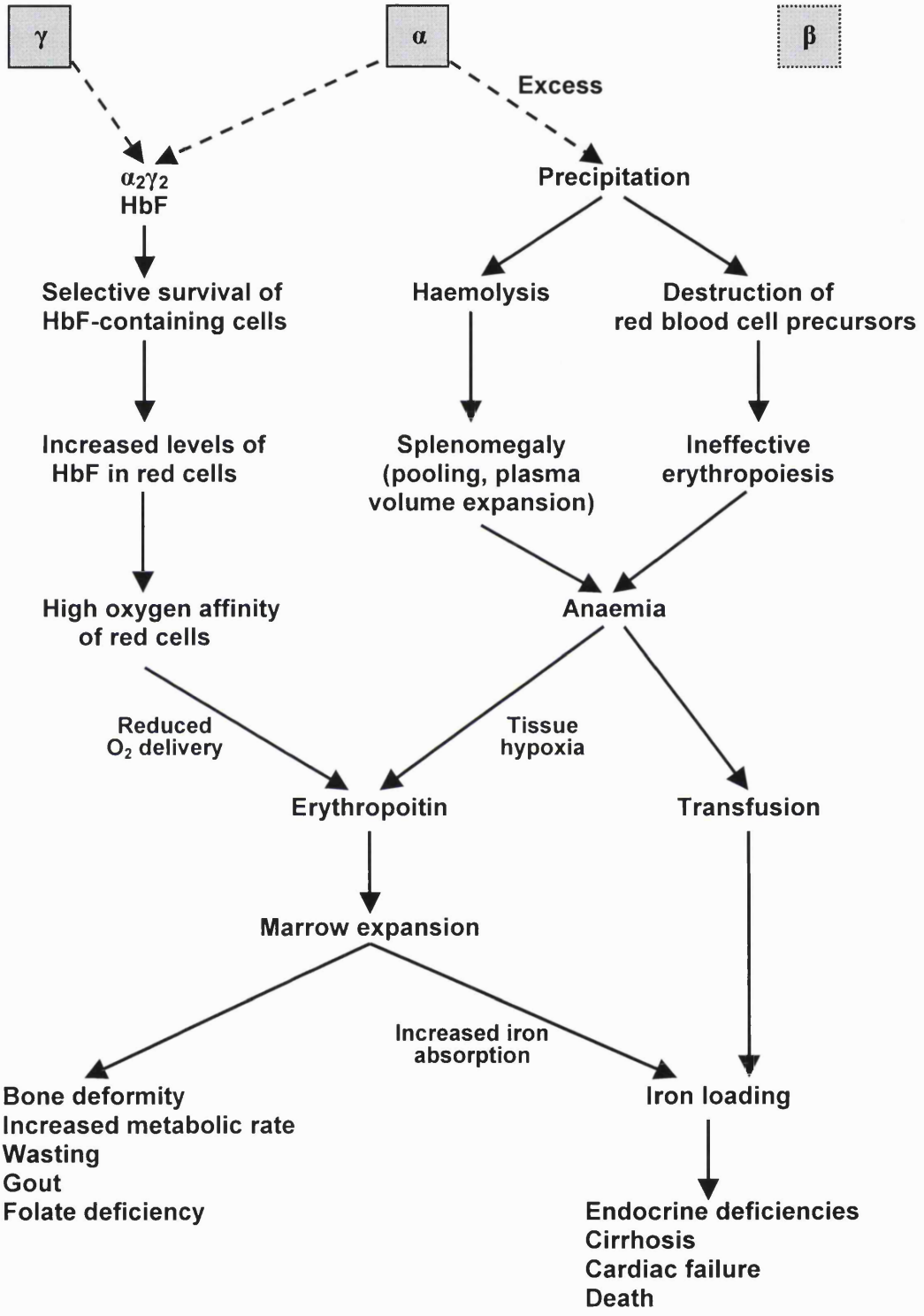


Figure 3.3 The pathophysiology of β-thalassaemia (Weatherall, 1998).

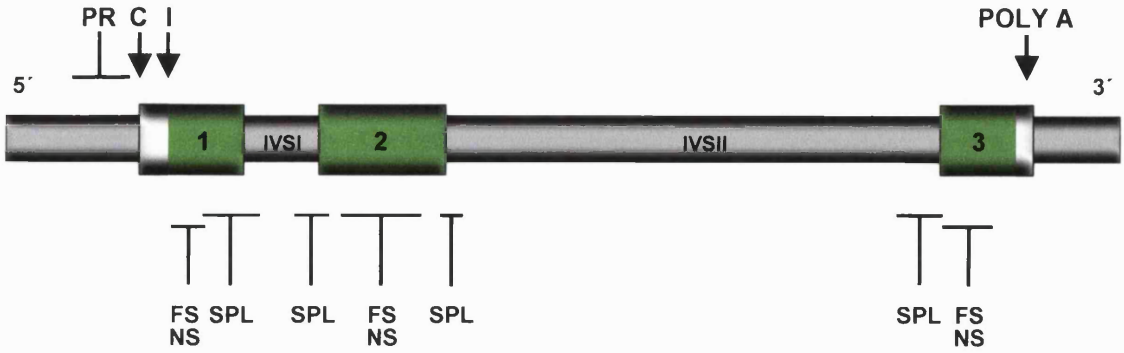


Figure 3.4 Major β-thalassaemia mutations. The β-globin gene consists of three exons (green) and two introns (IVSI and IVSII, grey). PR=promoter region, C=CAP site, I=initiation codon, FS=frameshift, NS=nonsense, SPL=splice site, POLY A=RNA cleavage and polyA addition site (Weatherall, 2001).

Major mutations in the populations where β-thalassaemia is common are listed in **Table 3.1**. The mutations IVSI-110 (G→A), codon 39 (C→T), IVSI-6 (T→C) and IVSI-1 (G→A) are common in the Mediterranean region (Amselem *et al.*, 1988; Di Marzo *et al.*, 1988; Diaz-Chico *et al.*, 1988; Kazazian *et al.*, 1984; Rosatelli *et al.*, 1985). Six mutations, including IVSI-5 (G→C), -619bp del, FS8-9 (+G), IVSI-1 (G→T), FS41-42 (-TCTT) and codon 35 (-C), account for over 90% of Indian and Pakistan populations (Thein *et al.*, 1988). FS41-42 (-TCTT), IVSII-654 (C→T), codon 17 (A→T) and –28 (A→G) mutations are the major mutations in Chinese and Southeast Asians (Kazazian *et al.*, 1986). A few frequent mutations with a number of rare ones are particular to each region (Flint *et al.*, 1998). However, it has been estimated that there are only approximately 25 major mutations which are responsible for β-thalassaemia alleles in all at risk populations (Huisman, 1990).

Table 3.1 Spectrum of β-thalassaemia mutations in at risk populations.

Gene mutations	New mutation nomenclature	Population (%)					
		Greeks/ Italians (Kazazian *et al.*, 1984)	Sicilians (Di Marzo *et al.*, 1988)	Turks (Diaz-Chico *et al.*, 1988)	Spanish (Amselem *et al.*, 1988)	Indians/ Pakistanis (Thein *et al.*, 1988)	Chinese/ Southeast Asians (Kazazian *et al.*, 1986)
-28 (A→G)	-78A>G						16.7
FS8(-AA)	25-26delAA			13.8			
FS8-9(+G)	27-28insG					19.6	
codon 17(A→T)	52A>T						12.8
IVSI-1(G→A)	IVS1+1G>A	9.5		8.5			
IVSI-1(G→T)	IVS1+1G>T					13.7	
IVSI-5(G→C)	IVS1+5G>C					22.6	
IVSI-6(T→C)	IVS1+6T>C	10.8	28.9	23.4	15.5		
IVSI-110(G→A)	IVS1+110G>A	33.5	26.8	42.6	8.6		
codon 35(-C)	238delC					4.9	
codon 39(C→T)	248C>T	27.9	36.1		63.8		
FS41-42(-TCTT)	255-258del					11.8	35.9
IVSII-1(G→A)	IVS2+1G>A	7.6					
IVSII-654(C→T)	IVS2+654C>T						25.6
IVSII-745(C→G)	IVS2+745C>G	5.7					
-619bp del	1065-1683del					20.6	

Almost all mutations can be identified using various PCR based methods (Sutcharitchan and Embury, 1996). These analyses are useful for prenatal diagnosis of β-thalassaemia. The initial PCR technique used to detect the specific mutations involved restriction fragment length polymorphism (RFLP) (Pirastu *et al.*, 1989), allele-specific oligonucleotide probe hybridisation (ASO) (Ristaldi *et al.*, 1989) and reverse dot-blot hybridisation (Maggio *et al.*, 1993; Winichagoon *et al.*, 1999). Another popular method for identifying the particular defects is amplification refractory mutation system (ARMS) (Old *et al.*, 1990) and its modifications (Chang *et al.*, 1995; Chehab and Kan, 1989; Tan

et al., 1994). For distinguishing the unknown mutations, denaturing gradient gel electrophoresis (DGGE) is the most commonly used technique (Cai and Kan, 1990). Other methods, including single stranded conformation polymorphism (SSCP) (Orita *et al.*, 1989) and chemical mismatch cleavage (CMC) (Cotton *et al.*, 1988) are also useful tools.

Clinical management of β-thalassaemia major is mostly based on symptomatic treatment (Olivieri, 1998). Regular blood transfusion has markedly improved the patients' prognosis. However, infection with HIV or Hepatitis B or C is a common complication of blood transfusion. Splenectomy may be required in particular cases; however, it may lead to acceleration of iron overloading and post-splenectomy infection. Iron-chelating therapy is very useful in improving the survival from the fatal transfusional iron overloading. The only curative treatment is bone marrow transplantation; however, it is an expensive and risky procedure and a serious complication is graft-versus-host disease. Therefore, it is not a routine treatment in every hospital.

Several countries have introduced programmes to control the incidence of new cases by offering screening for heterozygotes, genetic counselling, and PND with termination of pregnancy in affected cases (Petrou and Modell, 1995; Tongsong *et al.*, 2000b). PND for β-thalassaemia was initially performed by globin chain synthesis analysis of fetal blood which was taken by fetoscopy or placental aspiration (Kan *et al.*, 1975). However, the current preferred method involves DNA analysis from CVS or amniocytes (Cao *et al.*, 1998). These approaches are currently used and have helped in reducing new cases in many countries. As an alternative, several PGD techniques for β-thalassaemia have been developed employing a variety of mutation detection methods

including restriction enzyme digestion (Kuliev *et al.*, 1998; Ray and Handyside, 1996), single stranded conformation polymorphism (SSCP) (El-Hashemite *et al.*, 1997) and denaturing gradient gel electrophoresis (DGGE) (Kanavakis *et al.*, 1997). Successful diagnosis, and the birth of healthy children, has been accomplished using some of these techniques (Kanavakis *et al.*, 1999; Kuliev *et al.*, 1999), however contamination (**Section 1.5.3**) and allele drop out (ADO, the failure to amplify one of the alleles in a heterozygous cell; **Section 1.5.2**) remain significant problems for most protocols.

3.1.2 α-Thalassaemia

α-Thalassaemia (http://www.ncbi.nlm.nih.gov/entrez/; 141800 and 141850) is also a recessive disease and displays a particularly high incidence in Southeast Asia and the Mediterranean (Higgs *et al.*, 1989). Although the α-thalassaemias (possibly the most common single gene disorder in the world) are more common than the β-thalassaemias, due to the lethal feature of the most severe form (homozygote affected) which leads to intrauterine death and the carrier state being generally asymptomatic, they do not obtain much attention on health care. The pathogenesis is caused from a deficiency of α-globin chain synthesis. The α-globin genes on chromosome 16 may be defective for both loci (namely α^0- or α-thalassaemia 1) or just one locus (namely α^+- or α-thalassaemia 2). The combination of α-globin gene defects leads to 4 levels of clinical characteristics, including asymptomatic carriers of α-thalassaemia 2 or 1, Hb H disease and Hb Barts hydrops fetalis, with 3, 2, 1 and 0 loci of functional α-globin genes, respectively (**Figure 3.5**).

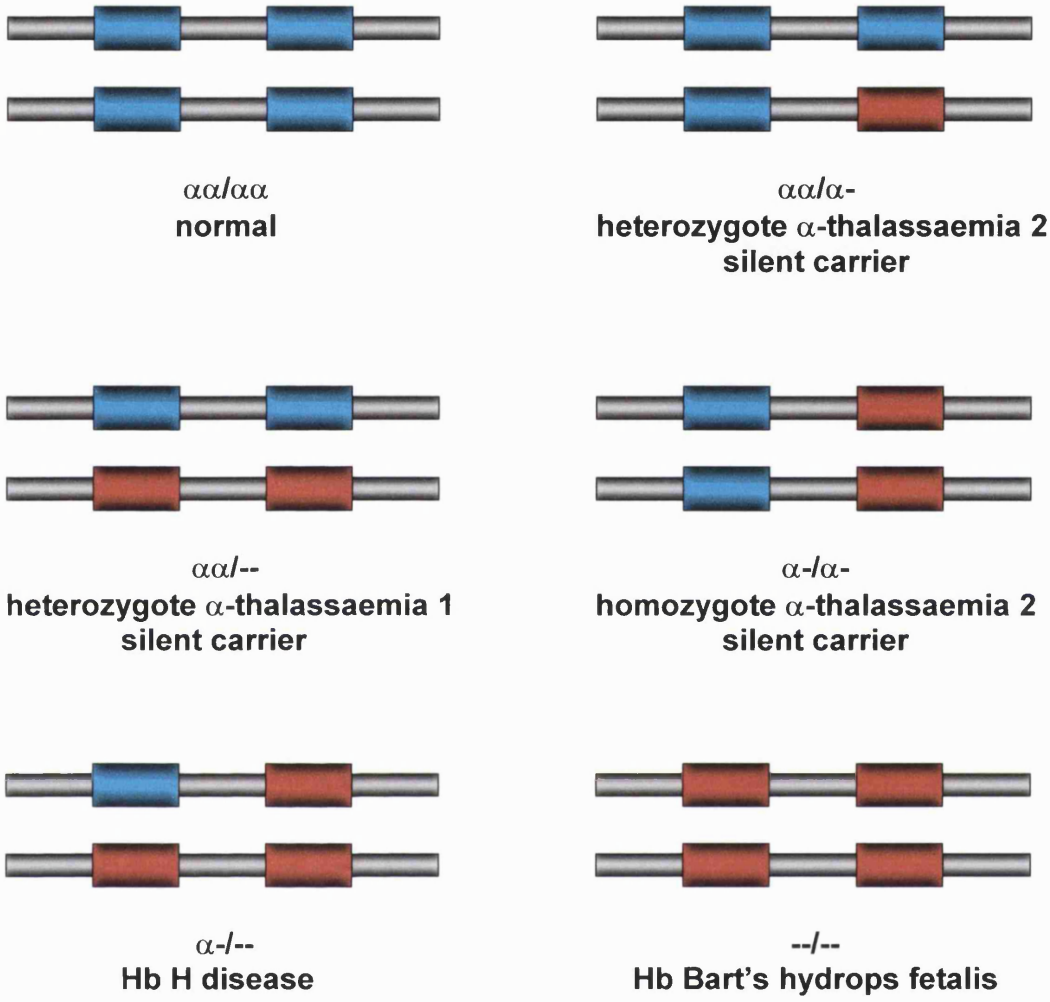


Figure 3.5 Various forms of α-thalassaemia from different combinations of normal α-globin and α-thalassaemia 1 and 2 genes. The normal α-globin genes are in blue and the deleted α-globin genes are in red.

The individuals with Hb H disease, which is the compound heterozygous state for α-thalassaemia 1 and 2, possess a significant deficiency of α-globin chains, subsequently a relative excess of β-globin chains which gives rise to the Hb H (β_4). Hb H disease patients have a moderate anaemia, rarely have severe anaemia or need special care and are relatively healthy. However, when they marry a Hb H disease subject or a

heterozygote individual for α-thalassaemia 1, there is a 50% chance of having a Hb Bart's hydropic fetus.

The homozygote state for α-thalassaemia 1 leads to the absence of α-globin chain synthesis in fetuses, consequently the excess of γ-globin chains which forms Hb Bart's (γ_4). Both Hb H and Hb Bart's possess high oxygen affinity, like myoglobin, do not release oxygen to the tissues and cause asphyxia. Both Hb H and Hb Bart's are soluble and do not precipitate in the bone marrow. This results in less red cell precursor destruction and more functional erythrophoresis than in β-thalassaemia. However, Hb H is unstable, precipitates in aged red cells and causes inclusion bodies. These abnormal red cells are entrapped in the microcirculation and spleen, subsequently leading to a shorter red cell life and splenomegaly. Therefore, the anaemia is more likely to be caused from haemolysis than in β-thalassaemia (Weatherall, 1995b).

The nature of α-globin gene mutations involves deletions of one or two α-globin genes, point mutations, deletions or insertions (Higgs *et al.*, 1989). α-Thalassaemia 1 is usually caused from large deletions in the α-globin gene cluster which involve both α-globin genes. The Southeast Asian deletion ($--^{SEA}$) is common in Southeast Asian and Chinese populations (Fucharoen and Winichagoon, 1987), while the Mediterranean deletion ($--^{MED}$) is prevalent in Italy, Greece and Sardinia (Di Rienzo *et al.*, 1986; Paglietti *et al.*, 1986; Pirastu *et al.*, 1982; Tzotzos *et al.*, 1986). In addition to the major SEA and MED mutations, there are approximately 20 other deletion mutations and approximately 20 non-deletion mutations with much lower incidences (Flint *et al.*, 1998). For α-thalassaemia 2, deletions are also found more often than point mutations. The 3.7kb deletion ($-\alpha^{3.7}$) is found in the Mediterranean, Africa, Middle East, India

subcontinent and Oceania regions, and the 4.2kb deletion ($-\alpha^{4.2}$) is frequent in the Southeast Asia and Pacific regions (Flint *et al.*, 1998). Approximately 30 non-deletion types with rare incidences have been reported (Bernini and Harteveld, 1998). However, as most data were obtained from the analysis of prenatal samples, the real figures may be much larger than those reported.

Fetuses with the most severe form cannot synthesise normal α-globin chains. This leads to the abolition of heme-heme interactions and formation of haemoglobin that is incapable of efficient oxygen transfer. Such fetuses develop massive edema due to heart failure from severe anaemia, namely Hb Bart's hydrops fetalis syndrome, and almost always die in utero or soon after birth (Weatherall, 1995a). Mothers carrying these fetuses are likely to develop potentially life-threatening obstetric complications, e.g. eclampsia, pre-eclampsia, dystocia and obstetric haemorrhage. Therefore, the target of prenatal diagnosis for Hb Bart's hydrops fetalis syndrome is to prevent the serious maternal mortality and morbidity. Prenatal diagnosis for α-thalassaemia has traditionally been performed by assessing globin chain synthesis in fetal blood or by Southern blotting after CVS (Old *et al.*, 2000). A more recent technique, which involves PCR to detect large α-thalassaemia deletions, is known as gap PCR (Bowden *et al.*, 1992; Ko *et al.*, 1992). A PGD protocol using nested gap PCR has been proposed (Chang *et al.*, 1996).

3.1.3 Aims

The first aim of this study was to find an appropriate versatile mutation detection method for thalassaemias that can identify more than one mutation at a time. This strategy would be useful in saving time spent on developing a new technique for each different

mutation. Moreover, it would also greatly benefit the detection of compound heterozygote genotypes which are not uncommon in thalassaemias. The second objective was to develop sensitive and specific PGD protocols for different types of thalassaemia mutations, i.e. substitution, small and large deletions, using various PCR techniques. PGD protocols for two β-thalassaemia mutations, i.e. IVSI-110 and codon 41-42 and one α-thalassaemia mutation, SEA deletion, have been developed. A polymorphic marker linked to the β-globin gene, HUMTH01, was included in the PGD protocols as multiplex PCR for contamination detection and back up linkage analysis results (for β-globin mutations only). The protocols were tested using single buccal cells and single human blastomeres, so that they are ready for clinical application. The resulting PGD protocols can be used for a wider range of mutations with or without minor modification. The last aim was to develop a widely applicable PGD protocol for thalassaemias using fluorescent single cell sequencing and minisequencing (SNaPshot™), and to compare its efficiency with the traditional protocols.

3.2 Materials and methods

3.2.1 Mutation detection of β-thalassaemia

After amplifying with the outer and inner β-thalassaemia primers (**Section 2.2.5.2** and **Table 3.2**), known DNA samples of β-thalassaemia mutations that can be found in the UK, including IVSI-1 (G→A), IVSI-1 (G→T), IVSI-5 (G→C), IVSI-6 (T→C), IVSI-110 (G→A), FS8-9 (+G), codon 41-42 (-TCTT), codon 39 (C→T) and haemoglobin sickle, were tested extensively in comparison to normal DNA samples using SSCP with a wide variety of experimental conditions (**Section 2.2.7.3**).

Table 3.2 The details of the primers used for thalassaemias.

Primers	Sequences (5′-3′)	Fluorescent labelled	Location	Size of the product (bp)	Concentration (μM)	Annealing temperature (°C)	References
Outer β-thal*							
forward	5′-GCA ACC TCA AAC AGA CAC C-3′	-	11p15.5	279	0.2	60	(El-Hashemite *et al.*, 1997)
reverse	5′-CAA AGA ACC TCT GGG TCC AA-3′	-			0.2		
Inner β-thal*							
forward	5′-CTG AGG AGA AGT CTG CCG TT-3′	Cy5®	11p15.5	223	0.2	60	(El-Hashemite *et al.*, 1997)
reverse	5′-GGT AGA CCA CCA GCA GCC TA-3′	- / Cy5®			0.2		
bthalw1*							
forward	5′-GCA AGG TGA ACG TGG ATG A-3′	TET® (green)	11p15.5	364	0.2	60	OMIM
reverse	5′-CAC AGT GCA GCT CAC TCA GTG TG-3′				0.2		
bthalw2*							
forward	5′-ACC TCA CCC TGT GGA GCC AC-3′		11p15.5	432	0.2	60	OMIM
reverse	5′-GGT AGA CCA CCA GCA GCC TA-3′				0.2		
athalsea							
S1	5′-GTG TTC TCA GTA TTG GAG GGA A-3′	-	16p13.3		0.2	60	(Ko *et al.*, 1992)
S2	5′-GAC ACG CTT CCA ATA CGC TTA-3′	TET® (green)		287	0.2		
S3	5′-CTA CTG CAG CCT TGA ACT CC-3′	HEX® (yellow)		194	0.2		
HUMTH01							
forward	5′-AGG GTA TCT GGG CTC TGG-3′	6'FAM® (blue)	11p15.5	115-140	0.2	60	(Kuliev *et al.*, 1998)
reverse	5′-CTT CCG AGT GCA GGT CAC-3′	CY5®			0.2		

*Sequence mapping in **Figure 3.32**.

In order to find the optimal technique of mutation detection for each particular mutation, three analytical instruments and a number of gel components were tested. The experiments involved PhastSystem™ using 20% polyacrylamide gel (PhastGel® Homogeneous 20) in combination with 'Native' or 'DNA' Buffer Strips, GenePhor™ using 12.5% and 15% polyacrylamide gel (GeneGel® Excel and GeneGel® Clean), ALF Express™ using 0.5×, 0.3× MDE® acrylamide gel and 0.5× gel with 5% glycerol. Several running times (200Vh, 250Vh, 300Vh, 350Vh and 400Vh for PhastSystem™ and 1 h 30 min, 2 h and 2 h 30 min for GenePhor™) and temperature conditions (5°C, 10°C, 15°C and 20°C) were employed. In total, 42 PhastGel™ (12 lanes of each run), 12 GeneGel® (24 lanes of each run) and 27 F-SSCP runs (20 lanes of each run) were used.

3.2.2 PGD strategy for β-thalassaemia, IVSI-110 mutation

Adding HUMTH01 primers (Kuliev *et al.*, 1998) (**Table 3.2**) to the outer β-thalassaemia primers (El-Hashemite *et al.*, 1997) (**Table 3.2**) as a multiplex PCR (**Section 2.2.5.3**) provides the advantage of linkage analysis, in order to confirm direct mutation analysis from β-thalassaemia primers, in addition to contamination detection. The forward HUMTH01 primer was labelled with the blue fluorescent dye (Cy5®) (**Section 2.2.5.5**) to allow analysis to be performed on an automated fluorescence sequencer, the ALF Express™ (**Section 2.2.7.2.1**). The inner nested amplification reaction (**Section 2.2.5.4**) was carried out using an aliquot of the outer amplification products as the templates and the inner β-thalassaemia primers (El-Hashemite *et al.*, 1997) (**Table 3.2**). This resulted in the production of a fragment of 223 bp, 56 bp smaller than that produced by the outer β-thalassaemia primers. The PCR products from the inner amplification were analysed by 2% agarose gel electrophoresis (**Section 2.2.7.1**) and

SSCP analysis on GenePhor™ (**Section 2.2.7.3.2**). A control study comparing the efficiency of two polymerase enzymes, SuperTaq® and AmpliTaq Gold™, was conducted.

3.2.3 PGD strategy for β-thalassaemia, codon 41-42 mutation

The nature of the β-thalassaemia, codon 41-42 mutation is a 4 bp deletion (-TCTT) which can simply be identified using F-PCR (**Section 2.2.5.5**). The PGD protocol for the β-thalassaemia codon 41-42 mutation was designed such that fragments from both β-thalassaemia and HUMTH01 loci (labelled in green, TET®, and blue, 6-FAM®, respectively) could be analysed at the same time on an automated fluorescence sequencer, the ABI Prism™310 (**Section 2.2.7.2.2**) following a multiplex amplification (**Section 2.2.5.3**). The bthalw1 primers (**Table 3.2**) were employed for mutation detection giving a product size of 364 bp. The peak areas of the HUMTH01 alleles and the normal and mutant alleles of the β-globin gene of each sample were observed and scored for ADO and PA.

3.2.4 PGD strategy for α-thalassaemia, $--^{SEA}$ mutation

The α-thalassaemia, $--^{SEA}$ mutation is a large deletion defect within the α-globin gene cluster which removes both α1- and α2-globin genes. The traditional diagnostic method involves Southern blot which is not applicable for PGD. PCR amplification of the whole 20kb fragment in order to include the deletion is inefficient at the single cell level. Gap PCR (**Section 2.2.5.6**) is a wisely developed PCR based technique for molecule analysis of such a large deletion while maintaining the amplification efficiency. In the analysis of the α-thalassaemia, $--^{SEA}$ mutation, the primers S1 and S2 results in a PCR

product of 287 bp for the normal allele and the primers S1 and S3 generates a fragment of 194 bp for the mutant allele (**Figure 3.6**). The primers S2 and S3 were tagged with TET® (green) and HEX® (yellow/black) fluorescent dyes so that the normal and mutant alleles can be identified and distinguished on an ABI Prism™310. The polymorphic HUMTH01 primers, labelled with 6'FAM® (blue) fluorescent dye, were included as a multiplex reaction for contamination detection. The single step multiplex gap fluorescent PCR protocol was tested and optimised using single buccal cells of a heterozygote subject for the α-thalassaemia, $--^{SEA}$ mutation so that the amplification efficiency and ADO rates could be evaluated. The final optimised protocol was carried out on single human blastomeres donated for research.

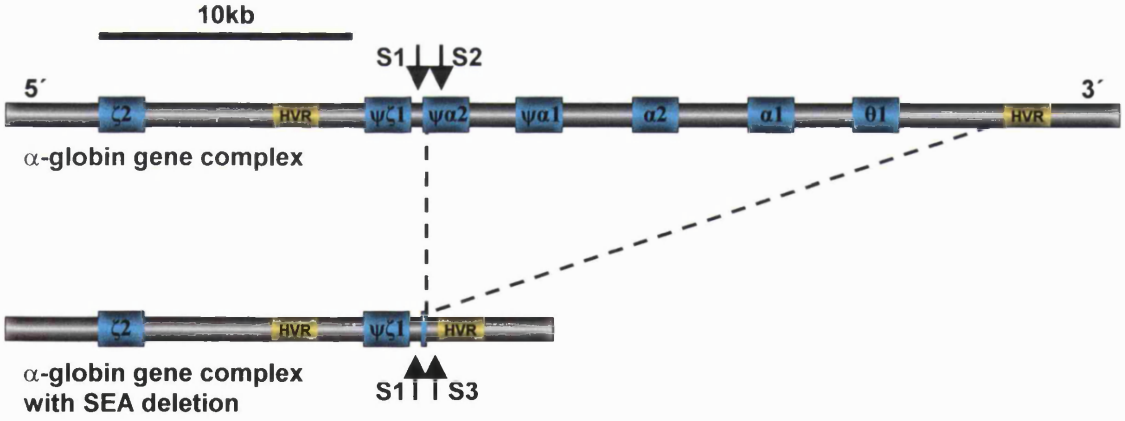


Figure 3.6 α-Globin gene complex and α-thalassaemia SEA mutation with mapping of gap PCR primers. For a normal genotype, S1 and S2 primers generate a PCR product of 287 bp, while S1 and S3 primers result in an amplified fragment of 194 bp for the SEA mutation with 20kb deletion.

3.2.5 Single cell sequencing

Single cell sequencing (**Section 2.2.7.4**) was applied to single cell PCR samples that had been previously shown to have equal amplification of both alleles (SA); preferential amplification of the normal allele (PAN); preferential amplification of the mutant allele (PAM); ADO affecting the normal allele (ADON); ADO affecting the mutant allele (ADOM) for β-thalassaemia, IVSI-110 (substitution) and codon 41-42 (-TCTT frameshift) mutations (**Section 3.2.2** and **3.2.3**). Isolated human blastomeres, derived from embryos donated for research, were also tested.

3.2.6 Single cell minisequencing (SNaPshot™)

Single cell PCR samples of heterozygote subjects with various β-globin gene mutations with known amplification characteristics, i.e. SA, ADOM, ADON, were processed with the minisequencing (SNaPshot™) protocol (**Section 2.2.7.5**). The β-globin gene mutations tested in this study involved β-thalassaemia IVSI-110 (**Section 3.2.2**), codon 41-42 (**Section 3.3.2**) and sickle. The ssIVSI110, sscd4142 or sssickle minisequencing primers were employed in the minisequencing reactions for the IVSI-110, codon 41-42 and sickle mutations, respectively (**Table 3.3**). The single cells from the sickle heterozygote subject were amplified using the bthalw2 primers (**Section 2.2.5.2** and **Table 3.2**). The protocol for IVSI-110 mutation was applied to single human blastomeres donated for research.

Table 3.3 The details of the primers used for single cell minisequencing.

Primers	Sequences (5′-3′)	Location	References
ssIVSI110	5′-ACT GAC TCT CTC TGC CTA TT-3′	11p15.5	OMIM
sscd4142	5′-CTA CCC TTG GAC CCA GAG GTT-3′	11p15.5	OMIM
sssickle	5′-GAC ACC ATG GTG CAC CTG ACT CCT G-3′	11p15.5	OMIM

3.3 Results

3.3.1 Mutation detection of β-thalassaemia

During the experiments, problems with analytic instruments and techniques were experienced and corrected. Firstly, the silver staining quality of the PhastGel® was so pale that the band patterns could not be analysed. Formaldehyde and gluteraldehyde were found to be critical for silver staining, these reagents serve as developer and sensitiser respectively. The original manual silver staining protocol (Harvey *et al.*, 1995) excluded the use of the glycerol solution and resulted in flaking of the GeneGel® surface within a few days, before a picture of the band pattern was taken. The introduction of the final glycerol solution step helped in gel preservation at room temperature for at least 2 months.

The amplified product of the IVSI-110 mutation using the inner β-thalassaemia primers could be identified on the Phast System™ using 20% PhastGel® and Native Buffer Strips with the conditions 15°C and 20°C, 350Vh (**Figure 3.7**). Using the GenePhor™ for the analysis of the amplified products using the outer and inner β-thalassaemia primers, only the IVSI-110 mutation showed a different band pattern from the normal (**Figure 3.8**). The optimal condition was to use a 12.5% polyacrylamide gel at 5°C for 1 h 30 min. The reproducibility of this analysis in identifying the IVSI-110 mutation is shown in **Figure 3.9**.

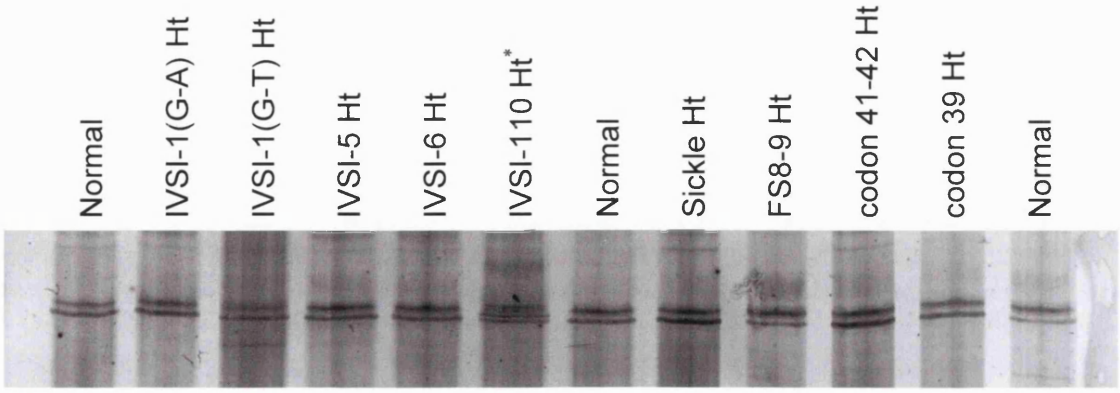


Figure 3.7 SSCP analysis results of the amplified products using inner β-thalassaemia primers from DNA samples of normal and IVSI-1 (G→A) Ht, IVSI-1 (G→T) Ht, IVSI-5 Ht, IVSI-6 Ht, IVSI-110 Ht, sickle Ht, FS8-9 Ht, codon 41-42 Ht and codon 39 Ht mutations on PhastSystem™ using 20% PhastGel® at 350Vh, temperature 20°C. *Only the band pattern of IVSI-110 mutation possesses four bands, while the others, including the normal, possess two bands. Ht = heterozygous.

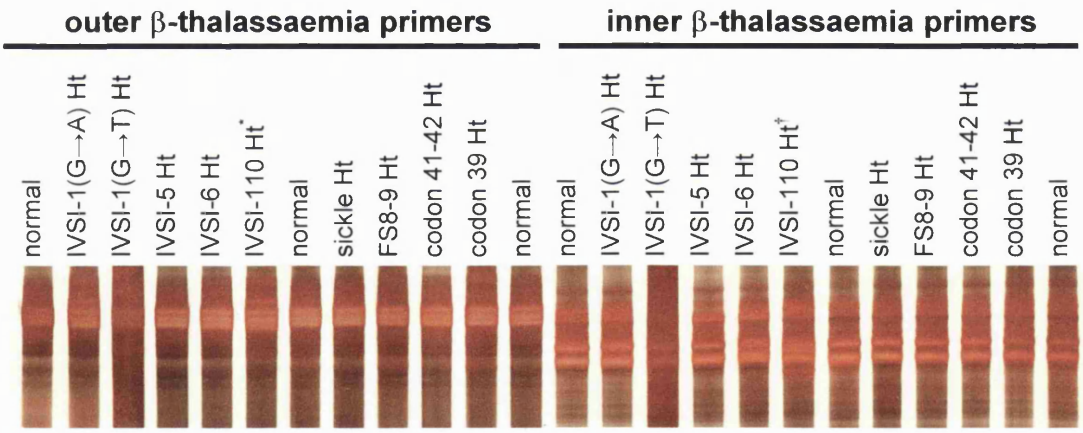


Figure 3.8 SSCP analysis of products amplified using outer and inner β-thalassaemia primers from DNA samples of normal, IVSI-1 (G→A) Ht, IVSI-1 (G→T) Ht, IVSI-5 Ht, IVSI-6 Ht, IVSI-110 Ht, sickle Ht, FS8-9 Ht, codon 41-42 Ht and codon 39 Ht mutations on GenePhor™ using 12.5% GeneGel® Excel, temperature 5°C, 1 h 30 min. Using outer set of primers, only IVSI-110* Ht mutation can be distinguished from normal, and when using inner set of primers, only IVSI-110† mutations show different band patterns from the normal. Ht = heterozygous.

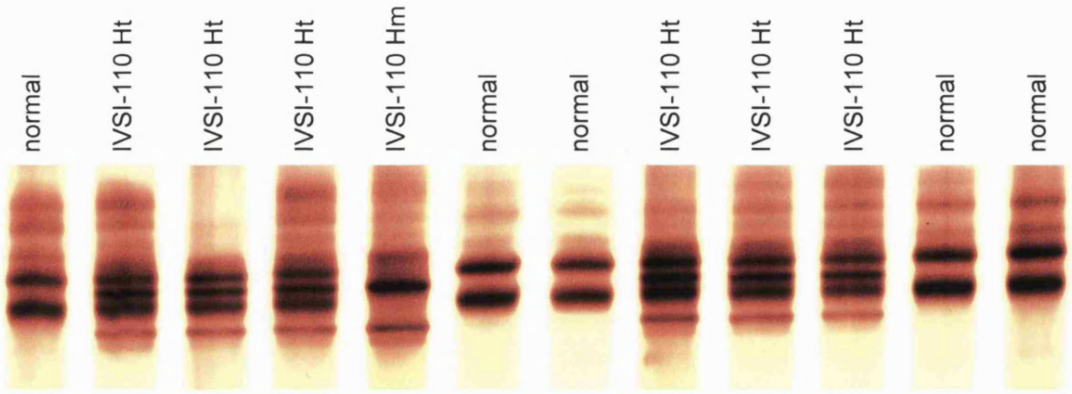


Figure 3.9 SSCP analysis results of the amplified products using inner β-thalassaemia primers from DNA samples of normal, IVSI-110 Ht and IVSI-110 Hm mutations from several different subjects on GenePhor™ using 12.5% GeneGel® Excel, 5°C, 1 h 30 min. The normal, IVSI-110 Hm and IVSI-110 Ht samples show two normal bands, two mutant bands and four (two of normal and two of mutant) bands, respectively. Ht = heterozygous, Hm = homozygous.

The analyses of F-SSCP using the ALF Express™ at several conditions were not informative for mutation detection, either with or without DNA size standards. The amplification using both labelled forward and labelled reverse primers, for visualisation ability of both sense and antisense strands on the ALF Express™, did not give better information than those using just the labelled forward primer. Amplified products from different normal samples failed to show the same peak (band) pattern and running time, neither did the different samples of the same mutation (**Figure 3.10** and **3.11**), i.e. the results failed to show a reproducible normal pattern.

In conclusion, from the analyses of the products amplified using the inner β-thalassaemia primers, the Phast System™ was useful for mutation detection of one β-thalassaemia mutation, i.e. IVSI-110; however, GenePhor™ provided a clearer and

simpler mutation detection technique for IVSI-110, while the ALF Express™ was not informative in this study.

