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The minisequencing method: an alternative strategy for preimplantation genetic diagnosis of single gene disorders

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We have applied a new method of genetic analysis, called 'minisequencing', to preimplantation genetic diagnosis (PGD) of monogenic disorders from single cells. This method involves computer-assisted mutation analysis, which allows exact base identity determination and computer-assisted visualization of the specific mutation(s), and thus facilitates data interpretation and management. Sequencing of the entire PCR product is unnecessary, yet the same qualitative characteristics of sequence analysis are maintained. The main benefit of the minisequencing strategy is the use of a mutation analysis protocol based on a common procedure, irrespective of the mutations involved. To evaluate the reliability of this method for subsequent application to PGD, we analysed PCR products from 887 blastomeres including 55 PGD cases of different genetic diseases, such as cystic fibrosis, β-thalassaemia, sickle cell anaemia, haemophilia A, retinoblastoma, and spinal muscular atrophy. Minisequencing was found to be a useful technique in PGD analysis, due to its elevated sensitivity, automation, and easy data interpretation. The method was also efficient, providing interpretable results in 96.5% (856/887) of the blastomeres tested. Fifteen clinical pregnancies resulted from these PGD cases; conventional prenatal diagnosis confirmed all the PGD results, and 10 healthy babies have already been born. Its applicability to PGD could be helpful, particularly in cases in which the mutation(s) involved are difficult to assess by restriction analysis or other commonly used methods.

Key words: allele drop-out/minisequencing/preimplantation genetic diagnosis/single cell PCR/single nucleotide polymorphism

Introduction

Preimplantation genetic diagnosis (PGD) is presently a valid alternative for couples at high risk of pregnancy with genetic anomalies. PGD enables unaffected embryos generated by IVF to be identified and transferred and it therefore permits couples to avoid termination of affected pregnancies.

Protocols for genotyping single cells for monogenic disorders are based on the PCR (Saiki *et al*., 1985; Li *et al*., 1988), which represents the only method sensitive enough to detect single gene mutations. Due to its sensitivity, PCR is highly prone to sources of error; thus precautions must be taken in its use for clinical diagnosis. Since the first PCR-based PGD cases were performed (Handyside *et al*., 1989; 1990; 1992), several inherent difficulties associated with single cell DNA amplification have become evident. They include potential sample contamination, total PCR failure, allelic drop-out (ADO, when one of the alleles fails to amplify to detectable levels), and preferential amplification (PA) of one of the alleles.

PGD continues to be a technical challenge, as only one or two blastomeres are available for analysis, which must be performed within 1 day. A major limitation of PGD practice comes from the need to develop single cell DNA analysis protocols. They should be sensitive enough to provide the greatest amplification efficiency, thus allowing the maximum number of embryos to be diagnosed. This is very important when PGD is performed for an autosomal dominant disease, in which 50% of the embryos could theoretically be affected. PGD protocols should also meet high standards of accuracy, have a low ADO rate and contamination controls, ensuring transfer of only unaffected embryos. Therefore a PGD protocol must be put through an extensive preclinical trial before it can be applied to clinical cases.

The goal of centres performing single cell DNA analysis is thus to optimize a strategy that maximizes efficiency, sensitivity, and reliability of the procedure, enabling interpretable and unambiguous results to be obtained.

Techniques involving non-automated gel analysis are successfully used for mutation screening in the majority of PGD cases to detect the presence or absence of restriction sites (Ray *et al*., 1999; 2000; Kuliev *et al*., 1998; 1999), electrophoretic mobility shift, as in single strand conformation polymorphism (SSCP) (El-Hashemite *et al*., 1997; Ioulianos *et al*., 2000) or in denaturing gradient gel electrophoresis (DGGE) (Kanavakis *et al*., 1999; Vrettou *et al*., 1999; Palmer *et al*., 2002). Computer-assisted highly sensitive mutation detection is also performed, for the above techniques, by means of fluorescent PCR (Van de Velde *et al*., 1999; Blake *et al*., 1999; De Vos *et al*., 2000; Abou-Sleiman, *et al*., 2002; Harper *et al*., 2002) and for allele specific amplification (ARMS: amplification refractory mutation system) (Moutou *et al*., 2001).

For diseases involving a heterogeneous spectrum of mutations identified, such as cystic fibrosis, β-thalassaemia or haemophilia A, the development of a mutation-based PGD strategy is not practical because it requires time and resources for standardization of PCR protocols unique for the specific mutations of interest. For these kinds of monogenic diseases, the use of a diagnostic strategy capable of

Table I. Description of genetic regions amplified and primers used

Disease	Gene	Exon	Outer primers (5′ to 3′)	Inner primers (5′ to 3′)	Annealing temperature (°C)
β-Thalassaemia	HBB	1	F-5′-CTGTCATCACTTAGACCTCA-3′ R-5′-TGGTCTCCTTAAACCTGCTTG-3′	F-5′-CATCACTTAGACCTCACCCTGT-3′ R-5′-TCTCCTTAAACCTGTCTTGTAACC-3′	60
		2	F-5′-ACTGGGCATGTGGAGACAGAGAAGA-3′ R-5′-TGTACCCTGTTACTTCTCCCCTTCC-3′	F-5′-TGGGTTTCTCATAGGCACTGA-3′ R-5′-AAAGAAAACATCAAGGGTCCC-3′	60
Cystic fibrosis	CFTR	3	F-5′-CTTGGGTTAATCTCCTTGGA-3′ R-5′-ATTCACCAGATTTCGTAGTC-3′	F-5′-TGTGTGAATCAAACTATGTTAAGGG-3′ R-5′-TCGTAGTCTTTTCATAATCACAAA-3′	50
		4	F-5′-CACATATGGTATGACCCTC-3′ R-5′-TTGTACCAGCTCACTACCTA-3′	F-5′-AGTCACCAAAGCAGTACAGC-3′ R-5′-GCTATTCCATCTGCATTCC-3′	54
		7	F-5′-AGACCATGCTCAGATCTTCC-3′ R-5′-CAAAGTTCATTAGAACTGATC-3′	F-5′-AGAACTGAAACTGACTCGGAAG-3′ R-5′-ATTGCTCCAAGAGAGTCATACC-3′	57
		10	F-5′-GCAGAGTACCTGAAACAGGA-3′ R-5′-CATTCACAGTAGCTTACCCA-3′	F-5′-GATAATGACCTAATAATGATGGGTT-3′ R-5′-GGTAGTGTGAAGGGTTCATATG-3′	56
		11	F-5′-CAACTGTGGTTAAAGCAATAGTGT-3′ R-5′-GCACAGATTCTGAGTAACCATAAT-3′	F-5′-ACTCTCTAATTTCTATTTTT-3′ R-5′-TTTTACATGAATGACATTTA-3′	55
		13	F-5′-CAGAACTCCAAAATCTACAGCC-3′ R-5′-TGCTCAGAATCTGGTACTAAGG-3′	F-5′-CATTAGAAGGAGATGCTCCTGT-3′ R-5′-ACAGCCTTCTCTCTAAAGGCTC-3′	54
		20	F-5′-GGTCAGGATTGAAAGTGTGCA-3′ R-5′-TATGAGAAAACTGCACTGGA-3′	F-5′-GTCACAGAAGTGATCCCATC-3′ R-5′-CTGGCTAAGTCCTTTTGCTC-3′	57
		21	F-5′-GGTAAGTACATGGGTGTTTC-3′ R-5′-CAAAAGTACTTGTCGCGCCA-3′	F-5′-ATTCATACTTTCTTCTTCTTTCT-3′ R-5′-CATTTGTGTTGGTATGAGTTAC-3′	55
Sickle cell anaemia	HBB	1	F-5′-CTGTCATCACTTAGACCTCA-3′ R-5′-TGGTCTCCTTAAACCTGCTTG-3′	F-5′-CATCACTTAGACCTCACCCTGT-3′ R-5′-TCTCCTTAAACCTGTCTTGTAACC-3′	60
Haemophilia A	F8C	8	F-5′-CCATATAGCCTGCAGAACAT-3′ R-5′-CGAGCCAGCTATGTTAG-3′	F-5′-AGTCTCTGGTATAGAACAGCC-3′ R-5′-AGTCTTCCGCTTCTTCATTA-3′	54
Retinoblastoma	RB1	13	F-5′-AGTATCCTCGACATTGATTTCT-3′ R-5′-CTATAGTACCACGAATTACAATGA-3′	F-5′-TTACCTCCTAAAGAACTGCAC-3′ R-5′-AGTACCACGAATTACAATGAAT-3′	53
Spinal muscular atrophy	SMN	7	F-5′-CATTAAAAGACTATCAACTTAATTTCTG-3′ R-5′-TAAGGAATGTGAGCACCTTCCTTC-3′	F-5′-AGACTATCAACTTAATTTCTGATCA-3′ R-5′-CACCTTCCTTCTTTTTGATTTTGT-3′	55

