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# Strategies and clinical outcome of 250 cycles of Preimplantation Genetic Diagnosis for single gene disorders

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**BACKGROUND: We report on our experience with preimplantation genetic diagnosis (PGD) for single gene disorders (SGDs), from 1999 to 2004, describing strategies and overall clinical outcome of 250 cycles in 174 couples for 23 different genetic conditions. METHODS: PGD cycles included 15 for autosomal dominant, 148 for autosomal recessive and 19 for X-linked SGDs. In addition, 68 cycles of PGD for SGDs were performed in combination with HLA matching. The strategy in each case used an initial multiplex PCR, followed by minisequencing to identify the mutation(s) combined with multiplex PCR for closely linked informative markers to increase accuracy. Linkage analysis, using intragenic and/or extragenic polymorphic microsatellite markers, was performed in cases where the disease-causing mutation(s) was unknown or undetectable. RESULTS: In 250 PGD cycles, a total of 1961 cleavage stage embryos were biopsied. PCR was successful in 3409 out of 3149 (92.4%) biopsied blastomeres and a diagnosis was possible in 1849 (94.3%) embryos. Four hundred and twenty-seven embryos were transferred in 211 cycles, resulting in 71 pregnancies (33.6% per embryo transfer), including 15 biochemical pregnancies, six spontaneous miscarriages, two ectopic pregnancies, which were terminated, and nine pregnancies which are still ongoing. The remaining pregnancies were confirmed to be unaffected and went to term without complications, resulting in the birth of 35 healthy babies. CONCLUSIONS: Minisequencing for mutation detection combined with multiplex fluorescence PCR for linkage analysis is an efficient, accurate and widely applicable strategy for PGD of SGDs. Our experience provides a further demonstration that PGD is an effective clinical tool and a useful option for many couples with a high risk of transmitting a genetic disease.**

*Key words*: clinical outcome/minisequencing/PGD/preimplantation HLA matching/single gene disorders

## Introduction

Couples at high risk of transmitting an inherited disease to their offspring have the option of undergoing prenatal diagnosis to allow the detection of the genetic disorder in the fetus. However, if the analysis reveals a genetically affected fetus, the only options available to couples are to have a child with a genetic disease or to terminate the affected pregnancy. This is a difficult and often traumatic decision, especially in advanced pregnancies. Many couples may also experience repeated pregnancy terminations in attempts to conceive a healthy child and might feel unable to accept further affected pregnancies.

PGD has been introduced as an alternative to prenatal diagnosis, to increase the options available for couples who have a known genetically transmittable disease, providing reassurance and a reduced anxiety associated with reproduction. The intended goal of PGD is to diagnose a specific genetic disease in embryos, obtained through IVF, before a clinical pregnancy has been established, by selecting and transferring to the mother only embryos found to be unaffected after mutation analysis. Consequently, PGD may spare the couple decisions regarding possible pregnancy termination, giving them the opportunity to start a pregnancy with the knowledge that their child will be unaffected by the specific genetic disorder (Braude *et al*., 2002; Sermon *et al*., 2004).

Following its first application in 1990 (Handyside *et al*., 1990), PGD has become an important complement to the presently available approaches for prevention of genetic disorders and an established clinical option in reproductive medicine. Since then, the numbers of centres performing PGD has risen steadily, along with the number of diseases that can be tested (International Working Group on Preimplantation Genetics, 2001; ESHRE PGD Consortium Steering Committee, 2002;

 

Sermon *et al*., 2005), and new applications and methodologies are introduced regularly. The range of genetic defects which can be diagnosed has expanded dramatically and now includes numerical and structural chromosomal abnormalities (Munnè *et al*., 2000; Wilton, 2002) and most of the common single gene disorders (SGDs) (Vandervorst *et al*., 2000; Pickering *et al*., 2003). The scope of PGD has also been extended to screening for chromosomal aneuploidies in IVF patients at increased risk, including advanced maternal age and repeated miscarriage (Verlinsky *et al*., 1999; Munnè *et al*., 2002; Rubio *et al*., 2003). More recently, HLA typing, with or without SGD diagnosis, has been introduced with the aim of recovering compatible stem cells from cord blood at birth for transplantation to an existing sick child (Verlinsky *et al*., 2001; Fiorentino *et al*., 2004, 2005; Van de Velde *et al*., 2004; Rechitsky *et al*., 2004).

Although a growing number of clinics are offering PGD, mainly for aneuploidy screening, the overall experience of using PGD for SGDs remains restricted to only a few centres worldwide. To date it is estimated that PGD has been applied in >50 different SGDs in >1000 cycles, resulting in the birth of >300 unaffected children (International Working Group on Preimplantation Genetics, 2001; ESHRE PGD Consortium Steering Committee, 2002; Sermon *et al*., 2005).

Here we present our experience of using PGD for SGDs from 1999 to 2004, describing strategies and overall clinical outcome of 250 PGD cycles performed for 23 different genetic conditions.

## Materials and methods

### *Case referrals and patient counselling*

A total of 174 couples were included in the PGD programme over a 6 year period (Table I). Maternal age ranged from 21 to 44, with a mean age of 31.8 ± 4.3 years. Referrals were received from IVF centres, geneticists and gynaecologists. Patients were counselled by a clinical geneticist, who first assessed the feasibility of carrying out the diagnosis of the genetic disease at single cell level. Genetic counselling consisted of reviewing the couple's genetic history and their reason(s) for requesting PGD, followed by an explanation of the PGD process for the specific genetic disorder and a discussion on the possible diagnostic options. A calculation of the genetic risk, the possible genetic outcomes, the success rates and the risk of misdiagnosis were also discussed. Finally the patients were referred to an assisted reproduction clinic to arrange the clinical aspects of the treatment.

During the initial consultation, a blood sample from both partners and affected children (when available) was taken as required for preclinical PGD work-up. Time needed to develop the test at single cell level averaged 1.5 months.

Written informed consent was obtained from all the couples, in which the possible risk of misdiagnosis was specified and confirmatory prenatal diagnosis for any ensuing pregnancy was always recommended.

### *Pre-clinical work-up*

Determination or confirmation of the genetic status of the couples was performed as previously described (Fiorentino *et al*., 2003). PCR amplification of each region of interest was performed using the outer oligonucleotide primers listed in Table II or reported elsewhere (Fiorentino *et al*., 2003, 2004, 2005; Iacobelli *et al*., 2003). Short tandem repeat (STR) markers (Table III), closely linked to the disease-causing genes, were included to avoid a possible misdiagnosis resulting from allele dropout (ADO). STR markers were also included to determine the copy number of chromosomes 21, 18 and 13, in patients of advanced maternal age (≥37 years of age).

The reliability of each protocol was evaluated before clinical application on single lymphocytes collected from both parents, as previously described (Fiorentino *et al*., 2003).

### *IVF and embryo biopsy procedure*

The majority of the patients were stimulated according to standard protocols (Fiorentino *et al*., 2003, 2004; Iacobelli *et al*., 2003). Other patients followed the specific protocols used by the referring clinics. In all cases, ICSI was used to reduce the risk of contamination with spermatozoan DNA. Selection of embryos for biopsy and the biopsy procedure were performed as described elsewhere (Fiorentino *et al*., 2003, 2004; Iacobelli *et al*., 2003), using either the acid Tyrode's protocol or a non-contact infrared laser for zona drilling. According to the biopsy policy, one blastomere was removed from embryos with <7 cells and two blastomeres from embryos with ≥7 cells. After biopsy, each blastomere was washed several time through drops of $Ca^{2+}$/$Mg^{2+}$-free EB-10 medium (Vitrolife, Gothenburg) and transferred into a sterile 0.2 ml PCR tube containing 5 µl of alkaline lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol). Blank controls from the wash drops for each blastomere were also included. Before proceeding to cell lysis, each tube was overlaid with one drop of PCR-grade mineral oil (Sigma-Aldrich, Milan, Italy). The samples were then placed on a rack, refrigerated by using wet ice, holding a 4°C temperature, and transported from the clinics to the PGD laboratory in Rome for genetic analysis. The transportation took 3 h, on average, and genetic analysis usually started within 6 h after the end of the biopsy procedure.

### *Cell lysis and multiplex PCR*

DNA analysis of blastomeres was carried out on the same day as biopsy. Before proceeding to multiplex PCR, cells were lysed by incubation at 65°C for 10 min. The alkaline lysis buffer was then neutralized by the addition of 5 µl of neutralization buffer (900 mmol/l Tris–HCl, 300 mmol/l KCl, 200 mmol/l HCl).

The technical details of the PCR protocols used for cystic fibrosis, β-thalassaemia, sickle cell anaemia, haemophilia A, retinoblastoma, spinal muscular atrophy, Charcot–Marie–Tooth type X and HLA matching have been described elsewhere (Fiorentino *et al*., 2003, 2004, 2005; Iacobelli *et al*., 2003). For the other diseases, a nested multiplex PCR assay was used. The first round of PCR contained the external primers for the amplification of the gene regions involved by mutations, STR markers linked to these regions for ADO detection and those used for detection of aneuploidies in patients with advanced maternal age. The first round of multiplex PCR was followed by a separate second round PCR for each locus. PCR conditions used were reported earlier (Fiorentino *et al*., 2003, 2004, 2005). The annealing temperature depended on the specific primers used (Table II).

### *Mutation analysis in single cells*

The strategy used for mutation analysis and mutations investigated are described in Table I. A fluorescent PCR protocol, involving multiplex amplification of one exon located in the deleted region and one non-deleted exon, was used for Duchenne muscular dystrophy. Mutation analysis for primary dystonia and myotonic dystrophy was performed by fluorescent amplification of the gene region surrounding the Cod.302 del GAG mutation in exon 5 of the *DYT1* gene and the CTG repeat region in the 3′-untranslated region of the *DMPK* gene, respectively. Linkage analysis was the strategy preferred for neurofibromatosis type 1 (NF1), using fluorescent primers listed in Table II, and for congenital adrenal hyperplasia (CAH), using the STR markers MIB, MIC-A, 62, TNFa, LH-1 and DRA-CA described elsewhere (Fiorentino