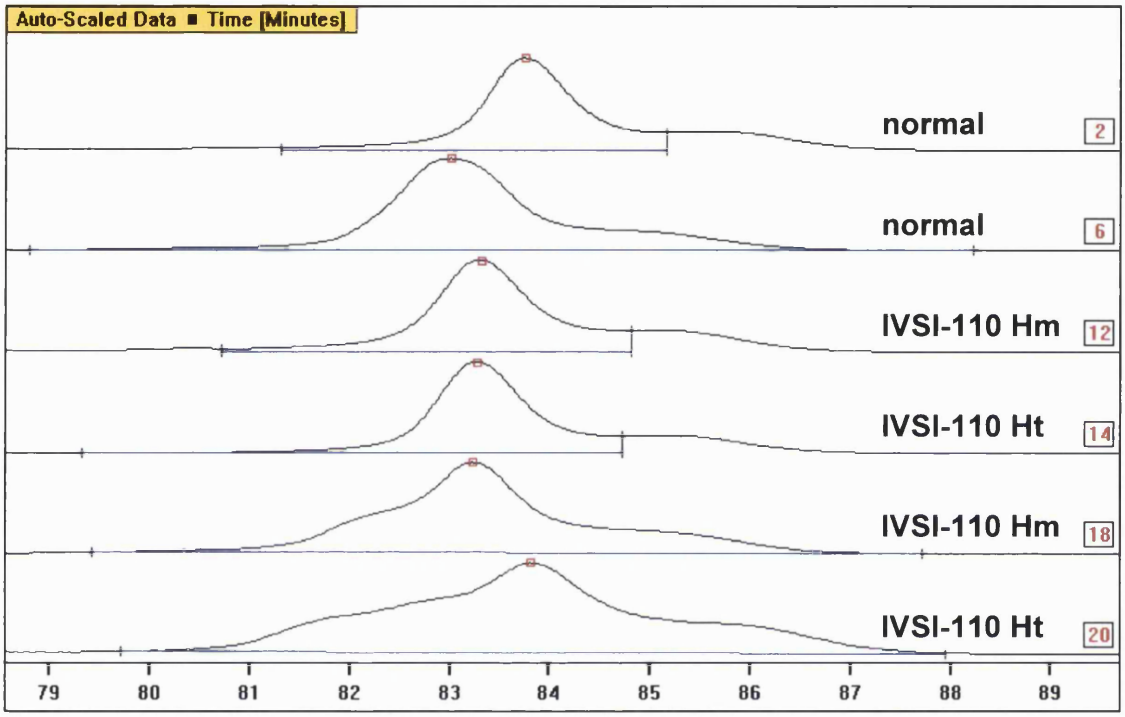


Figure 3.10 F-SSCP results of amplified products from DNA samples of normal (lanes 2 and 6), IVSI-110 homozygotes (Hm) (lanes 12 and 18) and IVSI-110 heterozygotes (Ht) (lanes 14 and 20) using inner β-thalassaemia primers on ALF Express™ using 0.5× MDE gel at 15°C. The amplified products of both normal samples (lanes 2 and 6) and both IVSI-110 Hm (lanes 12 and 18) fail to show the same running time. Moreover, the amplified products of both IVSI-110 Hm (lanes 12 and 18) and IVSI-110 Ht (lanes 14 and 20) show different peak patterns. The x and y axes are base and peak height units, respectively.

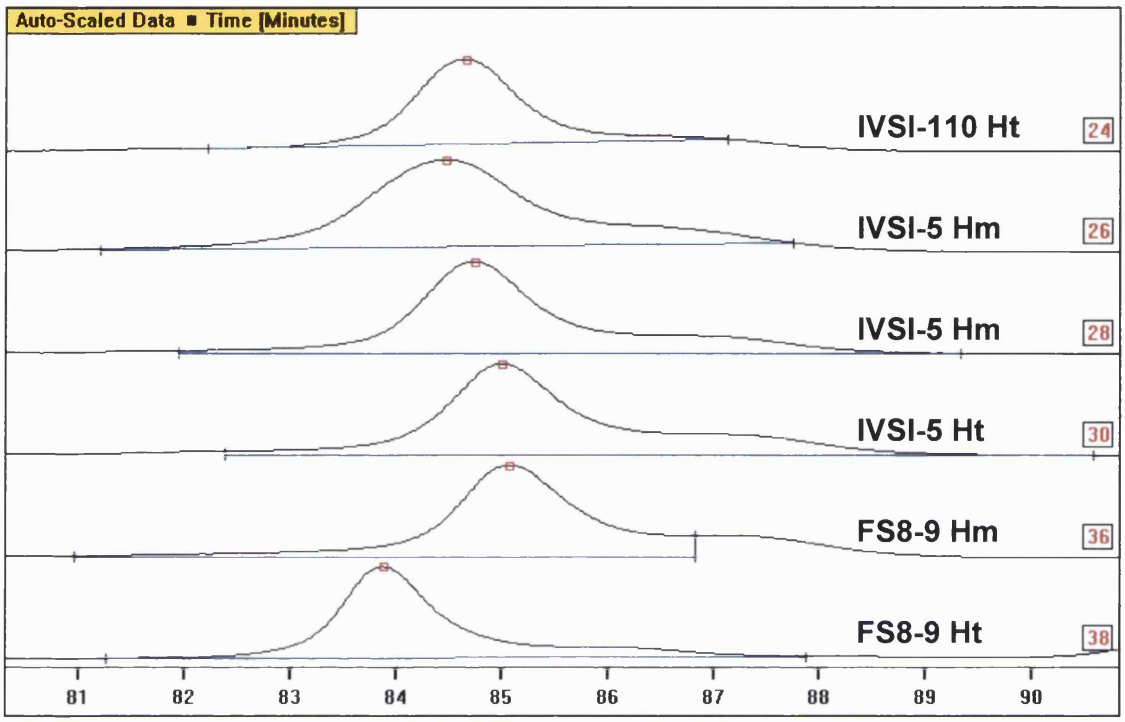


Figure 3.11 F-SSCP results of amplified products from DNA samples from IVSI-110 heterozygotes (Ht) (lanes 24), IVSI-5 homozygotes (Hm) (lanes 26 and 28), IVSI-5 Ht (lane 30), FS8-9 Hm (lane 36) and FS8-9 Ht (lane 38) using inner β-thalassaemia primers on ALF Express™ using 0.5× MDE gel at 15°C. The amplified products of both IVSI-5 Hm (lanes 26 and 28) fail to show the same running time, but are difficult to differentiate from that of IVSI-110 Ht (lanes 24). It is difficult to tell the difference between IVSI-5 Ht (lane 30) and FS8-9 Hm (lane 36) either. The x and y axes are base and peak height units, respectively.

3.3.2 PGD strategy for β-thalassaemia, IVSI-110 mutation

The protocol for single cell diagnosis of β-thalassaemia, IVSI-110 mutation was optimised and the final protocol is shown in **Figure 3.12**. The protocol was evaluated on approximately 700 single buccal cells of an IVSI-110 heterozygote subject (over 900 amplification reactions in total, including control clumps, negatives and studies on DNA samples) and was modified each step, including multiplex and nested PCR protocols and analyses, until the most efficient one was obtained. The amounts of the amplified normal and mutant alleles were evaluated by comparing the intensity of the corresponding bands and each sample was scored for amplification failure (AF), ADO, preferential amplification (PA) or symmetrical amplification (SA) of both alleles. Samples with appropriate or adequate amplification (AA) include those with PA and SA.

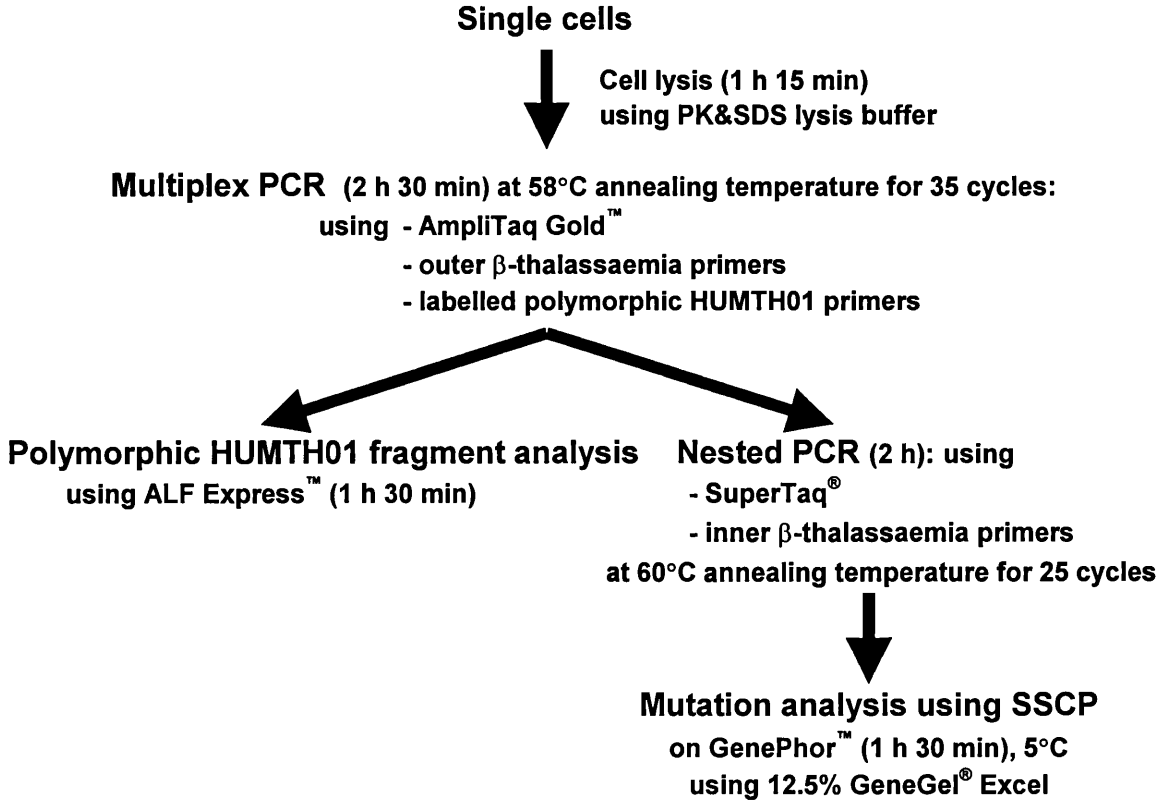


Figure 3.12 The optimal protocol for single cell diagnosis of β-thalassaemia, IVSI-110 mutation. The estimated time consumed for each step is given in the brackets.

The experiments were carried out on IVSI-110 heterozygote buccal cell samples so that the amplification efficiency of both normal and mutant alleles could be evaluated. The first 120 single buccal cell analysis which used SuperTaq® and the old stock of PK&SDS lysis buffer gave a surprisingly high amplification failure (31.7%) and ADO (35.8%) for the β-globin gene and total amplification failure of the HUMTH01 fragment, while the amplification from control clumps in the same PCR settings were impeccable. After an extensive work up, the problems with the β-globin gene amplification focused on the lysis buffer. The preliminary introduction of freshly prepared PK&SDS lysis buffer on 50 single buccal cells gave a more effective protocol with an AF rate of 18% and ADO rate of 18% for the nested products of the β-globin gene (**Table 3.4**). However, the amplification of the HUMTH01 fragment in a further 200 single cell multiplex PCRs analysed on a very sensitive ALF Express™ were still unsuccessful, while the HUMTH01 locus amplified successfully from cell clumps and it was possible to amplify the β-globin locus from single cells. Several compositions of PCR mixtures with different concentrations, i.e. 2×, 4× and 8×, of the primers and SuperTaq® were tested, but did not solve the problem. One experiment using the normal concentration but only a single set of the HUMTH01 primers (i.e. no multiplex) gave acceptable results of the HUMTH01 fragment analyses (**Figure 3.13**). The cause of this problem, therefore, pinpointed to the efficiency of SuperTaq® when doing multiplex amplification. Switching the polymerase enzyme from SuperTaq® to AmpliTaq Gold™ in the primary multiplex PCR step, and leaving all other conditions unchanged, caused a dramatic improvement in the HUMTH01 fragment amplification (**Figure 3.14**). This reflected the improved amplification efficiency of the repetitive sequences with 'hot start' afforded by AmpliTaq Gold™ and even more importantly the reduction in interaction between primers for different loci.

Table 3.4 Preliminary comparison of β-thalassaemia analysis results between two lysis buffers, old stock of PK&SDS and newly prepared PK&SDS, performed on single buccal cells of IVSI-110 heterozygote subject using the protocol for single cell diagnosis of β-thalassaemia.

β-Thalassaemia Analysis Results	Lysis Buffer		
	Old PK&SDS (n=120)	New PK&SDS (n=50)	
AA	39 (32.5%)	32 (64.0%)	Chi square = 14.44*, degrees of freedom =2, p value = 0.000*
ADO	43 (35.8%)	9 (18.0%)	
AF	38 (31.7%)	9 (18.0%)	

AA = appropriate or adequate amplification of both alleles, ADO = allele drop out, AF = amplification failure.

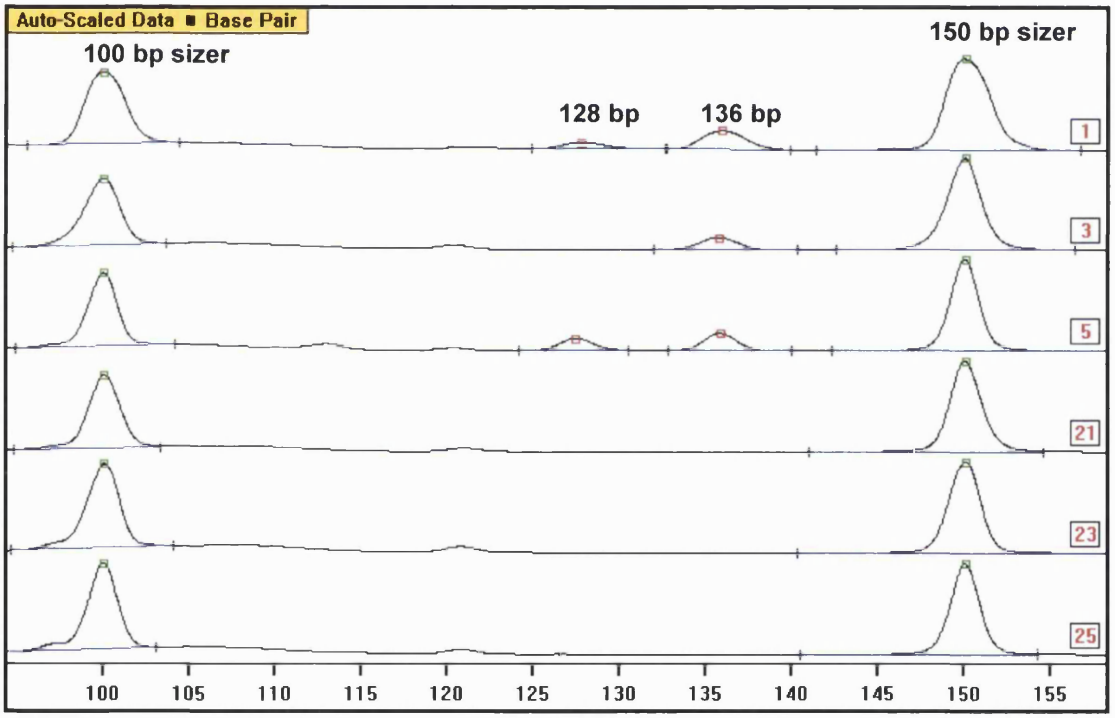


Figure 3.13 Results of HUMTH01 fragment analysis on ALF Express™ from single buccal cell products using SuperTaq®. Standard PCR using a single set of HUMTH01 primers only (lanes 1, 3 and 5) shows peaks of amplified products (PAL in lane 1 and 5, ADOS in lane 3), while multiplex PCR using both HUMTH01 and outer β-thalassaemia primers (lanes 21, 23 and 25) fails to show any peaks of the amplified products. (ADOS = allele drop out of the shorter allele, PAL = preferential amplification of the longer allele.) The x and y axes are base and peak height units, respectively.

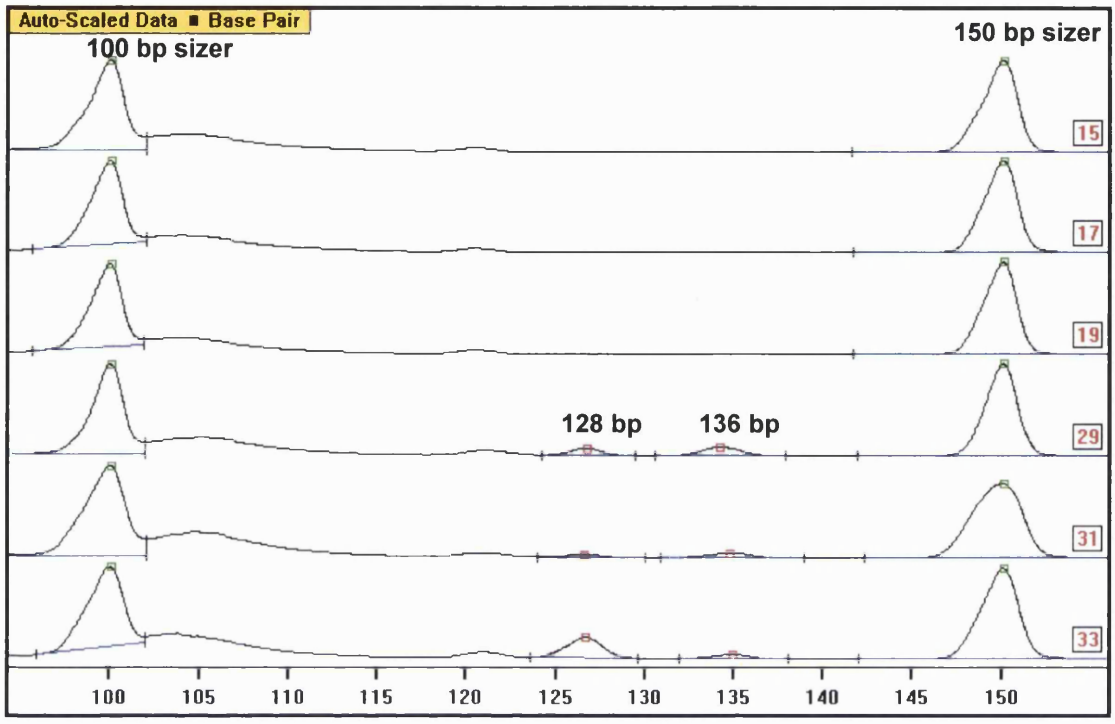


Figure 3.14 Results of HUMTH01 fragment analysis on ALF Express™ from single buccal cell multiplex products (HUMTH01 and outer β-thalassaemia primers); multiplex PCR using double concentration (2 units/25µl) of SuperTaq® and double concentration of HUMTH01 primers (lanes 15, 17 and 19) fails to show any peaks of amplified products, multiplex PCR using AmpliTaq Gold™ (lanes 29, 31 and 33) shows clear peaks of amplified products (SA in lanes 29 and 31 and PAS in lane 33). (SA = symmetrical amplification of both alleles, PAS = preferential amplification of the shorter allele.) The x and y axes are base and peak height units, respectively.

A subsequent control study was performed on 100 single cells to compare the efficiency between SuperTaq® and AmpliTaq Gold™ polymerases. Cells were amplified on the same thermal cycler and at the same time. SuperTaq® consistently failed to give any detectable amplification of the HUMTH01 locus, while AmpliTaq Gold™ showed successful amplification of both loci (**Table 3.5**). 100% amplification efficiency was

achieved with an 8% ADO rate for the HUMTH01 locus. Amplification failure of the β-globin fragment also tended to be higher when using SuperTaq® (18% vs 10% for AmpliTaq Gold™) but without statistical significance (Chi square=1.33, degrees of freedom=2, p value=0.514). ADO rates of the β-globin fragment from SuperTaq® and AmpliTaq Gold™ were 18% and 20%, respectively. Furthermore, if AmpliTaq Gold™ was used in the first round of nested PCR the fragments gave brighter bands on 2% agarose gels (**Figure 3.15**), reflecting the superior efficiency of the enzyme. However, as the secondary nested PCR provided acceptable β-globin amplification regardless of the polymerase enzymes used, SuperTaq® was continued to be used for this step of the PCR protocol for economical reasons.

Table 3.5 Control study of β-thalassaemia analysis and HUMTH01 analysis results between two polymerase enzymes, SuperTaq® and AmpliTaq Gold™, performed on single buccal cells of IVSI-110 heterozygote subject using the protocol for single cell diagnosis of β-thalassaemia.

Analysis Results	Taq Polymerase		
	SuperTaq® (n=50)	AmpliTaq Gold™ (n=50)	
β-thalassaemia primers			
• AA	32 (64%)	35 (70%)	Chi square = 1.33
• ADO	9 (18%)	10 (20%)	Degrees of freedom =2
• AF	9 (18%)	5 (10%)	p value = 0.514
HUMTH01 primers			
• AA	0 (0%)	46 (92%)	Chi square = 100.00*
• ADO	0 (0%)	4 (8%)	Degrees of freedom =2
• AF	50 (100%)	0 (0%)	p value = 0.000*

AA = appropriate or adequate amplification of both alleles, ADO = allele drop out, AF = amplification failure.

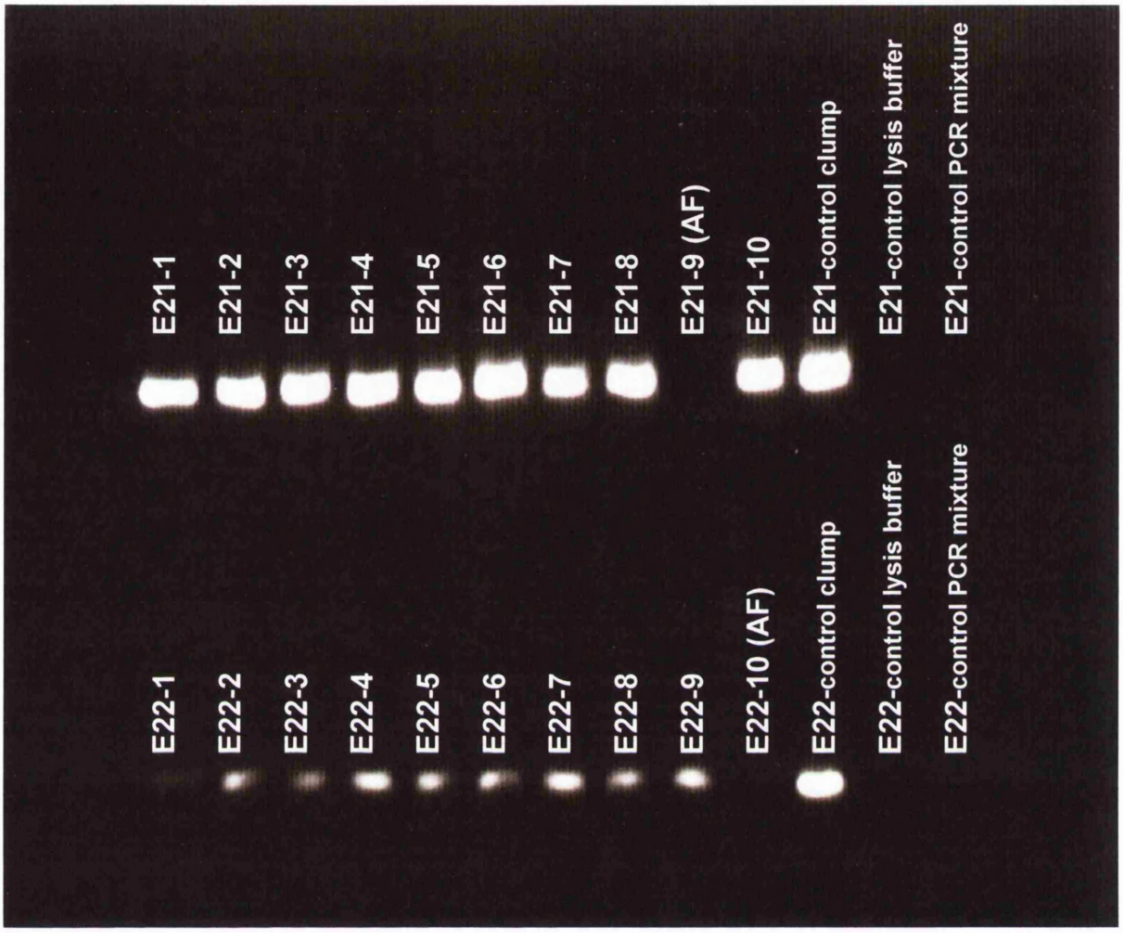


Figure 3.15 Results from 2% agarose gel electrophoresis analysis comparing single cell nested PCR products (using outer and inner β-thalassaemia primers). The bands of the amplification products using AmpliTaq Gold™ in the primary multiplex PCR step (lanes E21-) are thicker and brighter than those using SuperTaq® (lanes E22-) indicating the superior efficiency of the enzyme. (AF = amplification failure.)

The experiments optimising PCR protocols in order to obtain the appropriate amplified products for both HUMTH01 fragment and β-thalassaemia analyses revealed that the PCR protocol with too few cycles in the primary multiplex PCR step generated insufficient amplified HUMTH01 product for the analysis on ALF Express™ (**Figure 3.16**); while too many cycles gave rise to a darker background on GeneGel® and lead to

difficulty for mutation analysis. In conclusion, the optimal PCR protocol cycles were adjusted to 35 cycles for the primary multiplex PCR step and 25 cycles for the secondary nested PCR step.

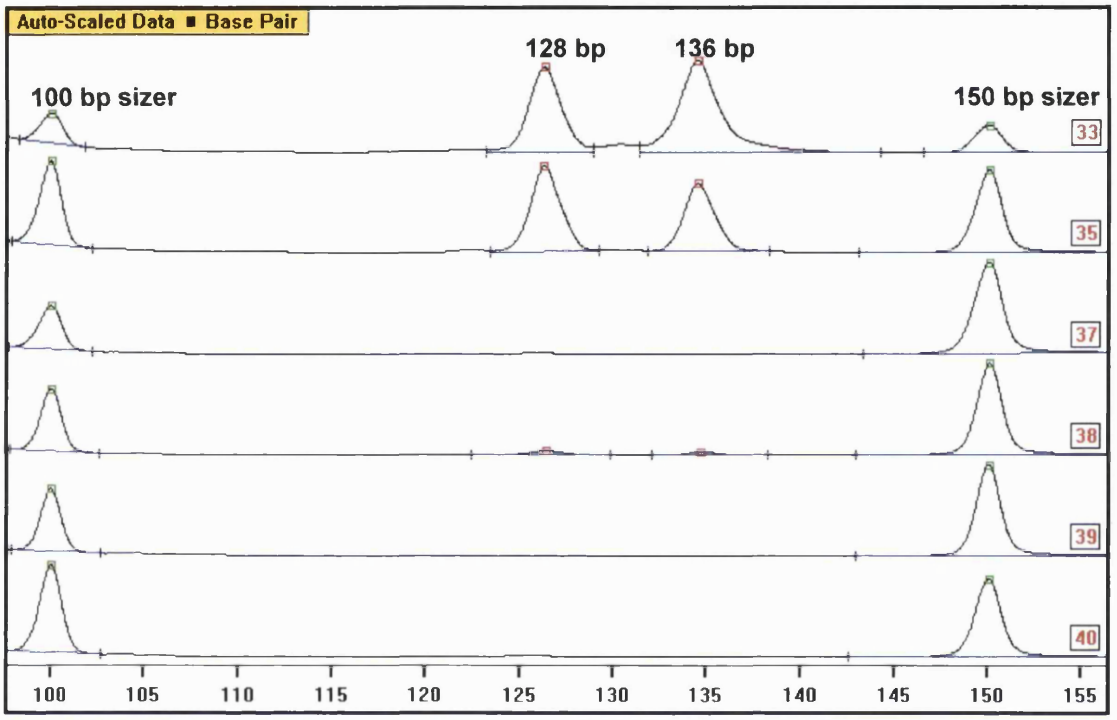


Figure 3.16 Results of HUMTH01 fragment analysis on ALF Express™ from single buccal cell multiplex products (HUMTH01 and outer β-thalassaemia primers); multiplex PCR performed for 35 cycles (lanes number 33 and 35) shows large peaks of amplified products, those performed at 30 cycles (lanes number 37 and 38) shows very small peaks in one sample, while those amplified at 25 cycles (lanes number 39 and 40) fail to show any peaks. The x and y axes are base and peak height units, respectively.

From 150 single cell PCRs, the optimised PGD protocol for β-thalassaemia, IVSI-110 mutation (**Figure 3.12**) using AmpliTaq Gold™ in the primary multiplex PCR step and SuperTaq® in the secondary nested PCR step gave amplification efficiencies of 96.7% (145/150) and 89.3% (134/150) for HUMTH01 and β-globin loci, respectively

(**Table 3.6**). The ADO rates were 9.0% (13/145) and 18.7% (25/134), respectively. The AF and ADO rates of β-globin fragments are higher than those of the HUMTH01 fragment with statistical significance (Chi square = 11.75, degrees of freedom =2, p value = 0.003*).

Table 3.6 Comparison of AF and ADO rates between HUMTH01 and β-globin genes from 150 multiplex amplified single buccal cells of IVSI-110 heterozygote subject using the protocol for single cell diagnosis of β-thalassaemia, IVSI-110.

Analysis Results	Genes		
	HUMTH01 (n=150)	β-globin (n=150)	
AA	132 (88.0%)	109 (72.7%)	Chi square = 11.75*
ADO	13 (8.7%)	25 (16.7%)	Degrees of freedom =2
AF	5 (3.3%)	16 (10.7%)	p value = 0.003*

AA = appropriate or adequate amplification of both alleles, ADO = allele drop out, AF = amplification failure.

Preferential amplification of β-globin was determined from the difference in thickness of the normal and mutant bands on the GeneGel® Excel, while the differential amplification of HUMTH01 fragment was identified by the size difference of both alleles peaks. Examples of HUMTH01 and β-thalassaemia, IVSI-110 mutation analyses with different amplification characteristics, including SA, ADO and PA are shown in **Figure 3.17** and **3.18**, respectively.

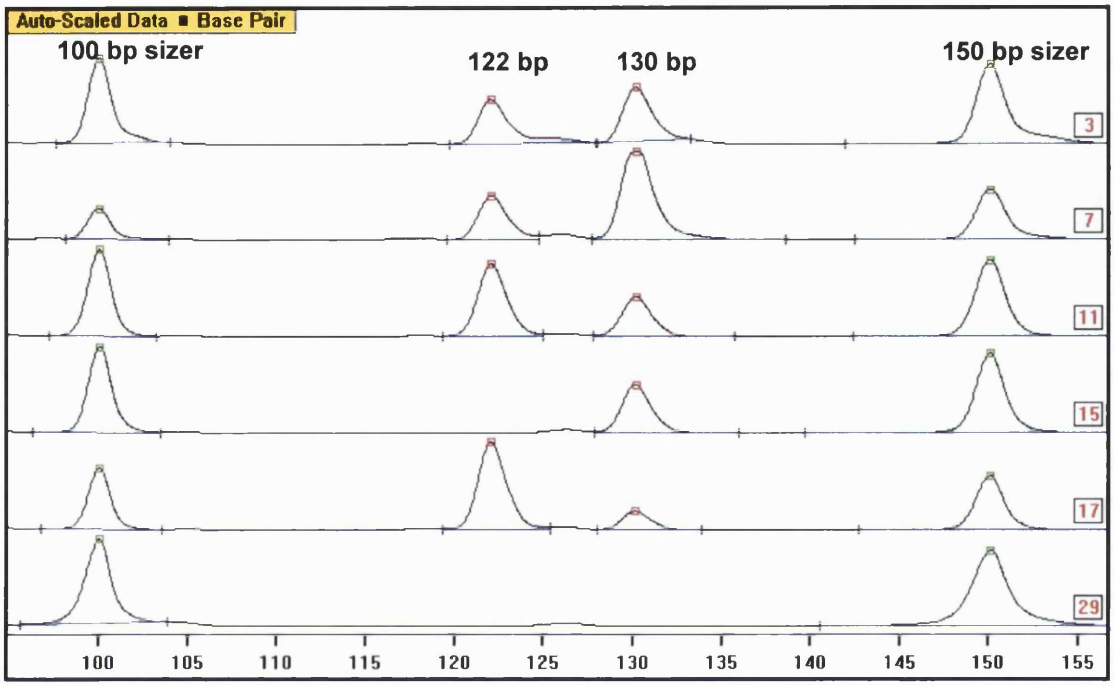


Figure 3.17 Results of HUMTH01 fragment analysis on ALF Express™ from single buccal cell (IVSI-110 heterozygote subject) multiplex products (using HUMTH01 and outer β-thalassaemia primers); show mild degree of PAL in lane 3, moderate PAL and PAS in lane 7 and 11, respectively, marked PAS in lane 17, ADOS in lane 15 and AF in lane 29. (ADOS = allele drop out of the shorter allele, PAL = preferential amplification of the longer allele, PAS = preferential amplification of the shorter allele, AF = amplification failure.) The x and y axes are base and peak height units, respectively.

The optimised PGD protocol for the β-thalassaemia IVSI-110 mutation was conducted on 24 single human blastomeres donated for research. The amplification efficiencies were 95.8% (23/24) and 91.7% (22/24) for HUMTH01 and β-globin loci respectively. ADO of the β-globin gene could not be calculated, as the embryos used in this study were not mutation carriers. As expected all amplified blastomeres gave a normal genotype after SSCP analysis of the β-globin gene. The HUMTH01 locus showed an 8.7% (2/23) ADO rate.

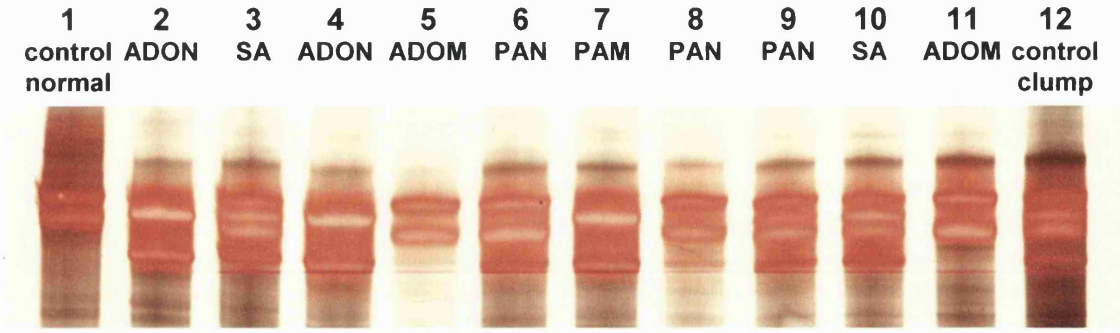


Figure 3.18 Results of β-thalassaemia analysis on GenePhor™ from single buccal cell (IVSI-110 heterozygote subject) nested products (using inner β-thalassaemia primers); show SA (lanes 3 and 10), PAN (lanes 6, 8 and 9), PAM (lane 7), ADON (lane 2 and 4) and ADOM (lans 5 and 11). (ADON and ADOM = allele drop out of the normal and mutant alleles, PAN and PAM = preferential amplification of the normal and mutant alleles, SA = symmetrical amplification of both alleles.)

3.3.3 PGD strategy for β-thalassaemia, codon 41-42 mutation

The examples of β-globin mutation detection using a fluorescent DNA sequencer (ABI Prism™310) in a family carrying the β-thalassaemia codon 41-42 mutation were demonstrated in **Figure 3.19**. Analyses of 50 single heterozygous cells using the optimised single step multiplex PGD protocol (**Section 3.2.3**) gave amplification efficiencies of 94% (47/50) and 92% (46/50) with ADO rates of 14.9% (7/47) and 23.9% (11/46) for the HUMTH01 and β-globin loci, respectively. The examples of β-thalassaemia, codon 41-42 analysis results with different characteristics, including SA, ADO and PA, are demonstrated in **Figure 3.20**. Twenty eight single blastomeres were analysed using the optimised protocol for the β-thalassaemia codon 41-42 mutation. All 25 blastomeres that were successfully amplified for the β-globin gene showed a normal genotype as expected (89.3% amplification efficiency). HUMTH01 primers exhibited a 96.4% (27/28) amplification efficiency and 7.4% (2/27) ADO rate.

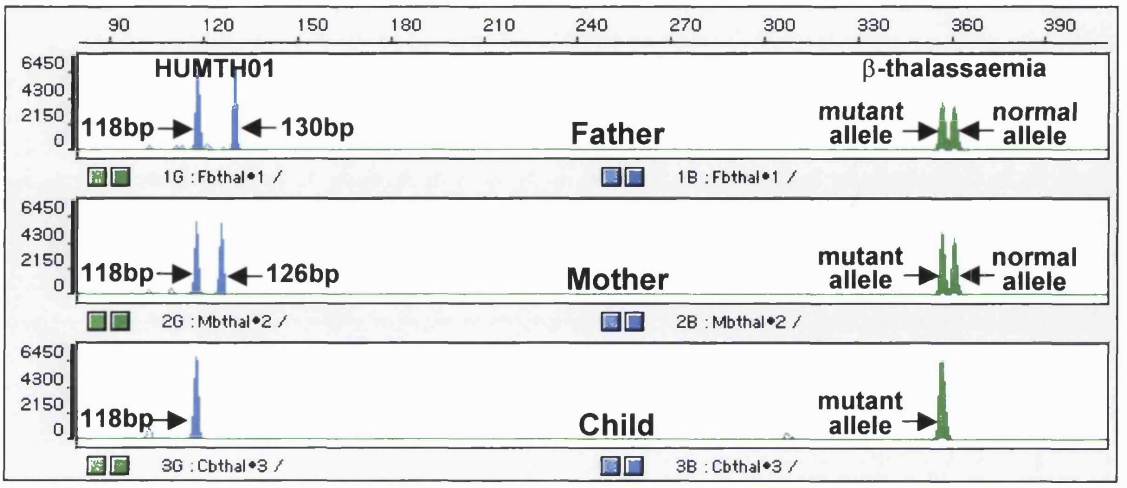


Figure 3.19 Detection of β-thalassaemia, codon 41-42 mutations using a fluorescent DNA sequencer (ABI Prism™310). Lanes 1G, 2G and 3G, analysis of a family carrying the codon 41-42 mutation using bthalw1 primers (labelled in green) and linked marker HUMTH01 (labelled in blue). Lanes 1G and 2G are heterozygote father and mother, respectively, lane 3G is the affected child. The mutant alleles of β-globin are linked to the 118 bp HUMTH01 alleles. The x and y axes are base and peak height units, respectively.

The buccal cell samples used in this study were not fresh, having been sent by post, and had spent several days at room temperature before isolation. Consequently they probably do not give an accurate representation of the performance of the protocols described. Those used in the β-thalassaemia IVSI-110 mutation studies were 1-2 days old, while those used to assess the codon 41-42 mutation were 5-6 days old and exhibited higher ADO rates. The use of fresh blastomeres substantially improved results, in some cases halving the incidence of ADO.