F = forward; R = reverse.

detecting a wide spectrum of mutations and compound genotypes is more feasible. Genotyping methods based on DGGE (Vrettou *et al*., 1999; Kanavakis *et al*., 1999; Palmer *et al*., 2002) or SSCP (El-Hashemite *et al*., 1997; Ioulianos *et al*., 2000; Harper *et al*., 2002; Abou-Sleiman, *et al*., 2002) have been used to facilitate mutation detection for the above anomalies, and have also addressed many of the inherent potential problems associated with PCR-based genotyping of single cells.

An alternative procedure to mutation-directed PGD protocols was proposed to overcome these problems: fluorescent multiplex PCR indirect diagnosis performed by the use of polymorphic markers, allowing identification of the pathogenic haplotype instead of the mutation (Dreesen *et al*., 2000; Moutou *et al*., 2002).

Our PGD strategy, instead, was based on the use of a single mutation analysis protocol that could be fluorescence-based (i.e. highly sensitive), computer-assisted (i.e. facilitating data interpretation and management), and involving the use of a common procedure for each mutation to be analysed.

Automated fluorescence-based DNA sequencing combines the above characteristics, allowing the identification and computer-assisted visualization of a specific mutation. Moreover, it enables the simultaneous analysis of more than one mutation in a single PCR fragment. However, while representing a valid genetic analysis technique, guaranteeing good interpretative reliability, its application to PGD analysis is unwieldy, time consuming, and requires good quality amplification products for analysis. Furthermore it requires experience for data interpretation.

In order to overcome some of these limitations, especially in the case of larger blastomere numbers, the application of a new mutation analysis method, based on a primer extension technique (Sokolov *et al*., 1990), primarily devised to detect single nucleotide polymorphisms (SNP), was investigated. This method, more generally known as minisequencing (Pastinen *et al*., 1997; Syvänen *et al*., 1999), permits identification of the specific mutations without sequencing the entire PCR product, yet it still maintains the same qualitative characteristics of sequence analysis.

The aim of this study was to evaluate the reliability of minisequencing for its following application in single cell DNA analysis. PCR products from 887 blastomeres from 55 PGD cases of different genetic diseases, such as cystic fibrosis, β-thalassaemia, sickle cell anaemia, haemophilia A, retinoblastoma, and spinal muscular atrophy (SMA), were analysed simultaneously with both traditional automated sequence analysis, routinely used in our laboratory for single cell mutation detection, and with the minisequencing method.

Materials and methods

Carrier detection

To determine or confirm the genetic status of the couples, genomic DNA was extracted from 200 µl of peripheral blood in EDTA according to the phenol-chloroform procedure (Sambrook *et al*., 2000). PCR amplification of each region of interest was performed using the outer oligonucleotide primers listed in Table I. Mutation analysis was carried out by direct sequencing of PCR products using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), according to the protocol provided by the manufacturer.

Minisequencing primers design

After mutation detection, minisequencing primers (Table II) were designed for each mutation investigated with the aid of Primer Express® software (Applied Biosystems) and initially tested on sequenced PCR products.

Table II. Description of mutations investigated and minisequencing primers used

Disease	Gene	Mutation investigated	Minisequencinq primers (5′ to 3′) for mutation detection	Minisequencinq primers (5′ to 3′) for ADO detection
β-Thalassaemia	HBB	-110 C→T	5′-CCTAGGGTGTGGCTCCACA-3′	5′-GCAGACTTCTCCTCAGGAGTCAG-3′
		-87 C→T	5′-GGAGTAGATTGGCCAACCCTAG-3′	
		Cod.8-AA	5′-TGCACCTGACTCCTGAGGAG-3′	
		IVS-I-1 G→A	5′-CCTGTCTTGTAACCTTGATACCAA-3′	
		IVS-I-6 T→C	5′-AGGCCCTGGGCAGGTTGG-3′	
		IVS-I-110 G→A	5′-GGCACTGACTCTCTCTGCCTATT-3′	
		Cod.39 C→T	5′-TGGTGGTCTACCCTTGGACC-3′	
		IVS-II-1 G→A	5′-CGTGGATCCTGAGAACTTCAGG-3′	
Sickle cell anaemia	HBB	Cod.6 A→T	5′-CATGGTGCACCGACTCCTG-3′	
Cystic fibrosis	CFTR	G85E	5′-ATGTTTTCTGGAGATTTATGTTCTATG-3′	5′-GGTTTTATTTCCAGACTTCACTTCTAATG-3′
		R117H	5′-GACCCGGATAACAAGGAGGAAC-3′	
		M348K	5′-ATTCTGCATTGTTCTGCGCA-3′	
		ΔF507	5′-GCCTGGCACCATTAAAGAAAATATC-3′	
		ΔF508(CTT)deletion	5′-CCTGGCACCATTAAAGAAAATATCAT-3′	
		ΔF508(TTT)deletion	5′-CTTCTGTATCTATATTCATCATAGGAAACACC-3′	
		G542X	5′-CAAGTTTGCAGAGAAAGACAATATAGTTCTT-3′	
		R553X	5′-TCACCTTGCTAAAGAAATTCTTGCTC-3′	
		2183 AA→G	5′-CTGTCTCCTGGACAGAAACAAAAA-3′	
		W1282X	5′-GTATCACTCCAAAGGCTTTCCT-3′	
		N1303K	5′-CCACTGTTCATAGGGATCCAA-3′	
Haemophilia A	F8C	intr.8 –27 G→A	5′-GGAGTCAGACAAACCAAACAATGT-3′	
Retinoblastoma	RB1	73868 A→G	5′-GGTTGTGTCGAAATTGGATCAC-3′	
Spinal muscular atrophy	SMN	exonic mismatch G(SMN^t) →A(SMN^c)	5′-CCTTTTATTTTCCTTACAGGGTTT-3′	

Desalted primers were purchased from MWG-Biotech (Germany). The guidelines for primer design included the following parameters: (i) design primers 18 nucleotides in length or greater with melting temperatures of ⩾45°C; (ii) check primers for possible extendable hairpin structures and for extendable dimer formation between primers; (i) high performance liquid chromatography purification of primers is recommended for oligonucleoides longer than 30 nucleotides; (iv) use primers that are complementary to the negative (–) DNA strand, if the positive (+) DNA strand is difficult to assay; (v) in multiplex reaction, primers must differ significantly in length so that overlap between the final products will be avoided. A difference of 4–6 nucleotides between primer lengths is recommended. The length of a primer can be modified by the addition of non-homologous polynucleotides at the 5′ end. Poly (dT), poly (dA), poly (dC) and poly (dGACT) are 5′ non-homologous tails which are predicted to have minimal secondary structures.

Trial PGD testing on single lymphocytes

In order to evaluate single cell amplification efficiency and ADO rate, all primers used for the detection of the mutations were first tested on single lymphocytes.

