

# Simple and Easy to Perform Preimplantation Genetic Diagnosis for $\beta$ -thalassemia Major Using Combination of Conventional and Fluorescent Polymerase Chain Reaction

## Abstract

**Background:** Thalassemias are the most common monogenic disorders in many countries throughout the world. The best practice to control the prevalence of the disease is prenatal diagnosis (PND) services. Extensive practicing of PND proved effective in reducing new cases but on the other side of this success high abortion rate is hidden, which ethically unfair and for many couples, especially with a previous experience of a therapeutic abortion, or moral concerns, is not a suitable choice. Preimplantation genetic diagnosis (PGD) is a strong alternative to conventional PND. At present PGD is the only abortion free fetal diagnostic process. Considering the fact that there are more than 6000 single gene disorders affecting approximately 1 in 300 live-births, the medical need for PGD services is significant. **Materials and Methods:** In the present study development of a PGD protocol for a thalassemia trait couple using nested multiplex fluorescent polymerase chain reaction (PCR) for the combination of polymorphic linked short tandem repeat (STR) markers and thalassemia mutations is described. Restriction fragment length polymorphism used to discriminate between wild and mutated alleles. **Results:** In PGD clinical cycle, paternal and maternal alleles for D11S988 and D11S1338 STR markers were segregated as it was expected. PCR product for IVSII-1 mutation was subsequently digested with Btscl restriction enzyme to differentiate normal allele from the mutant allele. The mother's mutation, being a comparatively large deletion, was detectable through size differences on agarose gel. **Conclusion:** The optimized single cell protocol developed and evaluated in this study is a feasible approach for preimplantation diagnosis of  $\beta$ -thalassemia in our patients.

**Keywords:**  $\beta$ -thalassemia, nested fluorescent polymerase chain reaction, polymorphic markers, preimplantation genetic diagnosis

## Introduction

$\beta$ -thalassemia (OMIM no. 613985) is a worldwide prevalent autosomal recessive inherited anemia. Various mutations in the  $\beta$ -globin gene leading to decreased production or complete absence of  $\beta$  chain of hemoglobin (Hb). Recent surveys suggest that about 400,000 infants are born with a serious Hb disorder each year. Although due to inappropriate registration and data collection in many countries the actual figure expected to be much higher than this, but with the available affected birth rate estimate, it is convincing that the disease is considerably affecting the global health resources.<sup>[1]</sup> The molecular basis of  $\beta$ -thalassemia is considerable number of mutations, reportedly to be more than 200, affecting the quantity of  $\beta$ -globin chains.<sup>[2]</sup>

$\beta$ -thalassemia major patients suffer from severe anemia with abnormally ineffective

erythropoiesis, erythroid marrow expansion, osteopenia, and bone deformities. Repeated blood transfusion for maintaining Hb level result in iron overload, and the need for chelation therapy. However, majority of patients will ultimately develop organ damage, in particular, the heart and liver, with reduced life quality and expectancy.<sup>[3]</sup>

In order to reduce the massive burden imposed to patients, their families and the entire society,  $\beta$ -thalassemia prevention programs have been launched and are active in almost all countries with high thalassemia prevalence.<sup>[4,5]</sup> Prenatal diagnostic (PND) testing using either chorionic villus sampling and/or amniocentesis, followed by termination of affected pregnancies are routinely practiced in many countries.<sup>[6]</sup> Despite the desirable achievements, large decrease in global birth rates of affected

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

**How to cite this article:** Salehi R, Khosravi S, Salehi M, Kheirollahi M, Khanahmad H. Simple and Easy to Perform Preimplantation Genetic Diagnosis for  $\beta$ -thalassemia Major Using Combination of Conventional and Fluorescent Polymerase Chain Reaction. *Adv Biomed Res* 2017;6:23.