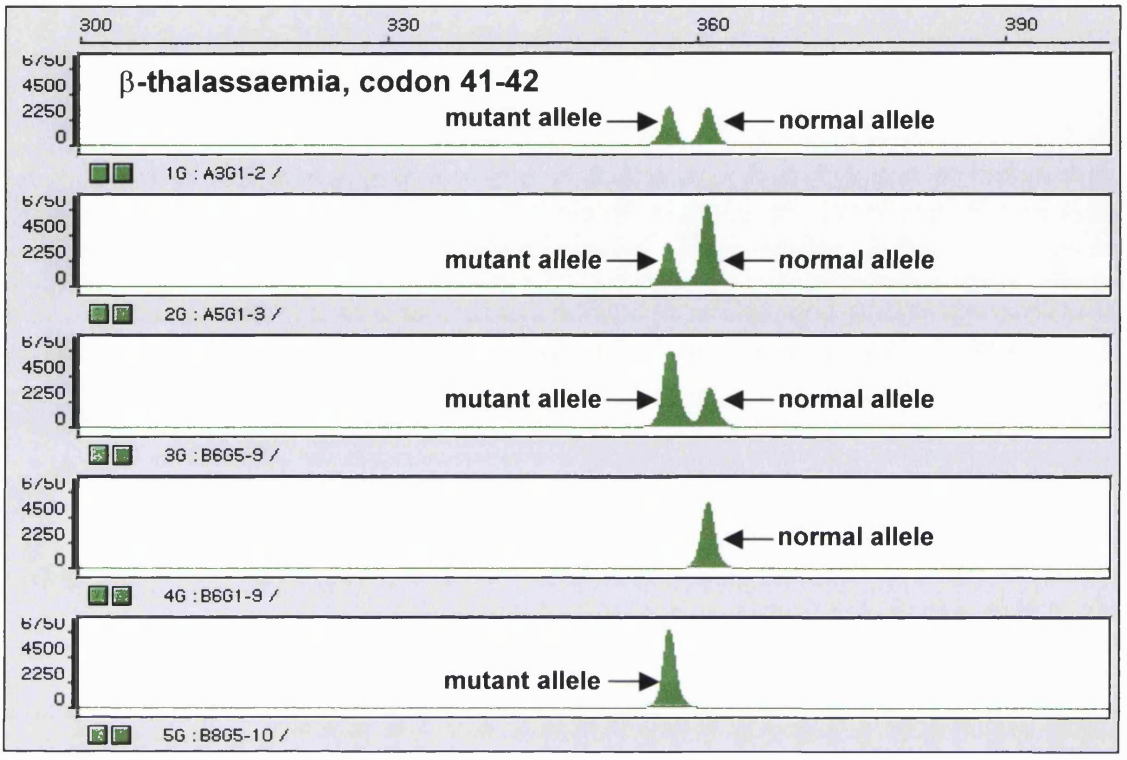


Figure 3.20 Results of β-thalassaemia, codon 41-42 analysis on ABI Prism™310 from single buccal cells of a heterozygote β-thalassaemia, codon 41-42 subject following multiplex F-PCR using bthalw1 (labelled in green) primers. Lanes 1G, 2G, 3G, 4G and 5G are amplified products with symmetrical amplification (SA), preferential amplification of the normal allele (PAN), preferential amplification of the mutant allele (PAM), allele drop out of the mutant allele (ADOM) and allele drop out of the normal allele (ADON), respectively. The x and y axes are base and peak height units, respectively.

3.3.4 PGD strategy for α-thalassaemia, --^{SEA} mutation

The examples of fluorescent gap PCR analysis in a family carrying the α-thalassaemia SEA mutation are shown in **Figure 3.21**. The peak sizes of the HUMTH01 alleles and the normal and mutant alleles of the α-globin gene of each

sample were recorded and scored for ADO and preferential amplification. Preliminary analysis of buccal cells from an α-thalassaemia SEA mutation carrier using fluorescent gap PCR alone gave an amplification efficiency of 90% (27/30) and ADO rate of 22.2% (6/27). When using multiplex PCR, with the addition of HUMTH01 primers, analysis showed 91.8% (45/49) and 81.6% (40/49) amplification efficiencies for HUMTH01 and α-thalassaemia respectively and ADO rates of 11.1% (5/45) and 37.5% (15/40). The PGD protocol for the α-thalassaemia, SEA mutation was carried out on 24 single blastomeres. Fluorescent gap PCR demonstrated 91.7% (22/24) amplification efficiency and gave a normal genotype in all amplified blastomeres, as expected. HUMTH01 primers gave 95.8% (23/24) amplification efficiency and 8.7% (2/23) ADO rates.

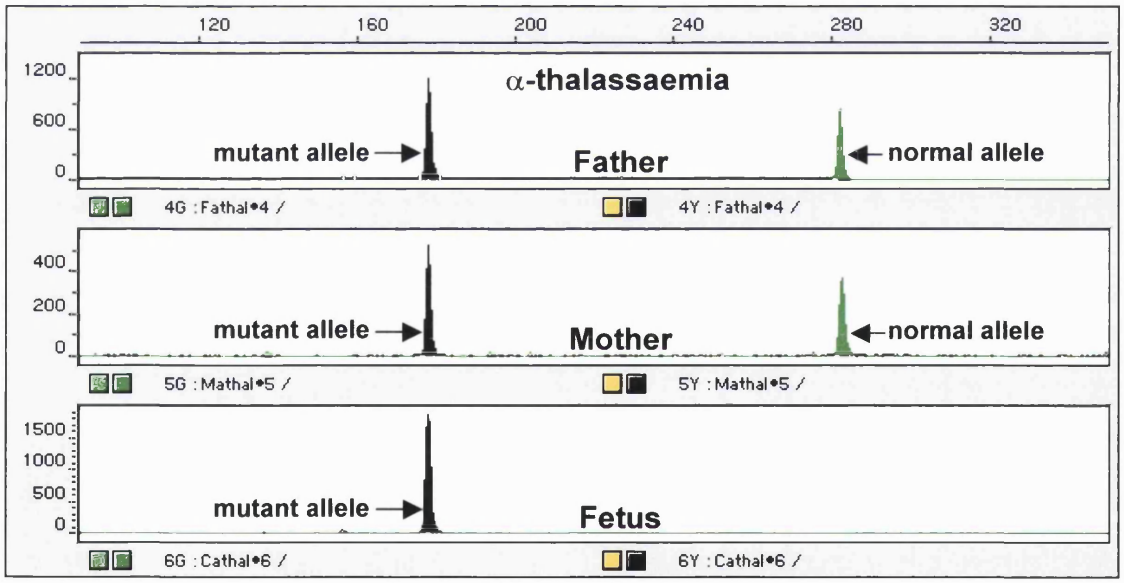


Figure 3.21 Detection of α-thalassaemia, SEA mutations using a fluorescent DNA sequencer (ABI Prism™310). Lanes 4G, 5G and 6G, analysis of an α-thalassaemia SEA mutation family following multiplex fluorescent gap PCR. Lanes 4G and 5G are heterozygote father and mother, respectively, lane 6G is the affected fetus. The x and y axes are base and peak height units, respectively.

3.3.5 Single cell sequencing

The single cell sequencing results of the β-thalassaemia IVSI-110 (G→A) mutation, which is a single nucleotide substitution, are demonstrated in **Figure 3.22-3.25**. In samples with symmetrical amplification (SA) of both alleles, sequencing allows detection of the normal (G) and mutant (A) nucleotides at position 110 of Intron 1 of the β-globin gene showing proportional size (**Figure 3.22**). Only normal (G) or mutant (A) nucleotides are shown at nucleotide 110 of Intron 1 for the ADOM and ADON samples, **Figure 3.23** and **3.24**, respectively. **Figure 3.25** shows both normal (G) and mutant (A) nucleotides with a bigger peak height of the normal (G) nucleotide than that of the mutant (A) for the PAN sample. The sequencing results of every sample correspond to the previously determined silver stained SSCP analysis (**Section 3.3.2**). Although the sequence analysis software mistyped some samples with preferential amplification, most could be correctly genotyped by eye. Cases with extreme PA, in which one allele could scarcely be detected above the background level of fluorescence, could not be typed with confidence, but were rare.

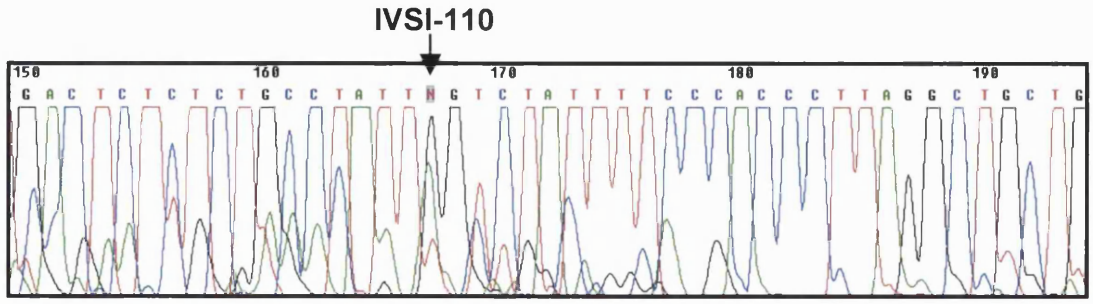


Figure 3.22 The single cell sequencing result of the β-globin gene shows both G and A nucleotides at IVSI-110 (arrow) in the β-thalassaemia, IVSI-110 (G→A) Ht sample with SA (from silver stained SSCP analysis). (SA = symmetrical amplification of both alleles; blue = C, green = A, yellow/black = G, red = T nucleotides.)

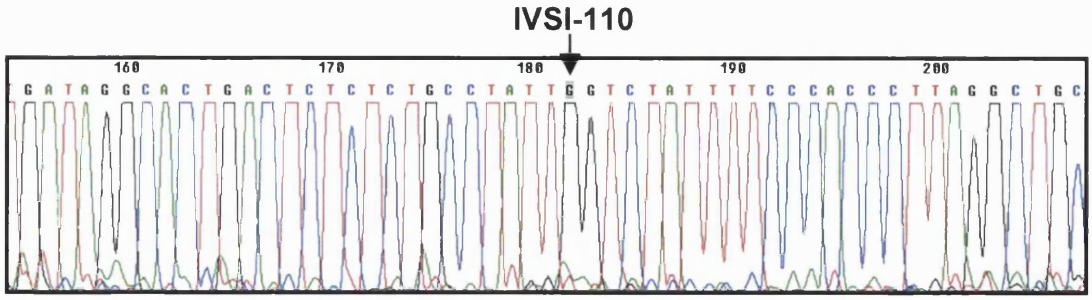


Figure 3.23 The single cell sequencing result of the β-globin gene shows only G nucleotide at IVSI-110 (arrow) in the β-thalassaemia, IVSI-110 (G→A) Ht sample with ADOM (from silver stained SSCP analysis). (ADOM = allele drop out of the mutant allele; blue = C, green = A, yellow/black = G, red = T nucleotides.)

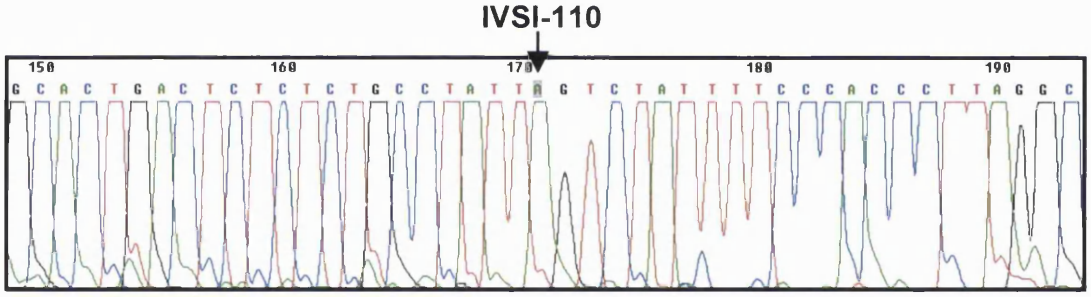


Figure 3.24 The single cell sequencing result of the β-globin gene shows only A nucleotide at IVSI-110 (arrow) in the β-thalassaemia, IVSI-110 (G→A) Ht sample with ADON (from silver stained SSCP analysis). (ADON = allele drop out of the normal allele; blue = C, green = A, yellow/black = G, red = T nucleotides.)

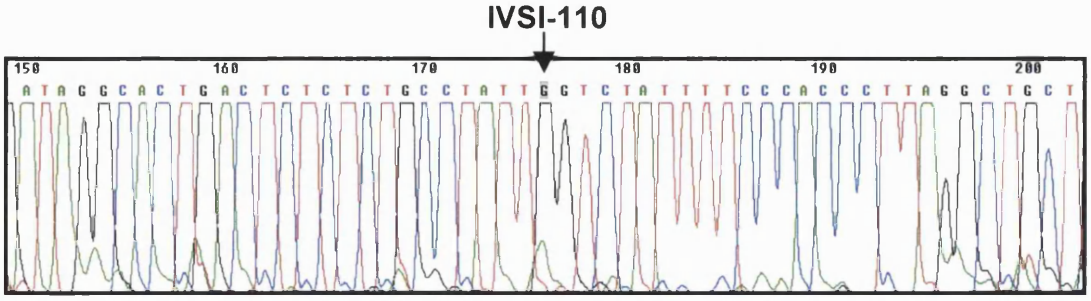


Figure 3.25 The single cell sequencing result of the β-globin gene shows both G and A nucleotides (G bigger than A) at β-thalassaemia, IVSI-110 (arrow) in the IVSI-110 (G→A) Ht sample with PAN (from silver stained SSCP analysis). (PAN = preferential amplification of the normal allele; blue = C, green = A, yellow/black = G, red = T nucleotides.)

In order to have clearer pictures of different amplification characteristics, including SA, ADO and PA, on the single cell sequencing results, the single buccal cell samples of a heterozygote subject with a frameshift mutation, β-thalassaemia codon 41-42 (-TCTT) mutation, which is a 4-nucleotide deletion, were employed. The PCR products with SA, ADON, ADOM, PAN and PAM from F-PCR analyses (**Section 3.3.3**) were processed with the single cell sequencing protocol. The sample with symmetrical amplification of both normal and mutant alleles shows the sequences of both alleles ('**TCTT**TGAGTCCTTTGGG' and 'TGAGTCCTTTGGGGATC') superimposed after the frameshift site with similar peak heights (**Figure 3.26**). Only the sequences of the mutant allele is present in the sample with ADON (**Figure 3.27**). In the sample with PAM, the sequences of both normal and mutant alleles can be visualised, but with the taller peak heights corresponding to those of the mutant allele than those the normal allele (**Figure 3.28**). These results support the previous genotyping results from GeneScan™ analysis (**Section 3.3.3**).

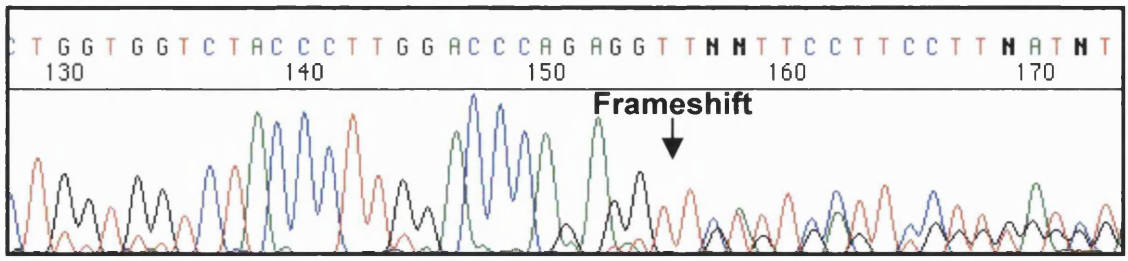


Figure 3.26 The single cell sequencing result of the β-globin gene shows the peaks of the normal (TCTTTGAGTCCTTTGGG) and mutant (TGAGTCCTTTGGGGATC) sequences, in the frameshift region (after the deletion point, arrow) in the β-thalassaemia, codon 41-42 (-TCTT) Ht sample with SA (from F-PCR analysis). (SA = symmetrical amplification of both alleles; blue = C, green = A, yellow/black = G, red = T nucleotides.)

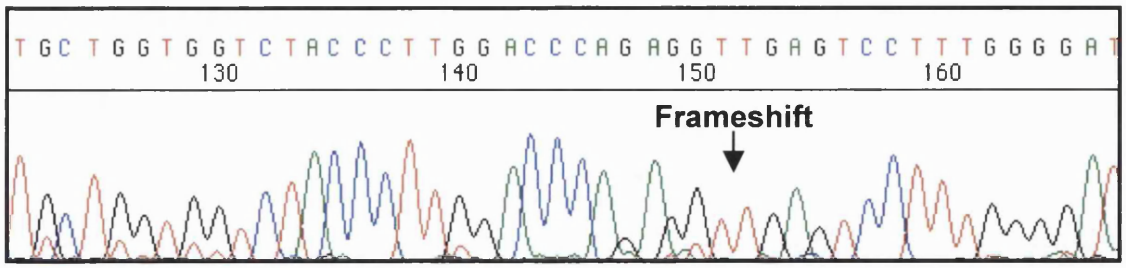


Figure 3.27 The single cell sequencing result of the β-globin gene shows only the peaks of the mutant (TGAGTCCTTTGGGGATC) sequences, in the frameshift region (after the deletion point, arrow) in the β-thalassaemia, codon 41-42 (-TCTT) Ht sample with ADON (from F-PCR analysis). (ADON = allele drop out of the normal allele; blue = C, green = A, yellow/black = G, red = T nucleotides.)

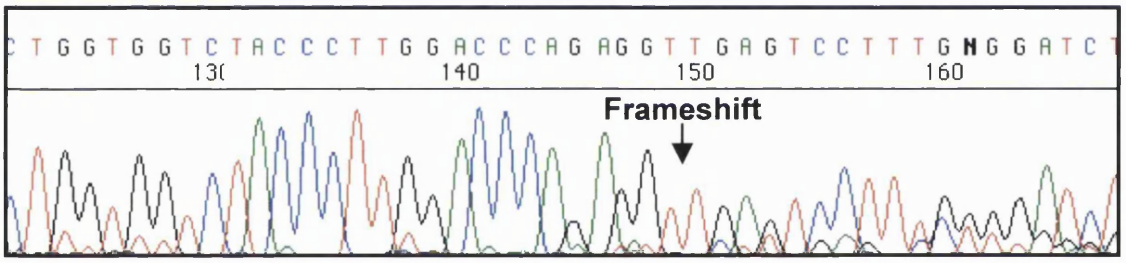


Figure 3.28 The single cell sequencing result of the β-globin gene shows the peaks of the normal (TCTTTGAGTCCTTTGGG) and mutant (TGAGTCCTTTGGGGATC) sequences (mutant allele bigger than normal allele), in the frameshift region (after the deletion point, arrow) in the β-thalassaemia, codon 41-42 (-TCTT) Ht sample with PAM (from F-PCR analysis). (PAM = preferential amplification of the mutant allele; blue = C, green = A, yellow/black = G, red = T nucleotides.)

These single cell sequencing protocols were applied to single human blastomeres donated for research and all blastomeres produced normal DNA sequence, indicating absence of any β-thalassaemia-causing mutations encompassed by the primers. The parents that donated the embryos were not suspected of having β-thalassaemia mutations and thus a normal DNA sequence was expected.

3.3.6 Single cell minisequencing (SNaPshot™)

The minisequencing (SNaPshot™) protocol (**Section 3.2.6**) was applied on single cell PCR products of the β-thalassaemia, IVSI-110 (G→A) Ht with known amplification characteristics (from SSCP analysis, **Section 3.3.2**). Both normal and mutant alleles are seen in the minisequencing result of the sample with equal amplification of both alleles (**Figure 3.29 a**). Only the normal and mutant alleles were seen in the minisequencing analysis of the samples with ADOM and ADON (**Figure 3.29 b** and **c**). This protocol was applied to single human blastomeres donated from the parents whom were not suspected of carrying β-globin mutations and the results showed only the normal allele as expected.

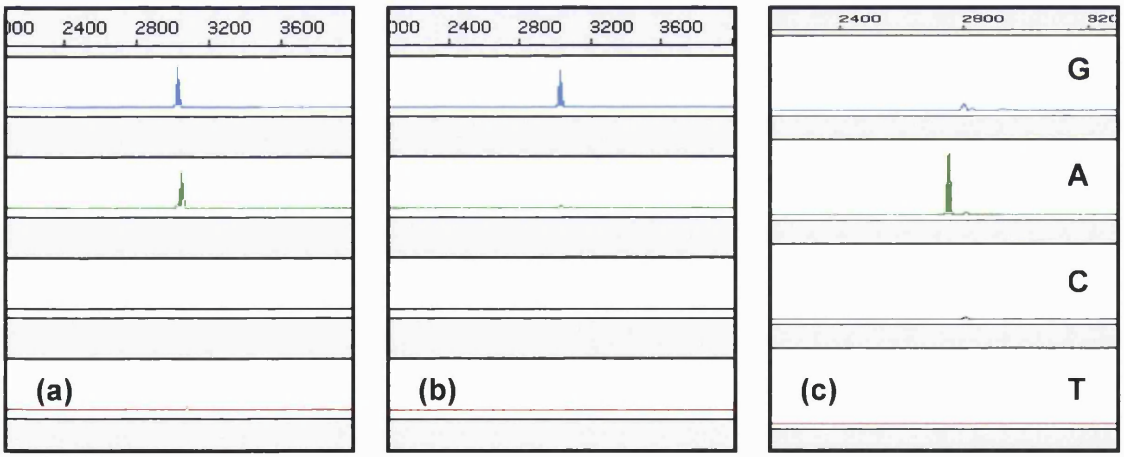


Figure 3.29 The single cell minisequencing results of the β-thalassaemia, IVSI-110 (G→A) mutation: **(a)** both G and A nucleotides are present in the Ht sample with symmetrical amplification (from silver stained SSCP analysis), **(b)** only G nucleotide is present in the Ht sample with ADOM, and **(c)** only A nucleotide is present in the Ht sample with ADON. (Blue = G, green = A, yellow/black = C, red = T nucleotides.)

The minisequencing protocol was also applied to the single cell PCR products of the β-thalassaemia, codon 41-42 Ht and sickle Ht mutations. Both single cell and clump cell samples of the heterozygote codon 41-42 genotype show both normal and mutant alleles, while the normal control sample shows only the normal allele in the minisequencing analysis (**Figure 3.30 a, b** and **c**). The minisequencing analyses of single cell and clump cell samples of the heterozygote sickle mutation exhibited both normal and mutant alleles (**Figure 3.31 a** and **b**). When the protocol was applied to normal control DNA, only the normal allele was seen as expected (**Figure 3.31 c**).

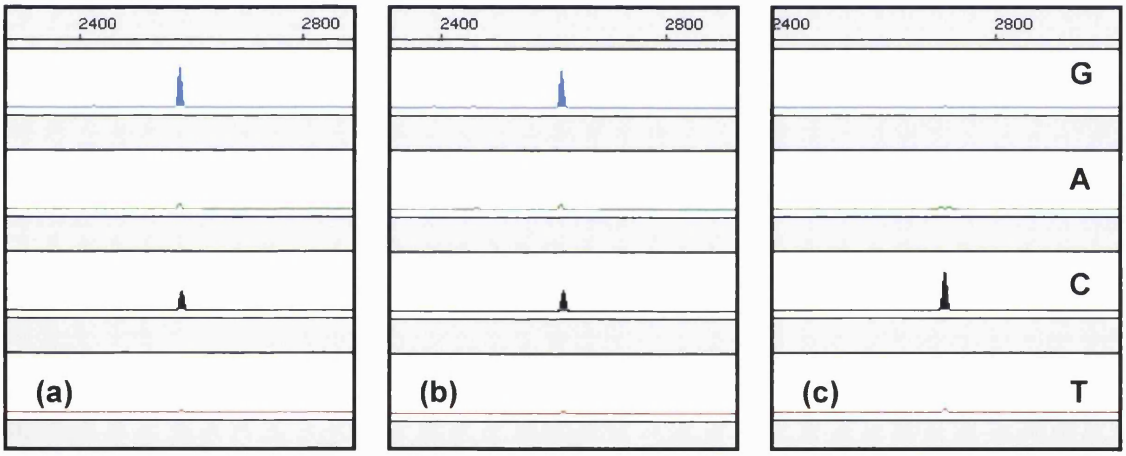


Figure 3.30 The minisequencing results of the β-thalassaemia, codon 41-42 Ht (normal C – mutant G) mutation: **(a)** Ht single cell sample shows both C and G nucleotides, **(b)** Ht clump cell sample shows both C and G nucleotides, and **(c)** normal control DNA sample shows only C nucleotide. (Blue = G, green = A, yellow/black = C, red = T nucleotides.)

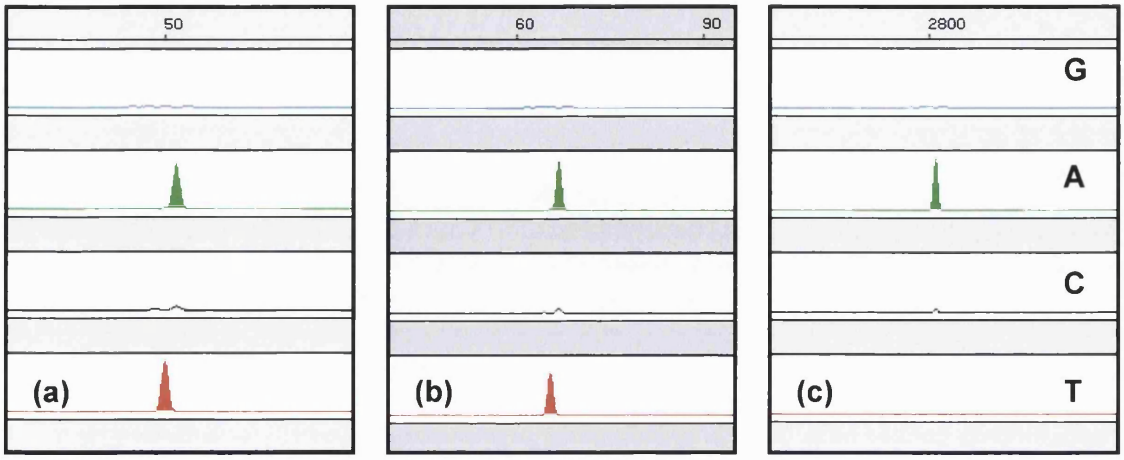


Figure 3.31 The minisequencing results of the sickle Ht (A→T) mutation: **(a)** Ht single cell sample shows both A and T nucleotides, **(b)** Ht clump cell sample shows both A and T nucleotides, and **(c)** normal control DNA sample shows only A nucleotide. (Blue = G, green = A, yellow/black = C, red = T nucleotides.)

3.4 Discussion

3.4.1 Mutation detection of β-thalassaemia

The mutations with size alteration, i.e. deletions and insertions, can be diagnosed simply by molecular analysis using traditional electrophoresis because of the difference in migration rate for DNA fragments of differing size. Single base pair substitution is the most problematic for molecular diagnosis as its migration characteristics on conventional gel electrophoresis are similar to the normal sequences. For this reason, various techniques, including restriction fragment length polymorphism (RFLP) (Old *et al.*, 1984), amplification refractory mutation system (ARMS) (Old *et al.*, 1990) and reverse dot-blot hybridisation (Winichagoon *et al.*, 1999) have been introduced for prenatal

diagnosis from uncultured amniocytes or chorionic villous samples of β-globin mutations. However, there are disadvantages with these methods; a different enzyme is needed for each mutation detection in RFLP; specific primers are used for each mutation in ARMS; and a particular allele-specific oligonucleotide (ASO) probe can hybridise with only one mutation in dot-blot hybridisation. A widely applicable analysis system that can detect several mutations at one time could allow diagnosis of multiple mutations within a single DNA fragment, thus simplifying the detection of compound heterozygotes. This is potentially useful for genes with a heterogeneous spectrum of mutations. In turn this should speed up the rate at which single cell diagnostic protocols can be devised. Single strand conformation polymorphism (SSCP) (**Section 2.2.7.3**) is a scanning technique that can identify the majority of single base pair changes, deletion and insertion mutations (Orita *et al.*, 1989) which can be useful for β-thalassaemia mutation analysis.

Silver stained SSCP analysis has been suggested as a quick and sensitive method for β-thalassaemia mutation detection, especially for mutations IVSI-1 (G→A), IVSI-1 (G→T), IVSI-5 (G→C), IVSI-6 (T→C) and IVSI-110 (G→A), FScodon 5 (-CT), FS8-9 (+G), FScodon 41-42 (-TCTT) and haemoglobin sickle (El-Hashemite *et al.*, 1997). The experiments in this study using the same sets of primers, PCR conditions and SSCP analysis conditions found that only the IVSI-110 mutation could be reliably identified using the reported technique. A wide range of additional SSCP conditions and different concentrations of polyacrylamide gels were also tested without a more favourable outcome. The results from this extensive investigation indicated that the methods published by El-Hashemite *et al.* (1997) were difficult to reproduce for some mutations. The lack of reproducibility suggests that the reported methods are not sufficiently robust for clinical application to any of the β-thalassaemia mutations, save IVSI-110.

The band patterns of SSCP analysis of IVSI-110 mutation in **Figure 3.9** demonstrate a good example of the SSCP concept, as a normal amplified DNA fragment consists of two strands when denatured, sense and antisense, which adopt different conformations determined by their DNA sequence. These conformations are subsequently expressed as two different bands on the polyacrylamide gel due to differences in mobility. In the same way, a fragment amplified from a mutant gene would show another two bands with different locations from the bands of the normal gene. As a result, four bands are observed in a heterozygote sample (**Figure 3.9**). However, it is possible that some different conformations share the same mobility, in which case the migration pattern on the gel gives fewer bands than expected. Additionally, some molecules produce more than one conformation and thus give more bands than expected, which can lead to a problematic interpretation of SSCP analysis. F-SSCP analysis on ALF Express™ was unsuccessful in identifying β-thalassaemia mutations despite several variations of conditions. This might be due to the property of the MDE® acrylamide gel. Experiments using other compositions of the polyacrylamide gel from different companies may succeed in the detection of the β-thalassaemia mutation.

Besides the DNA sequence of the gene fragments, the factors affecting SSCP analysis include temperature, running time and gel composition. Temperature appeared to be the most influential parameter as under the same gel composition and electrophoresis conditions, some mutant genes could be identified only at a particular temperature. The appropriate running time depends on the test fragment size. The longer fragment size usually needs a longer running time to be able to differentiate the different genotypes on the SSCP. Using 20% PhastGel®, the higher concentration of polyacrylamide gel was superior for the discrimination of the single base pair difference. This might be because of its higher resistance property. However, using a lower concentration polyacrylamide gel,

if running at a longer distance, i.e. 12.5% GeneGel®, the differentiation of the base pair differences was also possible and sometimes was even clearer due to the larger size of the gel.

Another important factor concerned the design of primers for the β-globin gene. When the β-thalassaemia primers were mapped to the β-globin gene sequences (**Figure 3.32**), the mutations IVSI-1, IVSI-5, IVSI-6 and IVSI-110 are included within the inner β-thalassaemia primers and the mutations IVSI-1, IVSI-5, IVSI-6, IVSI-110, FScodon 5, FS8-9 and haemoglobin sickle could be amplified by the outer β-thalassaemia primers. β-Globin is part of a family of closely related genes that share significant homology at the DNA sequence level. **Figure 3.33** shows the comparison of the sequences in promoter region, exon1, intron1 and exon2 of β-globin and δ-globin genes (http://www.ncbi.nlm.nih.gov/; g455025). From the 1,080 bp shown, the sequences of both genes match in 95.5% of nucleotide positions (highlighted). Therefore, some carelessly designed primers may amplify the δ-globin gene in addition to the β-globin gene. Amplified δ-globin gene fragments can interfere with the interpretation of mutation analysis in β-globin. The outer β-thalassaemia primers also amplifies the δ-globin gene (**Figure 3.34**) giving a fragment of 277 bp which is very close to the fragment amplified from the β-globin gene (279 bp) and may conceal the band patterns of the β-globin mutations. It was also noticed that the mutation FScodon 41-42, which was claimed by the published data to be successfully identified using this protocol (El-Hashemite *et al.*, 1997), was on top of the reverse outer β-thalassaemia primer (**Figure 3.34**); thus, the mutant gene would never be amplified. Therefore, the primers need to be re-designed for other mutations. The newly designed bthalw1 primers in this study are based on sequences unique to the β-globin gene.

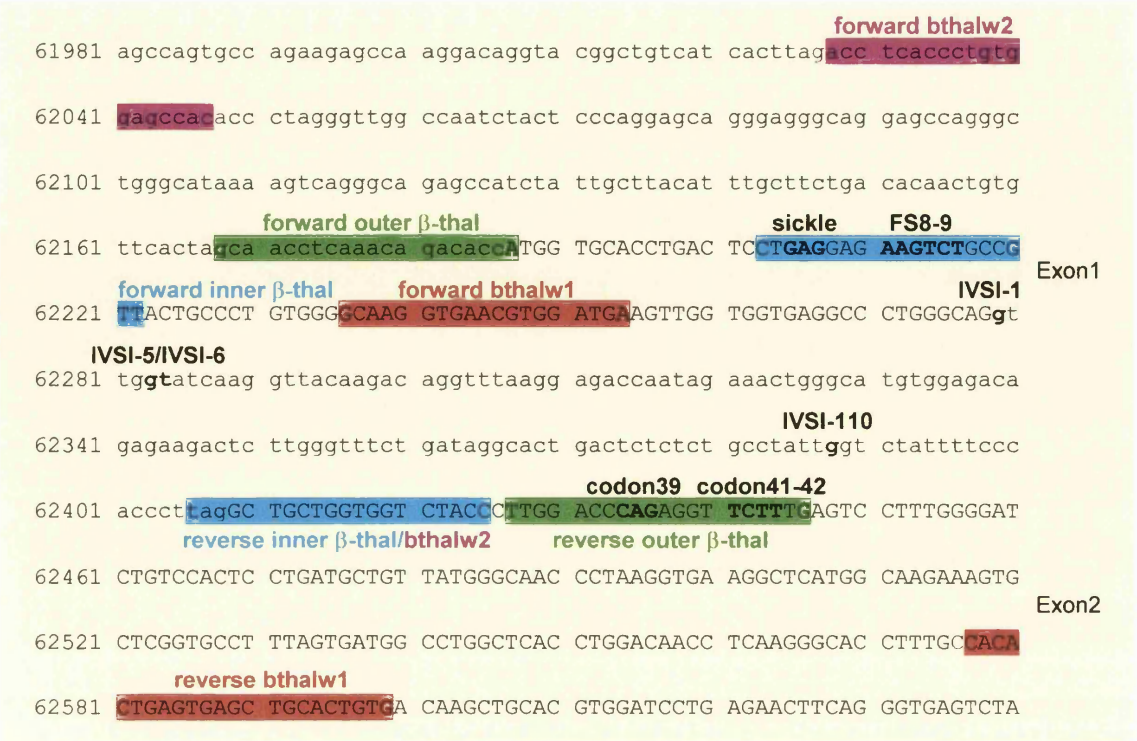


Figure 3.32 β-Globin gene sequences with mutations and primers mapping: green = outer β-thalassaemia primers, blue = inner β-thalassaemia primers, red = bthalw1 primers, pink/blue = bthalw2 primers (http://www.ncbi.nlm.nih.gov/; g455025).

Figure 3.33 The sequences of the promoter region, Exon1, Intron1 and Exon2 of the β-globin gene (black) compared with those of the δ-globin gene (green). Identical sequences of both genes are highlighted in red (http://www.ncbi.nlm.nih.gov/; g455025).

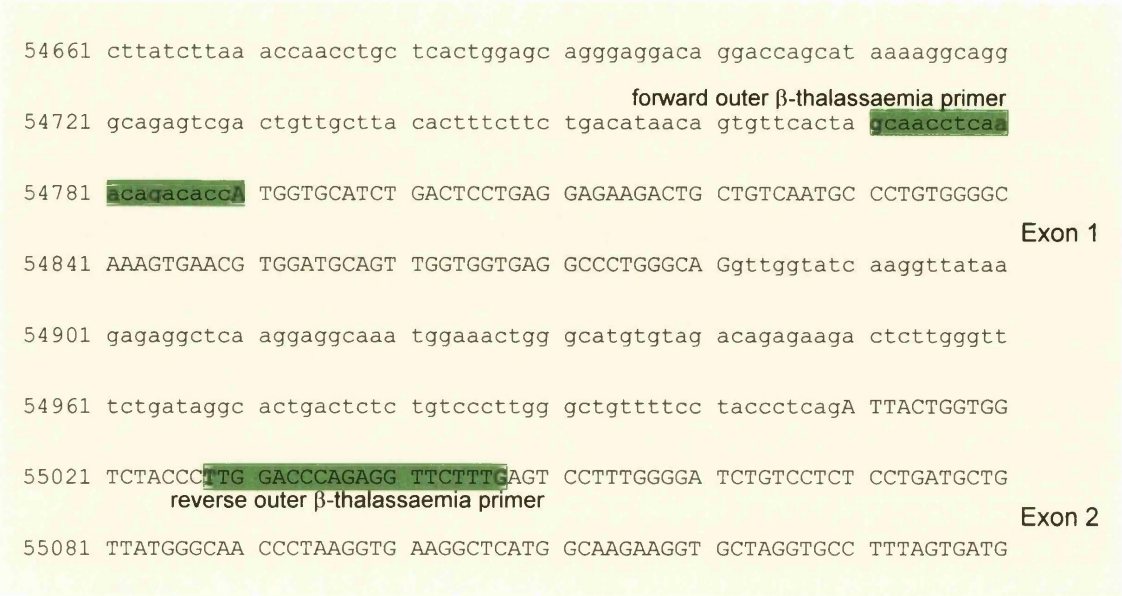


Figure 3.34 δ-Globin gene sequences with mapping of β-thalassaemia primers: green = outer β-thalassaemia primers sequences. (http://www.ncbi.nlm.nih.gov/; g455025).

In comparison of SSCP analysis using Phastsystem™ or Genephor™ (**Section 3.3.1**), the PhastSystem™ provided the advantages of the high concentration (20%) polyacrylamide used, which was suitable for identifying some mutations, while the concentrations of the polyacrylamide gel of the GeneGel® were 12.5% and 15%. However, the mini PhastGel® was quite small and could cause difficulty in handling and reading the band patterns, while the GeneGel® was much larger and provided clearer bands. Loading the very tiny amount of samples on to the PhastGel® surface needed a practiced technique, whereas direct transfer of the samples to the preformed wells of GeneGel® was simpler. Programming the electrophoresis file of the PhastSystem™ was sometimes confusing but the control panel of the GenePhor™ Electrophoresis Unit was straightforward. Preparing nine fresh staining solutions for each PhastGel® was rather tedious, and the convenient automated developing chamber occasionally caused problems in gel quality and spoiled the analyses. Fewer types of fresh solutions were needed for the

GeneGel®, but silver staining was performed manually. In conclusion, the detection of the mutation IVSI-110, which is the commonest β-thalassaemia mutation in the Mediterranean population (Flint *et al.*, 1998), could be achieved by SSCP analysis on both the PhastSystem™ and the GenePhor™. The GenePhor™ is a preferable instrument for SSCP analysis as it is less complicated and produces clearer results, while the PhastSystem™ is still useful because of the additional choices of high concentration polyacrylamide gels which may help in detecting more mutations.

F-SSCP analysis on ALF Express™ has been proved to be a sensitive and reliable tool for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (Ioulianos *et al.*, 2000) and cystic fibrosis mutation detection (Ioulianos *et al*, unpublished data); however, it failed to detect the β-thalassaemia mutations in this study (**Section 3.3.1**). Many different polyacrylamide gel compositions, gel temperatures and electrophoresis conditions were attempted. However, samples of the same genotype in the adjacent lanes showed different band (peak) patterns, while those of different genotypes sometimes showed an identical pattern. This indicated the difficulty in reproducibility of this technique using MDE® acrylamide gel. However, there are other compositions of polyacrylamide gels from different companies, which have not been tested in this study, that may be informative for β-thalassaemia detection. The use of the size standard in the F-SSCP analysis can help in aligning the band pattern in different lanes equally, subsequently improving the confidence in the band pattern determination when compared with the controls. F-SSCP application for β-thalassaemia diagnosis would provide the advantages of the automated fluorescence sequencer technology. The high sensitivity of fluorescent PCR means that the products from just one PCR amplification can be analysed without the need for nested amplification, as well as clear cut identification of

peaks. Applying this technique to the ABI Prism™377 and the ABI Prism™3100, which can visualise 4 fluorescent dyes, would increase the number of loci that can be simultaneously amplified.