Lymphocytes were isolated from 5 ml of unclotted blood in EDTA collected from male and/or female carriers of each couple using Ficoll-Paque density gradient separation (Amersham Pharmacia Biotech, Italy), according to the manufacturer's protocol. Lymphocytes were also collected from affected individuals, when available. The cell layer containing lymphocytes was removed and diluted with sterile phosphate-buffered saline to a suitable cell density for single cell isolation. Lymphocytes were then handled with a mouth-controlled fine heat-polished glass micropipette; the cells were selected and retrieved individually under visual control through an inverted microscope.

Fifty single lymphocytes for each PGD case were loaded into 0.2 ml tubes containing 5 μl of lysis buffer and subjected to PCR amplification as described below, followed by mutation analysis with both full sequencing and minisequencing methods to establish assay specificity. Twenty blank controls for each trial were also performed.

In order to evaluate the sensitivity of the minisequencing method, serial dilutions of a PCR product, previously typed as heterozygote for β-thalassaemia Cod.39 C→T mutation by using sequence analysis, were also performed starting from 100 to 1 ng. Each dilution was then subjected to minisequencing reaction using the primer listed in Table II, following the conditions described below.

IVF and embryo biopsy procedure

Induction of multiple follicular growth was performed by exogenous gonadotrophin administration following a desensitization protocol with long-acting GnRH analogues (Ferraretti *et al*., 1996). At 34–36 h post-hCG administration, oocytes were collected transvaginally via ultrasound guidance and incubated in Earle's balanced salt solution (EBSS) supplemented with 10% heat-inactivated maternal serum (MS), in a 5% CO_2 moist atmosphere at 37°C. Insemination was performed by ICSI or conventional IVF depending on sperm sample requirements (Gianaroli *et al*., 1996). Oocytes were checked at ~16 h post-insemination for the presence of pronuclei and polar bodies. Regularly fertilized oocytes were cultured individually in 100 μl drops of EBSS–15% MS and scored at 24 h time intervals. At 62–64 h post-insemination, embryos with ⩾6 cells and <50% fragmentation underwent embryo biopsy. Each embryo was manipulated in HEPES-buffered medium overlaid with pre-equilibrated oil. According to the internal biopsy policy, two blastomeres were removed from each embryo. The fundamental criteria for the selection of the blastomeres was the presence of a clear nucleus and the maintenance of cell integrity. A breach of 20–25 μm was opened in the zona pellucida with acidic Tyrode's solution; two blastomeres containing a clear nucleus were gently aspirated through the hole using a polished glass needle of 35–40 μm inner diameter. The biopsied embryo was extensively washed and transferred to blastocyst growing medium (Vitrolife, Sweden). After removal, each blastomere was washed twice through two drops of HEPES-buffered medium, transferred into sterile 0.2 ml PCR tubes containing 5 μl of alkaline lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol), and covered with mineral oil (Sigma–Aldrich, Italy) before subjection to cell lysis. For each embryo biopsied, a blank control was prepared from the wash drops by adding 5 μl of this medium to a sterile 0.2 ml PCR tube. All steps were double-checked, with a second biologist witnessing the correct labelling of dishes and tubes.

Cell lysis procedure

The 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) before proceeding to PCR.

PCR

The PCR strategy consisted of initial multiplex external amplification followed by nested PCR, specific for each region involving mutations. After cell lysis

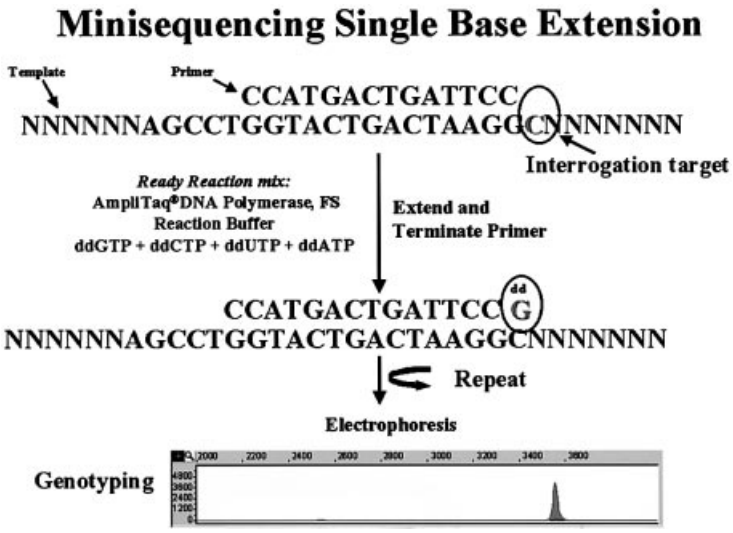

Figure 1. Minisequencing technique (see Materials and methods).

and neutralization, 1.5 mmol/l $MgCl_2$, 200µmol/l of each dNTP (Roche Diagnostic, Italy), 2.5 IU AmpliTaqPolymerase (Applied Biosystems), 10 pmol of each outer primer, were added to each tube, for a total volume of 50 µl. The first round of PCR involved a 96°C denaturation temperature in the first 10 cycles as a means to reduce ADO (Ray and Handyside, 1996), followed by a subsequent denaturation temperature of 94°C in 25 remaining cycles. Each round of PCR was preceded by an initial 4 min denaturation step at 94°C and followed by a final extension step of 10 min at 72°C. The extension temperature depended on the specific primers used (Table I). PCR positive heterozygous controls containing a single lymphocyte (two control cells for each genotype) isolated from carriers were also added.

For the second round of DNA amplification, 2 µl of the primary PCR reaction product were added to another tube containing 5 µl of 10×PCR Buffer II (500 mmol/l KCl, 100 mmol/l Tris HCl, pH 8.3; Applied Biosystems), 1.5 mmol/l $MgCl_2$, 200 µmol/l of each dNTP (Roche Diagnostic), 2.5 IU AmpliTaqPolymerase (Applied Biosystems), 10 pmol of each inner primer, in a total volume of 50 µl, and the tubes were cycled as above on a GeneAmp® PCR System 9700 (Applied Biosystems).

To monitor successful amplification, 5 µl of each PCR product was subjected to electrophoresis for 5 min at 150 V on 2% agarose gel in 1×Tris–borate/EDTA buffer stained with 0.5 µg/ml ethidium bromide.

To avoid participation in the subsequent primer-extension reaction, primers and unincorporated dNTP were removed from PCR products by performing Microcon 100 (Amicon, USA) purification, according to the manufacturer's protocol.

Mutation analysis was carried out only on positively amplified blastomeres, using two different techniques simultaneously: sequence analysis and minisequencing.

Sequence analysis

For sequence analysis, 20 ng of purified PCR products were sequenced by direct cycle sequencing using fluorescent-labelled dideoxy terminators (Big Dye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems), according to the manufacturer's protocol. The reaction conditions were as follows: 25 PCR cycles, a denaturation step of 10 s at 96°C, annealing for 5 s at 50°C and extension for 4 min at 60°C. Sequencing products were then purified using Centri-Sep™ columns (Princeton Separation) for unincorporated dye terminator removal, according to the protocol provided by the manufacturer. Five microlitres of each Centri-Sep™-purified product were then added to 15 µl of Hi-Di Formamide (Applied Biosystems), heat-denatured at 90°C for 4 min and run on ABI Prism® 310 automated DNA sequencer (Applied Biosystems). The sequences obtained were then compared with the wild type controls using Sequence Navigator® Software (Applied Biosystems) for mutation analysis.

Minisequencing

The minisequencing reaction was performed, starting from 10 ng of the same purified PCR product subjected to sequence analysis, using ABI Prism® SnaPshot Multiplex Kit (Applied Biosystems). The reaction volume was 10 µl, including 5 µl of Ready Reaction Premix and 10 pmol of each minisequencing primer. The reaction conditions were as follows: 25 PCR cycles, denaturation step of 10 s at 96°C, annealing for 10 s at 50°C and extension for 30 s at 60°C.