**Received:** January, 2015. **Accepted:** March, 2015.

**Rasoul Salehi,  
Sharifeh Khosravi,  
Mansour Salehi,  
Majid Kheirollahi,  
Hossein  
Khanahmad**

*From the Department of  
Genetics and Molecular Biology,  
School of Medicine, Isfahan  
University of Medical Sciences,  
Isfahan, Iran*

**Address for correspondence:**  
Dr. Rasoul Salehi,  
Department of Genetics and  
Molecular Biology, School of  
Medicine, Isfahan University of  
Medical Sciences, Isfahan, Iran.  
E-mail: r\_salehi@med.mui.ac.ir

Access this article online

Website: www.advbiores.net

DOI: 10.4103/2277-9175.201682

Quick Response Code:



babies is indicative of high abortion rate, which is not a suitable choice for many couples. For those who have the devastating experience of a previous abortion therapy following a PND with affected fetus or due to ethical or religious beliefs, an abortion-free choice should morally be available. Under these circumstances preimplantation genetic diagnosis (PGD) is the only possible alternative.<sup>[7]</sup> In PGD, although various method of sampling is available, but usually one or two blastomers are biopsies from the cleavage stage embryos for the purpose of genetic diagnosis. As the diagnosis takes place before embryo transfer, pregnancy termination is avoided.<sup>[8]</sup>

However, the main concern with PGD is the high possibility of misdiagnosis due to, mainly allele drop-out (ADO) and contamination. ADO is an extreme form of preferential amplification where one of the two alleles is fail to amplify. For example hemizygous loci under these circumstances result in complete absence of a polymerase chain reaction (PCR) product. Thus, the highest accuracy for PGD can only be achieved when the diagnosis is confirmed for both noninheritance of mutated allele(s) and presence of the wild allele(s). Multiplexing PCR for the mutation(s) with linked informative polymorphic short tandem repeat markers have shown to be quite helpful for the detection of both contamination and ADO.<sup>[9,10]</sup>

The causes of ADO are not well understood, but the most important issues receiving attention are: The cell lysis method, PCR conditions, the sequence of the template DNA, the size of the PCR products, and the degradation of target DNA molecule.<sup>[11-15]</sup>

Development of sensitive detection methods such as fluorescent PCR<sup>[16]</sup> followed by automated analysis using computer software and a higher denaturing temperature in PCR<sup>[11]</sup> are effective measures for reducing the rate of ADO, thereby improving the diagnostic accuracy.

In this study, we report development of simple and efficient multiplex single cell PCR protocol for one  $\beta$ -thalassemia trait couple using nested fluorescent PCR followed by restriction enzyme digestion for detection of the normal and mutated alleles. The protocol after extensive validations on single lymphocytes of patients and spare embryos were applied in clinical PGD cycles for this couple. Using complicated protocols needing sophisticated instrumentation or technology restrict the general use of PGD in developing countries. Simple protocols with desired level of sensitivity would be helpful to establish PGD setup in more centers and countries than being offered presently.

## Materials and Methods

### Patients

Here we describe the PGD clinical cycle offered to one of our couples already confirmed to be carrier of  $\beta$ -thalassemia mutation with a previous therapeutic abortion following

a PND procedure. Mother's mutation was IVSI-25 bp deletion and the father mutation was IVSII-1 (G>A).

### Ovarian stimulation and oocyte retrieval

Controlled ovarian stimulation was carried out in an agonist protocol, using gonadotrophin-releasing hormone analogues along with human menopausal gonadotropins or recombinant follicle-stimulating hormone.<sup>[17,18]</sup> Transvaginal ultrasound-guided oocyte harvest was performed 36 h after hCG administration.