3.4.2 PGD strategy for β-thalassaemia, IVSI-110 mutation

The important issues of concern when developing a PGD protocol are the selection of a method that can amplify from a single copy of the template, the limited timeframe for diagnosis (as the embryos have to be transferred to the uterus) and the accuracy of the test, as misdiagnosis is unacceptable. The previous PGD protocols for β-thalassaemia suggested ARMS (Sherlock *et al.*, 1998), RFLP (Kuliev *et al.*, 1999) and SSCP (El-Hashemite *et al.*, 1997). Molecular analyses using ARMS or RFLP need specific primers or restriction enzymes for each mutation. The preliminary attempt in applying ARMS using F-PCR for single cell diagnosis revealed nonspecific annealing between the normal primer and the mutant template when the samples were homozygote affected, which could lead to misinterpretation of homozygote affected samples. This occurred in 8% of cases (Sherlock *et al.*, 1998). Analysis by RFLP relies on the existence of restriction enzymes that can cleave the mutation site. Incomplete digestion may result in homozygous affected samples being diagnosed as heterozygote carriers. In order to avoid the transfer of affected embryos in the case of autosomal recessive disorders, the use of a restriction enzyme that digests the normal allele is recommended. In this case incomplete digestion will not result in the transfer of a homozygote affected embryo. Because of its reliance on physical properties of the DNA strand, rather than enzymatic action, and because it can identify multiple mutations, SSCP seems to be a superior technique for developing PGD protocols.

In addition to direct amplification of the causative mutation the protocols described included a highly polymorphic marker (HUMTH01) for contamination detection (**Figure 3.12**). The embryos produced by a given couple can only inherit a limited combination of alleles (one allele from each parent). If any biopsied blastomeres do not give one of these predictable combinations it is likely that they are contaminated. Indicators of contamination include the detection of an allele not present in either parent, or of three parental alleles rather than two (possibly sperm or cumulus cell contamination). Although the use of a single polymorphism falls a long way short of a true genetic fingerprint it does represent an extremely useful form of contamination detection. In the analysis of myotonic dystrophy this strategy has allowed the detection of contamination restricted to a single PCR tube (**Sections 4.3.3.1**). In the event that a couple share a HUMTH01 allele in common (i.e. are not fully informative) other polymorphic microsatellite loci can be substituted. The fact that HUMTH01 is closely linked to β-globin means that, in an informative family, it can also provide back up diagnostic information (Rechitsky *et al.*, 1998). This is useful in cases where ADO has affected the mutation site leading to confusing results. The probability of ADO affecting the amplification of the mutation site and HUMTH01 in the same reaction is low. As long as one of the loci is accurately amplified both alleles will be detected.

Experiences during the development of the PGD protocol for the β-thalassaemia, IVSI-110 mutation (**Section 3.3.2**) revealed that the type of *Taq* polymerase enzyme used significantly affects the efficiency of multiplex PCR at the single cell level. AmpliTaq Gold™ is a modified polymerase that remains inactive until exposed to high (denaturing) temperatures. Its success probably reflects superior amplification efficiency of repetitive sequences and the reduced interaction between primers afforded by 'hot-start'.

HUMTH01 and IVSI-110 fragments could be amplified separately using various polymerase enzymes, but multiplex was only possible using AmpliTaq Gold™, indicating that primer-primer interaction probably caused the amplification failure. In addition to the hot-start effect, another advantage of the heat activating property of the AmpliTaq Gold™ is that when exposed to a temperature of 94°C for 12 min, about 60% of the enzyme is activated. This meets the need of the amount of enzyme in the reaction tube where the starting templates possess low copy number at the very first cycles. The rest of the enzyme is gradually activated during the denaturation step of the thermal cycle program. This provides more active enzyme in the later cycles where there are more copies of the templates. When using conventional polymerase enzymes, all of the added enzyme is active from the start of the reaction. Towards the end of the reaction much of the *Taq* has become inactive, and yet there are more copies of the templates, which need more enzyme. Moreover, the comparison of the β- and δ–globin gene sequences (**Figure 3.33**) suggested that this reduced amplification efficiency in the first multiplex PCR step might result from the ability of the outer β-thalassaemia primers to amplify the δ–globin gene in addition to the β-globin gene. Thus, three loci were amplified in the same reaction, as a triplex PCR rather than a duplex PCR, and competed with each other for the polymerase enzyme. The problem was solved by switching to a more efficient enzyme. It suggested that primer-primer interactions were probably to blame. However, this situation only occurs in the presence of low copy number of the template, in particular a single copy, because multiplex-PCR using SuperTaq® worked well using DNA samples extracted from cell clumps as the template in the multiplex amplification using both sets of primers.

This study demonstrated the advantage of nested PCR (**Section 2.2.5.4**) in increasing the specificity of PCR, as the primary PCR step using outer β-thalassaemia

primers amplified both β- and δ-globin genes, while the secondary nested PCR using inner β-thalassaemia primers specifically amplified only the β-globin gene. Nested PCR can also prevent contamination by PCR products from the inner β-thalassaemia primers. The inner PCR amplifies fragments to extremely high levels. These build up in the lab where analysis is conducted. However they do not cause a significant contamination risk, as the outer set of primers cannot anneal to this fragment. However, the contamination by other genomic DNA or the amplified products from the outer set of primers would not be precluded using nested PCR. In these cases, the contaminant templates have the potential to be amplified as well as the intended templates and to superimpose upon or obscure the real results from every analysis. Therefore, the concept of confirming the results using two different analytical techniques would give the same misdiagnosed conclusion since the contaminant's DNA material would be present in all aliquots. The amplification of a highly polymorphic STR marker, as in the PGD protocol for β-thalassaemia, can help in identifying the presence of such contamination because, in most cases, the sample from an embryo would possess two alleles, one from each of the parents, and the presence of more than two alleles suggests contamination.

Amplification failure can result from a number of causes, including failure to transfer the single cell into the tube, choosing an anucleate cell, ineffective lysis or amplification conditions. The single cell can be checked under the microscope after transferring into the tube; although it is a labour intensive procedure. The efficiency of the cell lysis and PCR conditions were confirmed by the results of the other single cell PCRs from the same experiments. The amplification failure found in this study might be caused by the delivery of dead cells containing degraded DNA. However, it was noticed that the amplification failure rate of HUMTH01 was lower than that of β-globin (3.3%

and 10.7%, respectively). In some cases both alleles of the β-globin gene failed to amplify while the HUMTH01 locus was amplified successfully, or vice versa. Therefore, in the presence of an optimal PCR condition and DNA template, sometimes the β-globin gene just did not amplify.

The observed ADO rate of HUMTH01, as well as AF rate, was lower than that of the β-globin gene. It has been explained before that the analysis using F-PCR is more sensitive than traditional visualisation of the DNA bands on gels. Extreme cases of preferential amplification may resemble ADO when using less sensitive detection methods (Findlay *et al.*, 1995a). This explanation should apply to the occurrence of amplification failure in some cases. If so, the use of F-SSCP analysis (when ready) instead of the conventional silver staining SSCP analysis might reduce both ADO and AF of the β-globin gene and support this hypothesis.

The presence of ADO in β-thalassaemia analysis (**Figure 3.18**) would not lead to transfer of an affected embryo because in a homozygous affected sample ADO will not lead to the embryo being diagnosed as unaffected. A compound heterozygote could be misdiagnosed if only one mutation is detected; also, if the mutations in a compound heterozygote are both detected, but they are separated by a few hundred bp or more, necessitating separate amplification in a multiplex PCR. Then either amplified fragment could be affected by ADO of the mutant allele. This would give a heterozygous result despite the embryo being compound heterozygous (affected). For this reason the back up linkage analysis information from HUMTH01 would be very useful for confirmation of the mutational diagnosis results. However, for autosomal dominant disorders, the

occurrence of ADO is crucial because ADO of the mutant allele can lead to the transfer of a heterozygote embryo and the delivery of an affected child.

In conclusion, the PGD protocol for β-thalassaemia, IVSI-110 mutation (**Figure 3.12**) has been developed, optimised and tested using over 700 single cell PCRs. The optimised PGD protocol was performed by multiplex amplification of single cells with the outer β-thalassaemia primers and the fluorescent labelled HUMTH01 primers using AmpliTaq Gold™, aliquoting the amplified products from the primary reaction for secondary nested amplification by the inner β-thalassaemia primers using SuperTaq®, analysing the multiplex PCR product for the polymorphic HUMTH01 fragment on the ALF Express™ and mutation analysis of the nested amplified β-globin product by SSCP on the GenePhor™. The optimised PGD protocol gave rise to acceptable ADO and AF rates for both HUMTH01 and the β-globin gene and required a total of 8 h to perform. Multiplex amplification of the HUMTH01 fragment in addition to the β-globin gene is very useful in preventing misdiagnosis from ADO and contamination as well as providing linkage analysis back up results. However, it would be useful if the real causes of ADO and AF can be revealed and solved thus maximising the efficiency of the PGD protocol. The protocol was tested on human blastomeres donated for research with favourable results, Therefore, this PGD protocol for β-thalassaemia, IVSI-110 mutation is considered ready for clinical application.

3.4.3 PGD strategy for β-thalassaemia, codon 41-42 mutation

The PGD protocol for β-thalassaemia, codon 41-42 uses a single step multiplex fluorescent PCR protocol (**Section 3.3.3**) and thus provides rapid analysis without secondary nested amplification. This helps in not only halving the time used for the amplification, but also reducing the risk of contamination during mixture preparation (less amplification, therefore less potentially contaminating products formed, also PCR tubes are opened less). Moreover, the protocol provides highly sensitive and precise sizing of DNA fragments using the F-PCR technology (**Section 2.2.5.5**). This allows straightforward detection of deletions and insertions. The incorporation of the polymorphic linked HUMTH01 marker is useful for back up linkage analysis and contamination detection.

The amplification efficiency of HUMTH01 and the two β-globin gene fragments in both multiplex PCR strategies was comparable. However, ADO rates were high in amplifications of the codon 41-42 mutation. The high ADO rate might be a consequence of cell quality, as the buccal cells used had spent several days at room temperature and it was quite possible that their DNA was degraded. Evidence for this comes from the follow-up work conducted on fresh blastomeres. In this case ADO affecting the HUMTH01 locus was only half of that seen in buccal cells. It has been suggested that ADO rates are generally higher in blastomeres than other cell types (Kuliev *et al.*, 1998; Kanavakis *et al.*, 1999), so this difference was even more striking and indicates that there was a problem with the buccal cell sample. A clinical PGD case using fresh biopsied blastomeres is expected to provide superior amplification efficiency and lower ADO rates than those estimated from the buccal cells in this study. The presence of ADO in

β-thalassaemia analysis would not lead to the transfer of a homozygous affected embryo. However, compound heterozygotes could be misdiagnosed if the PGD strategy only allowed detection of one of the mutations, or if mutations were contained within separate amplified DNA fragments.

In a report (Kuliev *et al.*, 1998) which used RFLP following nested PCR, the β-globin gene showed an overall 94.2% (484/514) amplification efficiency and 9.1% (44/484) ADO rate in single fibroblasts with different thalassaemia mutations. However, in the same report the two-step polar body analysis of oocytes for the β-globin gene gave results for both first and second polar bodies in only 66.1% (78/118) with an ADO rate of 6.4% (5/78). The HUMTH01 results were obtained from both first and second polar bodies in 80% (8/10), with a 12.5% (1/8) ADO rate. In polar bodies analysis, it is important to obtain the results from both polar bodies as this allows recombination between the polymorphism and the mutation to be detected. The amplification efficiency of another linked marker to β-globin, 5′ Globin STR, in the same study was 71.1% (54/76) with a 3.7% (2/54) ADO rate. A clinical study on single biopsied nucleated blastomeres using denaturing gradient gel electrophoresis (DGGE) analysis after nested PCR demonstrated 61% (22/36) amplification efficiency and a 19% ADO rate for the β-globin gene (Kanavakis *et al.*, 1999). Therefore, the newly developed protocols in this study are expected to provide equivalent or better efficiency when compared to the previous reports, consequently leading to more diagnosed normal or carrier embryos to transfer and a better chance of achieving pregnancies.

In conclusion, the PGD protocol for β-thalassaemia, codon 41-42 mutation were designed and optimised. The protocol utilises a single step multiplex F-PCR method.

Tests of the protocol on single human blastomeres gave promising results. The multiplex amplification of a highly polymorphic locus (HUMTH01) in addition to the β-globin gene described here is very useful in reducing misdiagnosis caused by contamination and clarifies the diagnosis of cells affected by ADO. The two PGD protocols designed for β-thalassaemia with different types of mutations, i.e. single base pair substitution and deletion, reflect the potential for clinical application to a wide range of β-globin gene mutations. Therefore, these protocols may also be useful for other β-globin gene mutations that reside between these sets of primers with or without minor modification.

3.4.4 PGD strategy for α-thalassaemia, --^{SEA} mutation

The PGD protocol for α-thalassaemia, --^{SEA} mutation (**Section 3.3.4**) was designed and optimised using a single step multiplex gap F-PCR strategy. Gap PCR (**Section 2.2.5.6**) is a cleverly modified PCR technique for detecting a very large deletion, i.e. 20kb, without a reduction in amplification efficiency (Ko *et al.*, 1992). This study has the advantages of F-PCR and integrated F-PCR with gap PCR. The labelling of primers amplifying the normal and mutant alleles with different dyes allows the normal and mutant alleles to be clearly distinguished during the analysis. The addition of polymorphic HUMTH01 analysis is useful for contamination identification. In the case of an un-informative HUMTH01 locus, other informative microsatellites can be substituted. The study in single buccal cells of a heterozygote subject gave acceptable amplification efficiency and ADO rates, 90% and 22.2%, respectively. However, the higher ADO rate compared with other studies could be due to the quality of the samples. As for the study of β-thalassaemia, codon 41-42 mutation (**Section 3.3.3**), the buccal cell samples used in this study had spent several days at room temperature during transit. It is quite possible

that their DNA was degraded. The results from the study on fresh single blastomeres where the ADO rates of the HUMTH01 locus was lower, from 22.2% to 8.7%, confirmed this hypothesis. The previous PGD protocol for α-thalassaemia, $--^{SEA}$ mutation using semi-nested gap PCR and the traditional gel electrophoresis showed an amplification efficiency of 76.1% (Chang *et al.*, 1996). Therefore, this PGD protocol is comparable or better than the previous report. This is probably due to the different DNA extraction technique and the use of F-PCR technology. The promising results from the study in human blastomeres show that this PGD protocol can be clinically applied.

3.4.5 Single cell sequencing

It is essential that new PGD protocols be rigorously tested prior to any clinical application. In most cases this involves the isolation, amplification and testing of a significant number of cells of various genotypes. The significant investment of time and resources that this requires limits the number of new protocols that a PGD centre can develop in a given time. Because of this restriction, and also financial constraints, many PGD centres are forced to prioritise, only developing protocols that will be applicable to a large population of patients.

The number of centres offering PGD increases each year, however the range of diseases for which PGD is available expands at a disappointingly slow rate. Review of the literature reveals that PGD is applied to approximately four new diseases per year and that this rate of increase has remained static since 1998. Paradoxically there has been an increase in the number of new PGD protocols published, but most of these simply detail alternative strategies to existing PGD protocols. The reason for this is that most PGD

centres have concentrated on developing their own protocols for the most common mutations in their local population. For example, more than 10 different methods for detecting the common cystic fibrosis mutation, ΔF508, have been reported. The main reason for the slow increase in the number of diseases diagnosed at the preimplantation stage is the amount of time and resources required for design and testing of new PGD protocols. This makes it uneconomical to develop protocols for rare mutations. Any methodology that is applicable to multiple diseases/mutations, with little optimisation required, will accelerate the rate at which new methods can be developed.

For PGD the most popular have been restriction enzyme digestion, heteroduplex analysis and single strand conformational polymorphism (SSCP). Enzyme digestion is the most simple of these and requires the least work-up. However, many mutations do not disrupt enzyme recognition sites and therefore cannot be detected in this way. Heteroduplex analysis and SSCP have the advantage that a single protocol can detect multiple mutations within a given DNA fragment. Over 50% of DNA sequence alterations can be detected using heteroduplex analysis while for SSCP detection rates can be as high as 90%. However, this increased detection rate comes at the price of increased complexity. To achieve maximum detection rates for SSCP technical know-how is necessary and a significant number of experimental parameters must be optimised. This is a process that can only be achieved by trial and error. A protocol that allows detection of 100% of mutations within a DNA fragment, but requires little or no change of conditions for each mutation, would reduce the amount of time taken to develop new protocols. The greatest saving in time would come from PGD for diseases characterised by a heterogeneous spectrum of mutations. One such example is β-thalassaemia, for which several published PGD protocols already exist. In this study the bthalw1 primers used

encompass over 50 mutations in the β-globin gene. These primers are optimised for single cell amplification and could theoretically be used for amplification of any of these mutations.

In this study the use of DNA sequencing as a general protocol for mutation detection in single cells has been investigated. These methods allow the detection of virtually any mutation, with little or no adjustment of methodology. Because little optimisation is necessary the amount of time required for work-up of new PGD protocols should be reduced relative to methods such as SSCP, which can detect a range of mutations. This should free-up resources that can then be used for the development of additional PGD strategies. Theoretically sequencing should be particularly useful in cases of disease caused by a heterogeneous spectrum of mutations, as it will often be possible to design a single set of primers that allows the detection of multiple affected genotypes. Despite the potential of sequencing and minisequencing they have been little used for PGD and their efficacies at the single cell level have not been examined in detail. The reliability of these methods for the detection of a variety of important disease-causing mutations was tested, paying particular attention to the effect of preferential amplification and allele-specific amplification failure (ADO).

The single cell sequencing protocol using an automated laser fluorescence sequencer was established and evaluated in this study (**Section 3.3.5**). Fluorescent sequencing provides a quick and precise non-radioactive sequence analysis. Moreover, the advantage of the ABI Prism™377 sequencer in visualising up to 4 fluorescent dyes allows the sequencing reaction to be accomplished within a single tube and the sequencing product to be analysed on a single lane. Predictably, samples displaying equal

amplification of both alleles gave accurate sequencing results and it was possible to determine all genotypes correctly. As with other mutation detection techniques, such as SSCP, heteroduplex analysis and restriction enzyme digestion, heterozygous samples with ADO could not be distinguished from homozygous samples. The interesting samples for investigation were those showing preferential amplification of one of the two alleles. In some cases of heterozygous samples with PA, initially sequencing analysis software was unable to determine that two distinct sequences were present. However, with careful study of the critical nucleotide positions it was usually possible to recognize samples that had PA and were not truly homozygous. This was achieved by examining the level of fluorescence of the two possible nucleotides at sites affected by the mutation and determining which had fluorescence above background levels.

Clearly this is somewhat subjective and relies on the investigator judging whether or not the peaks corresponding to the two possible alleles are above background levels. For clinical application it would be necessary to err on the side of caution and only score peaks corresponding to 'normal' alleles if they were significantly above background levels. For the same reason peaks corresponding to 'mutant' alleles would have to be considered genuine even if scarcely above the background. Using these criteria it is inevitable that some heterozygous samples with extreme PA would be scored as homozygous affected, while some homozygous normal samples with unusually high background fluorescence would be scored as heterozygous. This incorrect genotyping would not cause an affected embryo to be diagnosed normal, but could lead to the accidental exclusion of healthy embryos.

Detection of both alleles in a sample with PA is easier to accomplish for samples with a frameshift mutation than a single base change because the sequencing analysis software manages to detect one of the four bases that are usually ambiguous when this stretch of DNA is heterozygous (**Figure 3.26**). This is because mismatches are seen at multiple positions downstream of the mutation site, thus giving more opportunities for obvious deviations above background fluorescence to be observed. Despite the correct diagnosis of most samples with PA, a small number could not be identified and resembled cells with ADO. Consequently the risk of misdiagnosis due to ADO affecting the mutant allele (primarily a problem for diagnosis of dominant disorders) is somewhat higher using this methodology.

The length of DNA sequence that could be read accurately varied depending on the primer set used, but was identical to that achieved following PCR amplification of larger DNA samples, and exceeded 200 bp in all cases. For amplifications using the bthalw1 primers more than 50 known β-thalassaemia mutations including the codon 41-42 mutation can be excluded, while use of the outer β-thalassaemia primers excluded over 20 mutations including IVSI-110.

In conclusion, the single cell sequencing protocol showed very promising data with accurate identification of disease causing mutations in single cells. This could provide the advantage of shortening the time required for the development of PGD protocols for new diseases or mutations. Multiple mutations can be sequenced if a whole genome amplification followed by specific PCR is used or if nested PCR with multiplex of the outer primers is employed. Additionally any other mutations or polymorphisms within a fragment are identified. This is particularly useful for the detection of compound

heterozygotes in which both mutations are in close proximity (i.e. can be amplified in a single amplicon). However, the important problem encountered when using traditional analysis techniques, ADO, still exists in this new method. The testing of the single cell sequencing protocol on single human blastomeres showed promising results. Therefore, after testing on more samples, single cell sequencing could be a useful PGD protocol for clinical application.

3.4.6 Single cell minisequencing (SNaPshot™)

The minisequencing protocol employs the same strategy as the standard fluorescent sequencing protocol, but extends only one nucleotide after the minisequencing primer. This gives rise to a shorter fragment size and consequently a faster analysis time for the electrophoresis, especially when using Fast Genescan® Gel which takes only 5 min for the electrophoresis on the ABI Prism™310, compared to the standard fluorescent sequencing method where the electrophoresis takes 40 min on the ABI Prism™310. However, the standard single cell sequencing allows the detection of more than one mutation encompassed within the same set of primers. Both protocols are useful for the detection of single nucleotide substitutions, deletions and insertions. A particular minisequencing primer, which locates just before the mutation, is used for each specific mutation. By changing the primers used in the PCR step and minisequencing primers, this protocol can be used as a versatile PGD protocol.

Single buccal cells of the subjects carrying single base pair substitution mutations, i.e. sickle and β-thalassaemia IVSI-110, and frameshift mutation, i.e. β-thalassaemia codon 41-42, were tested in this study providing satisfactory results compared with other

methods, i.e. SSCP. Similar to SSCP analysis of the β-thalassaemia IVSI-110, the ADO problem still existed when analysed by the single cell minisequencing protocol. Samples with equal amplification showed concordant results to the SSCP analysis. The application of single cell minisequencing on sickle and β-thalassaemia codon 41-42 mutation confirmed the reproducibility of the protocol. Minisequencing of normal control DNA demonstrated a normal result as expected. Therefore, single cell minisequencing has been shown to be another choice for a versatile PGD protocol. This will be very useful in reducing the time and expenses used for developing PGD protocols for new mutations.

3.4.7 Conclusion

Haemoglobinopathies are the world's most common class of single gene disorder and have a great impact on the quality of life for affected families. Thalassaemias were originally localised to specific parts of the world, i.e. Mediteranean, Middle East and Asia. This might be because of its malarial resistance advantage. However, due to the ease of travelling and population relocation they have now become significant in many countries where the disease was originally uncommon, e.g. the UK and the USA. The effective curative treatment, i.e. bone marrow transplantation, is a major procedure and cannot be offered routinely. The supportive management, i.e. blood transfusion, gives several serious complications from iron overload. Therefore, disease control by prevention of new cases is one of the most important methods for the combat of thalassaemias at present. Prenatal diagnosis of thalassaemias is now available worldwide; however, with the introduction of PGD a pregnancy can be started with the knowledge that the embryo is unaffected, thus eliminating the need for PND and termination of an affected pregnancy.

Three PGD protocols for β- and α-thalassaemias have been developed, optimised and assessed in this study. Major types of mutations, including substitution, small deletion and large deletion, were included demonstrating the potential for these protocols to be applied to a wide range of mutations with only minor modifications, i.e. primers to encompass the mutation, SSCP conditions. The single cell sequencing protocol has been developed and tested on two different types of mutations, substitution and small deletion, and provided satisfactory results. This protocol is very useful for compound heterozygote genotype detection where the mutations are close to each other. Moreover, this versatile form of mutation analysis could be applied to the detection of virtually any mutation, once the mutation site has been amplified by PCR. Unlike other techniques that allow the detection of multiple mutations, such as SSCP, little optimisation of the sequencing protocol is required for detection of individual mutations. Fluorescent minisequencing also offers the possibility of wide application. This technique provides the advantage of a faster analysis time compared to the sequencing protocol. However, a specific minisequencing primer is needed for each particular mutation. The impressive results from single human blastomeres show the potential for clinical application of these PGD protocols.

Future extension of this study will include the optimisation and testing of silver stained SSCP and F-SSCP for the detection of a wider range of β-thalassaemia mutations. For this purpose, more different gel compositions may be used. Modification of the primers used could also help by including more mutations within the amplified fragments. If accomplished, these techniques will be very useful for PGD of β-thalassaemia mutations. F-SSCP offers the advantage of providing a quicker and more sensitive method than the traditional silver stained SSCP. In addition, the promising results using

single cell sequencing and minisequencing protocols for β-thalassaemia IVSI-110 and codon 41-42 mutations should be followed up with more tests on single cells. If accuracy rates prove to be high, as anticipated, then these methods could be applied clinically. With the alteration of the primers used, it will also be interesting to apply the single cell sequencing and minisequencing protocols to other single gene mutations, to prove their potential versatility.

Chapter 4

Myotonic Dystrophy

4.1 Introduction

Myotonic dystrophy (DM; http://www.ncbi.nlm.nih.gov/entrez/; 160900, also known as dystrophia myotonica, myotonia atrophica and Steinert disease) is the most common form of inherited muscular dystrophy in adults and is a progressive autosomal dominant disorder (Shaw and Harper, 1989). In contrast to other types of muscular dystrophy, other organ involvement, i.e. eyes, heart, endocrine system, central and peripheral nervous systems, gastrointestinal organs, bone and skin, are found in the patients with DM in addition to myopathy. Earlier diagnoses were based on clinical symptoms, including myotonia (tonic muscle spasm with delayed relaxation), muscular weakness and atrophy, cataract, hypogonadism, frontal balding and changes of electrocardiography. Slit-lamp examination for lens changes, electromyography for myotonic discharges and immunoglobulin assays are useful for detecting subclinical cases. In contrast with other muscular dystrophies, DM first affects distal and then proximal muscles of extremities. There is also head, neck and extraocular muscle involvement. Muscle biopsy reveals non-specific changes, including central nuclei and ring fibres, necrosis, regeneration and increased collagen. Congenital DM is characterised by neonatal hypotonia, motor and mental retardation and a lack of facial expression (Harper, 1975). Respiratory insufficiency can be fatal. Antenatal findings involve polyhydramnios, talipes, and decreased fetal movements which reflect the neuromuscular failure of swallowing and movement. The mutant gene is almost always transmitted from the mother. However, paternal transmission of congenital DM has been reported (de Die-Smulders *et al.*, 1997; Nakagawa *et al.*, 1994).

The myotonin-protein kinase (DMPK) gene, which consists of 15 exons occupying over 13,000 bases of genomic DNA, encodes a polypeptide of 624 amino acids that functions as a member of a protein kinase family (Shaw *et al.*, 1993). The molecular genetic defect of DM involves an unstable expansion of a CTG repeat at the 3′ untranslated region (exon 15) of DM gene on the long arm of chromosome 19 (19q13.3) (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). The number of repeats relates to the age of onset and the severity of the disease (Harley *et al.*, 1993; Redman *et al.*, 1993): normal individuals possess 5-35 repeat copies, premutation 36-49, mildly affected 50-150, severely affected 100-1,000 and the most severe or neonatal form 2,000 or more copies (The International Myotonic Dystrophy Consortium (IDMC), 2000). The offspring have an earlier age of onset and a larger DM repeat length than their parents in almost all cases observed (Harley *et al.*, 1993). Anticipation, the progressively earlier onset, the increase in expansion and severity in successive generations, is frequently observed in association with the increased number of trinucleotide repeats (Mahadevan *et al.*, 1992). Intergeneration expansion was observed in all mother-to-congenital offspring pairs (Tsilfidis *et al.*, 1992). The phenomenon where an affected parent transmitted a normal allele to an offspring, namely reverse mutation, negative expansion or contraction, was also documented (Abeliovich *et al.*, 1993; Ashizawa *et al.*, 1994; Brunner *et al.*, 1993). The estimated risk of any heterozygous woman with DM to have a congenitally affected child is 3-9%, but the risk of having a second affected one is raised to 20-37% (Koch *et al.*, 1991). However, it has been noticed that the DM expansion has a preferential transmission with the overall transmission rate of 63% (Magee and Hughes, 1998). This type of segregation distortion is referred to as meiotic drive, which may help in maintaining the mutant DM alleles in the population.

A variation in the DM triplet repeat in sperm and somatic cells of the same individual was observed and named as gonosomal mosaicism (Jansen *et al.*, 1994). This phenomenon indicates that the intergenerational size changes of the unstable DM triplet repeat may occur during early embryonic mitosis. The discordance of repeat sizes in different tissues, i.e. skeleton muscle, cardiac muscle, liver, lung, brain cortex, kidney, small bowel, skin and bone, from a 20 weeks old DM fetus was also reported (Lavedan *et al.*, 1993). The expansion was observed to be greater in muscle than in the lymphocytes (Anvret *et al.*, 1993). Interestingly, there was a difference in size of amplification in affected identical twins (Dubel *et al.*, 1992). It was found that the repeat size in white blood cells of DM patients increased during a 5 years period, implying the mitotic instability of the CTG repeat throughout life (Martorell *et al.*, 1995). However, the level of the expansion was related to the original repeat length. Clinical symptoms were progressed in half of the observed patients. The linkage disequilibrium between the DM CTG repeats and an Alu insertion/deletion polymorphism in the DMPK gene was observed in the Japanese and European populations (Yamagata *et al.*, 1996). This finding indicates a common origin of the DM mutant alleles in both populations.

The mechanism of how the DM triplet repeat expansion gives rise to the clinical characteristics is still unknown. It was demonstrated that there was a reduced level of the mRNA and protein expression in association with DM, suggesting that a DMPK deficiency may be the cause of this disease (Fu *et al.*, 1993). However, the overexpression and underexpression of the *Dmpk* gene in mice failed to give the typical clinical features of DM (Harris *et al.*, 1996). This indicated that abnormal protein kinase activity is not the main cause of DM. It was postulated that the RNA produced by the mutant DMPK gene may influence the cellular processing of RNA produced by other genes, i.e. the gene

encoding DM locus-associated homeodomain protein (DMAHP) which is located immediately downsteam to the CTG repeat.

The molecular diagnostic test is useful for presymptomatic testing and PND. Early protocols for prenatal molecular diagnosis were performed using closely linked markers to the DM gene (Lavedan *et al.*, 1991; Norman *et al.*, 1989; Reardon *et al.*, 1992), and later using specific mutation analysis of the CTG mutation (Myring *et al.*, 1992). Reliable direct DM CTG repeat expansion analysis can be carried out by using a combination of Southern blot for the alleles larger than 100 repeats and PCR for the alleles of 5-200 repeats. PCR is a simpler and quicker technique than Southern blot; however, amplification efficiency of the fragments larger than 500 bp is poor. Therefore, the present PCR technique is only useful for exclusion basis. Successful PGD for DM based on PCR assays and analysis of the expansion on 4% agarose gel has been reported (Sermon *et al.*, 1997), and the subsequent information suggested that the application of fluorescent PCR and automated fragment analysis can improve both efficiency and accuracy (Sermon *et al.*, 1998a); however, a misdiagnosis has been documented (Sermon *et al.*, 1998b).

Essential management of DM is regular assessment for cardiac conduction defects by electrocardiogram. Cardiotropic drugs, i.e. procainamide, quinine and propanolol should be avoided, while the patients with heart block may need a pacemaker. In cases having an operation, the risks associated with general anaesthesia must be informed. Eye examination should be carried out regularly and lens extraction may be needed to improve vision from cataracts. Symptomatic treatment may be required for the patients who possess an impaired intestinal motility. Exercise of the particular muscles is useful

for their weakness; however, weakness of respiratory muscles should be observed as it can lead to nocturnal hypoventilation and aspiration pneumonia. Neonatal respiratory insufficiency is the most crucial complication for congenital DM.

This study aimed to develop a PGD protocol for DM without the risk of misdiagnosis. This includes the addition of a highly polymorphic marker, the primary function of which is to reveal when contamination has occurred. The diagnosis of a normal embryo in this protocol is based on the presence of two normal DM alleles from each of the parents. An affected embryo will show only one normal allele, as the mutant DM expanded allele is refractory to single cell PCR. Ideally, in informative families, as well as amplifying the normal DM alleles a polymorphic linked marker (APOC2) is amplified providing a back up diagnostic result. Furthermore, the highly polymorphic nature of this locus allows contaminating DNA not derived from the embryo to be identified. In families where the APOC2 genotypes are not fully informative, an unlinked short tandem repeat (STR) marker can be substituted for contamination detection. Working up PGD in two DM families has been carried out. As both families were not fully informative for the APOC2 locus, the unlinked marker, D21S1414, was substituted. Three clinical PGD cycles of DM were performed resulting in one singleton and one twin pregnancy, both of which had the PGD results confirmed by PND. Attempts to find an efficient condition for amplifying the mutant DM expansion at the single cell level were also carried out.

4.2 Materials and methods

4.2.1 PGD strategy for DM

Two PGD protocols for DM have been developed in this study using a single step multiplex F-PCR strategy (**Section 2.2.5.3** and **2.2.5.5**). The diagnosis of a normal embryo is based on the observation of two normal DM alleles, one from each of the parents. The mutant DM expanded allele is not detected by this protocol, as the fragment size of longer than 500 bp is refractory to single cell PCR; therefore, the diagnosis of an affected embryo will show only one normal allele. In addition to the amplification of the DM CTG repeat region, a linked or unlinked polymorphic marker was included for contamination detection and a back up linkage analysis result (for the linked marker only). Protocol 1 employed a combination of the DM primers (Brook *et al.*, 1992) (**Table 4.1**) and a polymorphic linked marker to the DM gene, APOC2 (Weber and May, 1989) (**Table 4.1**), which is situated approximately 4 cM upstream to the DMPK gene. The DM primers amplify the DM CTG repeat region within exon 15 of the DMPK gene (**Figure 4.1**). The inheritance and linkage of both the DM and the APOC2 genes were examined using DNA samples spanning 3 generations of the family FAP82 (**Section 2.1.4**). In case that the APOC2 genotypes are not informative in the test family, an unlinked marker on chromosome 21, D21S1414 (Sherlock *et al.*, 1998) (**Table 4.1**), was substituted in protocol 2. Experiments were carried out using different concentrations of primers and thermal cycle programs in order to obtain the optimal conditions for both protocols. Both optimised protocols were performed on single buccal cells of heterozygote subjects and the members of DM families 'A' and 'B' and single human blastomeres for scoring for the amplification efficiency and ADO rates.

Table 4.1 The details of the primers used for DM.

Primers	Sequences (5′-3′)	Fluorescent labelled	Location	Size of the product (bp)	Concentration (μM)	Annealing temperature (°C)	References
DM							
forward	5′-CTT CCC AGG CCT GCA GTT TGC CCA TC-3′	TET®	19q13.3	128-203	0.2	60	(Brook *et al.*, 1992)
reverse	5′-GAA CGG GGC TCG AAG GGT CCT TGT AGC-3′	Cy5®			0.2		
APOC2							
forward	5′-GGC TAC ATA GCG AGA CTC CAT CTC C-3′	6'FAM®	19q13.2	134-170	0.2	60	(Weber and May, 1989)
reverse	5′-GGG AGA GGG CAA AGA TCG ATA AAG C-3′	-			0.2		
D21S1414							
forward	5′-AAA TTA GTG TCT GGC ACC CAG TA-3′	6'FAM®	21q21	330-370	0.2	60	(Sherlock *et al.*, 1998)
reverse	5′-CAA TTC CCC AAG TGA ATT GCC TTC-3′	Cy5®			0.2		

Figure 4.1 DM primers (arrows) mapped to the CTG repeat region within exon 15 of the DMPK gene. By measuring the amplified fragment size, the copy number of the CTG repeat can be calculated.

For the optimal protocol, the PCR mixture consisted of 0.2μM of each primer, 200μM dNTPs, 1× GeneAmp® Buffer and 1.5U AmpliTaq Gold™ and was made up to a total volume of 25μl with distilled deionised water. The amplifications were performed with the conditions: 94°C 45 sec (96°C for the first 10 cycles), annealing at 60°C 45 sec and extension at 72°C 1 min for 40 cycles. These were preceded by denaturation at 94°C for 12 min to activate the AmpliTaq Gold™ enzyme. Single cell triplex PCR was also carried out using the same protocol and a combination of DM, APOC2 and D21S1414 primers (0.2μM concentration of each). The multiplex amplified products from single cells were each tagged with two different fluorochromes using labelled primers. This allowed analysis to be performed on an automated laser fluorescent sequencer (ALF Express™) and also an ABI Prism™310. Consequently it was possible to compare the performance of these two pieces of equipment. DM fragments were labelled with the green fluorescent dye (TET®) (forward primer) and a blue dye, Cy5® (reverse primer). APOC2 and D21S1414 fragments were both labelled with the blue fluorescent dye 6-FAM® (forward primers) and Cy5® (reverse primers).

4.2.2 Work up for DM PGD cases

Two families, A and B, were included in this study. Blood and buccal cells samples of the members of both families were collected and used for the work up. The genotypes of each member for DM, APOC2 and D21S1414 loci were identified. Single buccal cells of the members of both families were amplified using the PGD protocol for DM (**Section 4.2.1**). Neither family was informative for the APOC2 locus, therefore, protocol 2 was chosen for PGD.

4.2.3 Clinical DM PGD cases

Following IVF treatment, two PGD cycles were carried out in DM family A, and one in family B (**Section 2.2.2.2**). The single biopsied blastomeres (**Section 2.2.3.2**) were analysed using the optimal PGD protocol 2 of DM with the combination of DM and D21S1414 primers (**Section 4.2.1**). Normal embryos were chosen for transfer on day 4 post-fertilisation. The untransferred embryos were analysed using the same protocol for confirmatory diagnosis results.

4.2.4 Hi-fidelity PCR for DM expansion detection

In order to amplify the long template of the DM expansion, five high efficiency PCR systems, including AmpliTaq Gold™, *Pfu* DNA Polymerase, Expand Long Template (ELT) PCR System®, GC-RICH PCR System® and Extensor Hi-Fidelity PCR Kit® were tested. Eight different combinations of the polymerase enzymes, concentrations of PCR mixtures and thermal cycle programmes were carried out according to the suggested protocols of the particular enzymes and with some modifications detailed in **Table 4.2**. DNA and single cell samples of the normal subjects and DM carriers were employed for testing the protocols.

Table 4.2 PCR conditions of different PCR systems for long template amplification.