In the minisequencing technique, a primer extension reaction is performed, starting from a specific primer that is designed to anneal directly adjacent to the mutation site, by the incorporation of a single fluorescent dideoxynucleotide (ddNTP) which is complementary to the variant base in the template. Since the primer is designed to anneal directly adjacent to the variant base of interest, and the reaction mix does not include dNTP, incorporation occurs only at a single site. This process is repeated in successive rounds of extension and termination to generate the fluorescently labelled fragment for analysis. After the primer extension reaction, 1 µl of minisequencing product was mixed with 15 µl of Hi-Di Formamide and denatured for 4 min at 90°C. The samples were then resolved and detected by 15 min (per blastomere) of capillary electrophoresis on automatic DNA sequencer ABI Prism® 310, using POP-4™ polymer and 47 cm×50 µm capillaries. Thus, the mutation sites could reliably be differentiated among homozygotic wild types, homozygotic mutants or heterozygotes, by the dye-labelled ddNTP incorporated. To reveal the electrophoresis data, the peak signal was analysed with GeneScan® Analysis Software (Applied Biosystems); the dye colour of the fragment was used to identify the nucleotide of interest (Figure 1). For the minisequencing technique, colour was assigned to individual ddNTP as follows: green/A, black/C, blue/G, red/T. For sequencing, instead colours were assigned as follows: green/A, black/G, blue/C, red/T. The minisequencing reaction produces one (homozygote) or two (heterozygote) peaks depending on the genotype at this locus.

Confirmation of PGD results

After the clinical cases, non-transferred and non-frozen embryos (affected or morphologically incompetent) had the zona pellucida removed by acidic Tyrode's solution; the disaggregated blastomeres were collected in individual tubes and reanalysed to verify the PGD results. The whole embryo was put into a single PCR tube only when the reanalysis was performed on embryos at the blastocyst stage.

In cases in which pregnancies occurred, patients underwent conventional prenatal diagnosis to confirm the genetic status of the fetus.

Statistical analysis

Comparisons of results of minisequencing sequence analysis were performed using χ^2-test applying Yates' correction, 2×2 contingency table. Significance was assumed when $P \leqslant 0.05$.

Results

Lymphocyte testing

Efficiency and accuracy of PCR were evaluated in preliminary experiments on single lymphocytes with the known genotype of the male and/or female carriers of each couple. Results from PCR amplification for each of the loci investigated are shown in Table III. Among 650 single lymphocytes tested, 600 of them gave positive amplification. Amplification rates were generally high for all loci tested, ranging from 88 to 96%, with an overall amplification rate of 92.3%. Emphasis was put on the mutation analysis of lymphocytes to determine the ADO rate for each locus analysed. In total there were 51 occurrences of ADO; the ADO rates varied between the different loci investigated, ranging from 6.3 to 11.4%, with an average ADO rate of 9.3%. None of the 20 blank controls used for each locus-specific trial showed amplification.

Table III. PCR amplification efficiencies and allele drop-out (ADO) rates for different loci from single heterozygous lymphocytes

Gene	Exon	PCR amplification efficiency[a] (%)	ADO[b] (%)
HBB	1	47 (94)	4 (8.5)
	2	48 (96)	3 (6.3)
CFTR	3	45 (90)	4 (8.9)
	4	45 (90)	5 (11.1)
	7	46 (92)	4 (8.7)
	10	48 (96)	3 (6.3)
	11	46 (92)	5 (10.9)
	13	45 (90)	4 (8.9)
	20	47 (94)	5 (10.6)
	21	46 (92)	4 (8.7)
F8C	8	44 (88)	5 (11.4)
RB1	13	45 (90)	5 (11.1)
SMN	7	48 (96)	NE

[a]Number of positive amplifications obtained from 50 single lymphocytes analysed.
[b]Number of cells in which ADO occurred.
NE = not evaluated.

To determine the lower limit to which the minisequencing method could provide interpretable results, we tested six dilutions of a PCR product, previously typed as heterozygote for β-thalassaemia Cod.39 C→T mutation by using sequence analysis. Correct genotypes were obtained from 100 to 1 ng per minisequencing reaction with a progressive decrease of peak intensity, from an average peak height of 5748 relative fluorescence units (RFU) to 679 RFU. Data have shown that it is possible to obtain informative results with as little as 1 ng of PCR product (Figure 2).

Confident that each system used was sufficiently robust and accurate, we proceeded to perform PGD analysis.

Clinical PGD cases

The results of PGD cases are summarized in Table IV. A total of 580 regularly fertilized oocytes resulted after inseminating 701 oocytes; 534 cleaved (mean number 9.7 ± 3.0 per patient), and 496 (mean number 9.0 ± 2.9) were selected for embryo biopsy on the basis of regular development and morphology. A total of 992 blastomeres were biopsied; 970 cells (97.8%) were successfully biopsied, while 22 cells were lysed during biopsy procedure and were not analysed. Consequently, from 22 embryos only one cell was analysed (Figure 3).

PCR amplification was performed on 970 blastomeres obtaining positive amplification in 887 cells. Amplification failure resulted in 83 blastomeres. The efficiency of amplification on the blastomeres was similar to those obtained from single lymphocytes, ranging from 87.6 to 93.8%, with an overall amplification rate of 91.4%.

To test the accuracy and sensitivity of the minisequencing method in embryonic cells, the following mutation analysis was carried out simultaneously with both automated sequence analysis, routinely used in our laboratory for single cell mutation detection, and minisequencing. Interpretable results were obtained in 96.5% (856/887) of the blastomeres investigated with minisequencing, with respect to 86.7% (769/887) obtained with sequence analysis; the difference was statistically significant ($P < 0.001$). Non-interpretable results were

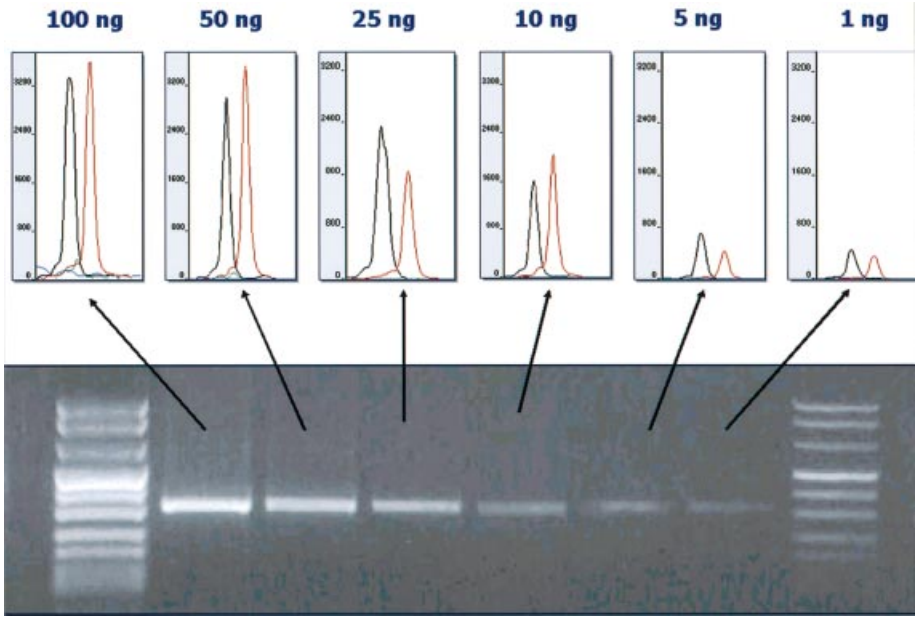

Figure 2. Minisequencing sensitivity test. Results of minisequencing reaction performed at different quantities of a PCR product, in order to determine the lower limit to which the method can provide interpretable results. The diluted PCR product resulted from amplification of a single lymphocyte isolated from a Cod.39 C/T β-thalassaemia mutation carrier. Data obtained have shown that it is possible to obtain good quality results with as little as 1 ng of PCR product. M1 and M2 are different amounts of mol. wt markers, 100 and 10 ng respectively.