### Intracytoplasmic sperm injection and embryo biopsy procedure

The details of the *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedure have been described previously.<sup>[19]</sup> Regardless of the sperm quality, ICSI was the method of choice rather than classical IVF to prevent DNA contamination with sperm DNA.<sup>[20]</sup>

### Lymphocyte preparation

Lymphocytes were separated by centrifugation through ficollpaque from 10 ml of blood with EDTA anticoagulant, as instructed by the manufacturer. The isolated lymphocytes were then washed in droplets of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium, supplemented with 15 mg/ml bovine serum albumin under a layer of mineral oil. Individual single cells were collected under an inverted microscope and transferred into a 0.2 ml thin-wall PCR tube, containing 2.5  $\mu\text{l}$  alkaline lysis buffer (200 mM KOH, 50 mM dithiothreitol). A few microliters of the medium from the last droplet were collected in a second PCR tube to serve as blank. Prior to PCR amplification, the tubes were heated at 65°C for 10 min, to lyse the cells. The blastomers were obtained from spare embryos that were not suitable for transfer or cryopreservation. The embryos were sequentially transferred to two droplets of acidic Tyrode's under oil, with a finely drawn Pasteur-pipette and left until the zona pellucid disappeared. The embryos were then transferred to a  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free medium and blastomers were separated by gentle up and down blowing. These single blastomers were then washed in fresh droplets of medium and transferred to PCR tubes containing 2.5  $\mu\text{l}$  of alkaline lysis buffer.

### Polymerase chain reaction conditions

Five linked microsatellite polymorphic markers flanking the Hb gene, namely D11S1338, D11S1760, D11S1997, D11S4181, and D11S988, were selected to use in this study. These markers proved to be highly informative; with high polymorphism information content score and a low recombination rate in Iranian population. Initial optimization of PCR protocol and informativity tests for selected markers were carried out on 100 ng genomic DNA from each individual. Subsequently, the method was applied to at least 50 single lymphocytes for the informative polymorphic markers combined with mutation specific primers and parameters such as contamination,

ADO and amplification rate were calculated. The efficacy of the method was tested on at least 10 single spare blastomers in the same way as it was carried out for the lymphocytes. Appropriate precautions were taken at all the stages to avoid contamination and appropriate genomic DNA and single cells from both heterozygous parents were included in every PCR round.

For PGD workout a two rounds triplex PCR protocol was used in which the product of first round was used as template for the second round. Primers for the first round PCR was a pair of  $\beta$ -globin specific oligonucleotide primers amplifying a larger region harboring the mutation of interest in combination with primers specific for the informative markers [Table 1]. The reaction mix for the first round contained 1x neutralization buffer (90 mM Tris-HCl pH 8.3, 30 mM KCl and 20 mM HCl) 50 mM KCl, 100 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatin, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 10 pmole of primers and 1.25 U AmpliTaq Gold. The second round PCR was performed separately for mutations and markers using 3  $\mu$ l of the first round triplex PCR as template for the second round PCR amplification. All labeled primers contained Cy5 label at their 5' termini [Table 2].

After second round PCR amplification, in order to identify paternal mutation, 10  $\mu$ l of PCR product was digested

**Table 1: Mutations of parents and the linked STR markers included in the study**

Mutations in parents' beta globin gene	STR markers evaluated	Selected STR markers used for PGD
Father: IVSII-1 (G>A)	D11S1338,	D11S1338
Mother: IVSI-25 bp del	D11S1760, D11S1997, D11S4181 and D11S988	D11S988

PGD: Preimplantation genetic diagnosis, STR: Short tandem repeat

by *Btscl* (*BseGI*) restriction enzyme for 2 h at 37°C. The reverse second round primer was modified in such a way to create restriction site on mutant allele for *Btscl* enzyme. Subsequently, digestion profiles were checked by agarose gel electrophoresis.

### Analysis of polymerase chain reaction products

Three microliters of the fluorescent labeled amplified DNA was mixed with an equal volume of loading dye (0.5% dextran blue in 100% formamide) and denatured by heating at 90°C for 5 min and immediately transferred on ice before its loading on 6% denaturing sequencing grade polyacrylamide gel (Amersham Biosciences, UK). Electrophoresis was done on an Automated Laser Fluorescence Express DNA sequencer (ALF express) from Pharmacia Biotech, Sweden. Fragment analysis was carried out using AlleleLinks software (Pharmacia Biotech) provided by the ALF express DNA sequencer manufacturer.