PCR System	AmpliTaq Gold™		*Pfu* DNA Polymerase		ELT* PCR System® System 3	GC-RICH PCR System®	Extensor Hi-Fidelity PCR Kit®		
							Buffer 1	Buffer 1	Buffer 2
Protocol	A	B	C	D	E	F	G	H	I
Templates	Genomic DNA	Genomic DNA	Genomic DNA	Genomic DNA	Genomic DNA	Genomic DNA	Genomic DNA	Genomic DNA/SC**	Genomic DNA/SC**
dNTP (mM)	0.2	0.8	0.2	0.8	0.5	0.2	0.8	0.8	0.8
PCR Buffer	1×	1×	1×	1×	1×	1×	1×	1×	1×
$MgCl_2$ (mM)	1.5	1.5	2	2	2.25	1.5	1.5	1.5	1.5
GC Resolution (mM)	-	-	-	-	-	1.0	-	-	-
Polymerase enzyme (u)	1.25	1.25	0.625	0.625	1.25	1	0.625	0.625	0.625
water	make up to 25 µl								
Primary denaturation	94°C, 12min×1	94°C, 12min×1	95°C, 2min×1	95°C, 2min×1	94°C, 2min×1	95°C, 3min×1	94°C, 2min×1	95°C, 2min×1	95°C, 2min×1
Denaturation	95°C, 45sec	95°C, 45sec	95°C, 45sec	95°C, 45sec	94°C, 10sec	95°C, 30sec	93°C, 10sec	95°C, 45sec	95°C, 45sec
Annealing	55°C, 45sec	55°C, 45sec	60°C, 30sec	60°C, 30sec	65°C, 30sec	65°C, 30sec	60°C, 30sec	55°C, 45sec	55°C, 45sec
Extension	72°C, 6min ×50	72°C, 6min ×50	72°C, 4min ×40	72°C, 4min ×40	68°C, 4min ×10	68°C, 3min 45sec ×10	68°C, 2min ×10	72°C, 2/5/ 10/20/30min ×50	72°C, 2/5/ 10/20/30min ×50
Denaturation					94°C, 10sec	95°C, 30sec	94°C, 20sec		
Annealing					65°C, 30sec	65°C, 30sec	60°C, 30sec		
Extension					68°C, 4min +20sec/cycle ×25	68°C, 3min 45sec +5sec/cycle ×25	68°C, 2min ×30		
Final extension	72°C, 10min ×1	72°C, 10min ×1	72°C, 5min ×1	72°C, 5min ×1	68°C, 7min ×1	68°C, 7min ×1	68°C, 7min ×1	72°C, 10min ×1	72°C, 10min ×1

*ELT = Expand Long Template **SC = Single Cells

4.3 Results

4.3.1 PGD strategy for DM

In addition to amplifying the triplet repeat region within the DMPK gene, the PGD protocol for DM was designed to incorporate a linked polymorphic marker flanking the DM gene (protocol 1). In cases that the APOC2 genotypes are not fully informative, an unlinked STR marker (D21S1414) can be substituted (protocol 2). If the parents share no alleles in common (i.e. the couple is fully informative) their embryos can only inherit one of four possible genotypes. Any deviation from these combinations of alleles is indicative of contamination. The CTG repeat region of DM is also very polymorphic; therefore it was usually possible to distinguish both alleles of normal heterozygote subjects on an automated DNA sequencer.

Prior to clinical application different concentrations of the DM and APOC2 primers and different PCR conditions were tested on approximately 800 single buccal cells (over 1,000 amplification reactions in total, including control clumps, negatives and the studies on DNA samples) and modified to achieve the optimal multiplex PCR protocol for both DM and APOC2 primers. The optimal multiplex PCR conditions employed 0.2μM of each primer, AmpliTaq Gold™ (rather than SuperTaq™ polymerase), 60°C annealing temperature for 40 cycles. Both fragments could be simultaneously analysed on the ABI Prism™310 using GeneScan® Analysis software. Inheritance and linkage of both the DM and the APOC2 genes were confirmed on DNA samples spanning 3 generations of family FAP82 (**Figure 4.2**). One recombination was detected in 14 meioses.

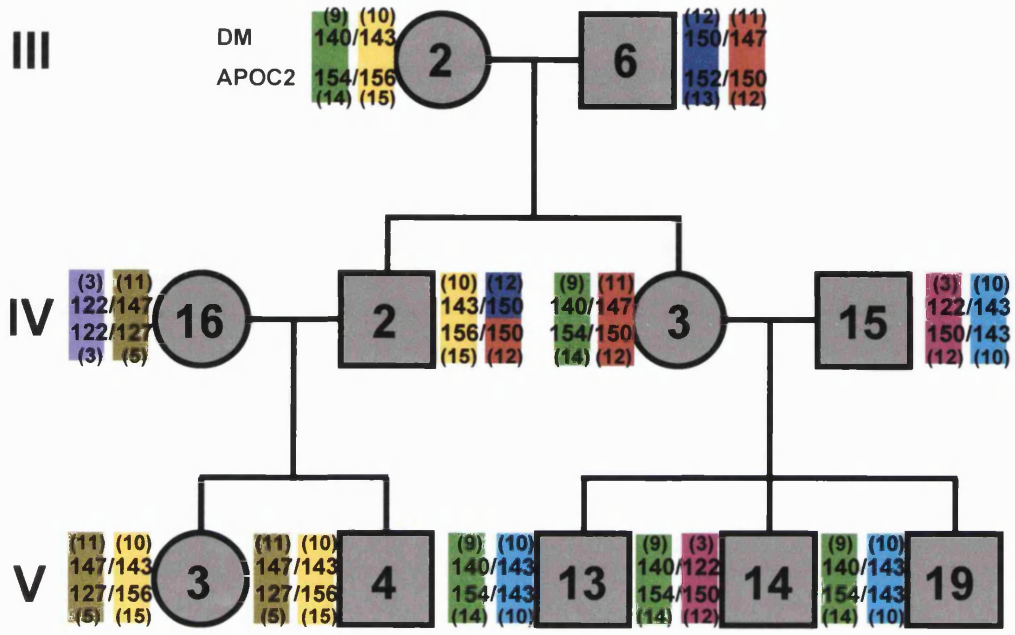


Figure 4.2 Inheritance of DM and APOC2 genotyping in three generations of the FAP82 family analysed by GeneScan®, ABI Prism™310. One recombination was found in either member IV2 or IV3. (number = base pair units and repeat copy number in brackets).

The peak area of the F-PCR analysis is indicative of the amount of the amplified product, in the same way as is the brightness of the band visualised after agarose gel electrophoresis. However, F-PCR allows for extremely accurate sizing of fragments and precise quantification of the amount of PCR product. The experiments aimed at optimising the multiplex PCR conditions for DM and the APOC2 or the D21S1414 primers provided an interesting observation. The DNA samples of subjects possessing large differences between the size of their DM alleles exhibited a remarkable preferential amplification (of the smaller allele) at every annealing temperature tested (**Figure 4.3**). Conversely, simultaneous amplification performed on the DM and the D21S1414 primers using DNA of a subject whose allele sizes were slightly different showed only a mild degree of preferential amplification (**Figure 4.4**).

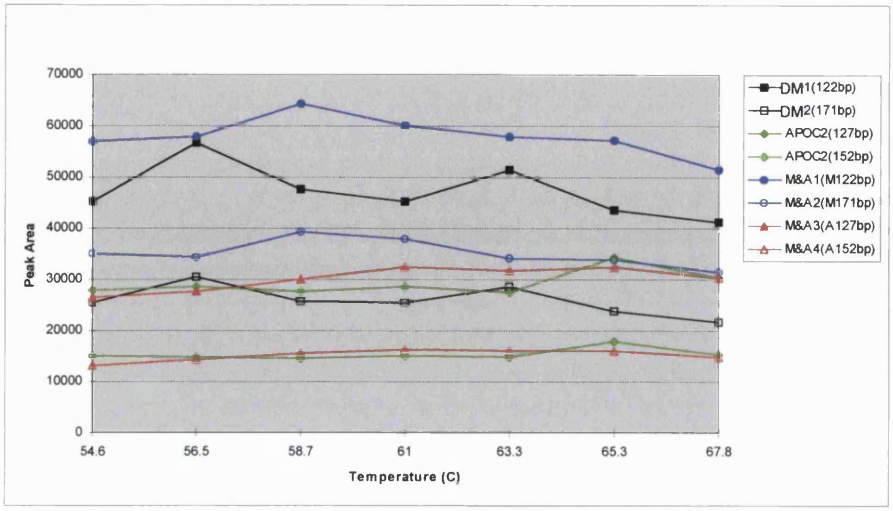


Figure 4.3 The histogram of the peak areas of amplified products from a DNA sample using DM, APOC2 and DM&APOC2 primers at several different annealing temperatures analysed on the ABI Prism™310. A remarkable preferential amplification of smaller alleles, 122 bp allele of DM gene and 127 bp allele of APOC2 gene, was noticed at every temperature. (DM = amplification using DM primers alone, APOC2 = amplification using APOC2 primers alone, M&A = multiplex amplification using both DM and APOC2 primers.)

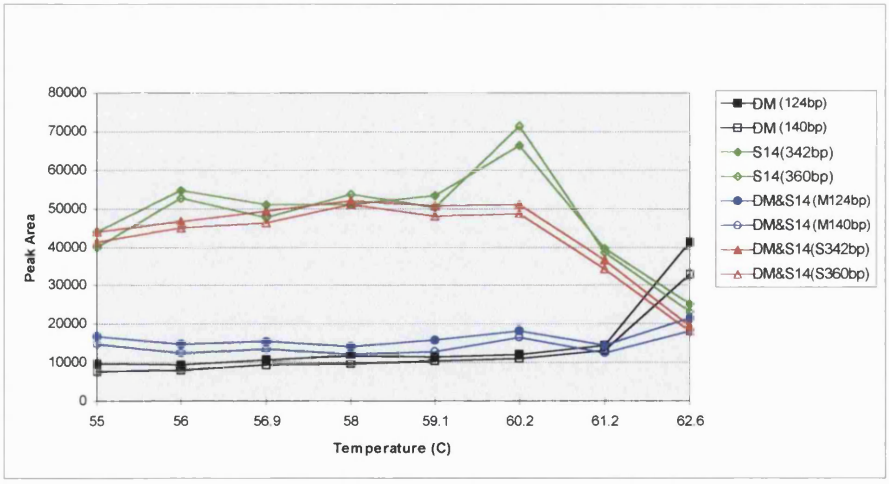


Figure 4.4 The histogram of the peak areas of amplified products from a DNA sample using DM, D21S1414 and DM&D21S1414 primers at several different annealing temperatures analysed on the ABI Prism™310. Preferential amplification was not obvious. (DM = amplification using DM primers alone, S14 = amplification using D21S1414 primers alone, DM&S14 = multiplex amplification using both DM and D21S1414 primers.)

In the amplification reaction of di-, tri- or tetranucleotide repeats regions, minor PCR products with 1-4 repeat copies shorter than the actual fragment can be found. The minor amplified product peaks, or 'stutter peaks', may be caused by polymerase slippage during the PCR extension step. Stutter peaks would not normally cause diagnostic error as they are readily identified, they are always one repeat unit smaller than the true allele and are relatively under-amplified. However, in cases where individuals have two alleles differing in size by just one repeat unit stutters may be somewhat confusing (see **Figure 4.5**).

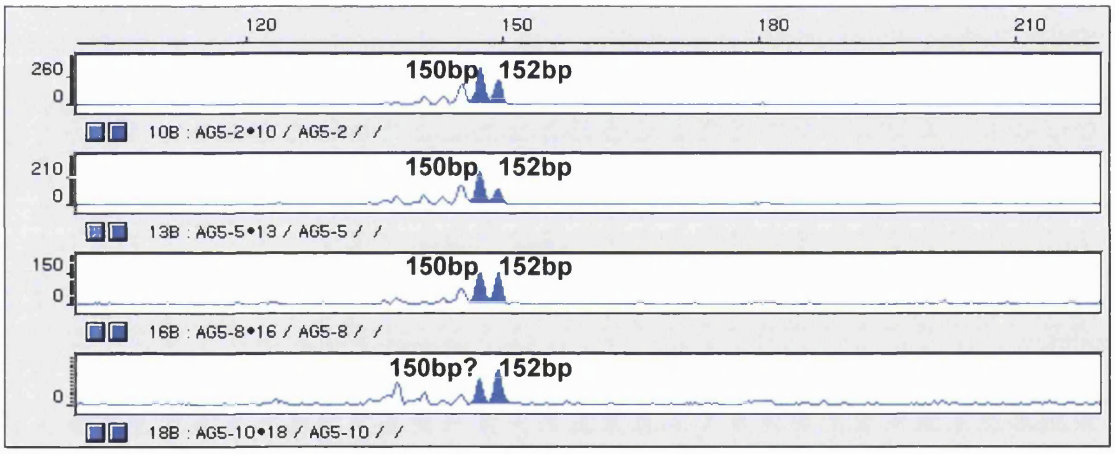


Figure 4.5 APOC2 marker results amplified from single buccal cells of a subject who possesses one copy (2 bp) difference of both alleles at this gene (150 and 152 bp) analysed on the ABI Prism™310 using GeneScan® analysis. Lanes 10B, 13B and 16B show varieties of peak patterns interfered by stutters; while peak pattern in lane 18B causes difficulty in concluding whether this is an appropriate amplification with preferential amplification of the longer allele or allele drop out of the shorter allele with a stutter from the 152 bp allele. The x and y axes are base and peak height units, respectively.

DNA polymerase enzymes often add a nucleotide, usually an adenosine, to the 3′ ends of the amplified fragments leading to PCR products one bp longer than the expected fragment size, the so-called Plus-A artefact. The Plus-A problem frequently causes split peaks. These would not cause misdiagnosis, but may confuse peak area calculation (**Figure 4.6**). However, after working up in the subsequent experiments, this problem could be reduced (**Figure 4.7**) by omitting the final 10 min extension step typically used in PCR protocols.

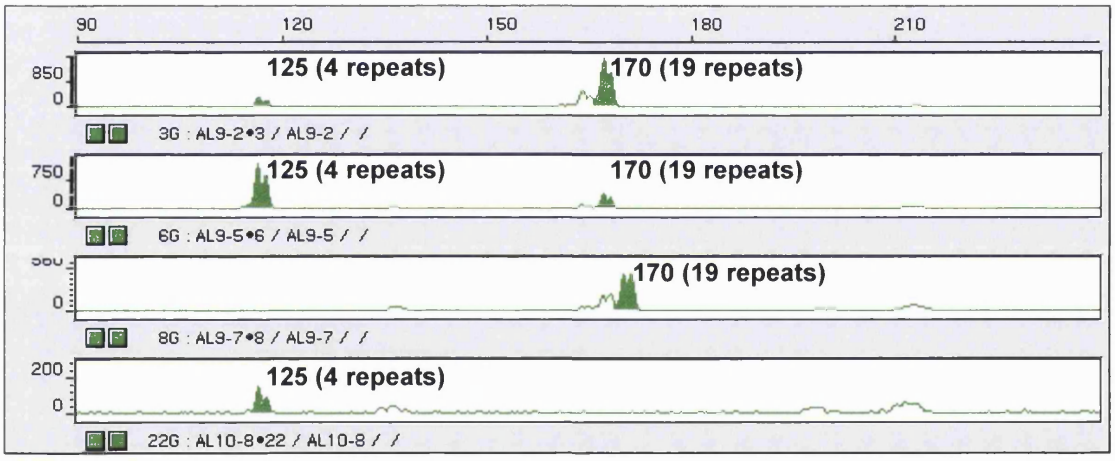


Figure 4.6 DM results amplified from single buccal cells of a subject who possesses a big difference (45 bp) of both alleles at this gene (125 and 170 bp) analysed on the ABI Prism™310 using GeneScan® analysis. Lanes 3G, 6G, 8G and 22G show PAL, PAS, ADOL and ADOS, respectively; split peak or Plus-A problem is also observed on every peak including stutters. The x and y axes are base and peak height units, respectively.

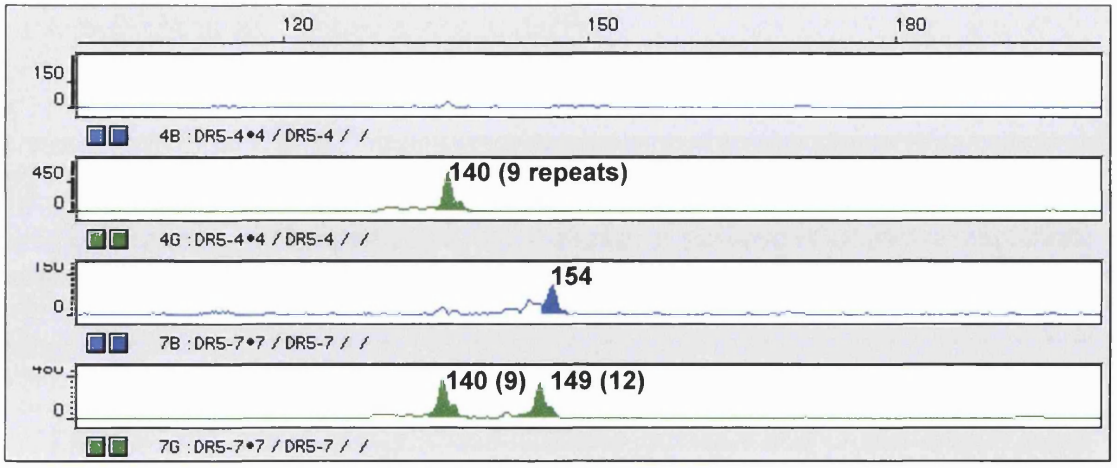


Figure 4.7 DM and APOC2 results multiplex amplified from single buccal cells analysed on the ABI Prism™310 using GeneScan® analysis. Lanes 4B and 4G are the analyses of APOC2 (lane 4B, blue) and DM (lane 4G, green) genes from the same single buccal cell sample, show AF of APOC2 fragment and ADOL of DM gene. The results from another cell, lanes 7B and 7G show ADOS of APOC2 fragment and SA of the DM gene. Split peak or Plus-A problem is reduced dramatically after the modification of the PCR protocol. The x and y axes are base and peak height units, respectively.

The reproducibility of the analysis results from the ABI Prism™310 was tested by programming the machine to analyse the same multiplex PCR product ten times. The standard deviations of the peak areas of four alleles (two of DM and two of the APOC2 genes) from ten analyses are between 2.60% and 6.08%, and those of the ratios between two alleles of the same gene are between 3.52 and 5.88%. Therefore, true preferential amplification in this study was defined as the samples having differences between both alleles of more than 10%.

The multiplex amplified products from single cells were each tagged with two different fluorochromes using labelled primers. This allowed analysis to be performed on

an ALF Express™ and also an ABI Prism™310. Consequently it was possible to compare the performance of these two pieces of equipment. DM fragments were labelled with the green fluorescent dye (TET®) (forward primer) and a blue dye, Cy5® (reverse primer). APOC2 and D21S1414 fragments were both labelled with the blue fluorescent dye 6-FAM® (forward primers) and Cy5® (reverse primers). The analyses of the same samples from both sequencers showed corresponding results. However, the ABI Prism™310 provided a flatter baseline and more precise fragment size results, while the ALF Express™ gave quicker results if more than 4 samples were analysed.

Analyses were carried out on 50 single buccal cells from normal heterozygote subjects. Amplification using DM primers alone provided results from 94% of cells with an ADO rate of 12.8% (**Table 4.3**). After these promising results, 100 single cell PCRs using a combination of DM and APOC2 primers (protocol 1) were attempted. These showed amplification in 95% of samples for both DM and APOC2 loci; ADO was observed in 9.5% of DM amplifications and 10.5% of APOC2 amplifications. Multiplex PCR analysis of 90 single buccal cells from DM carriers and affected patients (where only the normal allele can be detected) revealed 82.2% amplification efficiency for the DM primers. The reduced amplification efficiency observed when the carrier cells were tested could be due to the fact that all instances of ADO affecting the normal allele will resemble amplification failure. Multiplex amplification using DM and D21S1414 primers (protocol 2) on 50 normal heterozygous single buccal cells demonstrated 98% amplification efficiency for both sets of primers. The DM gene displayed a 4.1% ADO rate, while for D21S1414 it was 14.3%. The studies on single buccal cells indicate that the addition of a polymorphic marker to the DM gene analysis as multiplex PCR does not adversely affect the amplification efficiency or ADO rates.

Table 4.3 Summarised data showing amplification efficiencies and ADO rates of DM, APOC2 and D21S1414 loci from single buccal cells using singleplex (DM only) and multiplex (DM&APOC2 and DM&D21S1414) PCR.

Study	DM		APOC2		D21S1414	
	amplification efficiency	ADO	amplification efficiency	ADO	amplification efficiency	ADO
DM only	94% (47/50)	12.8% (6/47)				
DM&APOC2 (protocol 1)	95% (95/100)	9.5% (9/95)	95% (95/100)	10.5% (10/95)		
DM&D21S1414 (protocol 2)	98% (49/50)	4.1% (2/49)			98% (49/50)	14.3% (7/49)

Triplex PCR using DM, APOC2 and D21S1414 primers, which would detect an even greater number of contaminants, is also possible as shown in **Figure 4.8**.

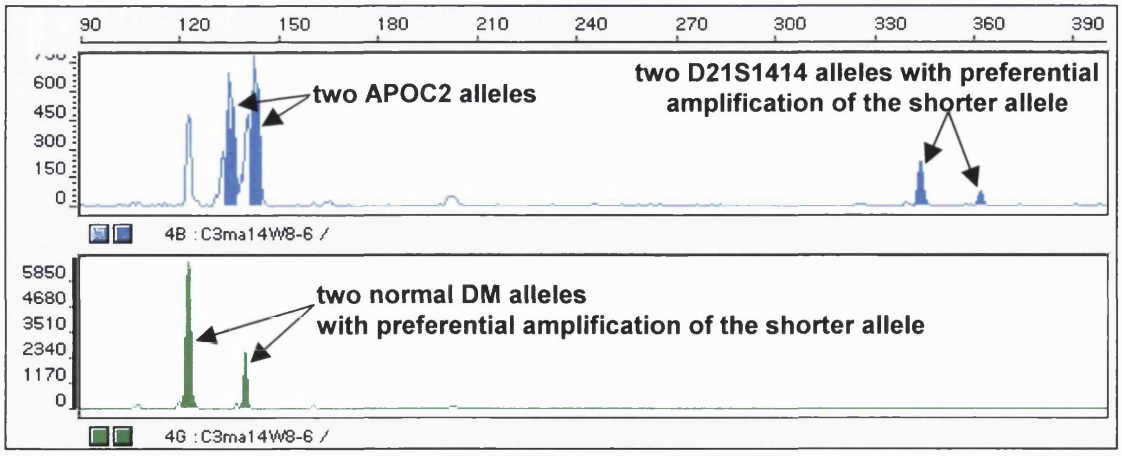


Figure 4.8 DM, APOC2 and D21S1414 results triplex amplified from a single buccal cell of a heterozygote subject for the 3 loci analysed on ABI Prism™310 using GeneScan analysis. Lane 4B (blue) shows 2 APOC2 alleles and 2 D21S1414 alleles, lane 4G (green) shows two normal DM alleles. The x and y axes are base and peak height units, respectively.

In both studies, there was no contamination in any of the final wash drop blanks, lysis-buffer-only negative controls or PCR reaction-mixture-only negative controls. One wash drop blank was taken for every 10 single buccal cells and one for every single blastomere, a lysis-buffer-only negative control and a PCR reaction-mixture-only negative control were taken for every experiment.

Multiplex F-PCR using DM and APOC2 primers was conducted on 31 spare single blastomeres donated for research and showed 93.5% (29/31) amplification efficiency for each locus. ADO rates were estimated to be 6.9% (2/29) and 10.3% (3/29) for DM and APOC2, respectively. Multiplex fluorescent analysis for DM and D21S1414 loci was carried out on 23 single blastomeres giving an amplification efficiency of 95.7% (22/23) for each locus. ADO rates for DM were 4.5% (1/22) while for D21S1414 they were 22.7% (5/22). However, chromosomal mosaicism can make ADO appear higher in this cell type (Delhanty *et al.*, 1997). All final wash drop blanks, lysis-buffer-only negative controls and PCR reaction-mixture-only negative controls were negative for DNA contamination.

4.3.2 Work up for DM PGD cases

PGD workup for two families, 'A' and 'B' (**Table 4.4**), at risk of having a congenital DM child was performed using DNAs extracted from blood and buccal cell samples from the members of both families. The 36-year old carrier mother, 'Am', of the family 'A' experienced two spontaneous miscarriages and two terminations of pregnancy following positive prenatal diagnosis of DM after the delivery of a congenital DM baby. The healthy father, 'Af', of this family was 38 years old, and the congenital affected son

was 8 years old with a history of operations correcting talipes of both feet. The carrier mother, 'Bm', of family 'B', whose father and brother were mildly affected, was 32 years old. She delivered one congenital affected baby and had two terminations of pregnancy after positive chorionic villous sampling for DM. The father 'Bf', of this family was 34 years old and healthy, and the congenital DM son was 9 years old.

Table 4.4 Summarised details of families 'A' and 'B'.

	Family 'A'	Family 'B'
Carrier	mother	mother
Mother age	35 years old	32 years old
Father age	38 years old	34 years old
Children	1 congenital DM son, 8 years old	1 congenital DM son, 9 years old
Obstetric history	2 spontaneous miscarriages 2 TOP following positive PND	2 TOP following positive PND
Family history	-	affected father and brother

DM = myotonic dystrophy, TOP = termination of pregnancy, PND = prenatal diagnosis

The results of F-PCR analyses and the numbers of CTG repeat in DM and AC repeats in APOC2 for each member of the two families are shown in **Figures 4.9-4.12**. There is only one normal allele (peak) of the DM gene of 'Am', 'Ac', 'Bm' and 'Bc', shown in **Figure 4.9** and **4.10**, because the mutant CTG expansion is longer than 500 bp and cannot be seen by the GeneScan® protocol. The expected genotypes of the DM and APOC2 genes in the offspring of both families are demonstrated in **Figure 4.10** and **4.12**.

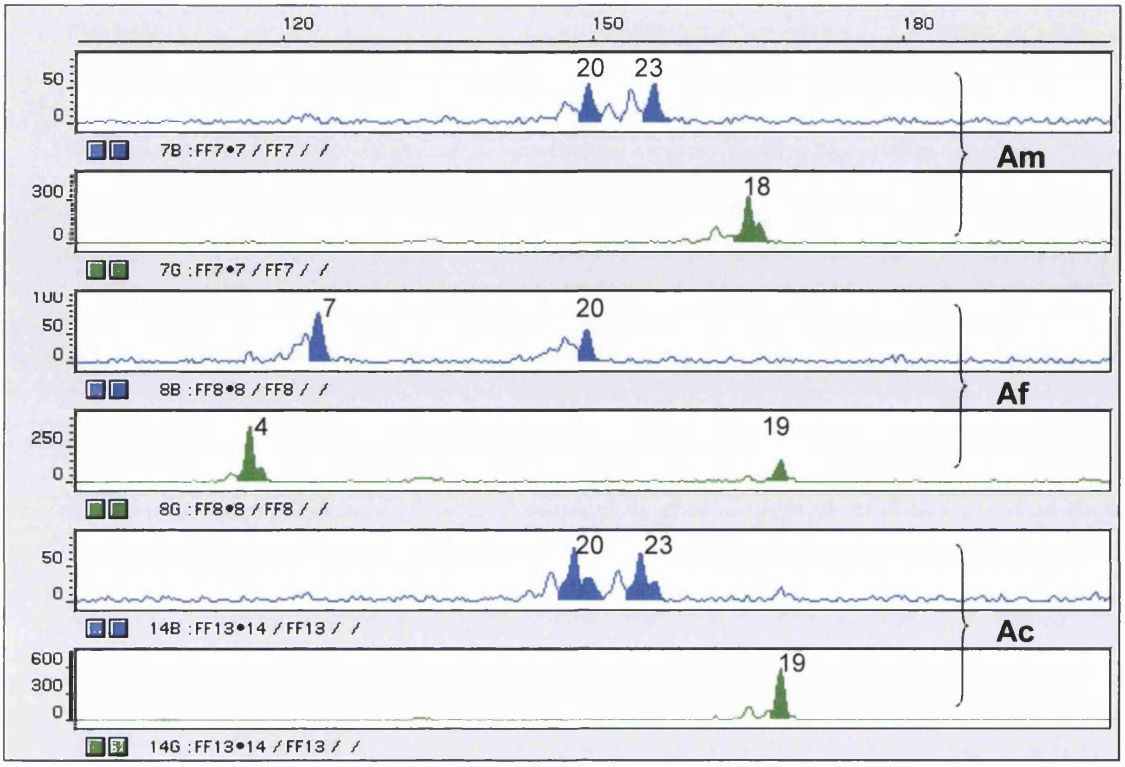
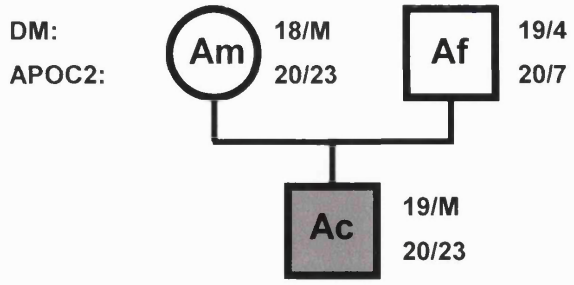


Figure 4.9 DM (green) and APOC2 (blue) genotypes of the 'A' family analysed on the ABI Prism™310, 'Am' = mother (lanes 7B and 7G), 'Af' = father (lanes 8B and 8G), 'Ac' = child (lanes 14B and 14G), were shown (number = repeat copy number). The x and y axes are base and peak height units, respectively.



The genotypes of all possible offspring

	a)	b)	c)	d)
DM:	18/19	18/4	M/19	M/4
APOC2:	20/20	20/7	23/20	23/7
Phenotypes:	NORMAL	NORMAL	AFFECTED	AFFECTED

Figure 4.10 The number of CTG repeat in the DM and AC repeat in the APOC2 genes in family 'A', 'Af' = father, 'Am' = mother, 'Ac' = child, (number = repeat copy number, M = mutant unstable CTG expansion). The genotypes of all possible offspring are also shown.

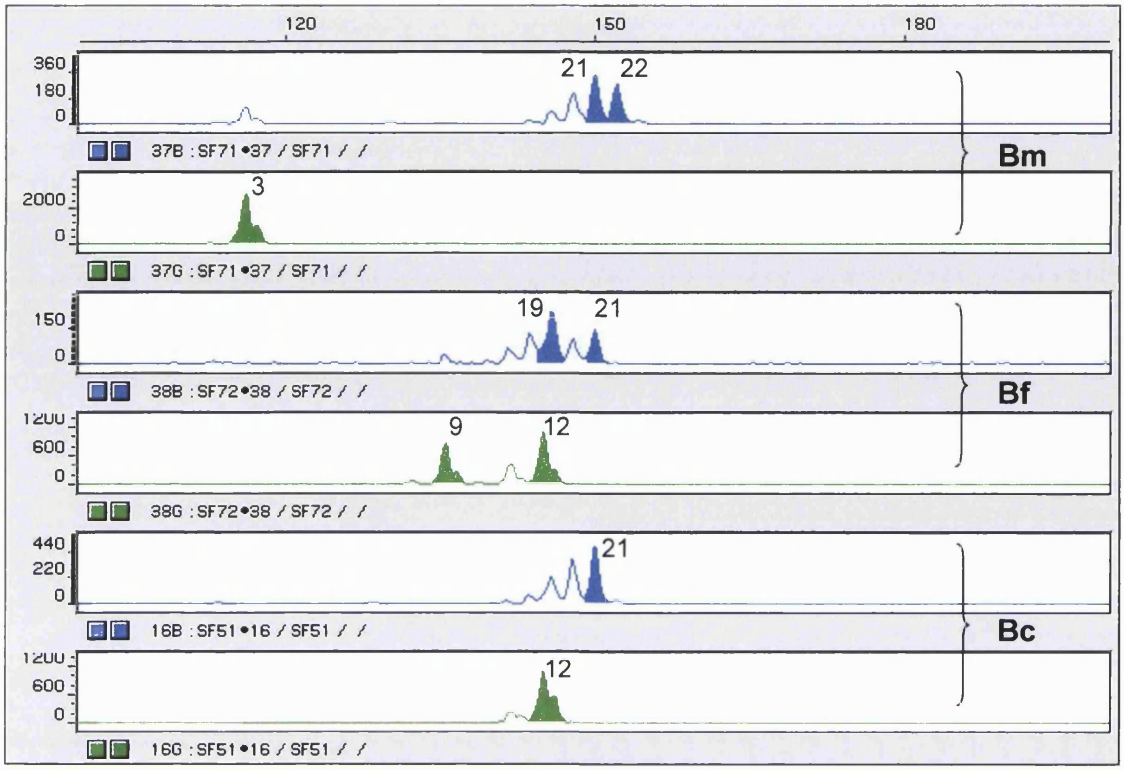
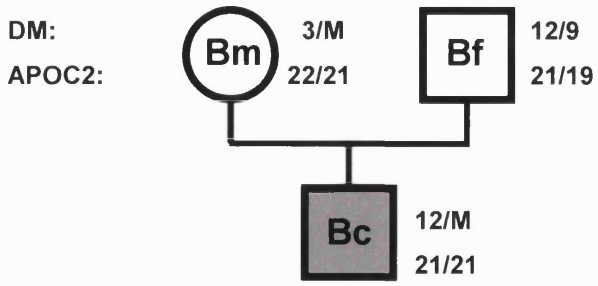


Figure 4.11 DM (green) and APOC2 (blue) genotypes of the 'B' family analysed on the ABI Prism™310, 'Bm' = mother (lanes 37B and 37G), 'Bf' = father (lanes 38B and 38G), 'Bc' = child (lanes 16B and 16G), were shown (number = copies number). The x and y axes are base and peak height units, respectively.



The genotypes of all possible offspring

	a)	b)	c)	d)
DM:	3/12	3/9	M/12	M/9
APOC2:	22/21	22/19	21/21	21/19
Phenotypes:	NORMAL	NORMAL	AFFECTED	AFFECTED

Figure 4.12 The number of CTG repeat in the DM and AC repeat in the APOC2 genes in family 'B', 'Bf' = father, 'Bm' = mother, 'Bc' = child, (number = repeat copy number, M = mutant unstable CTG expansion). The genotypes of all possible offspring are shown.

The single buccal cells used in optimising and testing the PGD protocol were derived from members of both families, including 120 cells from each of 'Af' and 'Bf' and 30 cells from each of 'Am', 'Ac', 'Bm' and 'Bc'. The data from all 360 single buccal cells of the members of both families showed an amplification failure rate of 13.6% and 12.8% and an ADO rate of 10.0% and 16.9% for DM and APOC2 genes, respectively (**Table 4.5**). The details of amplification failure and ADO rates of the single buccal cells of each member of both families are shown in **Table 4.5**. The higher amplification failure and ADO rates of the single buccal cells of 'Ac' might be because of the longer time from the sample collection until single cell isolation as the sample was at room temperature for 7 days during transit, consequently, the buccal cell sample contained more dead cells.

Table 4.5 The data from 360 single buccal cells of the members of both families, 'Af' = father, 'Am' = mother, 'Ac' = child of 'A' family and 'Bf' = father, 'Bm' = mother, 'Bc' = child of 'B' family, including all PCR protocols, shows amplification failure and allele drop out rates of the single buccal cells of each member for DM and APOC2 genes.

RESULTS	SUBJECTS						
	'Af'	'Am'	'Ac'	'Bf'	'Bm'	'Bc'	Total
n	120	30	30	120	30	30	360
DM							
• AA	78.3%	93.3%	46.7%	81.7%	70.0%	83.3%	77.8%
• ADO	19.0%	-	-	8.4%	-	-	10.0%
• AF	3.4%	6.7%	53.3%	10.8%	30.0%	16.7%	13.6%
APOC2							
• AA	83.3%	66.7%	40.0%	70.8%	46.7%	100.0%	72.5%
• ADO	11.5%	28.6%	50.0%	14.1%	30.0%	-	16.9%
• AF	5.8%	6.7%	20.0%	17.5%	33.3%	-	12.8%

AA = appropriate/adequate amplification, ADO = allele drop out, AF = amplification failure

A blind study was carried out on 30 single buccal cells, 10 from each of the 'A' family's members. Three normal samples (10%) gave inconclusive results due to ADO and would have been excluded if they had been embryos undergoing PGD. Using this protocol ADO would not result in the transfer of an affected embryo, only a reduction in the number of embryos available for transfer. During the blind study, no affected cells gave a result that could be interpreted as coming from an unaffected embryo.

The APOC2 genotypes of both families were not fully informative (i.e. the parents of families 'A' and 'B' shared the '20' and '21' APOC2 alleles, respectively, **Section 4.3.2**). In some cases an unaffected embryo with ADO at the APOC2 locus would resemble an embryo with an affected genotype. No embryo giving an affected result at APOC2 or DM loci would be considered for transfer and consequently some unaffected embryos could be excluded from transfer. It is inevitable that this loss would ultimately cause a reduction in pregnancy rates. A further problem when using polymorphisms that are only partially informative is that contamination with parental DNA could go undetected (e.g. **Figure 4.12**, if affected genotype 'c' had maternal contamination it would look identical to unaffected genotype 'a'). Using a fully informative marker, contamination would be revealed by the presence of three alleles rather than the usual two. The use of a microsatellite marker for contamination detection was considered to be of greater importance than for linkage analysis results. Therefore, the polymorphic D21S1414 marker, which was fully informative in family A (**Figure 4.13**), was substituted. As almost all cases of DM are transmitted from the mother, maternal contamination, most likely caused from inadvertent sampling of cumulus cells during embryo biopsy, is a significant problem. Sperm DNA contamination is unlikely due to the use of ICSI for fertilisation. Therefore, the D21S1414 marker was used for maternal

contamination detection in family B, in spite of the fact that the D21S1414 marker was not fully informative in this family (**Figure 4.14**).

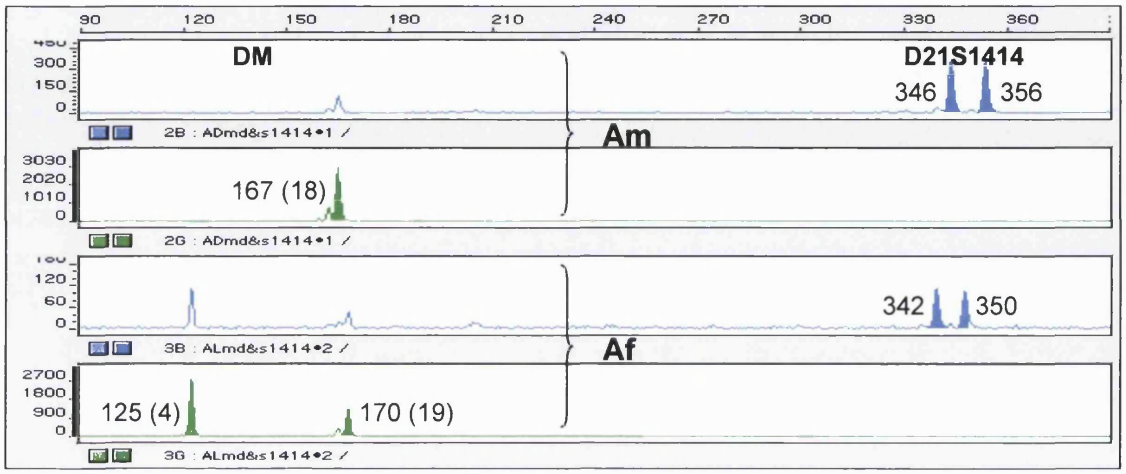


Figure 4.13 D21S1414 (blue) and DM (green) genotypes of the 'A' family analysed on the ABI Prism™310: lanes 2B and 2G are D21S1414 and DM genotypes of the 'Am' mother, lanes 3B and 3G are those of the 'Af' father, respectively (number = base pair units and repeat copy number, respectively). The x and y axes are base and peak height units, respectively.