**Table I.** Details of PGD cycles for single gene disorders performed according to their inheritance pattern

| Disease | Gene | Cycles ($n$ = 250) | Couples ($n$ = 174) | Mutation/genotype investigated | Detection method |
|---|---|---|---|---|---|
| Autosomal dominant | | 15 | 8 | | |
| Holt–Oram syndrome | *TBX5* | 1 | 1 | W401X | Minisequencing |
| Myotonic dystrophy | *DMPK* | 6 | 3 | 5–500/11–20; 14–340/5–5; 5–8/11–750 | Fluorescent PCR |
| Primary dystonia | *DYT1* | 2 | 1 | Cod.302 del GAG | Fluorescent PCR |
| Neurofibromatosis type 1 | *NF1* | 2 | 1 | * | Linkage analysis |
| Retinoblastoma | *RB1* | 3 | 1 | 73868 A→G | Minisequencing |
| Spastic paraplegia 3 | *SPG3A* | 1 | 1 | R415W | Minisequencing |
| Autosomal recessive | | 148 | 107 | | |
| β-Thalassaemia | *HBB* | 105 | 75 | –110 C→T, –87 C→T, –30 T→A, Cod.5–CT, Cod.6–A, Cod.8–AA, Cod.8/9+G, IVS-I-1 G→A, IVS-I-5 G→C, IVS-I-6 T→C, IVS-I-110 G→A, IVS-I-130 G→C, Cod.39 C→T, Cod.41/42-TTCT, Cod.44-C, Cod.82-G, IVS-II-1 G→A, IVS-II-654 C→T, IVS-II-745 C→G, Poly A | Minisequencing |
| Cystic fibrosis | *CFTR* | 25 | 19 | G85E, R117H, M348K, ΔF507, ΔF508, G542X, R553X, 2183 AA→G, F1052V, T1057A, W1282X, N1303K | Minisequencing |
| Congenital adrenal hyperplasia | *CYP21A2* | 1 | 1 | nt656 A-C→G | Linkage analysis |
| Familial Mediterranean fever | *MEVF* | 2 | 1 | M694V, V726A | Minisequencing |
| Mucopolysaccharidosis type IIIA—Sanfilippo syndrome | *SGSH* | 3 | 1 | M88T | Minisequencing |
| Mucopolysaccharidosis type VI—Maroteaux–Lamy syndrome | *ARSB* | 1 | 1 | L321P | Minisequencing |
| Sickle cell anaemia | *HBB* | 5 | 3 | Cod.6 A/T | Minisequencing |
| Spinal muscular atrophy | *SMN1* | 6 | 6 | $SMN^t$ Ex. 7 del | Minisequencing |
| X-linked | | 19 | 10 | | |
| α-Thalassaemia mental retardation syndrome | *ATRX* | 1 | 1 | IVS3+1 G-T | Minisequencing |
| Charcot–Marie–Tooth type X | *CMTX* | 2 | 1 | H94Q | Minisequencing |
| Duchenne muscular dystrophy | *DMD* | 5 | 2 | Del Ex. 5, Del Ex. 45–47 | Fluorescent PCR |
| Glucose-6-phosphate dehydrogenase deficiency | *G6PD* | 1 | 1 | S188F | Minisequencing |
| Haemophilia A | *F8* | 5 | 2 | intr.8–27 G→A, R1966X | Minisequencing |
| Haemophilia B | *F9* | 3 | 1 | G190D | Minisequencing |
| Lesch–Nyhan syndrome | *HPRT* | 1 | 1 | R169X | Minisequencing |
| Wiskott–Aldrich Syndrome | *WAS* | 1 | 1 | T353C | Minisequencing |
| Other | | | | | |
| SGD + HLA matching | HLA | 68 | 49 | | FluorescentPCR + Minisequencing |

*Unknown mutation.

**Table II.** Description of genetic regions amplified and primers used

| Disease | Gene | Exon/marker | Outer primers (5′ to 3′) | Inner primers (5′ to 3′) | Annealing temperature (°C) |
|---|---|---|---|---|---|
| α-Thalassaemia mental retardation syndrome | *ATRX* | 3 | F-5′-TGAGAATGGGTTTGTGGAGTTA-3′<br>R-5′-TCAGTAGCCTTCGACACATACA-3′ | F-5′-GTTTGTGGAGTTATAGGTATTG-3′<br>R-5′-CATATGCTCTACTTGGTTAATC-3′ | 52 |
| Familial Mediterranean fever | *MEVF* | 10 | F-5′-AGGGCTGAAGATAGGTTGAA-3′<br>R-5′-AGGTTGGAGACAAGACAGCA-3′ | F-5′-GCCCAGAGAAAGAGCAGC-3′<br>R-5′-ACAGCATGGATCCTGGGA-3′ | 60 |
| Spastic paraplegia 3 | *SPG3A* | 12 | F-5′-CAGACCAAACACCTGCAACTTA-3′<br>R-5′-GACTACAAACAGTGTGGCTGGG-3′ | F-5′-ATCTGTGAAGCTATTCCGAGGG-3′<br>R-5′-GCTGCATGGAAGATATTTTTGC-3′ | 56 |
| Glucose-6-phosphate dehydrogenase deficiency | *G6PD* | 6 | F-5′-TGATGCAGCTGTGATCCTCA-3′<br>R-5′-TGAGGTTCTGCACCATCTCC-3′ | F-5′-TCATCGTGGAGAAGCCCT-3′<br>R-5′-GGTAGTGGTCGATGCGGT-3′ | 60 |
| Haemophilia B | *F9* | 6 | F-5′-TCTAGTGCCATTTCCATGTG-3′<br>R-5′-CAGCTGAGCTCCAGTTTTGA-3′ | F-5′-ACTTCTAAGCTCACCCGTGC-3′<br>R-5′-AAGTACCTGCCAAGGGAATT-3′ | 55 |
| Holt–Oram syndrome | *TBX5* | 9 | F-5′-AAGTGAGCGGAGAAGTGCT-3′<br>R-5′-CAGCCTAGAGGACATCAGCT-3′ | F-5′-TGGTAGGGTAGCCTGTCCA-3′<br>R-5′-GAGGACATCAGCTGCAACA-3′ | 60 |
| Lesch–Nyhan syndrome | *HPRT* | 7 | F-5′-AATGCCCTGTAGTCTCTCTGTA-3′<br>R-5′-CTCTCTACAAATATTCTCTAAG-3′ | F-5′-TCTCTCTGTATGTTATATGTCA-3′<br>R-5′-TAAGCCATTTCACATAAAACT-3′ | 53 |
| Maroteaux–Lamy syndrome | *ARSB* | 5 | F-5′-CATCCTCATGCCAAGACCTT-3′<br>R-5′-TACCTGATGGTTTTCCACAC-3′ | F-5′-ATAACGGAGGGCAGACTTT-3′<br>R-5′-CTTTGTGCCATTGGTGTGT-3′ | 60 |
| Sanfilippo syndrome | *SGSH* | 3 | F-5′-AGGGCCATGGGAGAACAGG-3′<br>R-5′-TCTGCCTGCCTCTGTGCCT-3′ | F-5′-CCTGGACCTCATGCGCCTT-3′<br>R-5′-ACACCAGCTTGGCTGAGCA-3′ | 62 |
| Wiskott–Aldrich syndrome | *WAS* | 3 | F-5′-ACTGTGCCTCCCACCCTACAC-3′<br>R-5′-GTCCTCGTCTGCAAAGTTCAGC-3′ | F-5′-TACACCTCTCCAGGCTGGTC-3′<br>R-5′-GCAACTTTCCCATCTCCTCTC-3′ | 60 |
| Duchenne muscular dystrophy | *DMD* | 5 | F-5′-TATTATTGCAACTAGGCATT-3′<br>R-5′-TACACATTTGTTTCACACGT-3′ | F-5′-CAACTAGGCATTTGGTCTCT-3′<br>R-5′-FAM-TGTTTCACACGTCAAGGGTA-3′ | 53 |
| | | 45 | F-5′-GGAAATTTTCACATGGAGCT-3′<br>R-5′-CATTCCTATTAGATCTGTCG-3′ | F-5′-GTACAACTGCATGTGGTAGC-3′<br>R-5′-HEX TATTAGATCTGTCGCCCTAC-3′ | 53 |
| | | 48 | F-5′-AGAGCAGTTAAATCATCTGC-3′<br>R-5′-GCCTATTGTGGTTATCCTGA-3′ | *<br>R-5′-HEX-TGAATAAAGTCTTCCTTACC-3′ | 53 |
| Myotonic dystrophy | *DMPK* | 1 | F-5′-AACGGGGCTCGAAGGGTCCTTGT-3′<br>R-5′-TTCCCAGGCCTGCAGTTTGCCCA-3′ | F-5′-FAM-GGTCCTTGTAGCCGGGAAT-3′<br>R-5′-TTTGCCCATCCACGTCAG-3′ | 65 |
| Primary dystonia | *DYT1* | 5 | F-5′-CCCTGGAATACAAACACCTA-3′<br>R-5′-ACCGTTTTGCAGCCTTTATC-3′ | F-5′-FAM-TATCCGAGTGGAAATGCAGT-3′<br>R-5′-AGCCTTTATCTGAGAAAACT-3′ | 55 |
| Neurofibromatosis type 1 | *NF1* | Intron 26 | F-5′-TGGTGGCTAATACCTATAAC-3′<br>R-5′-ATTACAGGCATGAGCCACT-3′ | F-5′-HEX-GGTGAGAGAATTGCTGGAGG-3′<br>* | 52 |
| | | Intron 27 | F-5′-CTTGAAGCCCAGTTTCTTTA-3′<br>R-5′-CTAAGTGTAAACGCAAAGT-3′ | F-5′-FAM-AAGTATGCAGTTTTCCAGA-3′<br>R-5′-AACATTAACAACAAGTACC-3′ | 52 |
| | | Intron 38 | F-5′-AGATCACGCCACTGCACTCC-3′<br>R-5′-CCCCATTAGCACCCCTCCTA-3′ | F-5′-FAM-ACAGAGCAAGACCCTGTCTC-3′<br>R-5′-CCCCTCCTAACATTTATTAACC-3′ | 52 |
| | | Alu | F-5′-ACTGCATTCTAGCCTGAGTGA-3′<br>R-5′-GGATACTATTACGTTGCAATT-3′ | F-5′-HEX-CAGAGCAAGACTCTGTATGA-3′<br>R-5′-CAATGAGAAATGTTTTGGAA –3′ | 52 |

F = forward; R = reverse.
*A heminested approach was used. FAM-HEX are fluorescent dyes.

**Table III.** Oligonucleotide primer sequences for the first- and second-round amplification of selected informative STR markers linked to the disease causing genes