### Embryo transfer procedure

Embryo transfer into the uterus was carried out on day 3–5 postinsemination as in regular IVF cycles.

### Results

Incorporation of fluorescent labeled primers into the PCR products makes the subsequent automated analysis of the fragments possible, through an excitation/detection system. The DNA fragments are recorded as peaks of variable amplitudes corresponding to their concentrations. Using polymorphic linked markers amplification along with the patient's mutation, provide the opportunity to ascertain about ADO, contamination and probable recombination. Each triplex PCR was applied on at least 50 single lymphocytes and the rates of amplification, contamination and ADOs were determined. Efficiency and reproducibility of the results were carried out on spare blastomers as well.

**Table 2: Primers, restriction enzymes, digestion specificity and fragment sizes following restriction digests**

First round nested PCR primers (mutation + markers)	Second round PCR primers for mutations	Restriction enzyme used	Digestion specificity and fragment size (bp)
$\beta$ -globin locus F: 5'-CTTAGGGTTTGAAGTCAACTC-3' R: 5'-CTGTACCCTGTTACTTCTCCCCTC-3'	Mother's mutation F: 5'-GCAAGGTGAACGTGGACGAAG-3' R: 5'-AACAGCATCAGGAGTGGACAG-3'		
Microsatellite locus D11S988 F: 5'-GTAAGGATGCCCTCAAGTGG-3' (Fl)	Father's mutation F: 5'-AGGTTGGCATCAAGGTTACAAGACA-3' <i>Btscl</i>		Mutant allele digestion only; 360 bp +136 bp
D11S1338 F: 5'-TGGTGTCAGAAATCTGATGG-3' (Fl) R: 5'-TGCTACTTATTTGGAGTGTGAA-3'	R: 5'-ACGATCCTGAGACTTCCACACTGA-3' ( <i>BseGI</i> )		
Fl: 5' Cy5 fluorescent label			

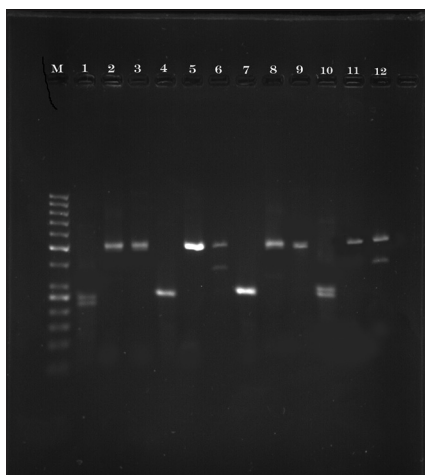
The average amplification rate for beta globin gene fragment was 96% on single lymphocytes when D11S988 and D11S1338 markers were amplified along with the mutation. Polymorphic marker amplification rate was 98% and 90% on single lymphocytes for D11S988 and D11S1338 markers, respectively. The amplification rate was slightly lower on spare blastomers compared to that of single lymphocytes. The results of the clinical PGD cycles are summarized in Table 3.

In PGD clinical cycle PCR product for IVSII-1 mutation was subsequently digested with Btscl restriction enzyme. As it is evident in Figure 1, a 496 bp undigested product is corresponding to the normal allele and restriction fragments of 360 and 136 bp correspond to mutant allele. For maternal mutation, the size differences were sufficiently large that would readily detectable using agarose gel electrophoresis system [Figure 1]. However, paternal and maternal alleles

**Table 3: Summary of PGD clinical cycle offered for this family**

Oocytes, embryos and diagnosis statistics	Numbers
Oocytes retrieved	12
Oocytes fertilized after ICSI	8
Embryos biopsied	8
Embryos diagnosed as	
Normal (N/N)	3
Carrier (N/M)	0
Affected (M/M)	3
Embryos without a diagnosis or with ADO	2
Embryos transferred	1
Outcome	Singleton