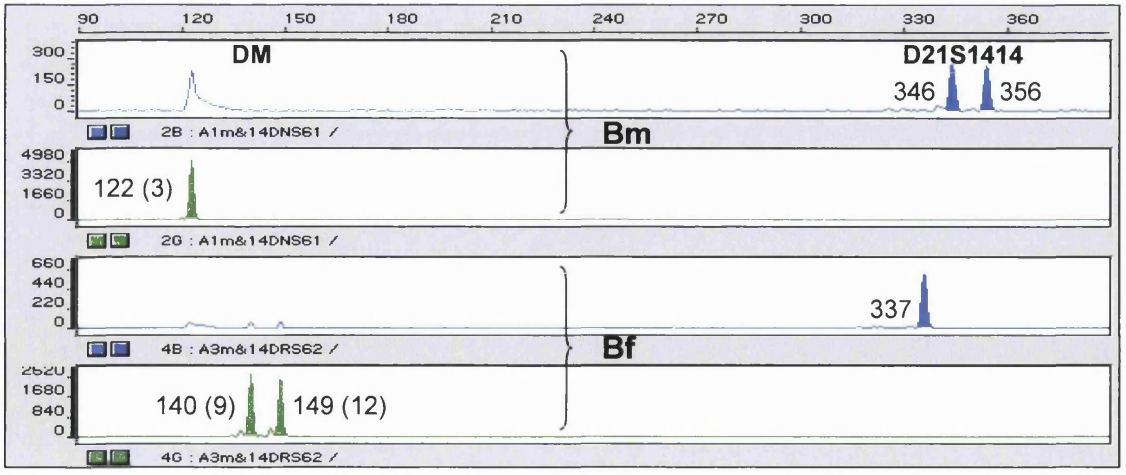


Figure 4.14 D21S1414 (blue) and DM (green) genotypes of the 'B' family analysed on the ABI Prism™310: lanes 2B and 2G are D21S1414 and DM genotypes of the 'Bm' mother, lanes 4B and 4G are those of the 'Bf' father, respectively (number = base pair units and repeat copy number, respectively). The x and y axes are base and peak height units, respectively.

4.3.3 Clinical DM PGD cases

Two PGD cycles for DM were carried out in family 'A' and one cycle in family 'B'. The single step multiplex F-PCR protocol 2 (**Section 4.2.1**) was employed in both families. The patients underwent routine IVF procedures and ICSI was employed for fertilisation in order to prevent sperm DNA contamination (**Section 2.2.2.2**). The embryos were biopsied on day 3 and the single blastomeres used for the diagnosis (**Section 2.2.3.2**).

4.3.3.1 Cycle 1, family A

PGD cycle 1 for family 'A' gave 15 oocytes, 14 were sperm-injected and 10 embryos were of sufficient quality for biopsy on day 3 post-fertilisation. Molecular analyses using multiplex F-PCR revealed 3 embryos to be affected, 2 normal, 1 contaminated and 4 with unclear results (two were suspected to be normal, while the other two were considered likely to be affected) (**Table 4.6**). The examples of the embryonic morphology prior to biopsy and the analysis results of two normal (embryos A4 and A8) and one affected (embryo A15) embryo are shown in **Figure 4.15-4.17**, respectively. The two normally diagnosed embryos (embryos A4 and A8) were chosen for transfer. The whole untransferred embryos were analysed further using the same protocol as used for the actual PGD. The embryos with ambiguous results that were thought to be normal (embryos A2 and A10) were proved to be normal. All three embryos diagnosed as affected (embryos A1, A3 and A5) and the two that were unclear but suspected to be affected (embryos A11 and A15) were confirmed as affected.

Table 4.6 Preimplantation genetic diagnosis results of family 'A', cycle 1.

Embryo number	Embryo grade (No. of cells) prior to biopsy (day 3)	No. of cell taken	Cell No.	DM results	D21S1414 results	Notes	Diagnosis	Embryo grade (No. of cells) after biopsy (day 4)	Outcomes	Confirmatory results
A1	2 (6)	2	A1.1 A1.2	A AF	diallelic AF	no nucleus seen	A	2 (6)	UT	A
A2	2^+ (7)	2	A2.1 A2.2	N with PA of paternal allele or A with a small artefact peak two normal alleles or one normal plus a stutter	diallelic diallelic		unclear, likely to be N	2^+ (5)	UT	N
A3	1 (8)	2	A3.1 A3.2	A A	diallelic diallelic		A	morula	UT	A
A4	2^+ (7)	2	A4.1 A4.2	N N	diallelic monoallelic		N	1^- (7)	ET	-
A5	1 (4)	1	A5.1	A	diallelic		A	compacting (4)	UT	A
A6	2 (4)	1	A6.1	two normal alleles or one normal plus a stutter	triallelic	incomplete washing step due to lysing cell	maternal DNA contaminated	2^- (4)	UT	N, diallelic
A8	1^- (6)	2	A8.1 A8.2	N N	diallelic diallelic		N	2^+ (7)	ET	-
A10	2 (5)	1	A10.1	two normal alleles or one normal plus a stutter	diallelic		unclear, likely to be N	2^+ (5)	UT	N
A11	2^+ (7)	2	A11.1 A11.2	A A?	diallelic diallelic		likely to be A	2 (6)	UT	A
A15	1 (8)	2	A15.1 A15.2	A? A	diallelic diallelic		likely to be A	morula	UT	A

DM = myotonic dystrophy; D21S1414 = STR marker; AF = amplification failure; PA = preferential amplification; A = affected for DM locus; N = normal for DM locus; UT = untransferred; ET = embryo transferred.

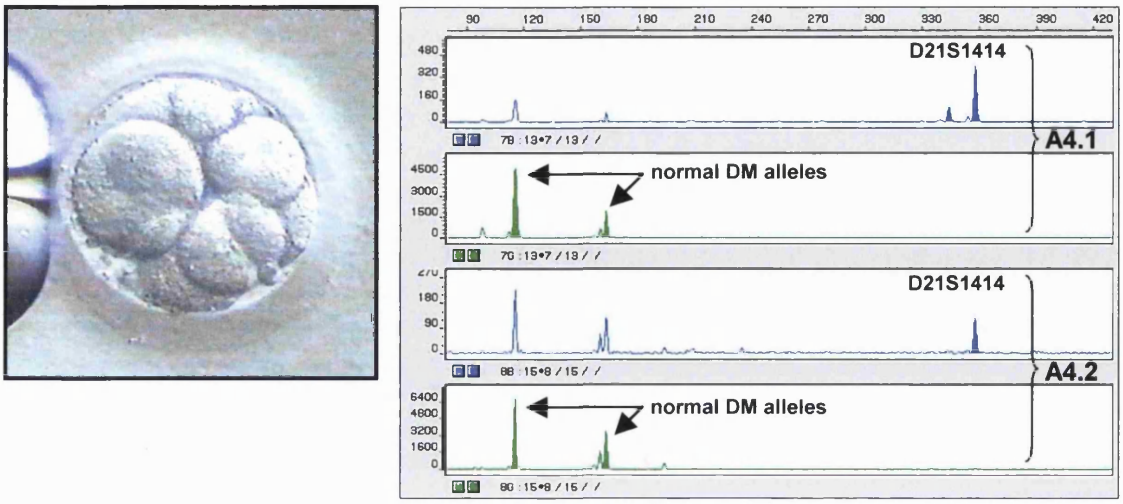


Figure 4.15 Morphology of embryo A4 (7-cell; left) prior to biopsy with the PGD of DM results (right). Both biopsied blastomeres (A4.1 and 4.2) showed two normal DM alleles (green), one from each of the parent. Blastomere A4.1 exhibited two D21S1414 alleles (blue), one from each of the parent, while blastomere A4.2 showed only one allele, probably due to ADO. This embryo was diagnosed as normal and chosen from transfer. The x and y axes are base and peak height units, respectively.

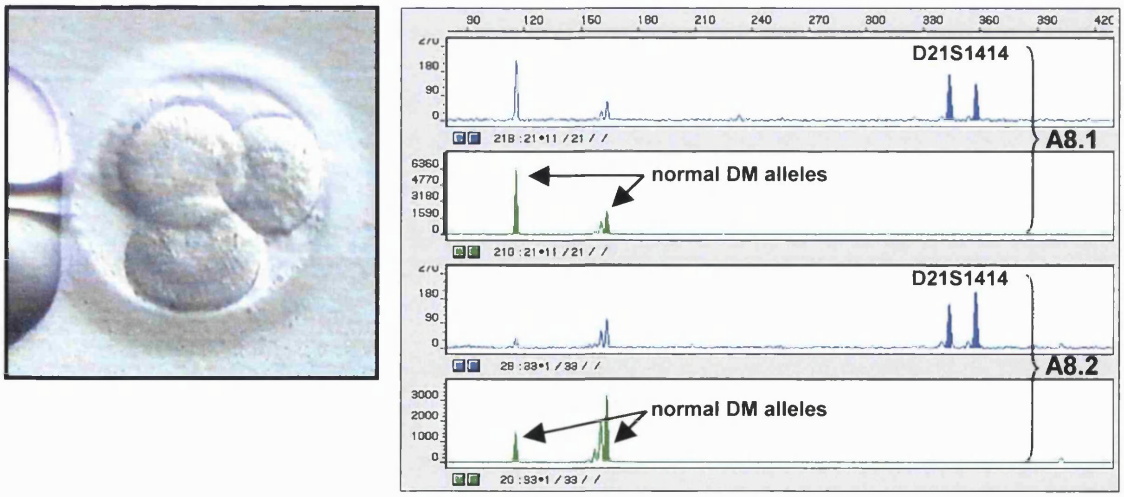


Figure 4.16 Morphology of embryo A8 (6-cell; left) prior to biopsy with the PGD of DM results (right). Both biopsied blastomeres (A8.1 and A8.2) showed two normal DM alleles (green) and two D21S1414 alleles (blue), one from each of the parent. This embryo was diagnosed as normal and chosen from transfer. The x and y axes are base and peak height units, respectively.

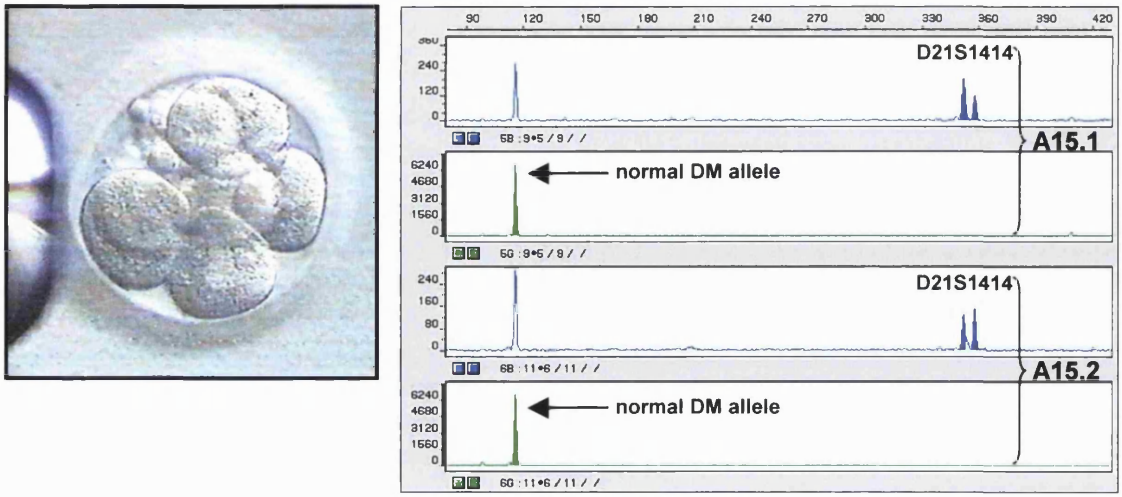


Figure 4.17 Morphology of embryo A15 (8-cell; left) prior to biopsy with the PGD of DM results (right). Both biopsied blastomeres (A15.1 and A15.2) showed only one normal DM allele (green) from the father and exhibited two D21S1414 alleles (blue), one from each of the parent. This embryo was diagnosed as affected and was not transferred. The x and y axes are base and peak height units, respectively.

Analysis of the biopsied cell from embryo 6 indicated the presence of both the mothers D21S1414 alleles as well as one allele from the father. This suggested that the embryo was either trisomic for chromosome 21, having inherited an additional copy of this chromosome from the mother or alternatively that the sample was contaminated with maternal DNA. Confirmatory analysis of the remaining blastomeres from this embryo revealed disomy for chromosome 21 and thus contamination is the most likely explanation in this instance. The cell had begun lysing soon after the biopsy and was transferred to the PCR tube after just one wash in PBS, rather than 3-4 washes, which may explain why contamination occurred in this case.

In embryos A5, A6 and A10 only one cell was analysed due to low cell number at the time of biopsy. However, the accuracy of diagnosis, predicted from the analysis of

single buccal cells, is still very high (>94%). It is better to get a result from as many embryos as possible even if the accuracy for some is slightly diminished. Unfortunately no pregnancy was obtained from this PGD cycle; therefore, the patient decided to undergo another PGD cycle (**Section 4.3.3.2**).

4.3.3.2 Cycle 2, family A

Ten oocytes were collected and sperm-injected in PGD cycle 2 for family 'A'. Six developed into good quality day-3 embryos suitable for biopsy. Multiplex fluorescent PCR analysis of the biopsied single blastomeres showed two normal (AA3 and AA9) and 3 affected (AA7, AA8 and AA10) embryos (**Table 4.7**). Both anucleated blastomeres from embryo AA6 gave no result. The whole untransferred embryos were analysed by the same protocol as used for PGD for confirmatory results. The later analysis of embryo AA6 revealed an unaffected embryo with three D21S1414 alleles (two maternal), possibly due to this embryo having three copies of chromosome 21 (Down's syndrome) (**Figure 4.18**). Both normally diagnosed embryos were transferred and one clinical pregnancy (singleton) was obtained. The CVS result confirmed the PGD, and a normal baby girl was delivered. Further analyses of the untransferred embryos confirmed the results of the original diagnosis.

Table 4.7 Preimplantation genetic diagnosis results of family 'A', cycle 2.

Embryo number	Embryo grade (No. of cells) prior to biopsy (day 3)	No. of cell taken	Cell No.	DM results	D21S1414 results	Notes	Embryo grade (No. of cells) after biopsy (day 4)	Diagnosis	Outcomes	Confirmatory results
AA3	2^+ (7)	2	AA3.1 AA3.2	N N	diallelic diallelic		morula	N	ET	-
AA6	2 (6)	2	AA6.1 AA6.2	AF AF	AF AF	no nucleus seen no nucleus seen	3 (5)	no result	UT	N, triallelic for D21S1414 marker
AA7	1^- (6)	2	AA7.1 AA7.2	A A	diallelic diallelic		morula	A	UT	A
AA8	2 (6)	2	AA8.1 AA8.2	A A	diallelic monoallelic		compacting (6)	A	UT	A
AA9	1^- (8)	2	AA9.1 AA9.2	N N	diallelic diallelic		2^+ (7)	N	ET	-
AA10	1^- (7)	2	AA10.1 AA10.2	A A	diallelic diallelic		morula	A	UT	A

DM = myotonic dystrophy; D21S1414 = STR marker; AF = amplification failure; PA = preferential amplification; A = affected for DM locus; N = normal for DM locus; UT = untransferred; ET = embryo transferred.

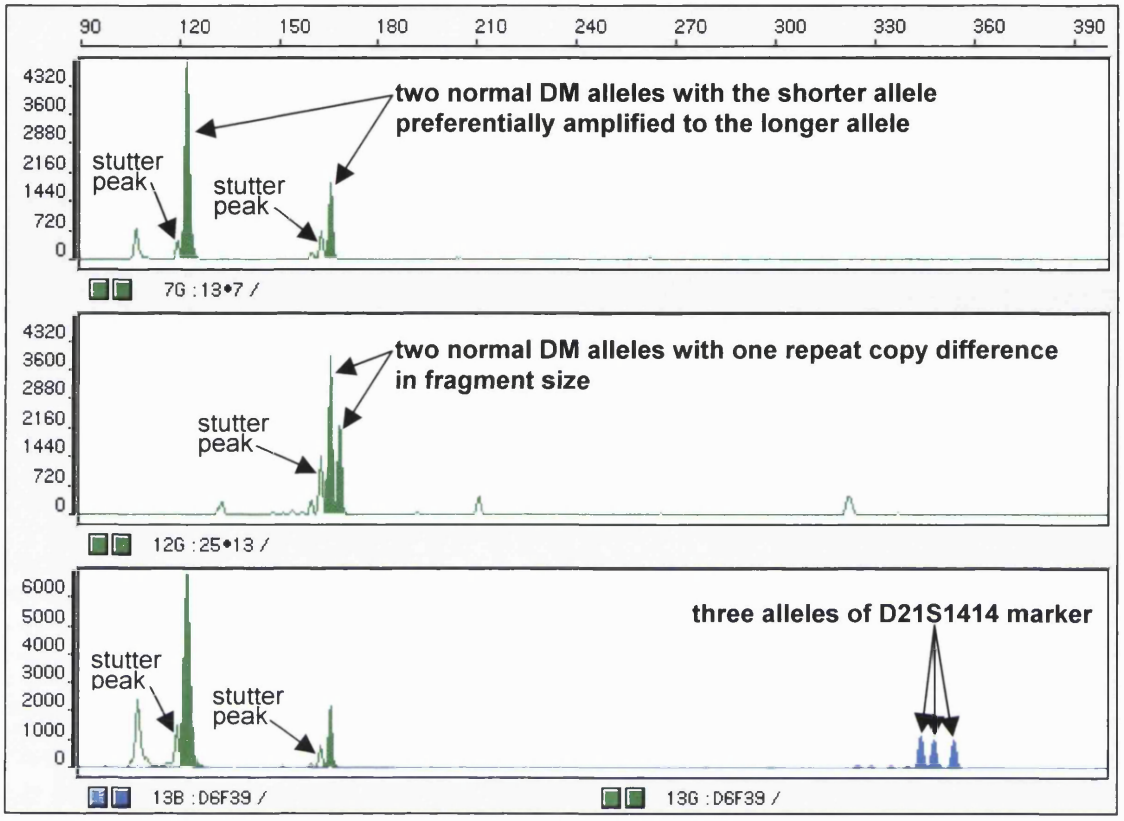


Figure 4.18 Result from GeneScan analysis™ on ABI Prism™310 for DM and D21S1414 primers multiplex amplified from single blastomeres (lanes 7G: blastomere A4.1 and 12G: blastomere A6.1) and a whole embryo (lane 13B: embryo AA6). Lane 7G shows the amplified DM product of a normal blastomere with the longer allele less efficiently amplified than the shorter allele. Stutter peaks were also exhibited by both alleles. Lane 12G shows PCR product of a normal blastomere which possesses two normal alleles with one repeat copy difference in size. This peak pattern can be confused with stutter peaks. Lane 13B shows multiplex analysis result from the un-transferred embryo AA6. Three alleles of the polymorphic marker on chromosome 21 (D21S1414) were present. This suggests that the embryo was trisomic or that contamination with maternal DNA had occurred. The x and y axes are base and peak height units, respectively.

4.3.3.3 Cycle 1, family B

During cycle 1 for family 'B' 15 oocytes were collected and sperm-injected giving rise to 14 good quality embryos suitable for biopsy on day 3. Two blastomeres were taken from each embryo and the analysis revealed three normal (embryos B2, B4 and B6), ten affected (embryos B5, B7, B8, B9, B10, B11, B12, B13, B14 and B15) and one embryo for which the diagnosis was ambiguous (embryo B1) (**Table 4.8**). The three normally diagnosed embryos were selected for transfer. Blastomere B1.1, from the ambiguous embryo, showed one normal paternal allele, while blastomere B1.2 gave one normal maternal allele. Therefore, embryo B1 might be normal with ADO results in both analysed blastomeres. The analysis of the rest of the embryo showed a normal genotype.

Follow up analyses were performed on the untransferred embryos. For this purpose whole embryos were transferred to PCR tubes and subjected to the same protocol as used for the actual PGD. The initial diagnosis was confirmed in all cases. The three blastomeres that failed to give results (blastomeres number B8.2, B10.2 and B15.1) were from affected embryos. No contamination was detected. Following this PGD cycle a twin pregnancy was achieved, CVS was carried out and confirmed that both twins were free of DM and two healthy baby girls were delivered.

Table 4.8 Preimplantation genetic diagnosis results of family 'B', cycle 1.

Embryo number	Embryo grade (No. of cells) prior to biopsy (day 3)	No. of cell taken	Cell No.	DM results	D21S1414 results	Notes	Diagnosis	Embryo grade (No. of cells) after biopsy (day 4)	Outcomes	Confirmatory results
B1	1^- (9)	2	B1.1	A	AF		ambiguous,	2^+ (8)	UT	N
			B1.2	N with ADO	monoallelic		likely to be N			
B2	1^- (8)	2	B2.1	N	monoallelic		N	blastulating	ET	-
			B2.2	N	diallelic					
B4	1^- (8)	2	B4.1	N	diallelic		N	morula	ET	-
			B4.2	N	diallelic					
B5	1^- (8)	2	B5.1	A	diallelic		A	compacting	UT	A
			B5.2	A	AF					
B6	1^- (7)	2	B6.1	N	diallelic		N	compacting (8)	ET	-
			B6.2	N	AF					
B7	2^+ (8)	2	B7.1	A	diallelic		A	blastulating	UT	A
			B7.2	A	diallelic					
B8	2^+ (7)	2	B8.1	A	AF		A	(4)	UT	A
			B8.2	AF	diallelic					
B9	1^- (8)	2	B9.1	A	diallelic		A	compacting	UT	A
			B9.2	A	diallelic					
B10	2^+ (6)	2	B10.1	A	monoallelic		A	2^+ (4)	UT	A
			B10.2	AF	AF	no nucleus seen				
B11	1 (8)	2	B11.1	A	diallelic		A	1^- (9)	UT	A
			B11.2	A	diallelic					
B12	1^- (8)	2	B12.1	A	diallelic		A	morula	UT	A
			B12.2	A	diallelic					
B13	1^- (7)	2	B13.1	A	diallelic		A	compacting (5)	UT	A
			B13.2	A	diallelic					
B14	2^+ (7)	2	B14.1	A	diallelic		A	compacting	UT	A
			B14.2	A	diallelic					
B15	1^- (7)	2	B15.1	AF	monoallelic		A	2^+ (5)	UT	A
			B15.2	A	diallelic					

DM = myotonic dystrophy; D21S1414 = STR marker; AF = amplification failure; PA = preferential amplification; A = affected for DM locus; N = normal for DM locus; UT = untransferred; ET = embryo transferred.

Summarising the data from the 57 single blastomeres biopsied during these three cases, the DM gene was successfully amplified in 51 (89.5%) and the D21S1414 locus was amplified in 49 (86%). Interestingly the amplification efficiencies of the DM and D21S1414 loci in the 53 blastomeres that were confirmed nucleated were 96.2% (51/53) and 92.5% (49/53) respectively. For 36 cells a confirmatory analysis had been achieved by reanalysis of the embryos that were not transferred to the mother. No misdiagnoses were recorded. The confirmatory data also enabled ADO rates to be calculated. ADO affected 3.9% (2/51) of DM amplifications and 12.2% (6/43) of D21S1414 amplifications. With the exception of the single cell from embryo A6, no contamination was observed. All of the PCR blanks were negative.

4.3.4 Hi-fidelity PCR for DM expansion detection

Eight PCR protocols (A-H) utilising five high efficiency PCR systems (**Section 4.2.4**) were employed in an attempt to amplify the mutant triplet repeat expansion within the DM gene. Initially these protocols were applied to the genomic DNA of two DM carriers, 'DM1' and 'DM2'. Every protocol succeeded in amplifying the normal alleles, 100-200 bp in size. However, only protocols H and I, using 30 min extension time, enabled detection of extra bands, approximately 800 bp in size (**Figure 4.19**). The analysis on the ABI Prism™310 and ALF Express™ exhibited the same results as agarose gel electrophoresis, but provided a more precise fragment size determination for the normal 167 and 122 bp alleles (for DM1 and DM2, respectively) and mutant 797 and 776 bp alleles (for DM1 and DM2, respectively).

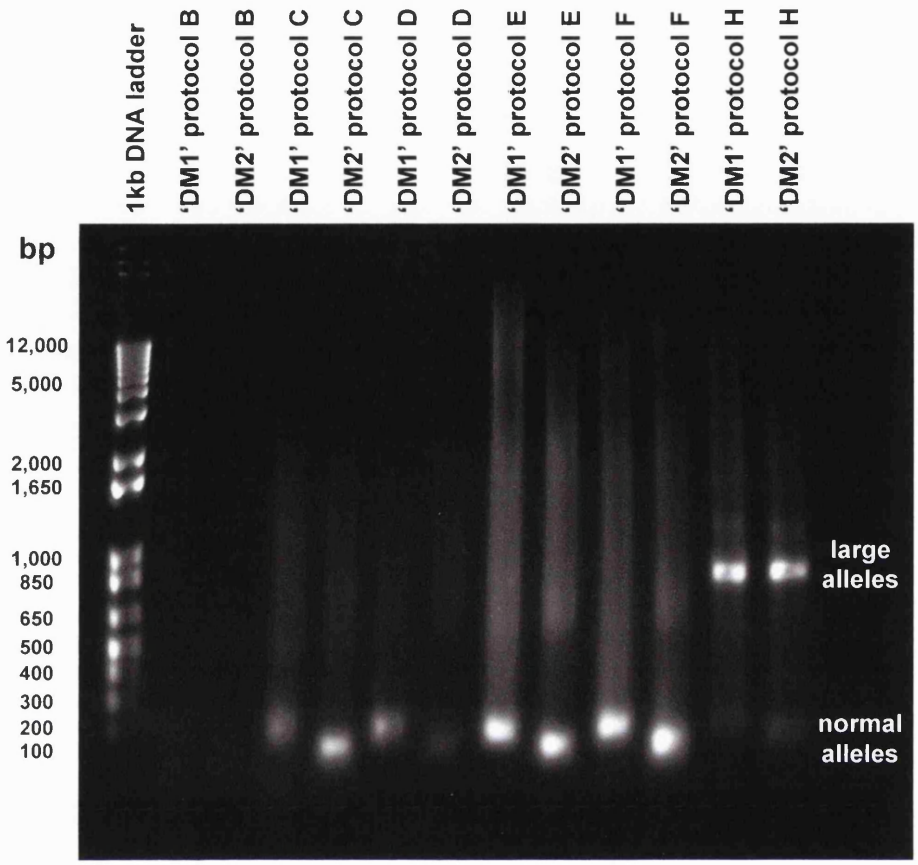


Figure 4.19 Electrophoresis analysis of the amplified products from genomic DNA of the DM carriers, 'DM1' and 'DM2', using different protocols, A, B, C, D, E, F, G and H, (details in **Table 4.2**) on 2% agarose gel. All protocols show the normal DM alleles of 100-200 bp, but only protocol H that also shows the large DNA fragments of approximately 800 bp in length.

The successful protocols for amplifying large triplet repeat alleles from genomic DNA, protocols H and I, were applied to single buccal cells from affected patients. The preliminary experiments failed to show the same results as those performed on genomic DNA and only protocol I, which uses a different PCR buffer to that of protocol H, was successful. However, the first use of protocol I, with a 30 min extension time and 50 cycles of the main PCR protocol, took 26 h to complete. Some PGD centres that prefer to

transfer embryos on day 3 or 4 post-fertilisation might consider this protocol too lengthy for clinical application.

In order to shorten protocol I the extension times of 2, 5, 10, 20 and 30 min were compared. When genomic DNA was amplified the normal alleles were present in all experiments, while the large fragments were only detected in those with 5, 10, 20 and 30 min extension times. However, successful application to single cells required extension times of at least 10 min. With 10 min extension time, protocol I was tested by amplifying single cells over 30, 35, 40, 45 and 50 cycles. Both normal and large fragments could be visualised by 1% agarose gel electrophoresis if amplified for at least 45 cycles. Therefore, the optimal single cell PCR for both normal and mutant expansion alleles comprises a 10 min extension time and 45 cycles, lasting a total of 10 h. This fits within a time frame suitable for application to PGD. However, the use of fluorescent PCR requires fewer cycles and provides more accurate sizing. The analysis on the automated laser fluorescent sequencer demonstrated that the large fragment is less efficiently amplified than the normal allele, but both could be readily identified.

Unfortunately application of the optimised protocol I to more DNA and single cell samples revealed the same large amplified fragments in the control normal samples as well as affected individuals. Therefore, the observed large amplified fragments were not the mutant DM expanded alleles, but were artefacts. These might have been due to annealing of one or both of the DM primers at an additional non-specific site. The fact that the fragment amplified is in approximately the same size range as the mutant repeat expansion is coincidental. Although the new protocol was unable to amplify the expanded repeat it is clearly capable of amplifying DNA fragments over 1kb in size from single cells.

4.4 Discussion

4.4.1 PGD strategy for DM

Accurate molecular analysis of the DM repeat expansion can be performed by using Southern blotting. However, this method needs a high copy number of the DNA template, and therefore cannot be applied for PGD. Alternatively, PCR is a fast and efficient technique for the molecular diagnosis of DM. However, the interpretation is based on the exclusion principle as the amplification of the fragment size larger than 500 bases is inefficient, therefore only the normal alleles can be examined. When applying to PGD where contamination and ADO problems are crucial, precautions must be taken during interpretation in order to obtain an accurate diagnosis.

Previous reports of PGD of DM described analysis using traditional gel electrophoresis (Sermon *et al.*, 1997) and later F-PCR (Sermon *et al.*, 1998a). However, a misdiagnosis occurred using the latter PGD protocol (Sermon *et al.*, 1998b), probably due to maternal DNA contamination (Vandervors *et al.*, 2000). As the normal allele of the DM gene is very polymorphic in the number of triplet repeat copies and the mutant allele, which consists of hundreds of triplet repeat copies, can not be amplified and visualised, a diagnosis of unaffected was made only when two normal alleles (one from each of the parents) were detected.

The polymorphic characteristic of the DM gene provides the advantage of identifying the presence of both normal DM alleles in the normal samples and some cases of contamination. However, in the case of an affected cell or abnormal cell with ADO,

where only one allele is present, the occurrence of contamination may be concealed and, moreover, may lead to misdiagnosis. Therefore, the PGD protocols in this project aimed to detect the presence of contamination by amplifying a linked marker in the same region as the DM gene or an unlinked polymorphic marker on another chromosome.

The hypervariable STR along with the polymorphic CTG repeat in DMPK, provides a simple form of genetic fingerprint. However, the use of the highly polymorphic microsatellite marker flanking the DM gene, APOC2, offers the added advantage of back up diagnosis by linkage analysis (Protocol 1). This marker is situated within the human apolipoprotein C2 (APOC2) gene and has a reported heterozygosity of 0.80 (Weber and May, 1989). The APOC2 gene is situated approximately 4 cM upstream to the DMPK gene with a maximum lod score (Z) of 7.877 at 4% recombination (θ) (Shaw *et al.*, 1985). In addition, the linkage information can also predict the presence of the mutant DM expanded allele in the embryo, which cannot be obtained by direct PCR analysis of the DM gene. This allows differentiation between the affected embryo and normal embryo whose DM results are complicated by ADO. Therefore, complete diagnosis can be drawn from more normal embryos, giving rise to a larger number of embryos for transfer and a better chance of pregnancy. This strategy is also useful for couples who have a DM allele in common where without linkage analysis results, only half of the normal embryos can be identified.

An unlinked polymorphic marker (D21S1414) can be substituted for the APOC2 in a family whose APOC2 genotypes are not fully informative (protocol 2) for contamination detection only. The D21S1414 locus is unlinked to DMPK gene, and consequently provides no information on inheritance of the DM mutation, but its highly

polymorphic nature allows it to serve as a very basic form of DNA fingerprint. The reported heterozygosity of the D21S1414 locus is 0.88 (Sherlock *et al.*, 1998).

In this study analysis was performed using the automated laser fluorescent sequencers: the ALF Express™ and the ABI Prism™310. The high accuracy of F-PCR using an automated fluorescent DNA sequencer, which allows differentiation of a single base pair differences in size, makes this PGD protocol more specific than those using traditional gel analysis. Furthermore, the high sensitivity of F-PCR can detect even a tiny amount of the amplified fluorescent product. This benefits the PGD protocol as nested PCR was unnecessary. The amplified products of both genes could be analysed immediately after one multiplex PCR; therefore, the PGD protocol was faster than the reported method (Sermon *et al.*, 1997). The PCR method was optimised for maximum efficiency and the PGD protocol developed and tested using 800 single cells (over 1,000 PCRs in total).

The split peak problem is caused by the addition of an extra nucleotide to the 3′ ends of the amplified fragments during the amplification reaction. The added nucleotide is usually an adenosine, and hence the phenomenon is known as plus-A. The plus-A products are usually shown as an extra peak one bp longer than the expected PCR products on the automated laser fluorescent sequencer. This problem would not affect the determination of DM or the STR marker genotypes, but may lead to peak area miscalculation and could theoretically confuse the diagnosis of mutations involving a 1 bp deletion or insertion. The split peak pattern can be minimised by either encouraging or suppressing the nucleotide addition. In this PGD protocol, it was decided to minimise the

plus-A peak by simply omitting the final extension step. This gave a satisfactory outcome.

The technique of labelling the forward primers with fluorescent dyes for the ABI Prism™310 and the reverse primers with a dye detectable by the ALF Express™ offers back up results and comparison of both types of analytical instrument. The peak patterns given by each sample were found to be identical for both machines. The ALF Express™ is a gel-based electrophoresis system that provides results for up to 40 samples within 2-3 h depending on the size of the fragments. In order to avoid the problem of spillage of the samples from one well to its neighbour, the samples were loaded in every other well; therefore, up to 20 samples can be run at a time. The ABI Prism™310 utilises capillary-based electrophoresis with a single capillary. Analysis can be carried out for only one sample at a time and takes approximately 30 min per sample. The ABI Prism™310 can visualise up to 4 fluorescent dyes (colours) at a time simplifying analysis of multiple products. The red colour is spared for a size standard that is run with every sample making sizing extremely accurate. The electrophoretograms from the ABI Prism™310 possess fewer artefact peaks than those from the ALF Express™. However, some artefact peaks are present in both instruments. Most irregular baseline peaks were probably due to the unhomogeneous composition of the media (gel/matrix). The ABI Prism™310 provides an advantage over the ALF Express™ in terms of a flatter baseline, clearer distinct peaks, and more precise fragment size calculation. However, if more than six samples are to be tested the analysis time is longer than that using the ALF Express™. The use of the gel based ABI Prism™377 or the 16-capillary ABI Prism™3100 automated sequencers can allow quicker results.

The optimised PGD protocol 1 and 2 of DM gave rise to acceptable amplification efficiency for DM, APOC2 and D21S1414 loci (95-98%, 95% and 98%, respectively; **Table 4.3**). The denaturation temperature was programmed to 96°C during the first 10 cycles in order to reduce the incidence of ADO in single cell PCR (Ray and Handyside, 1996). The ADO rates of DM, APOC2 and D21S1414 loci in this study (4.1-9.5%, 10.5% and 14.3%, respectively; **Table 4.3**) were within the range previously observed in studies of different genes (Ray and Handyside, 1996). The optimised PGD protocol 1 and 2 were tested using single buccal cells from the members of both families and other heterozygote subjects. The study on single human blastomeres showed impressive reliability. A blind study was performed using the single buccal cells of members of family 'A'. This revealed that 10% of normal embryos would give ambiguous results or would be diagnosed affected because of ADO or amplification failure. This would lead to a small reduction in the number of embryos available for transfer. However, no misdiagnosis of an affected cell was observed.

Multiplex analysis of the DM gene and the APOC2 linked marker (Lavedan *et al.*, 1991; Norman *et al.*, 1989) in a fully informative family, is an ideal PGD protocol for DM. The hypervariable nature of both loci allows most DNA contaminants to be detected in a sample specific fashion. Thus sporadic low-level contamination, which may affect a small proportion of PCR tubes, does not escape detection. A secondary benefit of using APOC2 is that it is linked to the DM locus and can therefore provide back up diagnostic information (Ao *et al.*, 1998; Rechitsky *et al.*, 1999). This is particularly useful for parents that share an identically sized DM allele. Using previous protocols half the unaffected embryos produced by such a couple were indistinguishable from affected embryos.

In conclusion, the optimised protocol 1 for PGD of DM was performed by multiplex amplification of the trinucleotide CTG repeat fragment in the DM gene and the highly polymorphic AC repeat sequence within the APOC2 gene, which is a flanking marker to the DM gene. For this purpose AmpliTaq Gold™ and fluorescent labelled primers were used and fragment sizes analysed on ABI Prism™310. The diagnosis of normal embryos would be based on the presence of two normal DM alleles and the confirmed information from the APOC2 linked marker. In the families where the APOC2 genotypes are not fully informative, an unlinked marker, D21S1414, can be substituted for contamination detection as protocol 2.

4.4.2 Work up for DM PGD cases

Unfortunately the parents in this series have an APOC2 allele in common and consequently some contaminants derived from parental cells could go undetected. Therefore, the D21S1414 marker, which is fully informative in family 'A' and acceptable semi-informative in family 'B', was substituted for APOC2. This marker cannot be used for linkage analysis as it is situated on chromosome 21, however contamination was considered to be the greatest threat to accurate diagnosis. Since all embryos are fertilised by ICSI to reduce the risk of sperm DNA contamination, the major risk of contamination comes from maternal DNA, in particular cumulus cells. This has added significance in the case of DM, as most cases are maternally inherited and it is the mother's normal allele that is diagnostic, being indicative of an unaffected embryo. The reported misdiagnosis of DM is believed to have been caused by contamination with maternal DNA (Vandervors *et al.*, 2000).

4.4.3 Clinical DM PGD cases

The first PGD cycle of family 'A' gave rise to 10 embryos for biopsy. Two good quality embryos with normal results were chosen for transfer. However, no pregnancy was achieved. Six embryos were generated in the second PGD cycle of family 'A' and two good quality embryos with two normal DM alleles were transferred resulting in a singleton pregnancy. The PGD result was confirmed by PND and a female baby was born in May 2001. The first PGD cycle of family 'B' provided 14 embryos. Three good quality embryos which showed two normal DM alleles were chosen for transfer giving rise to a twin pregnancy. The PND confirmed the PGD results and two DM-free baby girls were delivered in June 2001.

Although all of the PCR blanks (57 final wash drop blanks, 5 lysis-buffer-only negative controls and 5 PCR reaction-mixture-only negative controls) used during these three PGD cycles were free of contamination, a single contamination (with maternal DNA) was detected in one of the 57 blastomere amplifications. This would have gone undetected using previously reported PGD protocols. To further maximise diagnostic accuracy it was decided to sample two blastomeres from all embryos composed of six or more cells. The biopsy of two cells rather than just one does not seem to have adversely affected the embryos potential for further development, as evidenced by the two pregnancies from these three PGD cycles. A study comparing the implantation of the embryos following the biopsy of one, two or three blastomeres in 188 PGD cycles showed that taking two cells did not compromise the pregnancy rates (Van de Velde *et al.*, 2000).