| Disease | Gene | STR markers | Outer primers (5′ to 3′) | Inner primers (5′ to 3′) | Heterozygosity | References |
|---|---|---|---|---|---|---|
| Holt–Oram syndrome | *TBX5* | D12S1646[c] | F-5′-CACTCCATTGCTGGCTAGTTTC-3′<br>R-5′-GAAGCCCTTGCTGGGTAAGA-3′ | F-5′-GCATGGAAAGGCTTAGCGAATA-3′<br>* | 0.72 | Dib *et al.* (1996) |
| | D12S1330[a] | F-5′-GGGCAACAGAGCAAGACTCCAT-3′<br>R-5′-AATTCCCCGTCTCCATCTTGTG-3′ | *<br>R-5′-GGGAACGGTTGTTTTCTCAGGT-3′ | 0.78 | Dib *et al.* (1996) |
| | D12S354[a] | F-5′-TGGTGGTTCTGGGTCAGATAAAA-3′<br>R-5′-TCTCAGGAAAGGGAACCAATTGT-3′ | F-5′-ATTTCTGCTGGCCTAATAATGCC-3′<br>* | 0.74 | Gyapay *et al.* (1994) |
| Myotonic dystrophy | *DMPK* | D19S207[a] | F-5′-CGGTGTTTGAACCCTCGCTG-3′<br>R-5′-GCCTGAGTGACAAAGCGACG-3′ | F-5′-ACCCTCGCTGCAGACCACAC-3′<br>* | 0.78 | Jansen *et al.* (1993) |
| | D19S112[a] | F-5′-GAAAAAGATGGACCCACAGAAA-3′<br>R-5′-CCAAAGACTTTGTCTAATCCACG-3′ | F-5′-ACTGAAAGACACGTCACACTGGT-3′<br>* | 0.86 | Jansen *et al.* (1992) |
| | D19S219[b] | F-5′-GTTGCAGTGAGCCAAGATTGTG-3′<br>R-5′-TTCTGAGACAGATTCCCAGGGT-3′ | F-5′-TAGGCAATAGAGCGAGAATCCG-3′<br>* | 0.77 | Gyapay *et al.* (1994) |
| Primary dystonia | *DYT1* | D9S1831[b] | F-5′-AGGCTGAGCCAGGAGAATGG-3′<br>R-5′-AAGTCAGCCGATAGGCAGGC-3′ | *<br>R-5′-TTTGCCCACCACAGCCTATT-3′ | 0.81 | Dib *et al.* (1996) |
| | D9S63[a] | F-5′-CCACAAAAGAAAGTCACAATCC-3′<br>R-5′-TTATAATGCCGGTCAACCTT-3′ | F-5′-CACCGGAAGTTACTCTAGTCTAAA-3′<br>* | 0.88 | Henske *et al.* (1993) |
| Retinoblastoma | *RB1* | D13S1307[a] | F-5′-AATAGACTCCAAACCAGCCTATG-3′<br>R-5′-TTGCATTAGACACAGTTGACCAC-3′ | *<br>R-5′-CCTCATCTACTCCTTCAAACAGAC-3′ | 0.71 | Dib *et al.* (1996) |
| | D13S153[c] | F-5′-GCATTGTTTCATGTTGGTGTACG-3′<br>R-5′-AGGTCTAAGCCCTCGAGTTGTG-3′ | F-5′-AACCGACTCCTGTTTCTCCTCC-3′<br>* | 0.82 | Gyapay *et al.* (1994) |
| | D13S118[b] | F-5′-CTACTGACATTTGCTCCATAGGAC-3′<br>R-5′-TATATAACTTGTGTGACAGCAGGC-3′ | F-5′-CAGACATCAGAGTCCTTACAATGTT-3′<br>* | 0.72 | Deka *et al.* (1995) |
| | D13S168[b] | F-5′-TCCTTAACAGCCTAGCCCAGTG-3′<br>R-5′-CTGCTTGCTTGTGCCTATGTTC-3′ | F-5′-CATGATGCTGGACTGGACATTT-3′<br>* | 0.77 | Gyapay *et al.* (1994) |
| Spastic paraplegia 3 | *SPG3A* | D14S978[a] | F-5′-AAGAAGGAAAATACATTGGCCC-3′<br>R-5′-TGGCTACATGATGGACATTCAC-3′ | *<br>R-5′-CCAATACAGGCAACCTCAACTT-3′ | 0.84 | Dib *et al.* (1996) |
| | D14S1031[b] | F-5′-GATAAATACTGCACACTTGGGC-3′<br>R-5′-TGACAGAGTGAGATTCAGCCTC-3′ | *<br>F-5′-GCACACTTGGGCTACACTAAATT-3′ | 0.65 | Dib *et al.* (1996) |
| | D14S984[b] | F-5′-GGGAACATATATTCAGGGCAACT-3′<br>R-5′-CCTGGTGACAGAGTGAGACTCTT-3′ | F-5′-CCAGGTTGCAGTCCTCTATTTT-3′<br>* | 0.71 | Dib *et al.* (1996) |
| | D14S269[b] | F-5′-GTTGGGTATGTGGCTATTTCCA-3′<br>R-5′-ATTGATTCCTGATGATAACCGG-3′ | *<br>R-5′-TTGACAGGTACATTGTTGCTGG-3′ | 0.69 | Gyapay *et al.* (1994) |
| β-Thalassaemia/<br>sickle cell anaemia | *HBB* | D11S4146[a] | F-5′-AAAAACACGAGGTTAAGCAGAG-3′<br>R-5′-CTACCAAACATGATTCCTAGGA-3′ | F-5′-GGTTAAGCAGAGTTTAATAGGC-3′<br>* | 0.70 | Dib *et al.* (1996) |
| | D11S988[a] | F-5′CACAGAAAATAGTTCAGACCACCAT-3′<br>R-5′CTCATCACAAAAAGTGTCCAGAGAA-3′ | *<br>R-5′-TGGGACAAGAGAAAGTTGAACATAC-3′ | 0.83 | GDB:195012 |
| | D11S4181[a] | F-5′-CTGGGCAACAAGAGTAAGTCTCT-3′<br>R-5′-CAACACTAAACATCCAGCTCAAAG-3′ | *<br>R-5′-CCTTAAGAACTGAGACCAAGAACA-3′ | 0.80 | Dib *et al.* (1996) |
| | D11S4149[b] | F-5′-TGAATTATACCCCTGACCAATCTG-3′<br>R-5′-CCATATATAGAATCACACTGGCCAA-3′ | F-5′-GGCTAAAAAGGCAACAGATAACATC-3′<br>* | 0.77 | Dib *et al.* (1996) |
| | D11S1331[b] | F-5′-TTCCATGAGAGGATACTGACTTTG-3′<br>R-5′-CTTCCTTCGTCTTTCTCACTTTTAC-3′ | F-5′-GATGTTTAGATGCACAAGACACAGA-3′<br>* | 0.70 | Gyapay *et al.* (1994) |
| | D11S1997[b] | F-5′-TTCCTAAGAAAGATAAAGCACCAG-3′<br>R-5′-GGACAAAATAAAGACCAGCTTTAC-3′ | *<br>R-5′-CAATTGACAGTGGATTTTTGAC-3′ | 0.78 | GDB:365121 |
| | D11S1338[b] | F-5′-AGAGTAGGAATAAGATGGTGTCAGA-3′<br>R-5′-CCACACAGATTCACTTAAAGCAA-3′ | F-5′-GCTACTTATTTGGAGTGTGAATTTC-3′<br>* | 0.74 | Gyapay *et al.* (1994) |
| | D11S1760[b] | F-5′-ACCCTGAGTGTCTTCAAAACTCA-3′<br>R-5′-CTGCATCATGACTTGAAAAACG-3′ | F-5′-ATCTCAAGTGTTTCCCCACAAC-3′<br>* | 0.76 | Dib *et al.* (1996) |
| Cystic fibrosis | *CFTR* | D7S677[a] | F-5′-ATTAATCATTCACTATGGGATAGC-3′<br>R-5′-GGAATTACAAGTCACTCTATACAAA-3′ | F-5′-GATAGCAAACAGAGATCCCTAAG-3′<br>* | 0.63 | Gyapay *et al.* (1994) |
| | IVS17b-TA[c] | F-5′-GCTGCATTCTATAGGTTATCAA-3′<br>R-5′-CACCAATATGAGTATGAAGAGG-3′ | *<br>R-5′-AATCACAGAATTGCTACTCGAC-3′ | 0.89 | Zielenski *et al.* (1991) |

**Table III.** Continued

| Disease | Gene | STR markers | Outer primers (5′ to 3′) | Inner primers (5′ to 3′) | Heterozygosity | References |
|---|---|---|---|---|---|---|
| | | IVS8[c] | F-5′-GGCCATGTGCTTTTCAAACTAA-3′<br>R-5′-CAGGACAGGAGTACCAAGAAGT-3′ | *<br>R-5′-AAGAAGAGGCTGTCATCACCAT-3′ | 0.65 | Morral and Estivill (1992) |
| | | IVS1[c] | F-5′-CTCCAGAGAAGTAGAACCAATG-3′<br>R-5′-CTTCAGACTCAAACTGGAACAT-3′ | *<br>R-5′-GGACTTGTTAGCCATCATAATC-3′ | 0.72 | Moulin *et al.* (1997) |
| | | D7S23[b] | F-5′-TGTTGAGAGACGTGCTAGCCTG-3′<br>R-5′-TTTACCTTGAGGCAGTTTTGGC-3′ | F-5′-TGGTCTCCAGCTCTAGCACAGA-3′<br>* | 0.83 | Richards *et al.* (1991) |
| Familial Mediterranean fever | *MEVF* | D16S3070[a] | F-5′-CCCACTTTATCCCCTAACCATA-3′<br>R-5′-GTGGTTTAAGAGAGCAAGGAGG-3′ | F-5′-GAATTACTTGAACACGGGAGGT-3′<br>* | 0.76 | Dib *et al.* (1996) |
| | | D16S3082[a] | F-5′-ATCAGAGAGGACCTGCGGAAAT-3′<br>R-5′-CCTGTACCTCTGCCATGTGTCT-3′ | F-5′-CTGCGGAAATAACGGTGACACT-3′<br>* | 0.88 | Dib *et al.* (1996) |
| | | D16S3072[b] | F-5′-GAAGGCTGAGGCAGGAGAAT-3′<br>R-5′-CAGTGAAGGCAGAAAACAGA-3′ | *<br>R-5′-CAGATGTAGCATCCTGGAGG-3′ | 0.80 | Dib *et al.* (1996) |
| | | D16S3027[b] | F-5′-GATTCCGCACATACATTTCCATC-3′<br>R-5′-GCATGAGTTGCTTTGGTTTTGA-3′ | *<br>R-5′-CCTTGAACTGTCTTTCCCACTCT-3′ | 0.88 | Dib *et al.* (1996) |
| Mucopolysaccharidosis type IIIA—Sanfilippo syndrome | *SGSH* | D17S928[a] | F-5′-GAGGTTGCAGCAAGCCAAGTT-3′<br>R-5′-GGAAAGGAGTATGTTTGGGTTTATG-3′ | F-5′-CAAGTTCACGCCATTGCACT-3′<br>* | 0.76 | Gyapay *et al.* (1994) |
| | | D17S784[b] | F-5′-GATGAGTCTCCTAAATGCTGGG-3′<br>R-5′-CCAATGCCTTGTGTCTGTATAGA-3′ | F-5′-CTGGGTTTGTGGGTGATGTTAA-3′<br>* | 0.78 | Gyapay *et al.* (1994) |
| | | D17S1822[b] | F-5′-TTGCACCACAAGATCAGGTCTC-3′<br>R-5′-CCGAGCCTAGGACTCCTTTCTC-3′ | F-5′-CCAGGAAAGCAGGACTCCATAC-3′<br>* | 0.70 | Dib *et al.* (1996) |
| | | D17S1806[b] | F-5′-GACACCCTAATCCAATTCATCCA-3′<br>R-5′-TGTAACGTCCACCAGCAGAGAC-3′ | F-5′-GATGTGCTTATTTGAAACCTGCAC-3′<br>* | 0.71 | Dib *et al.* (1996) |
| Mucopolysaccharidosis type VI—Maroteaux–Lamy syndrome | *ARSB* | D5S672[a] | F-5′-GCAACAGAGTGAGACCCCATCT-3′<br>R-5′-TCCTGAGTGAAGCCAAGAACCT-3′ | F-5′-CAAGCATCCAAAATCCAGTCAA-3′<br>* | 0.63 | Gyapay *et al.* (1994) |
| | | D5S1962[b] | F-5′-GCCTGACGAATTTTATGTCCAA-3′<br>R-5′-CCCACAAAGTTAAGAAAAGCCA-3′ | *<br>R-5′-CAGATGATTGTAATGTGCCCCA-3′ | 0.80 | Dib *et al.* (1996) |
| | | D5S1977[b] | F-5′-TCATCAGACTCAGAACCCATTCC-3′<br>R-5′-AGTGAACCATGATCACCCCATT-3′ | F-5′-CCTTTCAGACAGAATCTAAGGGAAA-3′<br>* | 0.78 | Dib *et al.* (1996) |
| | | D5S424[b] | F-5′-CATGGGAGTTCATTAGGGCTACT-3′<br>R-5′-GCAGTGACACAAAGAAGTTTCGTA-3′ | *<br>R-5′-AATCCTCCATGTATTCAATGCC-3′ | 0.77 | Gyapay *et al.* (1994) |
| Spinal muscular atrophy | *SMN1* | D5S610[a] | F-5′-GCCCAGCCTAAACTGAACTTTC-3′<br>R-5′-CCTAAAGTGCTGGAATTGCAAG-3′ | *<br>R-5′-CTTTTGTTAAGCTCCTCCAGTGA-3′ | 0.80 | GDB:199084 |
| | | D5S1556[b] | F-5′-TTTAAGGATCTGCCTTCCTTCC-3′<br>R-5′-CATGTTGCTTAGGCCTCGTCTC-3′ | F-5′-GGAGCTTACATTTACTTTTCCAAGG-3′<br>* | 0.87 | DiDonato *et al.* (1994) |
| | | D5S629[b] | F-5′-AGTTTGCAGTGAGCCGAGATCT-3′<br>R-5′-GTATATCTGCCCCGGTTTGTTC-3′ | F-5′-AGATCTCGTCATTGCACTCCAG-3′<br>* | 0.83 | Gyapay *et al.* (1994) |
| α-Thalassaemia mental retardation syndrome | *ATRX* | DXS1225[a] | F-5′-AGAGAAATTGGCAACACAAAGG-3′<br>R-5′-GCCACTAACAAGAAAATCCTGG-3′ | *<br>R-5′-ATCCTGGATGGAAGGACTAAAA-3′ | 0.74 | Gyapay *et al.* (1994) |
| | | DXS441[b] | F-5′-GAAAAGTAAGACTGAGAATGAACCC-3′<br>R-5′-GTCACTTCAGTTTTCCAAGACAAAG-3′ | F-5′-ATGTGGAGGAACGTATATCTGTCTG-3′<br>* | 0.76 | Ram *et al.* (1992) |
| Duchenne muscular dystrophy | *DMD* | DXS992[a] | F-5′-GAGGCAGGAGAATTGCTTGAAC-3′<br>R-5′-ATCCACTGGGACAGAAAGGGTT-3′ | F-5′-AGTGAGCCCAGATCACGCTACT-3′<br>* | 0.86 | Gyapay *et al.* (1994) |
| | | DXS1214[c] | F-5′-TAGAACCCAAATGACAACCA-3′<br>R-5′-TCGTTTATTAATGCCAGCGTAT-3′ | F-5′-AAGATAGCAGGCAACAATAAGA-3′<br>* | 0.79 | Gyapay *et al.* (1994) |