Table IV. Embryological data and clinical outcome

Disease	PGD cases	No. of oocytes inseminated	No. of normally fertilized oocytes (%)[a]	No. of embryos biopsied	No. of blastomeres analysed	Clinical pregnancies	No. of births
β-Thalassaemia	20	255	211 (82.7)	177	349	8	5
Cystic fibrosis	20	251	206 (82.1)	173	343	5	4
Sickle cell anaemia	4	49	42 (85.7)	36	71	1	0
Haemophilia A	5	66	55 (83.3)	53	105	1	1
Retinoblastoma	3	42	34 (81.0)	29	54	0	0
Spinal muscular atrophy	3	38	32 (84.2)	28	48	0	0
Total	55	701	580	496	970	15	10

[a]Values in parentheses are percentages.
PGD = preimplantation genetic diagnosis.

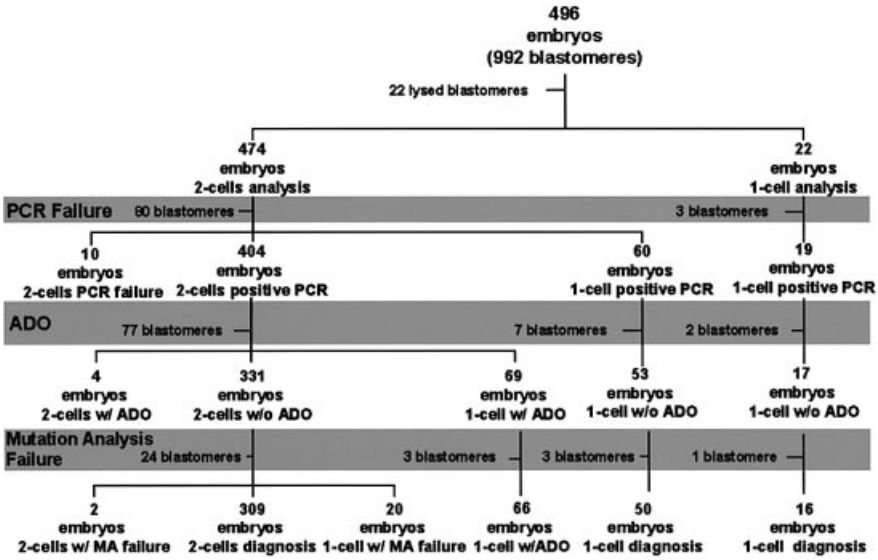

Figure 3. Clinical PGD results diagram. ADO = allele drop-out; MA = mutation analysis; w/ = with; w/o = without.

obtained in 31 blastomeres analysed with minisequencing, due to the presence of background extra-peaks covering the expected peaks constituting the blastomere genotype. In 118 blastomeres investigated with the sequencing method, instead, mutation analysis failure occurred due to the quality of the PCR products.

As represented in Figure 3, ADO was detected in 86/887 blastomeres, making it impossible to obtain a reliable diagnosis in nine embryos, since only one cell was available, and in four embryos due to ADO occurrence in both blastomeres. In 69 embryos ADO occurred in one of the two positively amplified blastomeres. The ADO rates varied between the different loci investigated, ranging from 6.3 to 12.2%, with an average ADO rate of 10.5% (Table V).

Mutation analysis failed in six embryos; in total no diagnosis was obtained for 32 embryos, whereas for 155 embryos the diagnosis was based on one blastomere only (Figure 3).

In all cases, single cell PCR positive heterozygous controls were diagnosed reliably. No contamination was detected in blank controls collected during the biopsy procedure nor in the blanks from the PCR reagents.

Reanalysis of non-transferred embryos

After the clinical cases, 136 non-transferred embryos (109 affected, 14 heterozygotes and 13 normal) were reanalysed to confirm the diagnosis. PCR amplification was performed on 480 blastomeres dissociated from the above embryos; positive amplification was obtained in 438 blastomeres. The amplification rates were similar to those obtained during clinical diagnosis, ranging from 87.3 to 93.2%, with an overall amplification rate of 91.3%. Interpretable results were obtained in 97.9% (429/438) of the above blastomeres investigated with minisequencing, with respect to 90.0% (394/438) obtained with sequence analysis.

ADO was detected in 44/438 blastomeres; the ADO rates varied between the different loci investigated, ranging from 7.7 to 12.9%, with an average ADO rate of 10.5% (Table VI).

The clinical diagnosis was confirmed in all non-transferred embryos reanalysed. Fifteen clinical pregnancies (15/55, 27%) resulted from the above PGD cases (Table IV), 10 healthy babies were delivered, while the remaining five pregnancies are ongoing and confirmed as healthy by prenatal diagnosis.

Comparison of minisequencing and sequence analysis for mutation analysis

Figure 4 compares results between the two methods used for mutation analysis from a PGD case of β-thalassaemia, in which the mutations involved were Cod.39 C→T (Figure 4A) and Cod.8-AA (Figure 4B). The result shows perfectly overlapping data. The result obtained from a blastomere in which Cod.39 C→T mutation was absent is represented at the top of Figure 4A, while a heterozygous blastomere for the same mutation is shown at the bottom. The minisequencing window (on the right) shows the presence of only one black peak

Table V. PCR efficiency, allele drop-out (ADO) rates and mutation analysis results for different loci from 970 biopsied blastomeres analysed during 55 PGD cases

Gene	Exon	No. of cells analysed	No. of cells with positive amplification (%)[a]	ADO[b] (%)[c]	Interpretable results[d] (%)[e]	
					Sequencing	Minisequencing
HBB	1	236	219 (92.8)	21 (9.6)	190 (86.8)	211 (96.3)
	2	184	170 (92.4)	16 (9.4)	149 (87.6)	166 (97.6)
CFTR	3	18	16 (88.9)	1 (6.3)	13 (81.3)	15 (93.8)
	4	21	19 (90.5)	2 (10.5)	16 (84.2)	18 (94.7)
	7	28	25 (89.3)	3 (12.0)	21 (84.0)	24 (96.0)
	10	102	94 (92.2)	8 (8.5)	83 (88.3)	91 (96.8)
	11	66	60 (90.9)	7 (11.7)	52 (86.7)	57 (95.0)
	13	28	25 (89.3)	3 (12.0)	22 (88.0)	24 (96.0)
	20	46	42 (91.3)	5 (11.9)	35 (83.3)	40 (95.2)
	21	34	31 (91.2)	3 (9.7)	26 (83.9)	30 (96.8)
F8C	8	105	92 (87.6)	11 (12.0)	84 (91.3)	90 (97.8)
RB1	13	54	49 (90.7)	6 (12.2)	40 (81.6)	47 (95.9)
SMN	7	48	45 (93.8)	NE	38 (84.4)	43 (95.6)

[a]Amplification rate.
[b]Number of cells in which ADO occurred.
[c]ADO rate.
[d]Number of cells in which interpretable results were obtained.
[e]Interpretable results rate.
NE = not evaluated.

Table VI. Results for the different loci investigated obtained after reanalysis of 136 non-transferred embryos

Gene	Exon	No. of cells analysed	No. of cells with positive amplification (%)[a]	ADO[b] (%)[c]	Interpretable results[d] (%)[e]	
					Sequencing	Minisequencing
HBB	1	103	96 (93.2)	10 (10.4)	87 (90.6)	94 (97.9)
	2	85	79 (92.9)	7 (8.9)	71 (89.9)	77 (97.5)
CFTR	3	9	8 (88.9)	1 (12.5)	7 (87.5)	8 (100.0)
	4	11	10 (90.9)	1 (10.0)	9 (90.0)	10 (100.0)
	7	13	12 (92.3)	1 (8.3)	11 (91.7)	12 (100.0)
	10	41	38 (92.7)	3 (7.9)	34 (89.5)	37 (97.4)
	11	26	24 (92.3)	3 (12.5)	21 (87.5)	23 (95.8)
	13	10	9 (90.0)	1 (11.1)	8 (88.9)	9 (100.0)
	20	21	19 (90.5)	2 (10.5)	17 (89.5)	19 (100.0)
	21	14	13 (92.9)	1 (7.7)	11 (84.6)	13 (100.0)
F8C	8	71	62 (87.3)	8 (12.9)	57 (91.9)	61 (98.4)
RB1	13	53	47 (88.7)	6 (12.8)	42 (89.4)	46 (97.9)
SMN	7	23	21 (91.3)	NE	19 (90.5)	20 (95.2)

[a]Amplification rate.
[b]Number of cells in which ADO occurred.
[c]ADO rate.
[d]Number of cells in which interpretable results were obtained.
[e]Interpretable results rate.
NE = not evaluated.