The use of the STR marker on chromosome 21 (D21S1414) could have the added benefit of providing copy number information on this chromosome in the cells sampled. From the quantitative fluorescent (QF) PCR concept (Mansfield, 1993), the alleles of a heterozygous short tandem repeat (STR) locus in a normal individual should amplify equally so long as the PCR is in the exponential phase of amplification. At an STR locus a trisomic subject has three alleles each amplified to the same extent (i.e. three different copies of the trisomic chromosome), or alternatively two alleles with one amplified twice as much as the other (i.e. two identical copies of the trisomic chromosome and one non-identical copy). This method has been used for the detection of trisomy in small numbers of fetal cells isolated from the transcervical canal (Sherlock *et al.*, 1997). However, when tested on single cells this method gave precise results in only 75% of amplifications (Sherlock *et al.*, 1998). Therefore, the D21S1414 marker cannot be used to reliably detect all trisomy 21 embryos. Nevertheless, monosomy 21 and tri-allelic trisomy 21 would be detected.

Normal samples with markedly preferential amplification of the fathers DM allele can resemble an affected sample. Unless the mother's unaffected allele is present at an appreciable level above the base line it is not considered safe to transfer the embryo. Preferential amplification of the paternal allele was seen in blastomere A2.1 and led to the exclusion of an unaffected embryo. Minor diagnostic problems were also encountered in blastomeres A11.2 and A15.1. In both of these cases a minute peak, similar in size to the mothers normal allele, was observed in addition to a large peak corresponding to a paternal allele. This gives the impression that the embryo could be unaffected, but have preferential amplification of the paternal allele. However, because the peak was not significantly above the background level of fluorescence (an allele must be at least 2

times bigger than the largest background peak) the cells were classified as likely to be affected and this diagnosis was confirmed by the second cell biopsied from each of these embryos.

Stutter peaks are a PCR artefact sometimes encountered when a repetitive DNA sequence is amplified. They manifest as a minor peak one repeat unit smaller than the genuine allele. Stutters rarely cause a problem for diagnosis, but in family 'A' the size of the two alleles in three normal blastomeres (blastomeres A2.2, A6.1 and A10.1) differed by only one repeat copy (**Figure 4.18**). Therefore, it was difficult to determine whether the smaller peak represented the smaller of two normal alleles in a normal sample or a stutter generated by the single normal allele of an affected sample. Unless a second cell is available for confirmatory analysis the results must be marked as unclear and the corresponding embryo must be excluded from transfer. The use of a linked marker, such as APOC2 would have helped to clarify these results.

In conclusion, a comprehensive PGD protocol for DM has been developed using single step multiplex analysis of the normal DM triplet repeat region and a polymorphic linked marker (APOC2) for back up linkage analysis and contamination identification. In cases where the linked marker is only partially informative, other polymorphic markers such as D21S1414 can be used for contamination detection. Triplex analysis using DM primers for the diagnosis of DM, APOC2 for backup linkage analysis and D21S1414 as an extra means of contamination detection was also successfully performed. However, more work is needed to optimise the protocol before triplex amplification can be clinically applied. The use of duplex PCR (DM and D21S1414 primers) for PGD of DM

in the present study resulted in 3 normal babies and indicates that this is an efficient and accurate protocol for PGD.

4.4.4 Hi-fidelity PCR for DM expansion detection

The most widely used PGD protocol for DM is based on the detection of the normal alleles. An embryo that has received a normal allele from the carrier parent cannot have also inherited the parent's mutant allele (except in extraordinary circumstances) and must therefore be unaffected. However, diagnosis in an uninformative family, where the parents share a normal DM allele of identical size, is not possible using this strategy. Furthermore, if ADO affects the carrier parent's normal allele in a blastomere from a normal embryo it will be misdiagnosed as affected. This leads to a reduced number of embryos available for transfer and a diminished pregnancy rate may result. In most cases of DM the mother transmits the mutation and as a result the accidental sampling of a cumulus cell(s) along with the biopsied blastomere can lead to a more significant misdiagnosis. In this case the mother's normal allele will be detected even if the embryo is affected, and can lead to an affected embryo being diagnosed as normal. A case of misdiagnosis for DM using PGD has been reported and may have been caused in this way (Sermon *et al.*, 1998b). Incorporation of a polymorphic marker linked to the DM gene provides a backup diagnosis in the event of ADO and the use of fingerprinting allows contaminating DNA (including cumulus cells) to be detected, thus reducing the chance of misdiagnosis. However, visualisation of both the normal and mutant DM allele would significantly improve all pre-existing protocols.

A molecular analysis protocol for DM that includes the examination of the unstable mutant expansion would benefit families that are uninformative using current techniques; such a protocol would be widely applicable. Moreover, a sample with maternal DNA contamination would be readily identified by virtue of it possessing two normal and one mutant DM alleles. Successful amplification of large repetitive DNA sequences using modifications of this protocol might also prove useful for diagnosis of other triplet repeat disorders (e.g. Huntington's disease, Fragile-X mental retardation, Freidreich's ataxia, spino-cerebellar ataxia, etc).

In addition to the diagnostic point of view, the CTG expansion analysis at the single cell level, when successful, could be applied to research into amplification and mosaicism. The study of CTG expansion in the gametes of the DM carriers or the affected DM individuals may reveal the timing of the amplification (mutation) of the unstable CTG repeats and the mechanism of preferential transmission of the mutant allele between parental sex. Mosaicism of the DM triplet repeat has been reported (Jansen *et al.*, 1994). The analysis of disaggregated single blastomeres from the untransferred affected embryos from PGD cycles may be useful in exhibiting the mechanism and timing of the mosaicism of the CTG repeat expansion.

Long template amplification was first investigated using genomic DNA, which is at a concentration analogous to that obtained from prenatal sampling. The successful Extensor Hi-Fidelity PCR Kit® was then further applied at the single cell level and optimised for speed, fidelity and amplification efficiency. The final thermal cycles take 10 h and agarose gel electrophoresis takes a further 30 min. Unfortunately the large amplified fragments turned out to be artefacts. The protocol did successfully amplify a

very large fragment from a single cell, so it could be useful for other large fragment amplification. If successful, this protocol could be used not only for prenatal, but also preimplantation diagnosis. Analysis using F-PCR provides a more precise measurement of allele size, and although polyacrylamide gel electrophoresis takes longer than agarose gel electrophoresis, this time is more than made up by virtue of the reduced number of PCR cycles necessary for detection of fluorescent PCR products.

During the search for an optimal long template amplification protocol, PCR-extension time was found to be a critical variable for amplification of large fragments. For amplification of normal alleles, 45 sec is sufficient (results not shown), while at least 5 min are needed for the efficient amplification of an 800 bp-long fragment from DNA and 10 min are required for single cell amplification. Failure to amplify the long fragment using the traditional PCR protocols reflects suboptimal PCR conditions for long template amplification. The results show that high concentrations of polymerase enzyme and primers are not the key to a high efficiency PCR protocol. More critical factors were thermal cycling conditions and combinations of polymerase enzymes used. It was observed that even in the optimised protocol I, the normal DM template is preferentially amplified relative to the large fragment, but both fragments were readily identified.

In conclusion, the concept of identifying both normal and mutant expanded alleles in the PGD protocol for DM is useful in preventing the risk of misdiagnosis. Moreover, this strategy can also be applied for studies regarding the nature of DM expansion. i.e. anticipation and mosaicism. Unfortunately, attempts in this study were not successful. Therefore, more work needs to be done in order to find an efficient hi-fidelity system and

appropriate conditions for single cell amplification. Known expansions of various sizes should be tested before any clinical application is contemplated.

4.4.5 Conclusion

Two PGD protocols of DM have been developed and optimised using the single step multiplex F-PCR strategy. The first protocol includes a polymorphic linked marker in addition to the DM triplet expansion analysis for a back up linkage analysis result and contamination detection. The second protocol employs an unlinked marker for contamination tracking only in the families that the linked marker is not fully informative as contamination is considered to be the crucial problem that can cause misdiagnosis. Three clinical PGD cycles were carried out in two DM families using protocol 2 giving rise to a singleton and a twin pregnancy. The PGD results of both pregnancies have been confirmed by PND, the baby girl of family 'A' was born in May 2001 and two baby girls of family 'B' were born in June 2001. Attempts to find a system for amplifying the DM triplet mutant expansion was not successful in this study. However, if succeeded it might be useful as an improved PGD protocol of DM and for the study of mosaicism event and timing of the extension of the triplet repeat disorders in the affected human embryos.

Chapter 5

Investigation of
Factors Affecting Single Cell PCR

5.1 Introduction

Of all the potential problems facing PGD using PCR, ADO (**Section 1.5.2**) has caused the most problems and has resulted in several misdiagnoses (Grifo *et al.*, 1994; Hardy and Handyside, 1992; Verlinsky, 1996). In most cases of misdiagnosis, the diseases being diagnosed were recessive (mostly cystic fibrosis), but the embryos were compound heterozygotes and the diagnostic protocols used only allowed the detection of one of the two mutations (Harper and Delhanty, 2000). If this 'diagnostic' mutation were subject to ADO then the embryos would appear normal regardless of their actual genotype. Compound heterozygosity can also lead to misdiagnosis in cases where the two mutations are both detectable, but cannot be encompassed by a single set of primers. In such cases PGD strategies sometimes employ multiplex amplification of two different fragments, each containing one of the mutations. If ADO affects the mutant allele in either of these fragments an affected embryo will be diagnosed as an unaffected carrier. On rare occasions when both sites are subject to ADO the embryo may even appear to be homozygous normal (Wells and Delhanty, 2001).

There are several theories as to the origin of ADO. Some have speculated that it is caused by DNA degradation; leading to PCR-refractory breaks in both DNA strands (Wells and Sherlock, 1998). Another possibility is that ADO results from inaccessibility of the DNA template due to imperfect PCR conditions or incomplete cell lysis. Methods for improving amplification efficiency and minimizing ADO have been proposed and include use of highly sensitive F-PCR (Findlay *et al.*, 1995a), increasing the PCR denaturation temperature (Ray and Handyside, 1996), and the use of different cell lysis

buffers (El-Hashemite and Delhanty, 1997). However, none of these measures consistently eliminates ADO. The most experienced PGD labs are generally able to reduce ADO rates to 5-15% (i.e. one of the two alleles affected in 5-15% of amplifications) (Harper and Wells, 1999). However, ADO rates higher than this are not uncommon and in rare cases they may exceed 40% (Ray and Handyside, 1996), significantly compromising diagnosis.

Preferential amplification is also common in single cell PCR affecting approximately one quarter of amplifications. In most cases there is an equal probability of either allele being over/under amplified, but in some cases preferential amplification is a consequence of an allele-specific difference in DNA sequence or fragment length. In this case one allele may be more often affected than the other. Preferential amplification is not of great diagnostic significance except in its most extreme forms. However, it is preferential amplification along with ADO that hinders the use of quantitative PCR for aneuploidy detection at the single cell level (Sherlock *et al.*, 1998).

Total amplification failure (AF; **Section 1.5.1**) is not an unusual problem of single cell PCR. It gives rise to the absence of results, resulting in fewer normally diagnosed embryos for transfer, or misdiagnosis if the interpretation of a normal result is based on the absence of the amplification (Hardy and Handyside, 1992). Another problem encountered during single cell PCR is contamination (**Section 1.5.3**) which can mostly be avoided by setting up PCR in a clean environment and using ICSI to reduce the risk of sperm DNA contamination. Maternal DNA contamination from cumulus cells can be identified by adding a highly polymorphic marker to the mutation analysis as a multiplex PCR (**Chapter 3** and **4**).

This study investigated factors that influence amplification efficiency and ADO rates in single cells using over 3,000 single cell PCRs. Single and multiplex PCR using different combinations of 7 sets of primers (**Section 5.2**) were carried out to investigate factors affecting PCR. Variables analysed included the effect of freezing and thawing, number of cells amplified, cell type and quality, preferential amplification, effect of multiplex PCR, ADO, AF, raised denaturing temperatures, altered thermal cycle times, duration of extension phase, duration of denaturing phase, DNA sequence composition and amplified fragment sizes.

5.2 Materials and methods

Single cell PCR was performed using single buccal cells from subjects heterozygous for one or more of the following seven loci: β-globin gene (two partially overlapping fragments encompassing a number of common mutations; 'bthalW1' and 'outer β-thalassaemia'), four microsatellite loci (APOC2, HUMTH01, D21S11, D21S1414), and the myotonic dystrophy triplet repeat region (DM) (**Table 3.2**, **4.1** and **5.1**). A single round of fluorescent multiplex PCR (F-PCR) was employed for the analysis of these fragments (**Section 3.2.3, 3.2.4** and **4.2.1**), the only exception being the outer β-thalassaemia fragment, which was detected by SSCP and silver staining following nested PCR (**Section 3.2.2**). Amplification efficiency, ADO and the amount of preferential amplification were assessed for each locus and compared to various physical or experimental parameters from over 3,000 single cell PCRs.

Table 5.1 Additional primers used for experiments comparing amplification efficiency.

Primers	Sequences (5′-3′)	Fluorescent labelled	Location	Size of the product (bp)	Concentration (μM)	Annealing temperature (°C)	References
D21S11							
forward	5′-TAT GTG AGT CAA TTC CCC AAG TGA-3′	6'FAM® (blue)	21q21	210-230	0.2	60	(Sharma and Litt, 1992)
reverse	5′-GTT GTA TTA GTC AAT GTT CTC CAG-3′	-			0.2		

Unless stated otherwise all cells used during this study were identical in terms of their treatment and the age of the sample. To control for any affects of freezing and thawing all cells were lysed and then frozen a single time, even if they were intended for immediate use. The thermal cycler and the reagents used for PCR were also kept constant for all experiments.

5.2.1 Freezing and thawing

The influence of freezing and thawing on amplification failure and ADO was assessed by comparing two groups of 50 buccal cells for amplification of the DM and D21S1414 loci. Both sets of cells were isolated from the same sample at the same time, but one group was lysed and then frozen prior to PCR, while the other group was lysed and then used immediately. The amplification efficiencies and ADO rates of both groups were counted and compared.

5.2.2 Amount of DNA necessary to avoid ADO

Experiments using precise numbers of isolated cells were employed to determine how much DNA (or how many cells) are necessary to entirely eliminate ADO. The ADO rates and amplification efficiencies were calculated for the DM and D21S1414 loci in 40 single cell amplifications, 40 two-cell amplifications and 40 three-cell amplifications.

5.2.3 Cell type/quality and the affect of DNA degradation

The amplification of various loci (DM, APOC2, D21S1414, β-globin) from 132 blastomeres was compared with identical reactions performed on 450 buccal cells.

5.2.4 Examination of preferential amplification

Multiplex fluorescent PCR was carried out using 100 single buccal cells from two subjects heterozygous for the DM triplet repeat and the polymorphic APOC2 locus. Thus, the amplification characteristics, amplification efficiency and ADO at both loci could be evaluated simultaneously.

5.2.5 Multiplex PCR

In the course of this study, four distinct single/multiplex PCR protocols were investigated. To demonstrate this empirically 50 single cells using D21S1414 primers and 50 more cells using a combination of D21S1414 and DM primers were amplified.

A study comparing the amplification of DM primers using 50 single cells to those of DM and APOC2 primers using 100 single cells was also carried out. The amplification efficiency of the DM locus in 50 single cells using DM primers and the 50 single cells using DM and D21S1414 primers was also analysed.

5.2.6 Deviation from expected amplification characteristics

The amplification characteristics of 950 single cell duplex PCRs were analysed to determine whether ADO and amplification failure affect loci independently, or whether failure or ADO at one locus increases the likelihood of failure or ADO at the second.

5.2.7 PCR cycling conditions

The affect of different denaturing temperatures and PCR cycle lengths on ADO and amplification efficiency was investigated by adjusting the PCR programme. The amplification of DM and D21S1414 loci from 100 cells, using denaturing temperatures of 94 °C throughout the programme was compared to a programme that employed a 96 °C denaturation for the first ten PCR cycles and 94 °C for the remaining cycles. Such programmes have been said to reduce ADO rates (Ray and Handyside, 1996).

Two hundred single buccal cells from subjects heterozygous for the DM triplet repeat and the polymorphic APOC2 locus were duplex amplified using one of three different thermal cycle programmes: denaturation/annealing/extension steps lasting 15sec/15sec/25sec, 30sec/30sec/45sec or 45sec/45sec/1min in order to investigate the effect of the thermal cycle times.

To ascertain which element of the cycle benefits most from an extended duration, different components were independently tested. An increase in extension time from 1 minute to two minutes (for the first 10 cycles only) was tested on 200 cells.

The effect of altering the length of the denaturing phase was also tested. One hundred cells were subjected to PCR for the D21S1414 and DM loci, half using denaturing times of 45 seconds and half using two minutes (for the first 10 cycles only).

5.2.8 DNA sequence composition, primers and chromatin structure

To determine whether DNA sequence influences the occurrence of ADO the base composition, melting temperature (Tm) and tendency to the form secondary structures was analysed for six amplicons (bthalw1, HUMTH01, outer β-thalassaemia, DM, APOC2 and D21S1414) and compared to their ADO rates

To further investigate the affect of differences in DNA sequence the amplification characteristics of D21S1414 and D21S11 were compared. These loci share 63% of their sequence, have the same GC composition and share a primer-annealing site. The only difference is that D21S1414 alleles are an average of 122 bp longer than D21S11 alleles. If differences in DNA sequence and/or chromatin structure were significant determinants of ADO, one would expect these loci to behave similarly. One hundred cells were isolated from the same sample, at the same time, and half were amplified with each set of primers.

5.2.9 Fragment length

Six hundred single buccal cells were amplified in order to study the relationship between the amplified fragment size and ADO and amplification efficiency of 6 loci, involving outer β-thalassaemia, bthalw1, HUMTH01, DM, APOC2, and D21S1414.

5.2.10 Definition of amplification characteristics

Symmetrical amplification - the two alleles are considered equally amplified. The quantity of each allele differs by less than 10%, as determined by analysis of the area of each fluorescent peak after electrophoresis.

Preferential amplification - the quantity of each allele differs by more than 10%. One allele is over amplified with respect to the other.

Amplification failure - neither allele can be detected; the locus has failed to amplify all together.

Allele dropout (ADO) - only one allele is detectable; the other has failed to amplify.

ADO rate - calculated by dividing the number of reactions displaying ADO by the number of reactions in which one or more alleles were detected (i.e. cases of total amplification failure are excluded from this calculation).

Amplification efficiency - defined as the proportion of tests in which one or more alleles is successfully amplified. This includes cases of ADO and preferential amplification, as well as cases of symmetrical amplification.

5.3 Results

5.3.1 Freezing and thawing

The experiment comparing the influence of freezing and thawing showed the average amplification rates for fresh cells of 94% for the two loci and ADO was seen in 19% of amplifications. Frozen cells were successfully amplified in 85% of reactions and displayed 30% ADO (**Table 5.2, Study A**). The difference between the two sets of cells is statistically significant (Chi square = 4.91 and 6.22, p value = 0.086* and 0.045*).

5.3.2 Amount of DNA necessary to avoid ADO

The amplification efficiencies and ADO rates provided from the study comparing 1:2:3 cells are given in **Table 5.2, Study B**. The dramatic reduction in ADO produced by the use of two cells verses one cell is statistically significant. For DM it reduced ADO from 20% to 3% (Chi square = 7.36, p value = 0.025*), while for D21S1414 ADO decreased from 28% to 5% (Chi square 29.15, p value = 0.000*). Use of three cells improved results further, with ADO rates approaching 0% and amplification efficiencies of ~100%. However, a statistical reduction in ADO for three cells verses two could not be demonstrated with the small number of cells used here.

Table 5.2 Studies A and B, control studies of different PCR protocols/conditions from duplex PCR. Single buccal cells from subjects heterozygous for each locus were used in order to maximise data regarding amplification efficiency and ADO rates.

Study	n	Cells treated prior to PCR	Raised denaturation temperature during the first 10 cycles	Microsatellite	Locus					
					DM			Microsatellite		
					AA	ADO	AF	AA	ADO	AF
A. Frozen and thawed effect			yes	D21S1414						
• Standard protocol from fresh cells	50	Fresh			44 (88%)	5 (10%)	1 (2%)	31 (62%)	14 (28%)	5 (10%)
• Standard protocol from frozen and thawed cells	50	Frozen			35 (70%)	13 (26%)	2 (4%)	20 (40%)	17 (34%)	13 (26%)
					Chi square = 4.91, Degrees of freedom = 2, p value = 0.086*			Chi square = 6.22, Degrees of freedom = 2, p value = 0.045*		
B. Comparison of 1:2:3 cell template		Fresh	yes	D21S1414						
• 1 cell	40				31 (78%)	8 (20%)	1 (3%)	21 (53%)	11 (28%)	8 (20%)
• 2 cells	40				39 (98%)	1 (3%)	0 (0%)	37 (93%)	2 (5%)	1 (3%)
• 3 cells	40				40 (100%)	0 (0%)	0 (0%)	38 (95%)	2 (5%)	0 (0%)
					1:2 cell - Chi square = 7.36, Degrees of freedom = 2, p value = 0.025*			Chi square = 29.15, Degrees of freedom = 4, p value = 0.000*		

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

5.3.3 Cell type/quality and the affect of DNA degradation

The experiments comparing the amplification from 132 blastomeres and 450 buccal cells using various loci (DM, APOC2, D21S1414, β-globin) revealed no significant differences between the amplification of the two cell types. Average ADO rates for buccal cells and blastomeres were 9.5% and 10.4%, respectively, while average amplification efficiencies were 96.2% and 94.7% (Chi square = 0.70, p value = 0.706; **Table 5.3, Study C**). More important than cell type was the condition of the cells. Fifty buccal cells were aged at room temperature for seven days prior to isolation and PCR amplification. After aging the HUMTH01 locus gave ADO rates 14.9% and an amplification efficiency of 94%. Fresh buccal cells gave even lower ADO rates for HUMTH01, 9.0%, and gave an amplification efficiency of 96.7% (**Table 5.4, Study D**). These differences could be the result of DNA degradation in the older samples.

Table 5.3 Study C, data comparing the amplification from 450 buccal cells and 132 blastomeres using various loci (DM, APOC2, D21S1414, β-globin).

Study C	n	Amplification Characteristics			
Cell Types		AA	ADO	AF	
Buccal cells	450	392 (87.1%)	41 (9.1%)	17 (3.8%)	Chi square = 0.70, degrees of freedom =2
Blastomeres	132	112 (84.8%)	13 (9.8%)	7 (5.3%)	p value = 0.706

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

Table 5.4 Study D, data of comparing the amplification of the HUMTH01 locus from aging buccal cells and fresh blastomeres.

Study D	n	Amplification Characteristics			
Cell Types		AA	ADO	AF	
Aging buccal cells	50	40 (80%)	7 (14%)	3 (6%)	Chi square = 2.01, degrees of freedom =2 p value = 0.366
Fresh buccal cells	150	132 (88%)	13 (8.7%)	5 (3.3%)	

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

For the amplification of blastomeres, the presence of a visible nucleus was also a critical factor in amplification efficiency. In clinical PGD cases (**Section 4.3.3**), amplification of the DM locus in 96.2% of nucleated cells (51/53) compared to 0% of anucleate cells (0/4) was observed. A similar effect was seen when amplifying the D21S1414 locus, which gave 92.5% amplification verses 0% in anucleate cells.

5.3.4 Examination of preferential amplification

The amplification efficiency and ADO of DM and APOC2 loci from 100 single cell multiplex PCRs of two heterozygote subjects were compared. It was noticed that in some multiplex amplified samples there was disparity between the amplification characteristics of the two loci. Amplification failure, ADO, or preferential amplification were sometimes observed at one locus while the other gave perfect results with symmetrical amplification, despite both loci having been amplified in the same reaction.

The amplification data for the two heterozygous patients is summarized in **Table 5.5, Study E**. Allele sizes ranged from 125bp-170bp. It was observed that the shorter alleles tended to be amplified with greater efficiency than the longer alleles, usually producing peaks of larger size. However, differences between the ADO rates of the shorter and longer alleles were not significant.

5.3.5 Multiplex PCR

Comparison of single cell amplification using individual pairs of primers with multiplex-PCR using combinations of primer-pairs revealed that in the vast majority of cases the addition of an extra set of primers did not significantly affect ADO rates or amplification efficiency. In the experiment comparing 50 single cells using D21S1414 primers with 50 more cells using a combination of D21S1414 and DM primers, amplification efficiency and ADO rates for D21S1414 were 70% and 32% when amplified alone and 74% and 34% when amplified in a duplex reaction (Chi square = 0.20, p value = 0.905; **Table 5.6, Study F**). Similar results were seen with amplification of the DM locus. This gave amplification efficiency and ADO rates of 94% and 12.8% respectively when amplified alone; 95% and 9.5% when amplified simultaneously with the APOC2 locus (Chi square 0.43, p value = 0.808; **Table 5.6, Study G**) and 98% and 4.1% when amplified with D21S1414 (Chi square = 3.41, p value = 0.182; **Table 5.6, Study H**).

Table 5.5 Study E, amplification results from single cell multiplex fluorescent PCR using DMPK and APOC2 primers with the thermal cycle program of 30sec/30sec/45sec. The data for both DMPK and APOC2 primers shows that the shorter alleles are more likely to dominantly amplify than the longer alleles regardless of how large or small the size difference is. On the other hand, the prevalence of allele dropout is similar for both short and long alleles.

Study E		DM primers		APOC2 primers	
Amplification Results		'S', 140/149bp (n=50)	'F', 125/170bp (n=50)	'S', 150/154bp (n=50)	'F', 128/152bp (n=50)
SA		13.0%	2.2%	7.1%	0
PA	• PAS	56.5% (41.1-71.1)*	63.0% (47.5-76.8)*	73.8% (58.0-86.1)*	72.7% (57.2-85.0)*
	• PAL	26.1% (14.3-41.1)*	21.7% (10.9-36.4)*	11.9% (4.0-25.6)*	15.9% (6.6-30.1)*
ADO	• ADOS	0	6.5% (1.4-17.9)*	2.4% (0.1-12.6)*	6.8% (1.4-18.7)*
	• ADOL	4.3% (0.5-14.8)*	6.5% (1.4-17.9)*	4.8% (0.6-16.2)*	4.5% (0.6-15.5)*
AF		8.0%	8.0%	16.0%	12.0%

* percentage (95% confidence interval)
AF = amplification failure, SA = symmetrical amplification of both alleles, ADO = allele dropout, ADOS = allele dropout of the shorter allele, ADOL = allele dropout of the longer allele, PA = preferential amplification, PAS = preferential amplification of the shorter allele, PAL = preferential amplification of the longer allele.

Table 5.6 Studies F, G and H, control studies of different PCR protocols/conditions from single/duplex PCR. Single buccal cells from subjects heterozygous for each locus were used in order to maximise data regarding amplification efficiency and ADO rates.

Study	n	Cells treated prior to PCR	Raised denaturation temperature during the first 10 cycles	Microsatellite	Locus					
					DM			Microsatellite		
					AA	ADO	AF	AA	ADO	AF
F. Comparison of Single/ Multiplex effect 1		Frozen	yes	D21S1414						
• Singleplex reaction (D21S1414)	50							19 (38%)	16 (32%)	15 (30%)
• Duplex reaction (DM & D21S1414)	50							20 (40%)	17 (34%)	13 (26%)
								Chi square = 0.20, Degrees of freedom = 2, p value = 0.905		
G. Comparison of Single/ Multiplex effect 2		Frozen	yes	APOC2						
• Singleplex reaction (DM)	50				41 (82%)	6 (12%)	3 (6%)			
• Duplex reaction (DM & APOC2)	100				86 (86%)	9 (9%)	5 (5%)			
					Chi square = 0.43, Degrees of freedom = 2, p value = 0.808					
H. Comparison of Single/ Multiplex effect 3		Frozen	yes	D21S1414						
• Singleplex reaction (DM)	50				41 (82%)	6 (12%)	3 (6%)			
• Duplex reaction (DM & D21S1414)	50				47 (94%)	2 (4%)	1 (2%)			
					Chi square = 3.41, Degrees of freedom = 2, p value = 0.182					

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

The only combination of loci that encountered problems during multiplex PCR contained primers for the β-globin ('outer β-thalassaemia') and HUMTH01 loci. In this case no HUMTH01 product was amplified. The problem could not be rectified despite the use of numerous different thermal cycling conditions in over 300 single cell PCRs. Ultimately efficient amplification of both loci was achieved, but only after a modified Taq polymerase (AmpliTaq Gold™) was employed. Use of this polymerase allowed 96.7% amplification efficiency with 9.0% ADO rates to be obtained for the HUMTH01 locus in 150 single buccal cells (**Table 3.6, Section 3.3.2**), not significantly different from the use of HUMTH01 primers alone. Similar results (amplification efficiency of 95.8% and 8.7% ADO rate) were obtained using 24 blastomeres. It is likely that interaction between the outer β-thalassaemia and HUMTH01 primers was responsible for the amplification failure, as both pairs of primers amplified with high efficiency when used alone, or in combination with other sets of primers. Although multiplex PCR may rarely cause a significant reduction in amplification efficiency, the phenomenon of ADO does not seem to be affected by the addition of an extra set of primers to the reaction mixture.

5.3.6 Deviation from expected amplification characteristics

The analysis of the amplification characteristics of pooled data of 950 single cell duplex PCRs were carried out (**Table 5.7, Study I**). On the basis of the amplification failure rates of individual loci it was predicted that failure of both loci should occur in 0.8% of amplifications. However, coincident amplification failure was actually observed in 3.7% of PCRs. This dramatic and highly statistically significant increase is presumably due to cases where the cell had been lost during transfer to the PCR tube, or where the DNA of the cell has been severely degraded, thus negating any amplification.

Table 5.7 Study I, pooled data of the amplification characteristics of 950 single cell duplex PCRs, determining the relationship of ADO and AF between the two loci.

Study I		Locus 1			
		AA	ADO	AF	Total
Locus 2	AA	491	83	12	586
	ADO	175	50	9	234
	AF	74	20	36	130
	Total	740	153	57	950

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

Not only was there an increased coincidence of amplification failures, but ADO also affected both loci more often than expected. Fifty amplifications displayed double ADO rather than the expected 38. However, even the large number of cells tested here were insufficient to reveal whether this difference was significant, or just a chance variation. It is difficult to assess coincidence of amplification failure at one locus and ADO at the other, as the excess of double amplification failures distorts the data. If cases of double amplification failure are removed from the data set, then a small (non-significant) increase in the coincidence of ADO and amplification failure is seen.

5.3.7 PCR cycling conditions

The study comparing the denaturing temperatures of 94 °C and the 96 °C denaturation for the first ten PCR cycles and 94 °C for the remaining cycles in the amplification of DM and D21S1414 loci from 100 cells were carried out. The

amplification efficiencies for DM and D21S1414 achieved using the 94 °C programme were 92% and 84%, while for the 96 °C programme they were 96% and 74%. ADO rates were 30% for both loci using the 94 °C programme, while for the 96 °C programme DM gave an ADO rate of 26% and D21S1414 34% (**Table 5.8, Study J**). The differences are not statistically significant (Chi square = 1.05 and 2.36, p value = 0.591 and 0.308 for the DM and D21S1414 loci, respectively) and indicate that an increase in denaturing temperature from 94 °C to 96 °C does not significantly reduce ADO.

The experiment using 200 single cells duplex PCRs comparing three different thermal cycle programmes: denaturation/annealing/extension steps lasting 15sec/15sec/25sec, 30sec/30sec/45sec or 45sec/45sec/1min was performed (**Table 5.8, Study K**). It was observed that the rates of amplification failure and ADO for both loci were gradually reduced with increasing PCR program length. Therefore, the longest thermal cycle programme in this study, 45sec/45sec/1min, showed the most efficient amplification results and the lowest ADO rate. The longest PCR programme displayed ADO rates of 6% and 4% for DM and APOC2 loci respectively, compared to 12% and 20% for the shortest programme. Although the difference was not statistically significant for the DM locus (Chi square = 6.38, p value = 0.173), the finding was highly significant for the APOC2 locus (Chi square = 18.71, p value = 0.001*). Thus it seems that longer PCR cycles provide superior amplification rates and reduced ADO.

Table 5.8 Studies J and K, control studies of different PCR protocols/conditions from duplex PCR. Single buccal cells from subjects heterozygous for each locus were used in order to maximise data regarding amplification efficiency and ADO rates.

Study	n	Cells treated prior to PCR	Raised denaturation temperature during the first 10 cycles	Microsatellite	Locus					
					DM			Microsatellite		
					AA	ADO	AF	AA	ADO	AF
J. Raised denaturation temperature		Frozen		D21S1414						
• Standard protocol with the raised denaturation temperature during the first 10 cycles	50		yes		35 (70%)	13 (26%)	2 (4%)	20 (40%)	17 (34%)	13 (26%)
• Standard protocol without the raised denaturation temperature	50		no		31 (62%)	15 (30%)	4 (8%)	27 (54%)	15 (30%)	8 (16%)
					Chi square = 1.05, Degrees of freedom = 2, p value = 0.591			Chi square = 2.36, Degrees of freedom = 2, p value = 0.308		
K. Thermal cycle programs		Frozen	yes	APOC2						
• 15sec/15sec/25sec	50				36 (72%)	6 (12%)	8 (16%)	28 (56%)	10 (20%)	12 (24%)
• 30sec/30sec/45sec	100				84 (84%)	8 (8%)	8 (8%)	78 (78%)	8 (8%)	14 (14%)
• 45sec/45sec/1min	50				45 (90%	3 (6%)	2 (4%)	46 (92%)	2 (4%)	2 (4%)
					Chi square = 6.38, Degrees of freedom = 4, p value = 0.173			Chi square = 18.71, Degrees of freedom = 4, p value = 0.001*		

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

The control study comparing the amplification efficiency using one and two min extension time (for the first 10 cycles only) in 200 single buccal cells showed no difference in the amplification efficiency and ADO rates between both groups for the DM locus; moreover, the AF and ADO rates appeared to be higher in the extra long extension time group for the D21S1414 locus (**Table 5.9**, **Study L** and **M**). The comparison of 45 sec and 2 min denaturing time (for the first 10 cycles only) were carried out on 100 single buccal cells using DM and D21S1414 primers (**Table 5.9**, **Study N**). For cells treated with the extended denaturing time the averaged ADO rates and amplification efficiencies for the two loci were 22% and 91% respectively. This compares to rates of 30% and 85% for cells denatured for just 45 seconds. Although there is an apparent trend towards improved amplification with increased denaturing time, this is not statistically significant (Chi square = 1.42, p value = 0.234).

5.3.8 DNA sequence composition, primers and chromatin structure

The analysis of 6 amplicons (bthalw1, HUMTH01, outer β-thalassaemia, DM, APOC2 and D21S1414) in comparison to their ADO rates in order to determine whether the DNA sequence influences the occurrence of ADO, the base composition, melting temperature (Tm) and tendency to the form secondary structures was carried out (**Table 5.10**, **Study O**). GC contents ranged from 33-63%, but no correlations related to ADO were identified. As well as the amplified fragments, the sites of primer annealing and flanking regions (200bp upstream and 200bp downstream) were also tested. No association between ADO rate and any of these characteristics were identified despite a wide range of Tm and GC contents. Loci containing repetitive sequences displayed no significant difference in ADO and amplification efficiency rates when compared with loci containing only unique sequence.

Table 5.9 Studies L, M and N, control studies of different PCR protocols/conditions from duplex PCR. Single buccal cells from subjects heterozygous for each locus were used in order to maximise data regarding amplification efficiency and ADO rates.

Study	n	Cells treated prior to PCR	Raised denaturation temperature during the first 10 cycles	Microsatellite	Locus					
					DM			Microsatellite		
					AA	ADO	AF	AA	ADO	AF
L. Extra long extension time 1		Fresh	yes	D21S1414						
• Extra long extension time (2 min)	50				46 (92%)	2 (4%)	2 (4%)	21 (42%)	17 (34%)	12 (24%)
• Control (1 min)	50				44 (88%)	5 (10%)	1 (2%)	31 (62%)	14 (28%)	5 (10%)
					Chi square = 1.66, Degrees of freedom = 2, p value = 0.435			Chi square = 5.10, Degrees of freedom = 2, p value = 0.078		
M. Extra long extension time 2 without the raised denaturation temperature		Frozen	no	D21S1414						
• Extra long extension time (2 min)	50				26 (52%)	20 (40%)	4 (8%)	12 (24%)	23 (46%)	15 (30%)
• Control (1 min)	50				31 (62%)	15 (30%)	4 (8%)	27 (54%)	15 (30%)	8 (16%)
					Chi square = 1.15, Degrees of freedom = 2, p value = 0.562			Chi square = 9.58, Degrees of freedom = 2, p value = 0.008*		
N. Extra long denaturation time		Frozen	yes	D21S1414						
• Extra long denaturation time (2 min)	50				40 (80%)	10 (20%)	0 (0%)	29 (58%)	12 (24%)	9 (18%)
• Control (1 min)	50				35 (70%)	13 (26%)	2 (4%)	20 (40%)	17 (34%)	13 (26%)
					Chi square = 2.72, Degrees of freedom = 2, p value = 0.256			Chi square = 3.24, Degrees of freedom = 2, p value = 0.198		

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

Table 5.10 Study O, the summerised data of the amplification efficiency and ADO rate of each gene are shown. The amplified fragment sizes and the locations in either light or dark G-bands are also stated.

Study O Primers	Locate in light/dark G-bands region	Fragment size (bp)	Sequence composition (%G:C)	Amplification efficiency	ADO rates	n
outer β-thalassaemia	light	279	52	89.3%	18.7%	150
bthalw1	light	364	52	92%	23.9%	50
DM	light	128-203	63	95%	9.5%	100
HUMTH01	light	115-140	47	96.7%	9.0%	150
APOC2	dark	134-170	49	95%	10.5%	100
D21S1414	dark	340-370	33	80%	34.4%	50

The control study comparing the affect of differences in DNA sequence the amplification characteristics of D21S1414 and D21S11 was performed on 100 single buccal cells. ADO and amplification efficiency rates were 32% and 70% for D21S1414 and 20% and 90% for D21S11, and thus differed significantly (Chi square = 11.13, p value = 0.004*; **Table 5.11, Study P**).

Table 5.11 Study P, control study of different amplified fragment lengths from singleplex PCR using single buccal cells from subjects heterozygous for each locus. All cells were amplified after being frozen and thawed once and the thermal cycle program contained the raised denaturing temperature during the first 10 cycles.

Study P Locus	n	Amplification characteristics AA	ADO	AF	
• D21S1414	50	19 (38%)	16 (32%)	15 (30%)	Chi square = 11.13,
• D21S11	50	35 (70%)	10 (20%)	5 (10%)	Degrees of freedom = 2, p value = 0.004*

AA = appropriate amplification, both alleles detected; ADO = allele drop out; AF = amplification failure

5.3.9 Fragment length

The amplification of microsatellite polymorphisms, in which alleles vary in length demonstrated that both longer and shorter alleles could be subject to preferential amplification. However, in the majority of cases it was the longer allele that is relatively under-amplified (11.9%-26.1% of reactions show preferential amplification of the long allele, 56.5%-73.8% show preferential amplification of the shorter allele; **Table 5.5, Study E**). Despite the differences in preferential amplification, ADO rates for long or short alleles of the same locus did not differ significantly. Although alleles of a given locus had no detectable difference in ADO rate, their sizes tend to fall within a relatively narrow range. The maximum size difference between alleles in this study was 45bp. Different loci, on the other hand, often vary considerably in fragment length and display clear differences in their ADO rates and amplification efficiencies (**Table 5.10, Study O**).