**Table III.** Continued

| Disease | Gene | STR markers | Outer primers (5′ to 3′) | Inner primers (5′ to 3′) | Heterozygosity | References |
|---|---|---|---|---|---|---|
| ‘ | | DXS1236[c] | F-5′-GCAAGTTTCTCTTCGTCGTTTAC-3′<br>R-5′-GAGCCATATGATACGATTCGTGT-3 | *<br>R-5′-AGATACAAGTTGGGAGGCTAACA-3′ | 0.91 | Clemens *et al*. (1991) |
| | | DXS1237[c] | F-5′-GAGGCTATAATTCTTTAACTTTGGC-3′<br>R-5′-GGTGGATTTTAATATTCAGGAAGCT-3′ | *<br>R-5′-CTCTTTCCCTCTTTATTCATGTTAC-3′ | 0.87 | Clemens *et al*. (1991) |
| | | DXS1238[c] | F-5′-GTGTGTGTATCCTTTTGTCATT-3′<br>R-5′-GCACACTTTATTTTAACTCATTG-3′ | F-5′-CTGTAATGAAAGTAAAAAATGCA-3′<br>* | 0.85 | Clemens *et al*. (1991) |
| | | DXS1242[b] | F-5′-GGACTCAACTTATCTTTCAGGT-3′<br>R-5′-GACAGCTGGTTTCATAGTTACA-3′ | *<br>R-5′-TATGAGTACTTGCACACAAAGC-3′ | 0.77 | Feener *et al*. (1991) |
| Haemophilia A<br>G6PD deficiency | *F8/GDPD* | DXS1107[a] | F-5′-GATTACAGGTGTGAGCCACCAC-3′<br>R-5′-TCCTAACCAGAGCAATTAGGCA-3′ | *<br>R-5′-GATGCCCACTCTCACCTATCCT-3′ | 0.72 | Freije *et al*. (1992) |
| | | DXS1108[a] | F-5′-GGAAGGAATATGAAAGCTTAGGG-3′<br>R-5′-GTGAAAGGAATATTTTCTGGGC-3′ | F-5′-GGGAGATAGGAATGATGGAGTG-3′ | 0.75<br>* | Freije *et al*. (1992) |
| | | F8 IVS22[c] | F-5′-AGTACTGGGAATGCACAGCCTA-3′<br>R-5′-TTGTCCAGAACCCAGACATGTC-3′ | *<br>R-5′-TCCCACGTTTTGGATACTAACG-3′ | 0.78 | Lalloz *et al*. (1994) |
| | | F8 IVS13[c] | F-5′-GGTTTTTCCCTCAGCACTTTAA-3′<br>R-5′-TGAATAAGCCTAGAGAATGCCA-3′ | F-5′-TCCCATTTGCATTCAACTGTAC-3′<br>* | 0.69 | Lalloz *et al*. (1991) |
| | | DXS8087[b] | F-5′-ACTTGCTCCCCTGGGTCATG-3′<br>R-5′-TCAAAGGCCAGCAGCATCAG-3′ | *<br>R-5′-GAGCACACACTCGGAACACG-3′ | 0.70 | Dib *et al.* (1996) |
| | | DXS15[b] | F-5′-TAATGAACCTCCACGTACCCAC-3′<br>R-5′-AAGCACAGTGTGAGTAGCATGC-3′ | *<br>R-5′-CCACCCAACTTCAACAATTATCC-3′ | 0.84 | Wehnert *et al*. (1993) |
| | | DXS1073[b] | F-5′-GAGAGACATGTAAAGTGGGCAA-3′<br>R-5′-AGATTAAATGTGGAGGCCTGAG-3′ | *<br>R-5′-GCCCTCTCCGAGTTATTACAAA-3′ | 0.75 | Dib *et al.* (1996) |
| Haemophilia B | *F9* | DXS1232[a] | F-5′-CCAACAGCCTAATAATGCTCTGG-3′<br>R-5′-TTTGGGAGATGAGAGATGGGAG-3′ | F-5′-CTGGAGCCATAAGATGAATGCA-3′<br>* | 0.74 | Gyapay *et al*. (1994) |
| | | DXS984[a] | F-5′-TGAGAATTTTTCTTTCTGTCTGCCA-3′<br>R-5′-GCCCTACTCCATTCCACACTGG-3′ | F-5′-TTTAATGGCAGCGTACATGCCC-3′<br>* | 0.72 | Gyapay *et al*. (1994) |
| | | DXS1192[b] | F-5′-CAGAGTTGCCAACTGCTGGAAC-3′<br>R-5′-TTTCTTCTTCCAGTGTGGTGCA-3′ | *<br>R-5′-GTGCAGGGAAGCCAAAAGATTG-3′ | 0.84 | Gyapay *et al*. (1994) |
| Lesch–Nyhan syndrome | *HPRT* | DXS8041[a] | F-5′-AACAGAGCAAGACTCCGTCTCAA-3′<br>R-5′-TTTCCTGCTACCTGCAATTCCT-3′ | F-5′-ATAATAACCGTCCCCCAAAACAC-3′<br>* | 0.70 | Dib *et al.* (1996) |
| | | IVS3[c] | F-5′-GCCCCCCAAGTAGTTTCTTAAAG-3′<br>R-5′-CCCTGTCTATGGTCTCGATTCA-3′ | F-5′-GGGCAGTAGCTTTCAGCTTAAAC-3′<br>* | 0.85 | Edwards *et al*. (1992) |
| Wiskott–Aldrich syndrome | *WAS* | DXS6849[a] | F-5′-TCAAACCTCCACTCCAAGTTGTT-3′<br>R-5′-TGATGCTGGAGGTTATTTTGAGC-3′ | *<br>R-5′-GGGAAGCTGAAGGGTTTTTAAA-3′ | 0.80 | Boycott *et al*. (1996) |
| | | DXS573[b] | F-5′-CACTAATCACTAAAGCCTTCCCAG-3′<br>R-5′-GCTTATGTGTTAGTAGGGTGAGACA-3′ | *<br>R-5′-AGAAAGGAATAGGGAGTGGGTG-3′ | 0.72 | Roustan *et al*. (1992) |

F = forward; R = reverse.
*A heminested approach was used.
[a]Telomeric to the gene. [b]Centromeric to the gene. [c]Intragenic
GDB number refers to the Genome Data Base (http://www.gdb.org) accession number.

*et al*., 2004). For the other genetic disorders, mutation analysis was performed using the minisequencing method, as previously reported (Fiorentino *et al*., 2003). Fluorescent fragments were analysed by 20 min of capillary electrophoresis on an automatic DNA sequencer ABI Prism 3100™ (Applied Biosystems).

### *Embryo transfer*

Embryo transfer was carried out on either day 4 or day 5. The number of the embryos transferred varied according to the number of unaffected embryos available after diagnosis, the age of the patient and the morphology of the embryos. When the quality was comparable, non-carrier embryos were transferred in preference to heterozygous embryos.

### *Confirmation of PGD results*

As specified in the informed consent, following successful embryo transfer, affected or morphologically incompetent embryos were collected in individual tubes and reanalysed to confirm the PGD results. Supernumerary unaffected embryos were cryopreserved if cleavage continued.

In cases in which pregnancies were achieved, patients were advised to undergo conventional prenatal diagnosis to confirm the genetic status of the fetus.

## Results

Over a 6 year period, 174 couples underwent 250 PGD cycles for 23 different indications (Table I). The mean maternal age was 31.8 ± 4.3 years, ranging from 21 to 44. Of the 174 couples included in the treatment, 122 underwent only one PGD cycle, 33 had two, 16 had three, two had four and one couple had six attempts. In total, 248 were stimulated cycles and two were frozen embryo replacement cycles.

The single gene defects investigated included autosomal dominant (15 cycles; eight couples), autosomal recessive (148 cycles; 107 couples) and X-linked disorders (19 cycles; 10 couples). A total of 68 cycles, for 49 couples, were also performed for SGDs combined with HLA matching.

Myotonic dystrophy was the most frequent autosomal dominant disease tested ($n = 6$; 40.0%). β-Thalassaemia was the most frequent autosomal recessive disorder ($n = 105$; 70.9%), followed by cystic fibrosis ($n = 25$; 16.9%), spinal muscular atrophy ($n = 6$; 4.1%) and sickle cell anaemia ($n = 5$; 3.4%). Duchenne muscular dystrophy ($n = 5$; 26.3%) and haemophilia A ($n = 5$; 26.3%) were the most frequent X-linked disorders investigated.

A total of 1961 embryos (mean number 7.8 ± 3.7 per cycle) were biopsied and two cells were removed in 1448 (73.8%) for analysis (Table IV). PCR amplification was successful in 3149 out of 3409 blastomeres (92.4%). Amplification failed for all the markers/loci tested in 260 blastomeres. The efficiency of amplification for the individual genetic conditions ranged from 80.0 to 100%, with an overall amplification rate of 92.4%. The ADO rates varied between the different loci/markers investigated, ranging from 0 to 12.9%, with an average ADO rate of 7.5% (data not shown).

Overall, 1849 (94.3%) embryos were successfully genotyped in the 250 clinical cycles. In 1231 embryos, the final diagnosis was obtained from the concordant results of two cells, whereas for 618 embryos the diagnosis was based on one blastomere only. No diagnosis was obtained for 112 embryos.

Embryos suitable for transfer where identified in 211 of the 250 cycles (84.4%). In nine PGD cycles, embryo transfer was cancelled because unaffected embryos were not found. Similarly, in 30 cycles of PGD for SGDs combined with HLA matching, transfer was cancelled because no unaffected HLA-matched embryos were found.

Following transfer of 427 embryos (mean 2.0 ± 0.8, range 1–3), 71 women had positive HCG levels (33.6% pregnancy rate per embryo transfer), 56 of which were confirmed with fetal sacs and heart beat (26.5% implantation rate per embryo transferred). Six pregnancies spontaneously miscarried within the first trimester and two ectopic pregnancies were terminated. Prenatal diagnosis was performed in the remaining pregnancies using either chorion villus sampling or amniocentesis. All pregnancies were confirmed to be unaffected; nine pregnancies are still ongoing, whereas the others went to term without complications, resulting in the birth of 35 healthy babies.

Two hundred and fifty-two untransferred embryos were reanalysed to confirm the diagnosis and this was successful in 231 (91.7%) embryos. The genotypes were concordant for all embryos (data not shown).

The largest group of PGD cycles (105 for 75 couples) was performed for β-thalassaemia. Of 871 embryos analysed, 829 (95.2%) yielded a successful diagnosis. There were 37 genotype combinations, involving the diagnosis of 20 different β-thalassaemia mutations. Genotype analysis involved detection of a single mutation in 48 cycles, and two mutations in 57 cycles. Two hundred and twelve unaffected embryos were transferred in 105 cycles, resulting in 35 pregnancies (33.3% pregnancy rate per embryo transfer) and the birth of 15 healthy children.