(normal allele, wild type base C) in the upper panel, and the presence of two differently-coloured peaks in the lower panel: one refers to the normal allele (black peak) and the other to the mutated allele (red peak, mutant base T). Instead, the result obtained from a blastomere normal for the mutation Cod.8 del-AA is shown at the top of Figure 4B, and a heterozygous blastomere for the same mutation at the bottom. The minisequencing window (on the right) shows only one green peak (normal allele, wild type base A) in the upper panel, and two differently-coloured peaks in the lower panel, one coming from the normal allele (green peak) and the other from the mutated allele (blue peak, mutant base G).

Figure 5 provides an example of multiplex minisequencing analysis resuts from a β-thalassaemia PGD case in which the mutations involved were Cod.39 C→T and Cod.8 del-AA. Figure 5A shows the result of a compound heterozygote blastomere for the two mutations investigated. The simultaneous presence of normal alleles (green and black peaks) and mutated alleles (red and blue peaks) can be observed. An example of a normal blastomere for these mutations is shown in Figure 5B, in which the normal genotype can be easily deduced by the absence of the mutated alleles. Figure 5C and D display two heterozygote blastomeres for Cod.8 del-AA and Cod.39 C→T mutations respectively. The primers used for this example of multiplex reactions are shown in Table II.

With minisequencing we were also able to perform multiplex mutation analysis and ADO detection in the same primer extension reaction and electrophoresis run. An example of these results is given in Figure 6. Simultaneous investigations of IVS-1 G→A and -87 C→T β-globin gene mutations were performed, together with an internal SNP located in exon 1, codon 2, of the same gene, inside the PCR fragment to be amplified. ADO is highlighted by the absence of the red

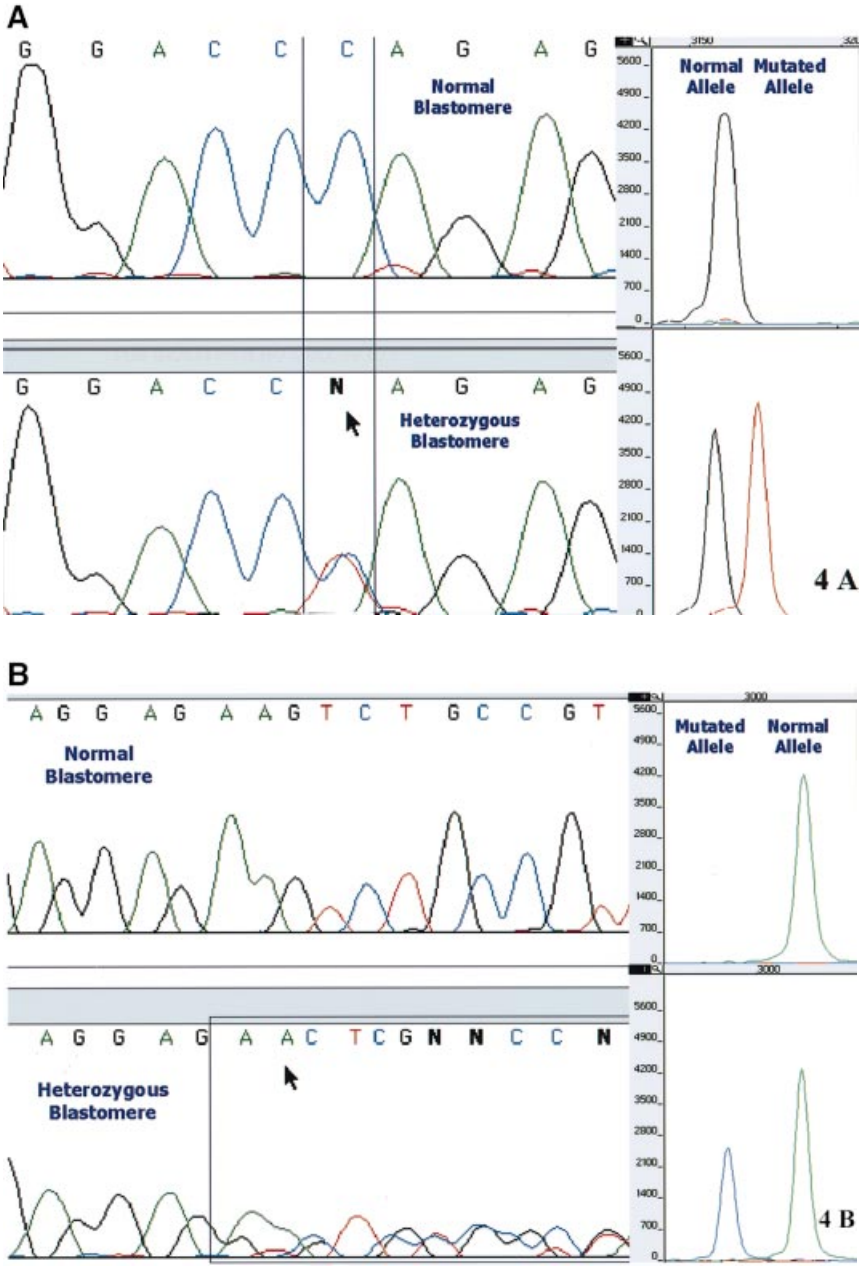

Figure 4. Comparison between sequence analysis, on the left, and the minisequencing method, on the right, for (**A**) Cod.39 C→T and (**B**) Cod.8 del-AA β-thalassaemia mutations. For the minisequencing technique, a colour is assigned to individual ddNTP as follows: green/A, black/C, blue/G, red/T; for the sequencing method, instead, the colours are assigned to individual ddNTP as follows: green/A, black/G, blue/C, red/T. The minisequencing reaction produces one (homozygote) or two (heterozygote) peaks depending on the genotype at this locus. The result obtained from a blastomere in which Cod.39 C→T mutation was absent is represented at the top of **A**, while a heterozygous blastomere for the same mutation is shown at the bottom. The minisequencing window (on the right) shows the presence of only one black peak (normal allele, wild type base C) in the upper panel, and the presence of two differently coloured peaks in the lower panel: one refers to the normal allele (black peak) and the other to the mutated allele (red peak, mutant base T). Instead, the result obtained from a blastomere normal for the mutation Cod.8 del-AA is shown at the top of **B**, and a heterozygous blastomere for the same mutation at the bottom. The minisequencing window (on the right) shows only one green peak (normal allele, wild type base A) in the upper panel, and two differently coloured peaks in the lower panel, one coming from the normal allele (green peak) and the other from the mutated allele (blue peak, mutant base G). For minisequencing, the *y*-axis represents the relative fluorescence units (RFU) of the detected fragments as they occurred over time; the *x*-axis represents time and is displayed by data points. Although minisequencing reaction products for a specific mutation site have the same size, the different electrophoretic mobility of each incorporated dye-labelled ddNTP allows visualization of two separate peaks and not just two superimposed peaks of different colour, as in the sequencing window.

peak (mutated allele of -87 C→T mutation) and the green peak (one allele of the SNP locus) (Figure 6B). The same procedure was followed for ADO detection in PGD cases involving mutation in the CFTR gene, interrogating an SNP located in exon 10, codon 470, determined by a G→A variation at nucleotide 1540 (data not shown), by using the primer indicated in Table II.