PGD: Preimplantation genetic diagnosis, ICSI: Intracytoplasmic sperm injection, ADO: Allele drop-out



**Figure 1: Agarose gel electrophoresis analysis of polymerase chain reaction (PCR) products of embryonic blastomer amplification.** The mother's mutation is IVS1-25 deletion and the father's one is IVSII-1 G>A mutation. A 496 bp PCR product was restricted using Btscl enzyme for discrimination of IVSII-1 mutation. The mutant allele digested in to 360 bp and 136 bp fragments and the normal allele remain uncut. Lane 1, 2, 3 are heterozygous mother, 4, 5, 6 heterozygous father, 7, 8, 9 normal embryo and 10, 11, 12 affected embryo. M is 50 bp ladder

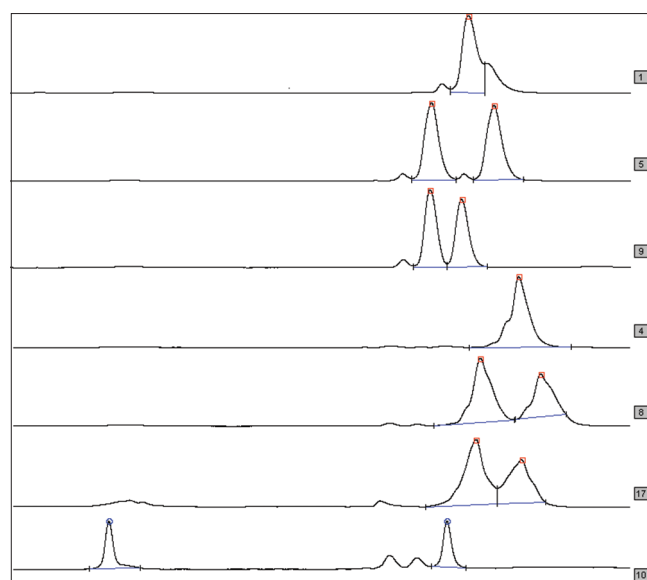
for D11S988 and D11S1338 were segregated as it was expected [Figure 2].

## Discussion

At present the application of PGD is covering a broad range of genetic and nongenetic conditions. Carriers of sex-linked, single gene and mitochondrial disorders, those with chromosomal aberrations, women age 35 and over or experiencing recurring pregnancy loss or those with more than one failed fertility treatment could be eligible cases for PGD. Inherited cardiac diseases,<sup>[21]</sup> cancers,<sup>[22,23]</sup> late-onset disease,<sup>[24]</sup> rare diseases<sup>[25,26]</sup> are some of the examples for this broad coverage.

$\beta$ -thalassemia as a most prevalent monogenic disorder in Iran, and many other countries throughout the world could benefit a lot from PGD, as an abortion free diagnostic procedure. Patients who are not willing to take the risk of abortion following PND with an affected fetus outcome benefit a lot from this strong alternative. The family presented here had experience of a previous abortion therapy and expressed their feeling as the most unpleasant experience of their life. The highly painful memories of the previous miscarriage made them to totally drop the idea of another pregnancy.

In PGD, since our starting material for diagnostic purpose is only a single cell, the strategy is predispose to several inherent pitfalls which considerably increase the risk of misdiagnosis comparing to the conventional PND. For any single cell PCR appropriate measures should taken to eliminate these pitfalls; the most devastating ones are contamination and ADO. Multiplex PCR with linked informative polymorphic markers



**Figure 2: Fragment analysis of selected markers using Automated Laser Fluorescence express instrument.** Lanes 1, 5 and 9 represent amplified polymerase chain reaction fragments of D11S988 microsatellite marker from maternal genomic DNA, paternal genomic DNA and normal embryonic blastomer respectively. Lanes 4, 6 and 17 represent the amplified fragments of D11S1338 marker from maternal genomic DNA, paternal genomic DNA and normal embryonic blastomer, respectively. Lane 10 is size marker

have shown to be quite helpful to overcome and detect these inherent treats of single cell amplification.<sup>[27]</sup>