Cystic fibrosis was the second most common indication for PGD in our experience. A total of 25 cycles were carried out for this disease, involving analysis of 147 embryos, of which 145 (98.6%) were successfully diagnosed. Eleven genotype combinations were tested, involving the diagnosis of 11 different cystic fibrosis mutations, the most common of which was DeltaF 508. Genotype analysis involved detection of one mutation in nine cycles, and two mutations in 16 cycles. Sixty unaffected embryos were transferred back to the patient in 24 cycles, resulting in seven clinical pregnancies (29.2%) and the birth of six unaffected children.

PGD for SGDs was also used in combination with HLA typing in 68 cycles performed for 49 couples. A total of 618 embryos were tested involving analysis of 1054 blastomeres, in 974 (92.4%) of which a successful amplification was obtained. Overall, 58 unaffected HLA-matched embryos were transferred to the patients in 38 of the 64 cycles performed (mean 1.5 ± 0.8 ). Twelve pregnancies were achieved (31.6% per transfer), four of which were only biochemical, one resulted in early abortion, and one ectopic pregnancy was terminated. From one twin and three singleton pregnancies, five healthy children were born, which were confirmed to be HLA identical to their affected sibling by HLA testing blood samples.

## Discussion

In the past two decades, knowledge of the molecular basis of genetic diseases, together with the development of new DNA technologies, has enabled early and exact diagnosis of an increasing number of congenital disorders and the identification

**Table IV.** Overall PGD data and clinical outcome.

| Disease | No. of embryos analysed | | | No. of blastomeres analysed | Cells with total PCR failure | Cells with a positive PCR | No. of embryos diagnosed | | | No. of transfers/ no. of embryos | Pregnancies | | | | | Children born |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 Cell biopsy | 2 Cells biopsy | Total | | | | 1 Cell | 2 Cells | Total | | HCG+ | Biochemical | Clinical | Miscarried | Ongoing | |
| Holt–Oram syndrome | 0 | 6 | 6 | 12 | 1 | 11 (91.7) | 1 | 5 | 6 (100.0) | 1/2 | 1 | 0 | 1 | 0 | 0 | 1 |
| Myotonic dystrophy | 10 | 20 | 30 | 50 | 5 | 45 (90.0) | 12 | 16 | 28 (93.3) | 3/6 | 1 | 1 | 0 | 0 | 0 | 0 |
| Primary dystonia | 0 | 17 | 17 | 34 | 3 | 31 (91.2) | 3 | 14 | 17 (100.0) | 2/6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neurofibromatosis type 1 | 1 | 5 | 6 | 11 | 1 | 10 (90.9) | 0 | 5 | 5 (83.3) | 1/1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Retinoblastoma | 0 | 20 | 20 | 40 | 3 | 37 (92.5) | 3 | 17 | 20 (100.0) | 3/7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Spastic paraplegia 3 | 2 | 10 | 12 | 22 | 1 | 21 (95.5) | 1 | 10 | 11 (91.7) | 1/1 | 1 | 0 | 1 | 0 | 1 | 0 |
| β-Thalassaemia | 256 | 615 | 871 | 1486 | 108 | 1378 (92.7) | 298 | 531 | 829 (95.2) | 105/212 | 35 | 6 | 29 | 4 | 5 | 15 |
| Cystic fibrosis | 30 | 117 | 147 | 264 | 18 | 246 (93.2) | 45 | 100 | 145 (98.6) | 24/60 | 7 | 0 | 7 | 0 | 1 | 6 |
| Congenital adrenal hyperplasia | 5 | 0 | 5 | 5 | 1 | 4 (80.0) | 4 | 0 | 4 (80.0) | 1/1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Familial Mediterranean fever | 0 | 4 | 4 | 8 | 0 | 8 (100.0) | 0 | 4 | 4 (100.0) | 1/3 | 1 | 1 | 0 | 0 | 0 | 0 |
| Sanfilippo syndrome | 4 | 22 | 26 | 48 | 5 | 43 (89.6) | 3 | 20 | 23 (88.5) | 3/9 | 2 | 2 | 0 | 0 | 0 | 0 |
| Maroteaux–Lamy syndrome | 0 | 6 | 6 | 12 | 2 | 10 (83.3) | 0 | 5 | 5 (83.3) | 1/2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sickle cell anaemia | 7 | 35 | 42 | 77 | 5 | 72 (93.5) | 4 | 34 | 38 (90.5) | 5/12 | 2 | 1 | 1 | 0 | 0 | 1 |
| SMA | 6 | 33 | 39 | 72 | 5 | 67 (93.1) | 9 | 29 | 38 (97.4) | 5/13 | 2 | 0 | 2 | 0 | 0 | 2 |
| ATRX syndrome | 0 | 2 | 2 | 4 | 0 | 4 (100.0) | 0 | 2 | 2 (100.0) | 0/0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Charcot–Marie–Tooth type× | 4 | 6 | 10 | 16 | 2 | 14 (87.5) | 2 | 6 | 8 (80.0) | 2/4 | 2 | 0 | 2 | 1 | 0 | 1 |
| Duchenne muscular dystrophy | 0 | 24 | 24 | 48 | 6 | 42 (87.5) | 6 | 18 | 24 (100.0) | 4/8 | 1 | 0 | 1 | 0 | 0 | 1 |
| G6PD deficiency | 0 | 15 | 15 | 30 | 2 | 28 (93.3) | 2 | 12 | 14 (93.3) | 1/3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Haemophilia A | 0 | 21 | 21 | 42 | 4 | 38 (90.5) | 4 | 17 | 21 (100.0) | 5/11 | 2 | 0 | 2 | 1 | 0 | 1 |
| Haemophilia B | 0 | 20 | 20 | 40 | 4 | 36 (90.0) | 4 | 16 | 20 (100.0) | 3/5 | 1 | 0 | 1 | 0 | 0 | 1 |
| Lesch–Nyhan syndrome | 0 | 7 | 7 | 14 | 2 | 12 (85.7) | 2 | 5 | 7 (100.0) | 1/2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Wiskott–Aldrich syndrome | 6 | 7 | 13 | 20 | 2 | 18 (90.0) | 5 | 4 | 9 (69.2) | 1/1 | 1 | 0 | 1 | 0 | 0 | 1 |
| SGD + HLA matching | 182 | 436 | 618 | 1054 | 80 | 974 (92.4) | 210 | 361 | 571 (92.4) | 38/58 | 12 | 4 | 8 | 2 | 2 | 5 |
| Total | 513 | 1448 | 1961 | 3409 | 260 | 3149 (92.4) | 618 | 1231 | 1849 (94.3) | 211/427 | 71 | 15 | 56 | 8 | 9 | 35 |

Values in parentheses are percentages.

of patients at increased risk of having affected offspring. In this context, PGD has become an established approach for early diagnosis of genetic disorders, providing the opportunity, for couples who have a known genetically transmittable disease, to start a pregnancy with the knowledge that their child will be unaffected by the specific disorder tested.

Although it is more than a decade since the first PGD for SGD was performed (Handyside *et al*., 1992), the complexity of the approach has so far limited its clinical application. PGD is a multidisciplinary procedure that requires combined expertise in reproductive medicine and molecular genetics (Geraedts *et al*., 2001). Thus, even if the numbers of centres performing PGD is increasing steadily, only a few centres worldwide are offering PGD for SGDs as a clinical service.

From 1999 we have been offering PGD for SGDs as a diagnostic service, and our experience over 6 years highlights many positive aspects and some potential limitations.

The first practical obstacle we encountered was to address the growing number of requests for PGD for different indications, some of which were rare diseases. In 15 cases performed for 14 different genetic conditions, the disease-causing mutations, carried by the patients, were also unknown. As a consequence, design and optimization of protocols for mutation screening of causative genes were necessary to determine the genetic status of the couples. Furthermore, for most of these genes, mutation detection was complicated by the presence of a large number of exons.

Another complication, associated with performing PGD for different genetic diseases, was represented by the time needed to optimize the specific protocols for each case. To validate a diagnostic single cell PCR protocol, before clinical application, extensive pre-clinical trials on single lymphocytes are necessary, in order to evaluate single cell amplification efficiencies and ADO rates for all the primers to be used in the procedure. Consequently, some of the couples included in the PGD programme had to wait several months before beginning the cycle. We partially addressed this limitation by using a PGD strategy characterized by a nested-PCR protocol producing amplicons analysable by the minisequencing technique (Fiorentino *et al*., 2003), avoiding the use of mutation-based strategies that require optimization of specific PCR protocols for each mutation to be analysed. The minisequencing approach has proven to be extremely flexible and appropriate for the analysis of a wide spectrum of mutations and compound genotypes (Figure 1). Its usefulness is evident particularly for the diagnosis of genetic diseases characterized by a large number of different mutations concentrated in a few exons (i.e. β-thalassaemia) or with a cluster of frequent mutations on a short distance (i.e. cystic fibrosis). Although each PGD case can involve the presence of different mutations and genotype combinations, the use of a panel of PCR primers tested pre-clinically enables a substantial shortening of the preliminary phase for each couple.

Minisequencing was applied in 234 PGD cycles for 17 different indications, involving the analysis of 49 different mutations and 65 genotype combinations (Table I). The strategy has proven to be extremely efficient, as it provides a high rate of interpretable results in blastomeres in which amplification is successful (98.7%). The accuracy of the genotyping method was established previously during the validation of the procedure

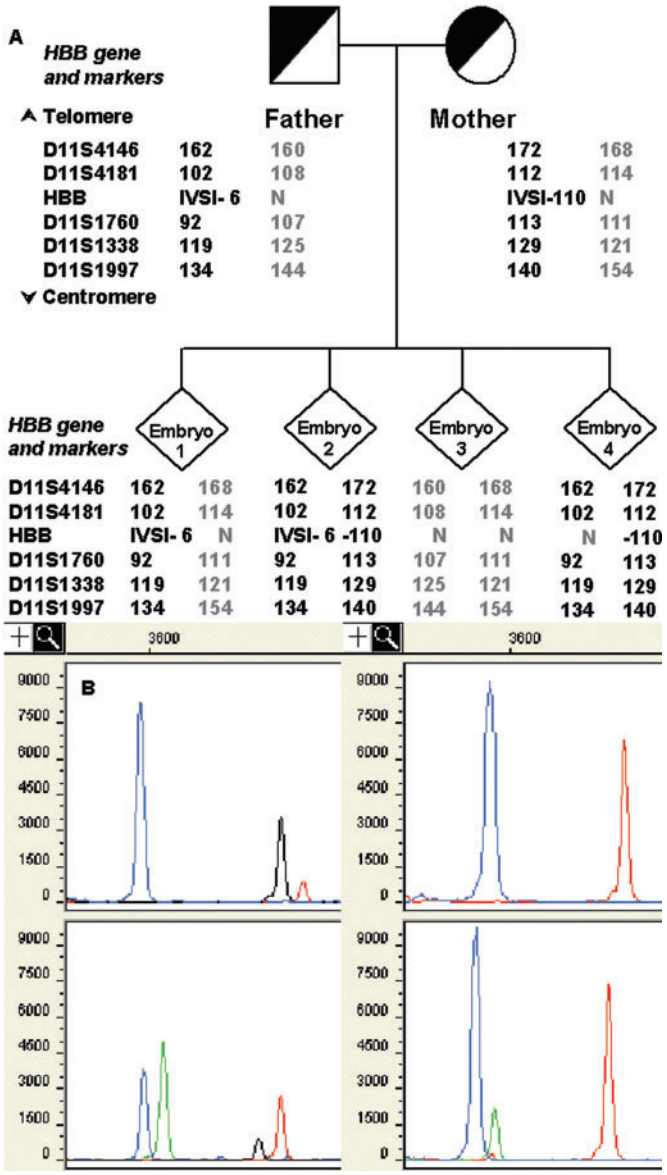


**Figure 1.** PGD for β-thalassaemia performed by using the minisequencing technique. (**A)** Pedigree of a couple carrying β-thalassaemia mutations and examples of different results of the *HBB* gene mutation analysis. Informative STR markers are ordered from telomere (top) to centromere (bottom). The numbers in STR markers represent the size of PCR products in bp. STR alleles linked to the paternal and maternal mutations are represented in bold. (**B)** Examples of minisequencing results obtained for the above case. The mutations of interest are IVSI-110 G-A and IVSI-6 T/C, analysed in a multiplex reaction format. The *y*-axis represents the relative fluorescence units (RFU) of the detected fragments; the *x*-axis represents time and is displayed by data points. Colour is assigned to individual ddNTPs as follows: green/A, black/C, blue/G, red/T. Mutation IVSI-110 G-A is shown on the left of the minisequencing window; the blue peak represents the normal allele (wild-type base G), the green peak (mutant base A) the mutated allele. Mutation IVSI-6 T/C is shown on the right; the red peak is the normal allele (wild-type base T) and the black peak (mutant base C) is the mutated allele. Embryo 1 (upper panel/left) is a carrier for the IVSI-6 T/C mutation; embryo 3 (upper panel/right) is normal; embryo 2 (lower panel/left) is compound heterozygote for the two mutations. Embryo 4 (lower panel/right) is also affected, although the minisequencing result shows a heterozygosity for mutation IVSI-110 G-A. In fact, linked STR markers highlight an ADO of the affected allele (black peak, mutant base C).