Discussion

Minisequencing chemistry is based on the single dideoxynucleotide extension of unlabelled oligonucleotide primers. The primer extension reaction is performed starting from the purified amplified target. Specific minisequencing primers, which are exactly one base short of

the mutation sites, are used for each mutation under investigation. Primers bind to the complementary templates in the presence of fluorescent-labelled ddNTP and AmpliTaq® DNA Polymerase. The polymerase then adds a single ddNTP at the 3′ end of each primer, complementarily according to the sequence. Since the reaction contains only template, primer, ddNTP and does not include dNTP, interruption of the reaction occurs after only one incorporation of a dideoxy terminator. This process is repeated in successive rounds of extension and termination, thus the resulting products, varying in colour, can then be analysed by electrophoresis (Figure 1). The colours of the final peaks are determined by the specific genotype at this locus, making it possible to identify the base variation. The mutation site can thus be reliably differentiated between homozygous wild type and mutant (one peak of a specific colour) or heterozygous. In the latter case, two differently coloured peaks occur in the electropherogram, one derived from the normal base and the other from the mutated base. The size of each peak is determined by the length of the primer, as well as by the specific dye-labelled ddNTP incorporated, whose chemical structure produces different electrophoretic mobility. This results in the visualization of two separate peaks in heterozygous samples and not just two superimposed peaks of different colours, as could be expected.

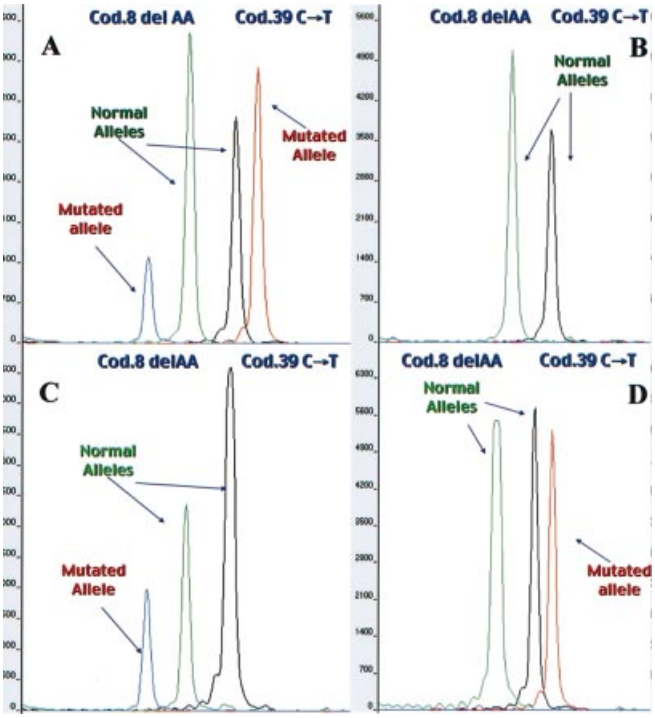

Figure 5. Minisequencing results of a β-thalassaemia PGD case, in which the mutations of interest were Cod.8 del-AA and Cod.39 C→T, analysed by a multiplex reaction. (**A**) Compound heterozygote blastomere for the two mutations; (**B**) normal blastomere; (**C**) heterozygote blastomere for Cod.8 del-AA; (**D**) heterozygote blastomere for Cod.39 C→T. The vertical scale shows the fluorescence intensity (RFU). The minisequencing primers used were as follows: 5′-$(T)_{10}$TGCACCTGACTCCTGAGGAG-3′ for Cod.8 del-AA mutation; 5′-$(T)_{16}$TGGTGGTCTACCCTTGGACC-3′ for Cod.39 C/T mutation. In our design, a 10 bp tail of dTTP was added to the former to its 5′ end, while a 16 bp tail of dTTP was added to the latter. Thus, multiplex single base extension produces minisequencing products that differ significantly in size, so that it is possible to distinguish easily the two different mutation sites in a single capillary electrophoresis run.

The minisequencing method involves the use of a fluorescence-based DNA mutation analysis protocol. When compared with conventional methods, fluorescence-based protocols offer many distinct advantages for clinical PGD. Firstly, fluorescence permits PGD sensitivity, as well as accuracy and reliability, to be substantially increased (Findlay *et al*., 1995; 1998). Secondly, packaged computer software allows easy data interpretation and management. Furthermore, fluorescence-based systems are highly amenable to multiplexing, which has great potential regarding detection of multiple mutation sites.

The present study demonstrated that this strategy can be accurate enough to distinguish different kinds of mutations, such as single nucleotide substitution and small deletions or insertions. In our experiments, the amplified PCR products from patients' DNA samples, single lymphocytes and clinical blastomeres matched the expected genetic status. For single lymphocytes, the mutation profiles were identical to the genotypes of the patients, while in DNA samples the minisequencing results perfectly overlapped those obtained with sequence analysis. The same was observed in PGD cases where there was an exact correspondence between minisequencing and sequencing results in the blastomeres investigated, thereby indicating that the

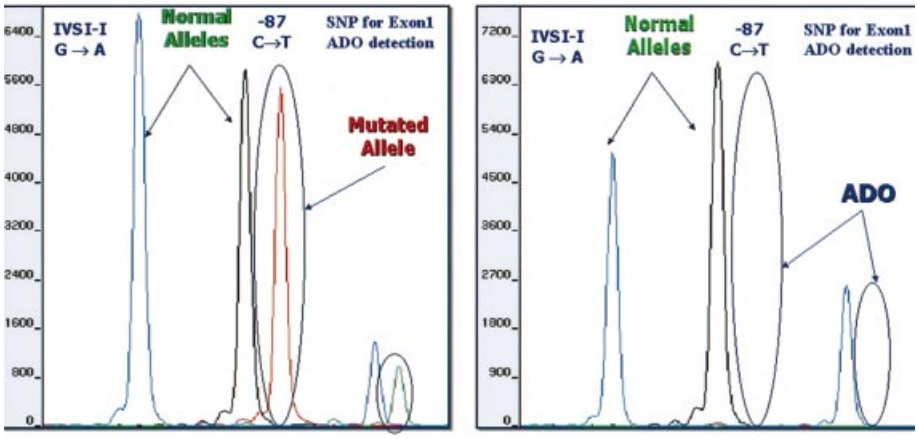

Figure 6. Allele drop-out (ADO) detection by means of the minisequencing method. Simultaneous investigation of IVSI-1 G→A, -87 C→T and of an internal single nucleotide polymorphism (C/T) located in exon 1, codon 2, of β-globin gene, inside the amplified PCR fragment. (**A**) Blastomere heterozygote for -87 C→T mutation without ADO, and (**B**) another blastomere from the same embryo with ADO of a mutated allele. ADO is highlighted by the absence of the red peak (mutated allele of -87 C→T mutation) and the green peak (one allele of the SNP locus). The following minisequencing primers were used: 5′-CCTGTCTTGTAACCTTGATACCAA-3′ for IVSI-1 G→A mutation; 5′-GGAGTAGATTGGCCAACCCTAG-3′ for -87 C→T mutation; 5′-GCAGACTTCTCCTCAGGAGTCAG-3′ for SNP. The latter primer was designed to anneal to the negative (–) DNA strand, because the positive (+) DNA strand is difficult to assay, thus the relative bases are G (blue peak) and A (green peak).

results obtained were reliable and corresponding to the true genotypes of the embryos (Figure 4).

Moreover, the results of PGD were confirmed by later reanalysis of non-transferred embryos and by prenatal diagnosis of the ensuing pregnancies. Fifteen clinical pregnancies (15/55, 27%) resulted from the above PGD cases (Table IV), 10 healthy babies were delivered, while the remaining five pregnancies are ongoing.

The minisequencing method has proven to be extremely efficient, as it provides a higher rate of interpretable results in the investigated blastomeres (96.5%, 856/887), compared to sequence analysis (86.7%, 769/887; $P < 0.001$).