So far various techniques adopted for PGD with different levels of complexity. These strategies are including real-time PCR with FRET hybridization,<sup>[28]</sup> direct single cell sequencing,<sup>[29]</sup> whole genome amplification,<sup>[30,31]</sup> minisequencing,<sup>[32]</sup> array-comparative genomic hybridization<sup>[33]</sup> and single nucleotide polymorphism microarrays.<sup>[34]</sup> However highly sophisticated procedures are not suitable for practicing in less developed centers.

De Rycke *et al.* compared the results obtained by nonfluorescent and fluorescent PCR for sickle cell anemia and  $\beta$ -thalassemia. The amplification efficiency dropped from 94% to 78% in conventional PCR, while ADO increased from 8% to 33%.<sup>[35]</sup> Findlay *et al.* obtained a low ADO rate of about 4% due to increased sensitivity using fluorescent PCR.<sup>[15]</sup>

In addition PGD can be combined with human leukocyte antigen (HLA) typing together with mutation detection to find an HLA compatible newborn for saving an affected sibling's life.<sup>[36]</sup> Preimplantation testing for HLA typing is an alternative approach of PGD with combined purpose to create a pregnancy with unaffected child and at the same time HLA-matched with the existing affected sibling. Achieving the most success in stem cell transplantation depend on finding an HLA identical healthy sibling to reduce the incidence of bone marrow transplantation complication, but because of limited number of children in families it is estimated that only 25% of patients are able to find an HLA matched donor among family members. Reports from 2 centers in Belgium and Italy for HLA typing combined with PGD covers the following diseases; sickle cell disease,  $\beta$ -thalassemia, Fanconi anaemia, Wiskott–Aldrich' syndrome, chronic granulomatous disease, Duncan syndrome, hyper IgM syndrome, mannosidosis alpha 1, Hurler syndrome, Gaucher disease, Bruton agammaglobulinaemia, Glanzmann thrombasthenia, adrenoleukodystrophy, and for HLA-only typing; acute lymphoblastic leukaemia, severe aplastic anaemia, Diamond–Blackfan anaemia and histiocytosis<sup>[37]</sup>

### Acknowledgments

The financial support of Vice Chancellor for Research, Isfahan University of Medical Sciences is greatly acknowledged.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