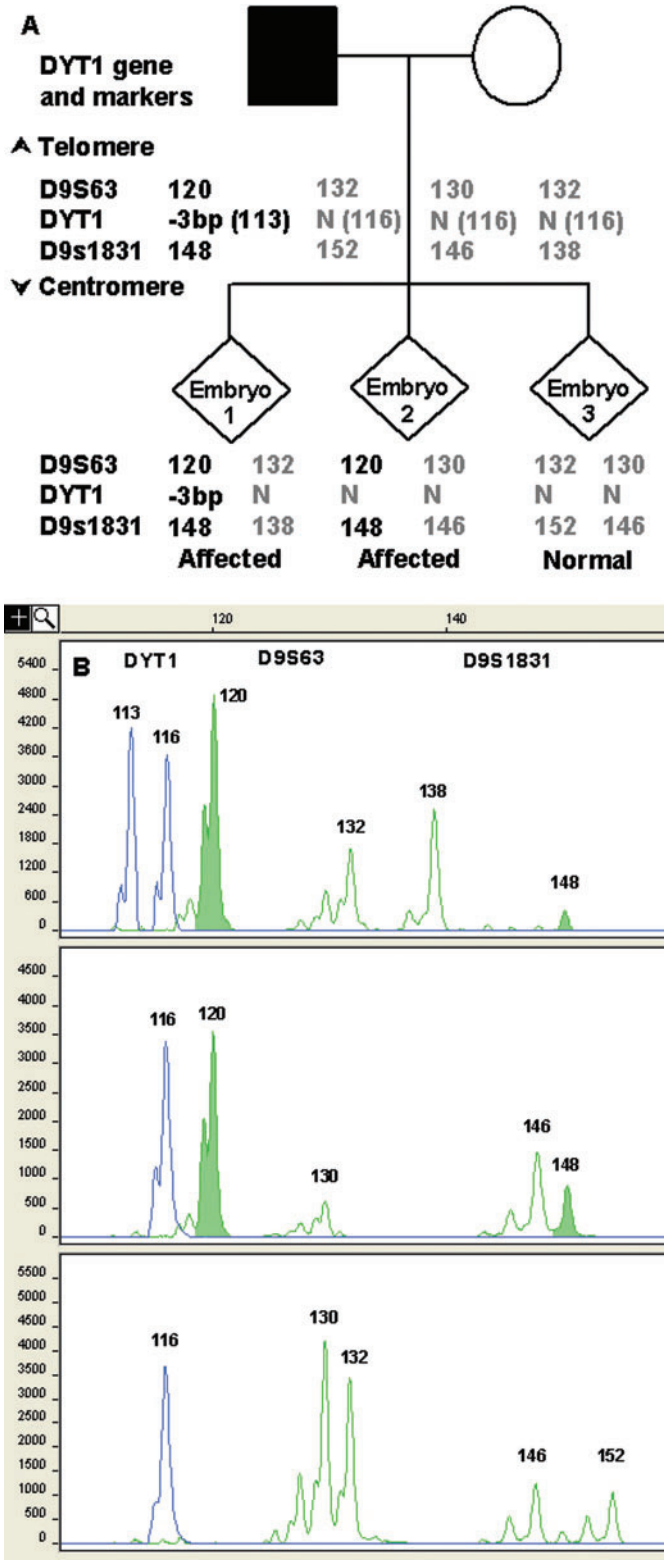


**Figure 2.** PGD for primary dystonia. (**A**) Haplotyping results of STR markers linked to the *DYT1* gene are numbered according to the size in bp. Numbers in bold represent alleles linked to the mutation. Examples of different results of *DYT1* mutation analysis from biopsied blastomeres are shown in the lower panel. (**B**) Capillary electrophoresis of fluorescent PCR products obtained after multiplex amplification of the *DYT1* region involved by mutation and a set of linked polymorphic markers. The *x*-axis shows the length of PCR products in bp and the *y*-axis shows the fluorescence intensity in relative fluorescence units (RFU). Mutation analysis was performed by determining the size of the PCR fragments, detecting the affected alleles (113 bp peak) because of the 3 bp difference from the normal allele (116 bp peak). On top of the electropherogram the marker name is located above the corresponding alleles (peaks). Numbers next to each peak represent the size of the allele (in bp). Alleles linked to the mutation are highlighted in green. The upper lane shows the results of an affected embryo (embryo 1). An affected embryo (embryo 2) in which the mutated allele (113 bp peak) of the *DYT1* gene has dropped out is shown in the middle lane. ADO occurrence has been evidenced from the presence of the affected haplotype (highlighted peaks). The lower lane shows the profile of a healthy embryo (embryo 3).

(Fiorentino *et al.*, 2003) and has also been monitored by later reanalysis of non-transferred embryos and by prenatal diagnosis of the ensuing pregnancies, confirming in all cases the clinical diagnosis obtained during PGD cycles.

To increase the level of diagnostic accuracy, hence reducing the chances of misdiagnosis, wherever possible two cells from each embryo were biopsied. Diagnosis was assigned only when both cells yielded concordant results or, as in cases where only one cell result was available (one cell biopsy or total amplification failure in one cell), when haplotype profiles, obtained from linked STR markers, and mutation analysis profiles were concordant. Despite the biopsy of two cells in most of the embryos investigated, pregnancy and implantation rates were apparently not significantly affected. In fact, pregnancy was initiated in 33.6% of the cycles, with embryo transfer and the average clinical pregnancy rate was 26.5%. These results are comparable with those reported for single gene disorders by other groups using a one-cell biopsy policy (Pickering *et al.*, 2003; Traeger-Synodinos *et al.*, 2003) and with the ESHRE PGD Consortium data (ESHRE PGD Consortium Steering Committee, 2002; Sermon *et al.*, 2005). On the other hand, our experience with the biopsy and analysis of two blastomeres demonstrates that this is an effective strategy especially when amplification failure of one of the two cells of the embryo occurs, increasing the number of successfully diagnosed embryos available for transfer.

Including a panel of polymorphic STR markers in the diagnostic assay, closely linked to the gene regions containing the disease-causing mutations, has also increased the robustness of the diagnostic procedure. In fact, determination of the specific STR haplotype associated with the mutation acts both as a diagnostic tool for indirect mutation analysis, providing an additional confirmation of the results obtained with the direct genotyping procedure, and as a control of misdiagnosis due to undetected ADO (Rechitsky *et al.*, 1999) (Figures 1 and 2). The multiplex STR marker system also provides an additional control for contamination with exogenous DNA, as other alleles, differing in size from those of the parents, would be detected (Piyamongkol *et al.*, 2001). The experience of this series of PGD cycles strongly suggests that PGD protocols for SGDs are not appropriate for clinical practice without including a set of linked STR markers; consequently, this strategy is currently followed for all our PCR protocols.

For myotonic dystrophy, characterized by the presence of a dynamic mutation involving the expansion of a trinucleotide repeat sequence, a multiplex fluorescent PCR protocol was used. Since the

large expanded alleles are refractive to PCR amplification, a general approach, allowing detection of only healthy alleles, was applied. The PGD strategy involved amplification of the DNA fragment around the repeated region and STR markers linked to this region for ADO detection (Sermon *et al*., 1998; Dean *et al*., 2001).

A multiplex fluorescent PCR protocol, including simultaneous testing of the *DYT1* gene mutation and a set of linked polymorphic markers, was also used for early-onset primary dystonia, an autosomal dominant disorder caused, in most of the cases, by a 3 bp deletion (codon 302) in the *DYT1* gene. Mutation analysis was performed simply by sizing PCR fragments, detecting the affected alleles because of the 3 bp difference from the normal allele (Figure 2). Because this mutation is responsible for >70% of primary dystonia cases, the PGD strategy used can be applied for most patients without the need for extensive pre-clinical preparatory work.

The single cell PCR protocol used for Duchenne muscular dystrophy, a disease characterized by large deletions of one or more exon(s) of the dystrophin gene in ~60% of Duchenne muscular dystrophy patients, involved multiplex amplification of one exon located in the deleted region, one non-deleted exon as an internal PCR control and a panel of informative polymorphic intragenic STR markers for ADO detection and discrimination of carrier embryos. Fluorescent PCR was preferred over conventional PCR (Ray *et al*., 2001; Girardet *et al*., 2003) because the sensitivity is several orders of magnitude higher, increasing accuracy and reliability (Findlay *et al*., 1995, 1998). Furthermore, fluorescence-based protocols are highly amenable to multiplexing, which has great potential when applied to simultaneous detection of mutation sites and linked STR markers (Figure 3).

PGD has also been used in our Centre for several indications beyond its original purpose, although their application is still controversial because they raise ethical issues. In 68 cycles, PGD was used not only to test embryos for a monogenic disorder, but also to select embryos for a non-disease trait, such as specific HLA genotypes, related to immune compatibility with an existing affected child in need of a haematopoietic stem cell transplant. A preimplantation HLA-matching assay, involving haplotyping of the HLA region by analysing different polymorphic STR markers located along the HLA complex, was optimized and clinically applied in 49 couples, with the purpose of selecting embryos HLA matched with the existing affected offspring. Using this approach, the HLA region was indirectly typed by segregation analysis of the STR alleles and comparison with the affected sibling (Fiorentino *et al*. 2004, 2005). The assay is highly accurate in the selection of HLA-matched embryos for transfer. Overall, eight clinical pregnancies have been established, resulting in the birth of five healthy HLA-matched children, representing one of the most extensive series of clinical cases and complementing other similar experiences on preimplantation HLA matching (Rechitsky *et al*., 2004; Van de Velde *et al*., 2004).