Sensitivity tests demonstrated that minisequencing can be powerful enough to be performed on very weak PCR products, obtaining informative results with as little as 1 ng of PCR product. Its elevated sensitivity explains the higher success rate obtained compared to sequence analysis (96.5 versus 86.7%), since with the latter method, mutation analysis failure may occur when it is performed on 'low quality' (i.e. smeared or very weak) PCR products. Non-interpretable results after minisequencing were obtained in 31 blastomeres due to the presence of background extra-peaks covering the expected peaks constituting the blastomere genotype. The presence of these artefacts was caused by sub-optimal purification of PCR products leading to incomplete removal of some PCR reagents, such as primers and dNTP. Regarding this point, it is important to keep in mind that minisequencing is designed as a primer-driven reaction, and is especially sensitive to the presence of residual primers (i.e. inner PCR primers). PCR primers that have not been removed can participate in the primer extension reaction and resemble signals derived from the minisequencing interrogation primer.

The minisequencing protocol involves computer-assisted mutation analysis, allowing exact base identity determination and computer-assisted visualization of the specific mutation(s), thus facilitating data interpretation and management, and reducing sources of error. This occurs without the need to sequence the entire PCR product, and the same qualitative characteristics of sequence analysis are maintained.

The main benefit of minisequencing is the use of a mutation analysis protocol based on a common procedure for each mutation to be analysed. This is very important for the PGD of genetic diseases characterized by a heterogeneous spectrum of mutations identified, such as cystic fibrosis, β-thalassaemia or haemophilia A. In fact, for these kinds of monogenic diseases, the use of a common procedure able to detect a wide spectrum of mutations and compound genotypes becomes more feasible. The development of different PGD protocols specific for each mutation to be analysed is thus avoided. With the use of minisequencing for mutation detection, the PGD strategy is the same for the diverse clinical cases involving different mutations: external and nested PCR, purification of PCR products, minisequencing reaction and capillary electrophoresis. The only difference between one PGD case and another that involves PCR amplification of the same DNA region, but with a different mutation panel, lies in the use of different minisequencing primers that can be easily designed and whose application does not require the need for extensive trial testing.

Another useful feature of minisequencing is that different mutation sites can be investigated simultaneously ('multiplexing'), even if they are located in different regions of the gene, depending on the number of primers used in the minisequencing reaction. In multiplex reactions, multiple primer/template combinations are used in a single tube reaction format, and products are analysed in a single electrophoresis run. However, the primers corresponding to different mutation sites need to differ significantly in size, so that users can distinguish different loci from the relative sizes of the final products. In fact, what is run out on the machine is the primer plus one nucleotide added during the single base extension. Adding non-homologous polynucleotide tails (for example poly-dTTP) of different lengths onto the primers equates to multiplex products having differing sizes. The size differential between products results in spatial separation of the peaks. If tails were not added to the primers and primers were of similar length, all the products would run out at the same time.

Using the minisequencing method, we were able to identify all mutation sites investigated with optimum discrimination in the same reaction and electrophoresis run. This feature is very useful and significantly reduces analysis time compared with the sequencing method. The primers for the multiplex minisequencing were designed to differ significantly in size so that it was possible to distinguish the two different mutation sites from the relative sizes of the final products. This was performed by adding Poly-T tails of different lengths to 5′ ends of each primer. In our design, for example, the minisequencing primer for Cod.8 del-AA mutation has a 10 bp tail of dTTP, while the minisequencing primer for Cod.39 C/T has a 16 bp tail of dTTP. Thus, multiplex single base extension gives minisequencing products of 31 and 37 bp respectively, that can easily be distinguished in a single capillary electrophoresis run resulting in spatial separation of the peaks (Figure 5).

Minisequencing could be particularly helpful in PGD cases where the mutation(s) involved is difficult to assess by restriction analysis because it does not alter a restriction endonuclease site. Point mutations that do not create or destroy a restriction site provide challenges to accurate genotyping in a single cell analysis context. Several techniques, such as ARMS and site-specific mutagenesis (SSM), have been used in such situations (Strom *et al*., 1998). The first method employs allele-specific primers for the mutant and normal sequence; the second assay involves synthesis of PCR primers creating an artificial restriction site. Additional techniques, such as SSCP, DGGE or sequence analysis, can be used. Minisequencing could represent an alternative approach applicable in these circumstances.

Another important feature of minisequencing is its adaptability in ADO detection as well. ADO is a major concern of single cell PCR; it results in the amplification failure of one allele. At present, there is not a single protocol that has been able to eliminate ADO completely. Advances in single cell genetic analysis have prompted different groups to address the problem of ADO. Multiplex PCR reactions, with the inclusion of one or more closely linked polymorphic markers have been used to act as an internal control for ADO (Wu *et al*., 1993; Ao *et al*., 1998; Kuliev *et al*., 1998; Rechitsky *et al*., 1998). Mutation analysis methods such as DGGE (Kanavakis *et al*., 1999;Vrettou *et al*., 1999) and SSCP (El-Hashemite *et al*., 1997) were also used for monitoring the occurrence of ADO by simultaneous detection of both alleles that contribute to the genotype. In practice, only embryos with a normal electrophoretic pattern are considered to be unaffected and suitable for transfer, preventing misdiagnosis even if ADO has occurred. Moreover, the use of multiplex fluorescent PCR was proposed to reduce apparent occurrence of ADO, since detection may be 1000-fold more sensitive compared to conventional PCR, distinguishing between cases of true ADO and extreme preferential amplification (Findlay *et al*., 1995; 1998).

Another reliable method for detecting ADO could be the simultaneous amplification, together with the mutations of interest, of an informative single nucleotide polymorphism (SNP). SNP account for 85% of the genetic variability in the human population and represent the most frequent type of DNA variation. They are thought to be present every several hundred bases, on average, throughout the human genome. As SNP are plentiful with respect to STR markers, it is sometimes easier to find an informative SNP inside a specific gene than a linked STR, despite the lower degree of heterozygosity

presented by the former. Furthermore, the identification and work-up of informative linked markers can be labour intensive and may not always be cost-effective for all diseases. Therefore, with the near completion of the human genome project, single nucleotide polymorphisms can become the marker of choice for ADO detection.

A protocol including simultaneous amplification of the region involved by mutation and an SNP marker located within the gene under investigation has already been used for ADO detection (Abou-Sleiman *et al*., 2002). Similarly, ADO evaluation could be performed by using SNP markers located inside the PCR fragment to be amplified. The latter strategy can be more informative than the former, because in this way it is possible to obtain direct evidence of occurred, and not only deduced, ADO.

With minisequencing, multiplex mutation analysis and ADO detection can be performed in the same primer extension reaction and electrophoresis run (Figure 6). In our PGD cases, when SNP on different genes investigated were not informative, we performed fluorescent PCR by using linked polymorphic markers. In the case of SMA diagnosis, ADO was not evaluated since amplification failure would not lead to the transfer of an affected embryo but only to misdiagnosis of a healthy heterozygous embryo, which would appear affected and would not be transferred. The latter possibility can be substantially reduced by separately genotyping two single blastomeres for each embryo.

Minisequencing is also practical for the detection of deletion or insertion mutations, associated with a single base substitution. Although the former mutation type can be analysed with other similarly sensitive methods, for example fluorescent PCR, we found it more practical to use a common procedure for both types of mutation and, as mentioned above, to investigate an SNP for ADO detection in the same reaction. Nevertheless, compared to other previously reported methods such as ARMS, SSCP and DGGE, minisequencing presents the inconvenience of requiring a purification step and an extra thermal cycling phase, that increase analysis and working time, and the risk of mishandling. The entire PGD procedure involving minisequencing takes an average time of 13 h, comparable with other reported techniques.

In conclusion, minisequencing, in combination with automated analysis and the possibility of multiplexing, simplifies single cell DNA analysis. Minisequencing has proven to be a very useful method in PGD analysis, due to its elevated sensitivity, automation, and easy data interpretation. Moreover minisequencing has been shown to be a reliable and generally applicable alternative for mutation-directed PGD protocols, irrespective of the mutations involved. Its applicability to PGD could be helpful, especially in cases where the mutation(s) involved is difficult to assess by restriction analysis or other commonly used methods.

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