To explore the influence of amplified fragment size on ADO and amplification efficiency, 6 loci (outer β-thalassaemia, bthalw1, HUMTH01, DM, APOC2, and D21S1414) were amplified from 600 single buccal cells. A significant increase in ADO and a small increase in amplification failure with fragment size were observed (**Figure 5.1** and **5.2**), suggesting that ADO, amplification failure and fragment length are intimately linked. During these studies particular attention was focussed on D21S1414 and D21S11, two loci that share extensive regions of sequence, but differ in length. As described in **Section 5.3.8** the longer D21S1414 locus displays significantly higher ADO than D21S11.

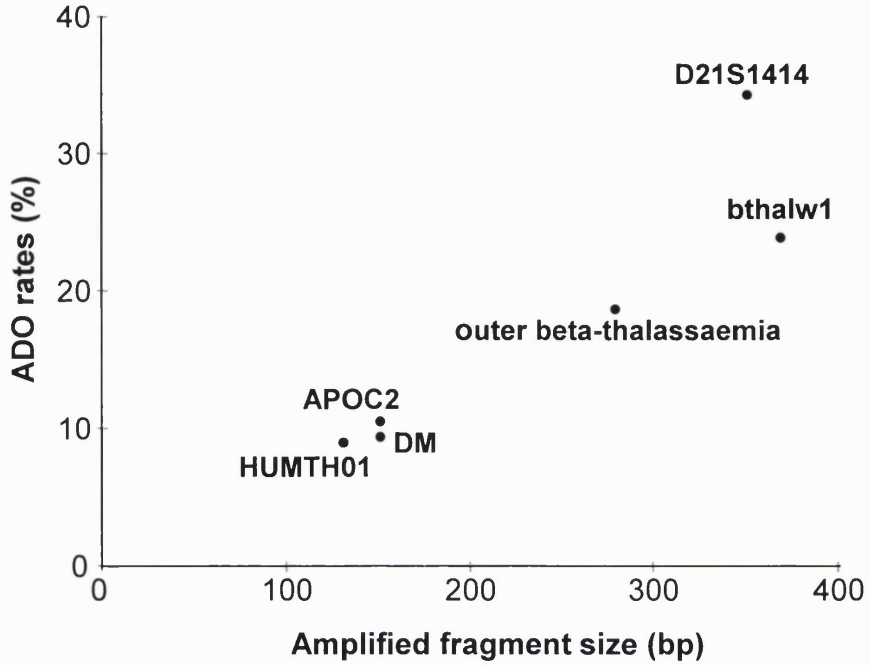


Figure 5.1 Relationship between amplified fragment size and ADO rates of single cell multiplex PCR from 6 different loci.

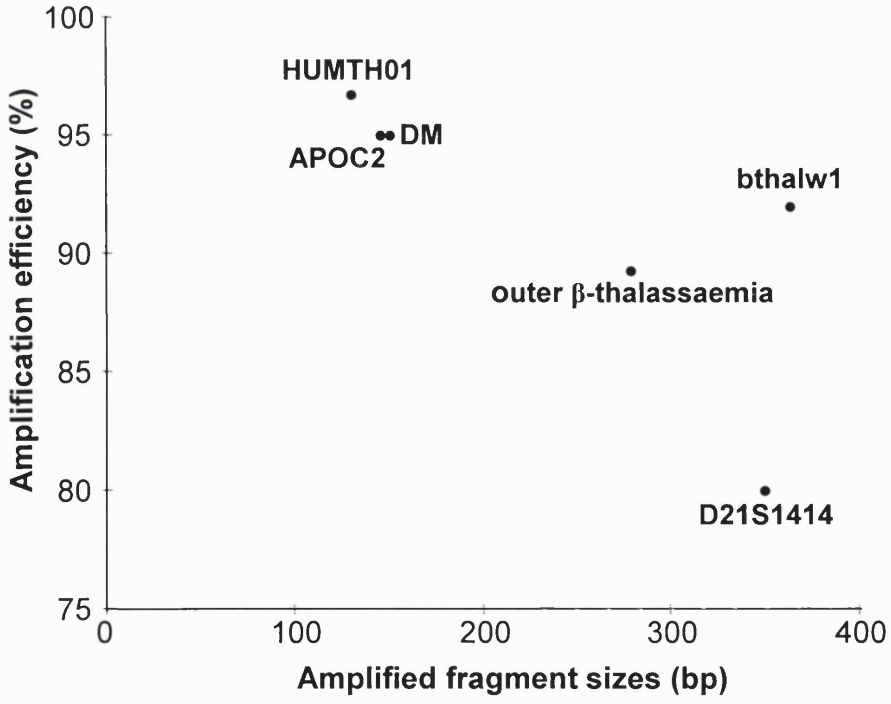


Figure 5.2 Correlation between amplified fragment sizes and amplification efficiency of single cell multiplex PCR from 6 different loci.

5.4 Discussion

Analysis in PGD, or other areas that utilises PCR amplification of single cells, should be acutely aware of the problem of ADO. Although the existence of ADO has been acknowledged for several years, progress in combating it has been slow and no way to entirely eliminate it has been discovered (Findlay *et al.*, 1995a). As a consequence most diagnostic strategies now employ multiple redundant analyses in order to ensure accuracy, the theory being that ADO is unlikely to affect all of the tests (Ao *et al.*, 1998) (also **Chapter 3** and **4**). There has been little prospect that this position will improve, largely because the basis of ADO is so poorly understood. Even review and inference from the many variant single cell PCR methods in the literature cannot assist in formulating reliable theories as to its origin, because ADO appears to be influenced by multiple factors. While two different methods might appear to be analogous they will certainly contain numerous subtle, but critical variations. In this study, systematic tests that control for all aspects likely to affect the incidence of ADO were employed. For example results are only compared between cells isolated at the same time, from the same sample, and amplified using identical reagents and the same PCR machine. To our knowledge this is the most rigorous treatment of the problem of ADO and amplification failure to date.

5.4.1 Freezing and thawing

Some variation in ADO rates which were observed in earlier work, could have been a consequence of differences in the number of times cells had been frozen and thawed. This is important, as often during the design and testing of new PGD protocols

cells from patients are stored in the freezer prior to testing. ADO rates are then inferred from work done on these cells, but this might not be representative of the ADO rates that will be observed when fresh blastomeres are amplified. There are two ways in which freezing could affect ADO and these highlight two of the principal factors suspected to cause this phenomenon. Firstly the accessibility of the DNA could be affected, as the freezing process might help to rupture the plasma and nuclear membranes thereby improving access to the DNA and possibly reducing ADO. Accessibility of the DNA has long been thought to affect the ADO rate (**Section 5.4.10**) (Wells and Sherlock, 1998). Alternatively, freezing and thawing might damage the DNA and consequently prevent amplification. This latter possibility is supported by the data in this study, which clearly indicates that freezing is detrimental. Analysis of two loci (DM and D21S1414) showed an increase in total amplification failure and an increase in ADO from 19% to 30% in cells frozen once (**Table 5.2, Study A**).

5.4.2 Amount of DNA necessary to avoid ADO

Most PGD units currently employ biopsy of 1-2 cells from the 8-10 cell embryo on day 3 post-fertilisation. The greatest reduction in ADO rates is achieved when the number of cells amplified is increased from one to two. Sampling of three cells provides a small further improvement. The data in this study suggests that for most loci it would only be necessary to take ~3 cells to achieve 100% amplification efficiency and ADO rates that approximate to zero (**Table 5.2, Study B**). The actual number of cells required appears to be predictable from ADO rates estimated from single cell PCR. For example 20% ADO rates, which were observed for the DM locus, can theoretically be reduced to ~1% if three cells are amplified (i.e. 0.2^3=0.008).

5.4.3 Cell type/quality and the affect of DNA degradation

Not only the number, but also the variety of cells amplified is said to influence ADO rates (Rechitsky *et al.*, 1998). Although some variation related to cell type (e.g. buccal cells and blastomeres) was observed, it was found that these discrepancies were minor in comparison to differences caused by the way the cells had been handled and treated. Theoretically blastomeres could give a higher than expected ADO rate due to the possibility that the cell sampled is haploid or monosomic (cytogenetic analyses predict 7% to 15% of blastomeres to be haploid) (Harper *et al.*, 1995; Kuo *et al.*, 1998). Anucleate cells from preimplantation embryos certainly display a higher frequency of total amplification failure than other cell types. However, the extremely high ADO rates in blastomeres relative to other cell types, reported by others (Rechitsky *et al.*, 1998), were not observed in this study (**Table 5.3, Study C**), suggesting that the differences these authors observed could have been due to factors other than cell type. Better results from blastomeres might have been obtained because those blastomeres used were always fresh. The use of fresh cells should give results analogous to the performance of blastomeres in a PGD case.

The existence of apoptotic or necrotic cells with high levels of degraded DNA could explain the higher than expected coincidence of ADO at both loci and of ADO affecting one locus while amplification failure affects the other. This is significant as the single cells used during the development of PGD protocols are often collected by mouthwash. Such cells are shed towards the end of their life or after death and may contain DNA degraded by apoptotic processes, digested by bacterial action, or damaged by the cells own enzymes following breakdown of internal membranes. The affect of poor

sample quality was illustrated by the comparison of buccal cells aged for seven days with fresh cells. The seven-day old cells were found to have increased levels of amplification failure and ADO rates were increased by 5.9% (**Table 5.4, Study D**). A relationship between cell quality, ADO and amplification failure has been noted previously (Ray *et al.*, 1998).

5.4.4 Examination of preferential amplification

Preferential amplification of one of the two alleles in a heterozygous cell is a common phenomenon. In cases of marked preferential amplification, the results may resemble ADO unless the use of sensitive detection methods reveals the under-amplified allele. Methods such as F-PCR might help in reducing ADO rates by detecting the weak signal of the less efficiently amplified alleles (Findlay *et al.*, 1995a). For qualitative analysis, as in PGD, the occurrence of preferential amplification is of little importance. However, it interferes with attempts to employ quantitative fluorescent PCR (QF-PCR) at the single cell level. QF-PCR has been successfully employed in prenatal testing for aneuploidy, but relies on both alleles being amplified proportionately (Mansfield, 1993). The data in this study (**Table 5.5, Study E**) and that of others suggests that less than 75% of single cells give accurate, quantitative amplification of alleles (Sherlock *et al.*, 1998). For the most part, the allele affected by preferential amplification is random, although there is a tendency towards preferential amplification of shorter DNA fragments in PCRs where the alleles differ in size (Walsh *et al.*, 1992). This is purely an affect of PCR reaction kinetics.

5.4.5 Multiplex PCR

The current trend towards the development of more sophisticated PGD protocols, employing multiple primer sets, has produced more robust methodologies for single cell testing. Multiplex PCR allows simultaneous amplification of mutation sites, linked polymorphisms and hypervariable markers. Such protocols are resistant to misdiagnoses caused by ADO and contamination (Kuliev *et al.*, 1999) (also **Chapter 3** and **4**). However, some groups have been concerned that the increased complexity of the reaction might adversely affect amplification of individual loci and thus be counterproductive. The analysis of 4 different primer combinations during this study, and the wider experience of single cell PCR, suggests that incompatible mixtures of primers will occasionally be encountered. However, combinations that severely impair amplification of one or more loci are rare and the effect is seldom sporadic, rather it affects every reaction and is thus easily identified during preliminary testing of new protocols. In some cases the use of a modified Taq polymerase enzyme overcomes problems in multiplex PCR. Importantly the addition of an extra set of primers was found to have little if any affect on ADO rates (**Table 5.6, Study F, G, H**). This confirms that multiplex PCR can be used with confidence at the single cell level, provided preliminary testing is undertaken.

5.4.6 Deviation from expected amplification characteristics

The analysis of duplex PCR conducted on almost 1,000 single buccal cells has shed further light on the relationship between ADO and total amplification failure and confirm that the two processes are linked (**Table 5.7, Study I**; see also **Figure 5.3**). More cells than expected were found to display a combination of ADO at one locus and amplification failure at the other, suggesting that the two phenomena may have causal factors in common. Cells with ADO at one locus also had a slightly higher probability than expected of suffering ADO at the second locus. Differences such as these might indicate that a small proportion of cells (~1%) are predisposed to unusually high levels of ADO. The existence of such cells will have a small impact on the accuracy of PGD protocols that utilise more than one diagnostic locus, making redundant diagnoses slightly less effective than anticipated.

The incidence of amplification failure affecting both loci in a single cell was higher than anticipated, occurring almost five times more often than predicted. Many cases of double amplification failure will undoubtedly result from loss of the cell during transfer to the PCR tube, failure of cell lysis, or the sampling of an anucleate cell. However, some of these cases might also result from DNA degradation, which is likely to be present in dead and apoptotic cells. A double stranded break in the DNA is refractory to PCR, as is the co-incidence of two single stranded breaks (one on the antisense strand and one on the sense strand) if they both fall between the primers used for DNA amplification. Depending on whether one allele or both is affected by such DNA damage ADO or total amplification failure will result. Theoretically DNA degradation could also cause some cases of preferential amplification. A single-strand break, present from the

beginning of the first PCR cycle is predicted to cause a 2:1 preferential amplification in favour of the undamaged allele. However, if breakage occurs in later cycles, the deviation from a 1:1 ratio will be progressively smaller.

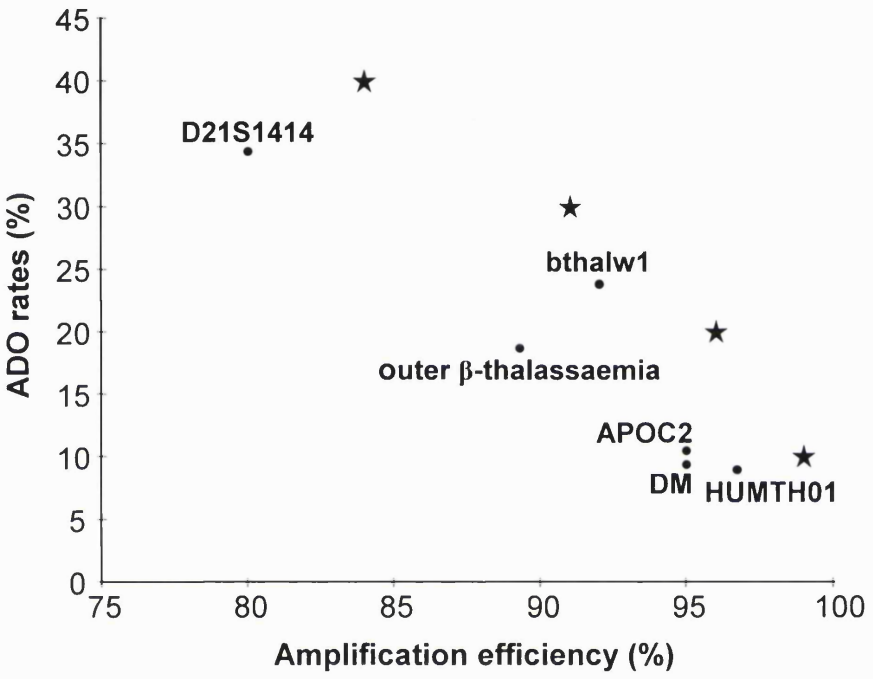


Figure 5.3 Relationship between amplification efficiency and ADO rates for 6 different loci amplified during single cell PCR. The amplification efficiency tends to have an inverse relationship with the ADO rates. The set of points corresponding to predicted amplification efficiency (AE), based on ADO^2, are shown as stars: 10% ADO=99% AE, 20% ADO=96% AE, 30% ADO=91% AE, 40% ADO=84% AE.

5.4.7 PCR cycling conditions

Of the possible PCR problems arising from sub-optimal reaction conditions, inadequate denaturation of DNA samples has been most strongly linked to the occurrence

of ADO. For this reason many PGD units now employ increased denaturing temperatures for the first 5-10 cycles of amplification. This modification is said to significantly reduce ADO rates (Ray and Handyside, 1996). However, in this study no reduction in ADO rates was found when comparing denaturing temperatures of 94 °C and 96 °C (**Table 5.8, Study J**). This indicates that the temperatures typically used in routine PCR are adequate for single cell PCR also and that no increase in denaturing temperature is necessary. Indeed, increased temperatures are likely to reduce the activity of the Taq polymerase, thus reducing the amount of PCR product generated. The improvement in ADO rates originally reported might have been observed because a wider range of temperatures was tested, 90°C, 93°C and 96°C. However, since denaturing temperatures lower than 94 °C are seldom used for PCR this observation might have less significance than originally believed. The observations in this study do not support the hypothesis that the higher ADO in long fragments is a consequence of incomplete denaturation.

Although an increase in denaturing temperature did not improve the results in this study (**Table 5.8, Study J**) there was an improvement when the length of cycles was increased (**Table 5.8, Study K**). Generally more rapid protocols are desirable because results are obtained more quickly. However, for diagnostic application accuracy is paramount. Independently increasing the length of different elements of the PCR programme (denaturation, annealing, extension) revealed that no individual component significantly improved the PCR (**Table 5.9, Study L, M, N**). Although there was a reduction in ADO when denaturing times were extended, this was not statistically significant. Theoretically an increase in denaturing time could improve accessibility of the DNA template to the PCR reagents, while an increased extension-phase could ensure that the polymerase had enough time to complete replication of the target.

To further explore the possibility that the melting temperature of the target affects ADO, and to determine whether base sequence or secondary structure have any influence, primer sites, flanking sequences and amplicons for melting temperature, sequence composition and propensity to form secondary structures were analysed. Comparison of seven different amplicons revealed no association between any of these factors and ADO rate (**Table 5.10, Study O**). The lack of any relationship between ADO and DNA sequence composition was also highlighted by the analysis of the D21S1414 and D21S11 loci (**Table 5.11, Study P**). As stated above, the ADO rates of these loci differ significantly despite the extensive sequence shared by these loci.

5.4.8 DNA sequence composition, primers and chromatin structure

The experiments using whole genome amplification performed on single cells have also indicated that the site of primer annealing and the local secondary structure are unlikely to influence most cases of ADO (Wells, Ao and Sherlock; unpublished observations). Whole genome amplification methods such as primer extension preamplification (PEP) and degenerate oligonucleotide primed PCR (DOP-PCR) initiate replication of a given locus from different sites each cycle, and are not dependent on the annealing of a primer to one specific location. This means that a heterogeneous group of fragments containing the sequence of interest, but with flanking sequences of varying length are produced. Theoretically this should overcome many problems specific to the sequence around and within the priming sites. However, it was observed that the use of WGA does not reduce the incidence of ADO (Wells, Ao and Sherlock; unpublished observations). Other studies have also suggested that PEP is as vulnerable to ADO as direct amplification (Hahn *et al.*, 1998).

It is also conceivable that large-scale chromosomal organization could affect the accessibility of the DNA and therefore amplification efficiency and ADO. This is harder to test for, but seems unlikely to be significant. The positioning of loci in light or dark G-bands certainly has no bearing on amplification rates (**Table 5.10, Study O**). Furthermore, ADO rates of adjacent loci can vary considerably. Such loci, separated by a few hundred or a few thousand base pairs, are likely to be in regions of similar chromatin organization and yet frequently have differing ADO rates (e.g. HUMTH01 9.0%, bthalw1 23.9%).

5.4.9 Fragment length

If DNA degradation is a significant cause of ADO and amplification failure then it seems likely that longer amplified fragments should exhibit higher levels of ADO. Breaks in the DNA strand are expected to occur at random positions, consequently the longer the DNA fragment the greater the chance of a break occurring between the two PCR primers. The comparison of ADO rates for loci reported in the literature is meaningless, as appropriate controls for this sort of analysis have not previously been employed. As such, the data in this study represents the first reliable assessment of the relationship between fragment size and ADO. Analysis of 6 different loci revealed that longer fragments do have higher ADO rates as well as more amplification failure when compared with small amplicons. This is shown in **Figures 5.1** and **5.2** and was further illustrated by the comparison of the amplification of D21S1414 and D21S11 loci (**Table 5.11, Study P**). These loci are almost identical, sharing most of their DNA sequence and one of their priming sites, but differ in size. D21S1414 fragments are an average length of 340 bp while the hemi-nested D21S11 fragments are an average of 220 bp. Despite their

similarities these loci have significantly different ADO rates. The incidence of ADO for D21S1414 is 32%, while for the shorter D21S11 fragments it is only 20% ADO. These results support the hypothesis that DNA damage is an important cause of ADO, but there are also other possible explanations. It would also be interesting to see if ADO could be eliminated when the amplified fragment size is as small as 30-40 bp.

The amplification efficiency of DNA fragments is also influenced by the time taken for the DNA polymerase to copy the target. A large fragment takes longer than a short fragment and, depending on the processivity of the enzyme, the action of several enzymes may be necessary to complete replication. Longer fragments also have a higher melting temperature and might denature less efficiently as a result. Sufficient denaturation of the sample DNA is essential for the proper annealing of primers and the subsequent replication of the template. These factors could occasionally prevent a fragment (particularly a long or G:C rich fragment) from being amplified during a PCR cycle and could explain some incidences of preferential amplification. However, to cause ADO amplification failure would have to repeatedly affect the same allele during the first few cycles.

5.4.10 Conclusion

Several studies have shown that the choice of cell lysis protocol can have a dramatic impact on ADO rates (El-Hashemite and Delhanty, 1997; Sermon *et al.*, 1995). This presumably reflects variation in the ability of different methods to ensure that the DNA template is made accessible to the PCR reagents. Most units involved in PGD use either proteinase K or alkaline buffers to achieve cell lysis (Ioulianos *et al.*, 2000; Ray *et*

al., 1998; Sermon *et al.*, 1998). These appear to reduce levels of ADO when compared to lysis in distilled water alone (El-Hashemite and Delhanty, 1997; Sermon *et al.*, 1995). Unfortunately the optimal methodology remains unclear, and data in the literature is contradictory. One study claimed that proteinase K digestion could accomplish a complete elimination of ADO (El-Hashemite and Delhanty, 1997). A later study also suggested a beneficial effect of proteinase K digestion (compared with alkaline lysis the use of proteinase K more than halved the ADO rate at the two loci tested), but was unable to replicate a 0% ADO rate (P. Abou-Sleiman, personal communication). A third study found that alkaline lysis was superior to proteinase K granting a dramatic reduction in ADO for two out of three loci tested (D. Wells, personal communication). Laboratories that have attempted comparison between lysis using proteinase K and alkaline buffers seem to achieve the best results with the technique that they already use routinely. This might suggest that neither technique is particularly robust, both performing poorly in the hands of those unfamiliar with their use.

It is clear from the data in this study that the phenomena of amplification failure and ADO are complex and are influenced by multiple factors. The two are, however, clearly related; high ADO is usually accompanied by increased amplification failure rates. It is logical that whatever causes ADO will sometimes cause amplification failure (i.e. ADO of both alleles). The incidence of amplification failure caused in this way is predicted to equal (ADO rate)^2. However, amplification failure affects 2-5% more amplifications than would be anticipated on this basis (**Figure 5.3**). This indicates that despite the link with ADO, most cases of total amplification failure are caused by factors other than those responsible for ADO. These include cell loss during transfer to the PCR tube, accidental sampling of an anucleate cell, and failure of cell lysis. Interestingly

analysis of duplex PCR reveals that simultaneous failure of both loci to amplify occurs in ~3 % more amplifications than predicted on the basis of their individual amplification efficiencies (**Table 5.7, Study I**), This falls within the 2-5% discrepancy range highlighted above and suggests that there is a baseline amplification failure rate (of 2-5%). Additional amplification failure is caused by the same factors that cause ADO and are locus specific. For most purposes the baseline amplification failure rate should be constant, but there may be some variation between labs (due to differences in micromanipulation and cell lysis techniques) and between samples (due to different amounts of DNA degradation).

The three most likely causes of ADO are incomplete/failed cell lysis, imperfect PCR-conditions and DNA degradation. It is likely that each of these influences ADO rates to a greater or lesser extent. The method of choice for cell lysis in this study utilizes proteinase K digestion, however not all investigators have achieved low ADO rates using this methodology (D. Wells, personal communication). The denaturing temperature used during PCR is also said to be important, although there was no correlation in this respect. It may be that temperatures above 94 °C, such as used in this study, are above the threshold necessary for efficient denaturation of most DNA templates.

Several lines of evidence indicate that some cases of ADO are caused by damage to the DNA strand. This is indicated by the increased ADO that was detected in old samples and in frozen and thawed samples (**Table 5.2, Study A**) and is also in keeping with the correlation observed between ADO and fragment size (**Figure 5.1** and **Table 5.11, Study P**). Extrapolation from the data in this study on ADO, amplification failure and fragment length suggests that amplification of fragments less than 40bp in length

could cause ADO rates to approach zero, and decrease amplification failure to the baseline (2-5%) level.

On the basis of the data in this study the best strategy for PGD would be to take 1-2 fresh, nucleated blastomeres, use an efficient lysis protocol, and then amplify small fragments containing the mutation sites. Because of the small size of the fragment it should be possible to complete amplification and electrophoresis extremely rapidly. In the event of persistent ADO this test could be performed as a multiplex PCR, allowing simultaneous amplification of a linked marker to provide a back up diagnosis.

In conclusion, this study investigated factors that influence amplification efficiency and ADO rates. For this purpose over 3,000 single cells were subjected to single or multiplex-PCR. Factors found to influence ADO were amplicon size, condition of sample (i.e. amount of DNA degradation), number of times the sample was frozen and thawed, length of the denaturing step during PCR, and the number of cells simultaneously amplified. Factors found to have no significant affect on ADO were local DNA sequence, denaturing temperature (94°C or 96°C), chromatin structure, and multiplex PCR as opposed to amplification of a single locus. Careful experimental design, with consideration of these causal factors, might allow a significant amount of ADO to be circumvented.

Chapter 6

Discussion and Conclusion

There is increasing interest in the use of PGD as an alternative to routine prenatal diagnosis. However, extensive development and testing of new protocols is necessary prior to clinical application. This is time-consuming and costly and has hindered the wider application of PGD. Budgetary restrictions have forced some PGD centres to concentrate on developing just a small number of protocols. Common diseases that have prevalent mutations are those usually chosen, as it is assumed that protocols designed will be of benefit to multiple families. Those for the detection of rare diseases and mutations, restricted to a small number of families, have been relatively neglected.

The primary objective of this study was to find a widely applicable analysis system for various mutations of β-thalassaemia using SSCP and to develop PGD protocols for different mutations of β- and α-thalassaemias using appropriate PCR applications, including multiplex and nested PCR, F-PCR, Gap PCR, SSCP, fluorescent sequencing and minisequencing (SNaPshot™). The second aim was to develop a PGD protocol of DM and to work up clinical PGD of DM for 2 families. The final objective was to investigate factors affecting single cell PCR using a variety of gene loci and PCR protocols in order to develop improved single cell PCR protocols in the future.

In **Chapter 3**, attempts to develop a versatile mutation detection system for β-thalassaemia mutations using silver stained SSCP could be accomplished for only one mutation, i.e. IVSI-110 (**Section 3.3.1**). However, the established silver stained SSCP for β-thalassaemia, IVSI-110, was successfully worked up for PGD (**Section 3.3.2**) and can be clinically useful as IVSI-110 is the commonest β-thalassaemia mutation in the Mediterranean (**Section 3.1.1**). F-SSCP failed to show its use for β-thalassaemia detection

in this study (**Section 3.3.1**); though, more gel compositions could be tried. In the future, the chance to test the application of F-SSCP using the capillary-based ABI Prism™3100 or gel-based ABI Prism™377 electrophoresis would provide the advantage of detecting up to 4 different fluorescent dyes. The higher sensitivity of F-SSCP compared to the traditional silver stained SSCP removes the need for nested PCR, which means a quicker amplification time.

In the thalassaemia project (**Chapter 3**), eight PGD protocols have been developed and tested using different combinations of various modern technologies. Some of these protocols can be applied to more mutations with or without modification. The PGD protocol for β-thalassaemia IVSI-110 mutation demonstrated the analysis of a substitution mutation using SSCP (**Section 3.3.2**), while the use of fluorescent gap PCR in the PGD protocol for α-thalassaemia, SEA mutation showed the modification of PCR for the diagnosis of a large deletion (**Section 3.3.4**). The single step multiplex F-PCR for β-thalassaemia codon 41-42 mutation (**Section 3.3.3**) incorporates a linked marker that provides back up diagnostic information and assists in the detection of DNA contaminations. Furthermore, after testing it can also be useful for other deletions or insertions of the β-globin gene which are included within this set of primers. The modification of primers used would allow the application of this protocol for more mutations. After the extensive work up in this study, the PGD protocols for β-thalassaemia IVSI-110 and codon 41-42 mutations and α-thalassaemia, SEA mutation are ready for clinical application.

One of the main factors in the design of new protocols is the choice of mutation detection technique. Currently the most popular are restriction enzyme digestion, SSCP

and heteroduplex analysis. However, each of these has limitations. The feasibility of using DNA sequencing (**Section 3.3.5**) and minisequencing (SNaPshot™; **Section 3.3.6**) for clinical diagnosis at the single cell level has been assessed in this study. Both protocols have exhibited their potential of being good widely applicable PGD protocols for disorders with substitutions, deletion and insertions. These strategies are very valuable for detecting the diseases caused from heterogeneous mutations, i.e. β-thalassaemia. The single cell sequencing protocol is particularly useful for the identification of a compound heterozygote sample where the two mutations are encompassed by the same set of primers. It could be concluded that both methods should be widely applicable and offer the potential for a significant reduction in the costs associated with the development of new PGD protocols. After scoring for the amplification efficiencies and ADO rates of the particular mutations using more single cells, these two methods would be ready for clinical application.

In the DM project (**Chapter 4**), two single-step multiplex F-PCR protocols were developed for PGD of DM (**Section 4.3.1**); one was clinically applied to three PGD cycles in two DM families resulting in two pregnancies with three DM-free babies (one singleton and one twin; **Section 4.3.3**). The DM genotypes of the babies have been confirmed by PND and all babies were delivered safely. The added linked polymorphic marker to the DM CTG repeat analysis (protocol 1) provides the advantage of backup linkage analysis results and contamination identification. However, in families where the linked marker is not informative, an un-linked marker is used (protocol 2) for contamination detection only. Both protocols would lead to a lower chance of misdiagnosis from foreign DNA contamination. Therefore, protocol 2 has been proved for its clinical use, while protocol 1 is ready for clinical application in the informative

families. Moreover, these DM PGD strategies may also be useful for PGD of other triplet repeat disorders with the modification of the primers and amplification conditions employed. Attempts to find a PCR system for the amplification of the mutant DM expansion from a single cell were not successful in this study. However, if accomplished, this method may be useful as another PGD protocol for DM and to learn the nature and mechanism of the DM triplet expansion. Therefore, more different hi-fidelity PCR systems should be tested in order to obtain an appropriate protocol for amplifying the mutant DM expansion with different lengths of the expansion.

The development of the various PGD protocols provided the chance to access patients' samples and spare human blastomeres and investigate factors affecting single cell PCR (**Chapter 5**). These resources were useful in a study exploring the nature of the amplification efficiency, PA and ADO. The analysis of the different gene loci in comparison to the amplification efficiency and ADO improved our understanding of single cell PCR. Factors found to influence ADO were amplicon size, sample condition, freezing and thawing, length of denaturing step and number of cells simultaneously amplified. This knowledge is essential for the development of future PGD protocols with improved efficiency. As amplicon size is one of the important factors affecting the amplification efficiency and ADO rate, it would be interesting to test the amplification efficiency and ADO rates of the smaller amplified fragment size, i.e. 30-40 bp. This size of the amplicon could markedly reduce or eliminate the amplification failure and ADO. Based on this knowledge, future PGD protocols should be designed to amplify the smallest fragment size possible.

In conclusion, PGD protocols for various types of mutations have been developed in this study. Most protocols have been tested and are ready for clinical application, and one has proved its clinical success. The mentioned strategies and the understanding of factors affecting single PCR can be useful for future PGD of a wider range of single gene disorders with an improved efficiency. Future work includes the test for the amplification efficiency and ADO rate of small amplicon single cell PCR system, i.e. 30-40 bp, optimisation of single cell sequencing and minisequencing (SNaPshot™) protocols for their clinical application, clinical application of DM PGD protocol 1 in an informative family and the search for an appropriate high fidelity PCR system for detecting both normal and mutant DM expanded alleles.

6.1 The future of PGD techniques

Since the first successful PGD in 1989 (Handyside *et al.*, 1989), a growing number of diseases detected by PGD have been reported (Wells and Delhanty, 2001). Misdiagnosis has been an important problem for both FISH and PCR diagnosis. Apart from the hybridisation efficiency, chromosome mosaicism is a major risk of misdiagnosis in FISH. Interphase FISH is limited by the number of chromosomes that can be analysed. Techniques for the full analysis of all chromosomes using interphase chromosome conversion and comparative genomic hybridisation (CGH) are being developed (**Section 6.1.2**). The success in applying whole genome amplification (WGA; **Section 6.1.1**) protocols using primer extension preamplification (PEP) (Zhang *et al.*, 1992) and degenerate oligonucleotide primed PCR (DOP-PCR) (Wells *et al.*, 1999) on single cells has been reported. The amplified products from WGA make the analysis of several loci in a single cell possible. Analysis methods after PCR are generally different from one

disease to another due to the nature of their mutations. Having a versatile mutation detection protocol would help in reducing time and expense spent in developing protocols for new diseases with minor modification.

6.1.1 The future of techniques to analyse single gene defects

Future development of PGD of single gene disorders aims at a more precise result with less chance of misdiagnosis as well as diagnosing more than one disorder from a single cell. In order to obtain maximum information from a single cell, WGA can be applied. Although multiplex PCR is less complicated and quicker, WGA produces much larger amounts of DNA for the analysis of several loci and other purposes, i.e. CGH, microarrays. This would be useful in patients carrying one or more single gene defects and who are also at risk of having a chromosomally abnormal offspring. PGD of familial adenomatous polyposis using PEP has been clinically performed (Ao *et al.*, 1998). It was demonstrated that DOP-PCR could provide enough DNA from a single cell for the analysis of more than 100 loci (Wells *et al.*, 1999). However, at present WGA is still not a popular technique for clinical PGD due to the complexity of the method and there are still few uses for WGA as the patients usually carry only one disorder. Microarrays or DNA chips offer the chance of detecting several mutations or polymorphisms at a time. All chips contain particular DNA fragments arrayed on a support, i.e. a glass slide or special membrane. The analyses used include short oligonucleotide probe hybridisation (dot blot), competing hybridisation (CGH) and minisequencing approaches. When clinically ready, microarrays (following WGA) may be a very useful tool for PGD.

6.1.2 The future of techniques to analyse chromosomes

As metaphase FISH is more efficient than interphase FISH, attempts have been made in order to induce metaphase formation of embryonic nuclei. The technique of nuclear conversion was successfully performed by fusing polar bodies or blastomeres into oocytes (Evsikov and Verlinsky, 1999; Willadsen *et al.*, 1999). The blastomere was injected into the perivitalline space of the enucleated human oocyte or abnormally fertilised zygote. An electric impulse was used to induce cell fusion and the injected blastomere was stimulated to enter metaphase by the factors within the ooplasm (Evsikov and Verlinsky, 1999). Metaphase nuclear conversion can also be induced by fusion with bovine oocytes and the cell cycle stopped using colcemid (Willadsen *et al.*, 1999). The resulting chromosomes are spread on a microscope slide or G-banded; however, whole chromosome paints are recommended for translocation detection. This allows the clinical application of a SKY (spectral karyotyping) (Schrock *et al.*, 1996) and M- (multi-fluorochrome karyotyping) (Speicher *et al.*, 1996) FISH, which include 24 chromosome specific paint probes labelled with different combinations of fluorochromes. Clinical use of SKY FISH was successfully performed on chromosomes from oocytes and polar bodies (Marquez *et al.*, 1998). However, the fusion of a human blastomere or polar body with an enucleated oocyte is not allowed in the UK.

CGH is a FISH related technique to examine the copy number of every chromosome in a single hybridisation (Kallioniemi *et al.*, 1992). This technique involves the labelling of the test and control DNAs with green and red fluorochromes, respectively, and hybridisation of both labelled DNAs to normal metaphase chromosomes on a microscope slide. In the case of a balanced choromosomal composition, the labelled

DNAs equally hybridised to the chromosomes, consequently giving a yellow colour. However, if the tested sample possesses an extra chromosome, for instance trisomy 21, the green DNA for chromosome 21 will be more intense than the red control DNA, resulting in a green colour. Conversely, if the tested sample has a chromosome deficit, a monosomy for example, the dominating red colour will be shown. This technique provides information of the chromosome regions with a resolution of at least 20Mb using computer analysis software. Therefore, CGH can also identify abnormalities with unbalanced translocations. However, this technique requires around 100ng to 1μg of test DNA which is equivalent to more than 10,000 cells, while only one or two cells can normally be obtained from PGD. The application of whole genome amplification (WGA) using degenerate oligonucleotide primed PCR (DOP-PCR) has allowed CGH to be performed on single human blastomeres (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). However, the present protocol takes longer than one day and so there is a need to quicken the protocol before it can be clinically used for PGD. Therefore, at present FISH is still the method of choice for PGD and PGS for identifying chromosome abnormalities.

6.2 Laws and ethics

The regulation of human embryo culture and research is varied from one country to another. In some countries, i.e. Germany, the restrictive laws against embryo research automatically prevent the application of PGD; though, PND and termination of pregnancy are legal in those countries (Viville and Pergament, 1998). In the UK, the Human Fertilisation and Embryo Act of 1990 permits embryo research up to 14 days post fertilisation for four purposes. These include the development of infertility treatment, the understanding of congenital disease, the improvement of contraceptive techniques and the

understanding of the causes of miscarriage. Therefore, it is possible to develop the methods for diagnosis of genetic disorders in preimplantation human embryos. However, all new diseases and the analysis protocols used need to be approved by the Human Fertilisation and Embryology Authority, while the application of PND is hardly regulated.

Embryo selection at the preimplantation stage following genetic diagnosis has caused numerous debates (Viville and Pergament, 1998). Attention has been drawn against the application of PGD for eugenics (the selection of characteristics that are not a disease, i.e. intelligence) (King, 1999). However, the inheritance of most of these characteristics are multi-factorial, consequently the molecular diagnosis is complicated. During the last decade, the main objective of PGD has been for avoiding babies with serious genetic defects. Most centres would decide not to perform PGD for non-medical reasons (Nagy *et al.*, 1998). However, there is the potential use of PGD for identifying sex of the embryos, and so sex selection according to the preference of the parents without a medical indication. This use of PGD creates a lot of argument, while it is being done in several PGD centres (Berkowitz, 1999). The birth of a PGD baby free from Fanconi anaemia and HLA matched to treat an older sibling suffering from Fanconi anaemia (Verlinsky *et al.*, 2000) was the first reported PGD application for saving the life of an existing patient. This case has stirred a great deal of debate worldwide regarding the use of PGD for the purpose of creating designer babies.

The opportunity to analyse human preimplantation embryos has revealed that less than half of normally developed embryos possesses normal chromosome components (Delhanty *et al.*, 1997). This knowledge leads to the idea of empirically applying PGD to all patients who receive IVF treatment as Preimplantation Genetic Screening (PGS) in the

hope to increase the pregnancy rates (Wells and Delhanty, 2000). However, this concept is still controversial in most centres. Although, there is no short term effect of taking 1-2 cells out of the embryos (Hardy *et al.*, 1990), and the PGD babies do not show a greater risk of neonatal problems or malformation than ICSI babies (ESHRE PGD Consortium Steering Committee, 2000), long term effects in their reproductive life and adulthood need to be observed.

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