In five cycles, PGD was performed for detection of inherited cancer predisposition. At-risk couples, who requested PGD for retinoblastoma and NF1, wished to avoid the birth of children with the inherited predisposition to malignancy, without having to face the option of possible pregnancy termination. In the NF1 patient, the predisposing germline mutation was unknown. Due to the difficulty associated with mutation screening of the

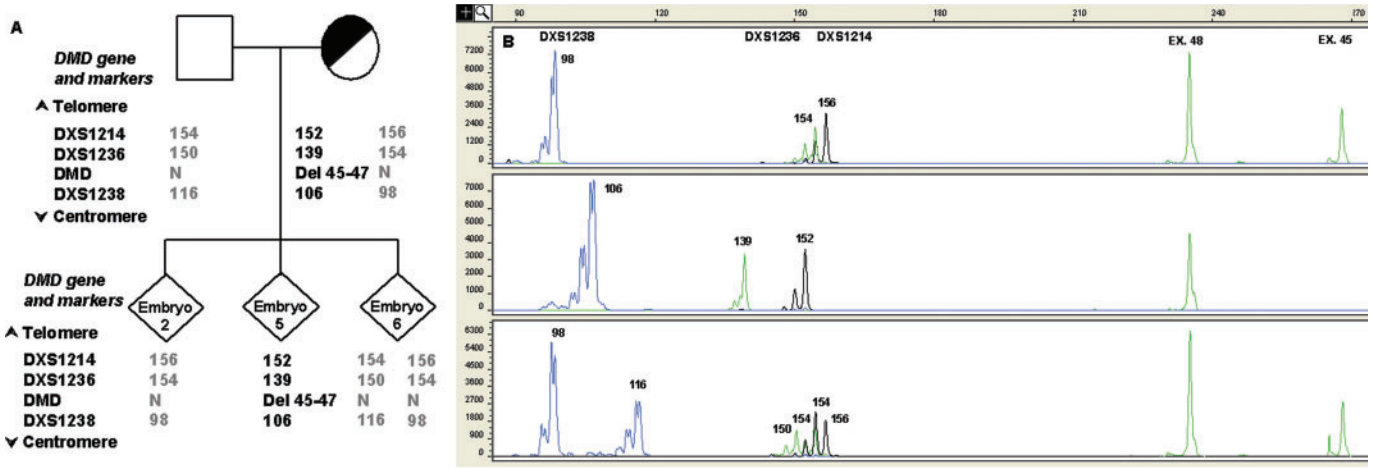


**Figure 3.** PGD for Duchenne muscular dystrophy (DMD). (**A**) Informative STR markers are ordered from telomere (top) to centromere (bottom). The numbers in STR markers represent the size of PCR products in bp. Numbers in bold are the alleles linked to the mutation. Examples of different results of DMD mutation analysis are shown in the lower panel. (**B**) Capillary electrophoresis of fluorescent PCR products obtained after multiplex amplification of the DMD region and a set of linked polymorphic markers. The *x*-axis shows the length of PCR products in bp and the *y*-axis shows the fluorescence intensity in relative fluorescence units (RFU). The single cell PCR protocol involved a multiplex amplification of one exon located in the deleted region (exon 45), one non-deleted exon (exon 48) used as internal PCR control and a panel of informative polymorphic intra-gene STR markers for ADO detection and discrimination of carrier embryos. The presence of the mutation (Del45–47) is highlighted by the absence of the peak corresponding to exon 45. The upper lane shows the result obtained from a normal male embryo (embryo 2), presenting the corresponding peak for exon 45 and carrying the normal STRs haplotype. An affected embryo (embryo 5), in which the signal from exon 45 is missing, also presenting the affected STRs haplotype, is shown in the middle lane. The lower lane shows the profile of a normal female embryo (embryo 6), presenting the corresponding peak for exon 45 and the healthy paternal and maternal haplotypes. Numbers next to each peak represent the size of the allele (in bp).

NF1 gene because of the presence of a large number of exons, linkage analysis was performed to determine the presence of the haplotype associated with mutation, thereby avoiding the need for direct mutation detection. After obtaining a family pedigree by testing family members, four polymorphic STR markers (Table II) located within the NF1 gene were used to determine which alleles were inherited along with the undetected mutation.

Linkage analysis was also the strategy used in the PGD cycle for CAH, performed in a consanguineous couple with an affected child, even though the disease-causing mutation is known. In this case, during the pre-clinical work-up, following mutation analysis of the *CYP21A2* gene, both partners were found to be homozygotes for the nucleotide 656 A-C→G mutation, without showing any clinical signs of the disease. Non-amplification of the normal allele at nucleotide 656 is a known phenomenon described in asymptomatic carriers (Day *et al*., 1996; Van de Velde *et al*., 1999). These putative nucleotide 656 G/G individuals are incorrectly typed due to dropout of the normal allele during PCR amplification. In order to overcome the ambiguity at nucleotide 656, instead of direct detection of the mutation, a PGD strategy involving linkage analysis through multiplex fluorescent PCR of six STR markers flanking the *CYP21A2* gene was developed (Figure 4). Because of the consanguinity of the couple, the affected child was showing a homozygous microsatellites profile (Figure 4). In order to exclude a multiple ADO occurrence leading to misdiagnosis of a healthy heterozygous embryo, which would appear affected and would not be transferred, a large number of STR markers was used. Although no pregnancy was achieved for this case, later reanalysis of untransferred embryos confirmed the results, indicating that this method could represent a reliable and flexible approach applicable to PGD of a wide spectrum of different genotype combinations causing 21-hydroxylase deficiency.

The described PCR protocols undertaken for this series of genetic conditions were efficient and accurate at single cell level. Results from both clinical cases and follow-up confirmation provided a high overall amplification rate (92.4%), with ADO rates ranging from 0 to 12.9%, depending on the PCR assay. These results are within the expected range for single cell PCR (Thornhill *et al*., 2005) and allowed an accurate genotyping of 94.3% of the embryos analysed.

Working as a separate PGD centre, in collaboration with different IVF centres, the embryo biopsy procedure was performed far from the laboratory where the diagnostic analysis was carried out. As a consequence, in most cases, blastomeres were transported over a long distance in lysis buffer in PCR

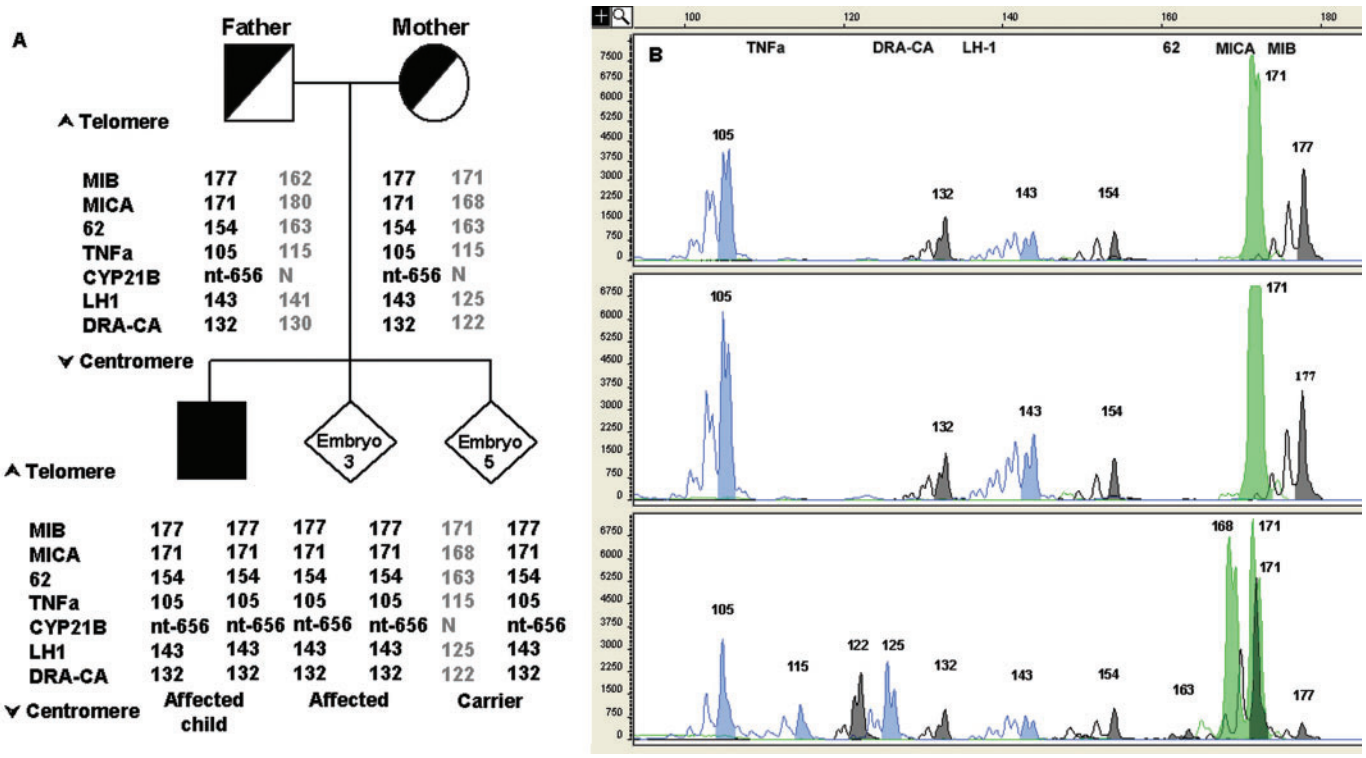


**Figure 4.** PGD for congenital adrenal hyperplasia (CAH) performed by linkage analysis. (**A**) Pedigree of a couple carrying CAH mutations and examples of different results of the *CYP21A2* gene mutation analysis. Specific haplotypes were determined by genomic DNA analysis of STR markers flanking the *CYP21A2* gene from father, mother (upper panel) and affected child (lower panel, left side, black square). Informative STR markers are ordered from telomere (top) to centromere (bottom). The numbers in STR markers represent the size of PCR products in bp. STR alleles linked to the paternal and maternal mutations are represented in bold. (**B**) Capillary electrophoresis of fluorescent PCR products after multiplex analysis of six STR markers flanking the *CYP21A2* gene. On top of the electropherogram the marker name is located above the corresponding alleles (peaks). Numbers next to each peak represent the size of the allele (in bp). The upper lane shows the STRs profile of the affected child. An affected embryo (embryo 3) is shown in the middle lane. The lower lane shows the profile of a carrier embryo (embryo 5), presenting both the affected and the normal haplotype.

tubes, refrigerated at 4°C. However, as demonstrated by the high overall amplification rate obtained, transportation does not seem to affect amplification efficiency and final diagnosis, determining only a delay of ~6 h in starting the PGD procedure, although we cannot exclude that the amplification failure observed with some of the blastomeres could be a consequence of cell loss, for example during transportation.

In conclusion, the clinical outcome of these cycles provides a further demonstration that PGD is an effective clinical tool for assisted reproduction and genetic screening, confirming the experience of other groups (Vandervorst *et al*., 2000; Harper *et al*., 2002; Pickering *et al*., 2003; Traeger-Synodinos *et al*., 2003; Verlinsky *et al*., 2004; de Boer *et al.*, 2004). From the patients' perspective, PGD is an important alternative to standard prenatal diagnosis. Low pregnancy and birth rates, and the high cost of the procedure, however, make it unlikely that PGD will supersede completely the more conventional methods of prenatal testing. PGD remains a complex combination of different technologies, that involves reproductive medicine as well as clinical and molecular genetics and requires the close collaboration of a team of specialists. Rapid advances in molecular genetics are likely to stimulate further use of PGD and to encourage a substantial increase in the range of genetic conditions for which PGD is offered. The accuracy of procedures will be improved and its clinical application will be simplified. In the future, PGD will play an increasing role as a specialized clinical procedure, becoming a useful option for many more couples with a high risk of transmitting a genetic disease, to prevent the birth of children affected by monogenic defects.

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## References

Boycott KM, Halley GR, Schlessinger D and Bech-Hansen NT (1996) A 2-megabase physical contig incorporating 43 DNA markers on the human X chromosome at p11.23–p11.22 from ZNF21 to DXS255. Genomics 33,488–497.

Braude P, Pickering S, Flinter F and Ogilvie CM (2002) Preimplantation genetic diagnosis. Nat Rev Genet 3,941–953.

Clemens PR, Fenwick RG, Chamberlain JS, Gibbs RA, de Andrade M, Chakraborty R and Caskey CT (1991) Carrier detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. Am J Hum Genet 49,951–960.

Day DJ, Speiser PW, Schulze E, Bettendorf M, Fitness J, Barany F and White PC (1996) Identification of non-amplifying CYP21 genes when using PCR-based diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia (CAH) affected pedigrees. Hum Mol Genet 5,2039–2048.

Dean NL, Tan SL and Ao A (2001) The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. Mol Hum Reprod 7,895–901.

de Boer KA, Catt JW, Jansen RP, Leigh D and McArthur S (2004) Moving to blastocyst biopsy for preimplantation genetic diagnosis and single embryo transfer at Sydney IVF. Fertil Steril 82,295–298.

Deka R, Jin L, Shriver MD, Yu LM, DeCroo S, Hundrieser J, Bunker CH, Ferrell RE and Chakraborty R (1995) Population genetics of dinucleotide (dC–dA)n·(dG–dT)n polymorphisms in world populations. Am J Hum Genet 56,461–474.

Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E et al. (1996) A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature 380,152–154.

DiDonato CJ, Morgan K, Carpten JD, Fuerst P, Ingraham SE, Prescott G, McPherson JD, Wirth B, Zerres K, Hurko O et al. (1994) Association between Ag1-CA alleles and severity of autosomal recessive proximal spinal muscular atrophy. Am J Hum Genet 55,1218–1229.

Edwards A, Hammond HA, Jin L, Caskey CT and Chakraborty R (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12,241–253.

ESHRE PGD Consortium Steering Committee (2002) ESHRE Preimplantation Genetic Diagnosis Consortium: data collection III (May 2001). Hum Reprod 17,233–246.

Feener CA, Boyce FM and Kunkel LM (1991) Rapid detection of CA polymorphisms in cloned DNA: application to the 5′ region of the dystrophin gene. Am J Hum Genet 48,621–627.

Findlay I, Ray P, Quirke P, Rutherford A and Lilford R (1995) Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation diagnosis of sex and cystic fibrosis. Hum Reprod 10,1609–1618.

Findlay I, Matthews P and Quirk P (1998) Multiple genetic diagnosis from single cells using multiplex PCR: reliability and allele drop-out. Prenat Diagn 18,1413–1421.

Fiorentino F, Magli MC, Podini D, Ferraretti AP, Nuccitelli A, Vitale N, Baldi M and Gianaroli L (2003) The minisequencing method: an alternative strategy for preimplantation genetic diagnosis of single gene disorders. Mol Hum Reprod 9,399–410.

Fiorentino F, Biricik A, Karadayi H, Berkil H, Karlikaya G, Sertyel S, Podini D, Baldi M, Magli MC, Gianaroli L et al. (2004) Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching. Mol Hum Reprod 10,445–460.

Fiorentino F, Kahraman S, Karadayi H, Biricik A, Sertyel S, Karlikaya G, Saglam Y, Nuccitelli A and Baldi M (2005) Short tandem repeats haplotyping of the HLA region in preimplatation HLA matching. Eur J Hum Genet 13,953–958.

Freije D, Helms C, Watson MS and Donis-Keller H (1992) Identification of a second pseudoautosomal region near the Xq and Yq telomeres. Science 258,1784–1787.

Geraedts JP, Harper J, Braude P, Sermon K, Veiga A, Gianaroli L, Agan N, Munne S, Gitlin S, Blenow E et al. (2001) Preimplantation genetic diagnosis (PGD), a collaborative activity of clinical genetic departments and IVF centres. Prenat Diagn 21,1086–1092.

Girardet A, Hamamah S, Dechaud H, Anahory T, Coubes C, Hedon B, Demaille J and Claustres M (2003) Specific detection of deleted and non-deleted dystrophin exons together with gender assignment in preimplantation genetic diagnosis of Duchenne muscular dystrophy. Mol Hum Reprod 9,421–427.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, Bernardi G, Lathrop M and Weissenbach J (1994) The 1993–94 Genethon human genetic linkage map. Nat Genet 7,246–339.

Handyside AH, Kontogianni EH, Hardy K and Winston RM (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 344,769–770.

Handyside AH, Lesko JG, Tarin JJ, Winston RM and Hughes MR (1992) Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. N Engl J Med 327,905–909.

Harper JC, Wells D, Piyamongkol W, Abou-Sleiman P, Apessos A, Ioulianos A, Davis M, Doshi A, Serhal P, Ranieri M et al. (2002) Preimplantation genetic diagnosis for single gene disorders: experience with five single gene disorders. Prenat Diagn 22,525–533.

Henske EP, Ozelius L, Gusella JF, Haines JL and Kwiatkowski DJ (1993) A high-resolution linkage map of human 9q34.1. Genomics 17,587–591.

Iacobelli M, Greco E, Rienzi L, Ubaldi F, Podini D, Nuccitelli A, Tesarik J, Baldi M and Fiorentino F (2003) Birth of a healthy female after preimplantation genetic diagnosis for Charcot–Marie–Tooth type X. Reprod Biomed Online 7,558–562

International Working Group on Preimplantation Genetics (IWGPG) (2001) Preimplantation genetic diagnosis—experience of three thousand clinical cycles. Report of the 11th Annual Meeting International Working Group on Preimplantation Genetics, in conjunction with 10th International Congress of Human Genetics, Vienna, May 15, 2001. Reprod Biomed Online 3,49–53.

Jansen G, de Jong PJ, Amemiya C, Aslanidis C, Shaw DJ, Harley HG, Brook JD, Fenwick R, Korneluk RG, Tsilfidis C et al. (1992) Physical and genetic characterization of the distal segment of the myotonic dystrophy area on 19q. Genomics 13,509–517.

Jansen G, Coerwinkel-Driessen M, Nillesen W, Brunner H and Wieringa B (1993) Dinucleotide repeat polymorphism at locus D19S207, close to the myotonic dystrophy (DM) gene. Hum Mol Genet 2,333.

Lalloz MR, McVey JH, Pattinson JK and Tuddenham EG (1991) Haemophilia A diagnosis by analysis of a hypervariable dinucleotide repeat within the factor VIII gene. Lancet 338,207–211.

Lalloz MR, Schwaab R, McVey JH, Michaelides K and Tuddenham EG (1994) Haemophilia A diagnosis by simultaneous analysis of two variable dinucleotide tandem repeats within the factor VIII gene. Br J Haematol 86,804–809.

Morral N and Estivill X (1992) Multiplex PCR amplification of three microsatellites within the CFTR gene. Genomics 13,1362–1364.

Moulin DS, Smith AN and Harris A (1997) A CA repeat in the first intron of the CFTR gene. Hum Hered 47,295–297.

Munnè S, Sandalinas M, Escudero T, Fung J, Gianaroli L and Cohen J (2000) Outcome of preimplantation genetic diagnosis of translocations. Fertil Steril 73,1209–1218

Munnè S, Cohen J and Sable D (2002) Preimplantation genetic diagnosis for advanced maternal age and other indications. Fertil Steril 78,234–236.

Piyamongkol W, Harper JC, Sherlock JK, Doshi A, Serhal PF, Delhanty JD and Wells D (2001) A successful strategy for preimplantation genetic diagnosis of myotonic dystrophy using multiplex fluorescent PCR. Prenat Diagn 21,223–232.

Pickering S, Polidoropoulos N, Caller J, Scriven P, Ogilvie CM and Braude P (2003) Strategies and outcomes of the first 100 cycles of preimplantation genetic diagnosis at the Guy's and St. Thomas' Center. Fertil Steril 79,81–90.

Ram KT, Barker DF and Puck JM (1992) Dinucleotide repeat polymorphism at the DXS441 locus. Nucleic Acids Res 20,1428.

Ray PF, Vekemans M and Munnich A (2001) Single cell multiplex PCR amplification of five dystrophin gene exons combined with gender determination. Mol Hum Reprod 7,489–494.

Rechitsky S, Strom C, Verlinsky O, Amet T, Ivakhnenko V, Kukharenko V, Kuliev A and Verlinsky Y (1999) Accuracy of preimplantation diagnosis of single-gene disorders by polar body analysis of oocytes. J Assist Reprod Genet 16,192–198.

Rechitsky S, Kuliev A, Tur-Kaspa I, Morris R and Verlinsky Y (2004) Preimplantation genetic diagnosis with HLA matching. Reprod Biomed Online 9,210–221.

Richards B, Reeves C and Horn GT (1991) Dinucleotide repeat polymorphism closely linked to the cystic fibrosis (CFTR) gene. Nucleic Acids Res 19,5798.

Roustan P, Curtis AR, Kamakari S, Thiselton D, Lindsay S and Bhattacharya SS (1992) Dinucleotide repeat polymorphism at the DXS559 locus. Hum Mol Genet 1,778.

Rubio C, Simon C, Vidal F, Rodrigo L, Pehlivan T, Remohi J and Pellicer A (2003) Chromosomal abnormalities and embryo development in recurrent miscarriage couples. Hum Reprod 18,182–188.

Sermon K, De Vos A, Van de Velde H, Seneca S, Lissens W, Joris H, Vandervorst M, Van Steirteghem A and Liebaers I (1998) Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease). Mol Hum Reprod 4,791–796.

Sermon K, Van Steirteghem A and Liebaers I (2004) Preimplantation genetic diagnosis. Lancet 363,1633–1641.

Sermon K, Moutou C, Harper J, Geraedts J, Scriven P, Wilton L, Magli MC, Michiels A, Viville S, De Die C. (2005) ESHRE PGD Consortium data collection IV: May–December 2001. Hum Reprod 20,19–34.

Thornhill AR, deDie-Smulders CE, Geraedts JP, Harper JC, Harton GL, Lavery SA, Moutou C, Robinson MD, Schmutzler AG, Scriven PN, Sermon KD and Wilton L (2005) ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)'. Hum Reprod 20,35–48.

Traeger-Synodinos J, Vrettou C, Palmer G, Tzetis M, Mastrominas M, Davies S and Kanavakis E (2003) An evaluation of PGD in clinical genetic services through 3 years application for prevention of beta-thalassaemia major and sickle cell thalassaemia. Mol Hum Reprod 9,301–307.

Vandervorst M, Staessen C, Sermon K, De Vos A, Van de Velde H, Van Assche E, Bonduelle M, Vanderfaellie A, Lissens W, Tournaye H et al. (2000) The Brussels' experience of more than 5 years of clinical preimplantation genetic diagnosis. Hum Reprod Update 6,364–373.

Van de Velde H, Sermon K, De Vos A, Lissens W, Joris H, Vandervorst M, Van Steirteghem A and Liebaers I (1999) Fluorescent PCR and automated fragment analysis in preimplantation genetic diagnosis for 21-hydroxylase deficiency in congenital adrenal hyperplasia. Mol Hum Reprod 5,691–696.

Van de Velde H, Georgiou I, De Rycke M, Schots R, Sermon K, Lissens W, Devroey P, Van Steirteghem A and Liebaers I (2004) Novel universal approach for preimplantation genetic diagnosis of β-thalassemia in combination with HLA matching of embryos. Hum Reprod 19,700–708.

Verlinsky Y, Cieslak J, Ivakhnenko V, Evsikov S, Wolf G, White M, Lifchez A, Kaplan B, Moise J, Valle J et al. (1999) Prevention of age-related aneuploidies by polar body testing of oocytes. J Assist Reprod Genet 16,165–169.

Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C and Kuliev A (2001) Preimplantation diagnosis for Fanconi anemia combined with HLA matching. J Am Med Assoc 285,3130–3133.

Verlinsky Y, Cohen J, Munne S, Gianaroli L, Simpson JL, Ferraretti AP and Kuliev A (2004) Over a decade of experience with preimplantation genetic diagnosis. Fertil Steril 82,302–303.

Wehnert M, Reiner O and Caskey CT (1993) Four STR polymorphisms map to a 500 kb region between DXS15 and DXS134. Hum Mol Genet 2,1503.

Wilton L (2002) Preimplantation genetic diagnosis for aneuploidy screening in early human embryos: a review. Prenat Diagn 22,512–518.

Zielenski J, Markiewicz D, Rininsland F, Rommens J and Tsui LC (1991) A cluster of highly polymorphic dinucleotide repeats in intron 17b of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Am J Hum Genet 49,1256–1262.

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