### References

- Williams TN, Weatherall DJ. World distribution, population genetics, and health burden of the hemoglobinopathies. *Cold Spring Harb Perspect Med* 2012;2:a011692.
- Hardison RC, Chui DH, Riemer CR, Miller W, Carver MF, Molchanova TP, *et al.* Access to a syllabus of human hemoglobin variants (1996) via the World Wide Web. *Hemoglobin* 1998;22:113-27.
- Olivieri NF. The beta-thalassemsias. *N Engl J Med* 1999 8;341:99-109.
- Abolghasemi H, Amid A, Zeinali S, Radfar MH, Eshghi P, Rahiminejad MS, *et al.* Thalassemia in Iran: Epidemiology, prevention, and management. *J Pediatr Hematol Oncol* 2007;29:233-8.
- Liao C, Mo QH, Li J, Li LY, Huang YN, Hua L, *et al.* Carrier screening for alpha-and beta-thalassemia in pregnancy: The results of an 11-year prospective program in Guangzhou Maternal and Neonatal hospital. *Prenat Diagn* 2005;25:163-71.
- Najmabadi H, Ghamari A, Sahebjam F, Kariminejad R, Hadavi V, Khatibi T, *et al.* Fourteen-year experience of prenatal diagnosis of thalassemia in Iran. *Community Genet* 2006;9:93-7.
- Järholmab S, Broberg M, Thurin-Kjellberga A. The choice of pre-implantation genetic diagnosis (PGD), a qualitative study among men and women. *J Reprod Infant Psychol* 2014;32:57-69.
- Kanavakis E, Traeger-Synodinos J. Preimplantation genetic diagnosis in clinical practice. *J Med Genet* 2002;39:6-11.
- Wells D, Sherlock JK. Strategies for preimplantation genetic diagnosis of single gene disorders by DNA amplification. *Prenat Diagn* 1998;18:1389-401.
- Sermon K. Current concepts in preimplantation genetic diagnosis (PGD): A molecular biologist's view. *Hum Reprod Update* 2002-Feb; 8:11-20.
- Ray PF, Handyside AH. Increasing the denaturation temperature during the first cycles of amplification reduces allele dropout from single cells for preimplantation genetic diagnosis. *Mol Hum Reprod* 1996;2:213-8.
- El-Hashemite N, Delhanty JD. A technique for eliminating allele specific amplification failure during DNA amplification of heterozygous cells for preimplantation diagnosis. *Mol Hum Reprod* 1997;3:975-8.
- Thornhill AR, McGrath JA, Eady RA, Braude PR, Handyside AH. A comparison of different lysis buffers to assess allele dropout from single cells for preimplantation genetic diagnosis. *Prenat Diagn* 2001;21:490-7.
- Vrettou C, Palmer G, Kanavakis E, Tzetis M, Antoniadis T, Mastrominas M, *et al.* A widely applicable strategy for single cell genotyping of beta-thalassaemia mutations using DGGE analysis: Application to preimplantation genetic diagnosis. *Prenat Diagn* 1999;19:1209-16.
- Findlay I, Ray P, Quirke P, Rutherford A, Lilford R. Allelic drop-out and preferential amplification in single cells and human blastomeres: Implications for preimplantation diagnosis of sex and cystic fibrosis. *Hum Reprod* 1995;10:1609-18.
- Findlay I, Quirke P, Hall J, Rutherford A. Fluorescent PCR: A new technique for PGD of sex and single-gene defects. *J Assist Reprod Genet* 1996;13:96-103.
- Van de Velde H, De Vos A, Joris H, Nagy ZP, Van Steirteghem AC. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1998;13:3160-4.
- Kolibianakis EM, Zikopoulos K, Verpoest W, Camus M, Joris H, Van Steirteghem AC, *et al.* Should we advise patients undergoing IVF to start a cycle leading to a day 3 or a day 5 transfer? *Hum Reprod* 2004;19:2550-4.

19. Van Landuyt L, De Vos A, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Blastocyst formation in *in vitro* fertilization versus intracytoplasmic sperm injection cycles: Influence of the fertilization procedure. *Fertil Steril* 2005;83:1397-403.
20. Liebaers I, Sermon K, Staessen C, Joris H, Lissens W, Van Assche E, *et al.* Clinical experience with preimplantation genetic diagnosis and intracytoplasmic sperm injection. *Hum Reprod* 1998;13 Suppl 1:186-95.
21. Kuliev A, Pomerantseva E, Polling D, Verlinsky O, Rechitsky S. PGD for inherited cardiac diseases. *Reprod Biomed Online* 2012;24:443-53.
22. Quinn GP, Vadapampil ST, Bower B, Friedman S, Keefe DL. Decisions and ethical issues among BRCA carriers and the use of preimplantation genetic diagnosis. *Minerva Med* 2009;100:371-83.
23. SenGupta SB, Vadapampil ST, Menon U. Preimplantation genetic diagnosis for hereditary cancers. *Adv Exp Med Biol* 2012;732:103-13.
24. Noble R, Bahadur G, Iqbal M, Sanyal A. Pandora's box: Ethics of PGD for inherited risk of late-onset disorders. *Reprod Biomed Online* 2008;17 Suppl 3:55-60.
25. Alberola TM, Vendrell X, Bautista-Llácer R, Vila M, Calatayud C, Pérez-Alonso M. Successful application of preimplantation genetic diagnosis for hypokalaemic periodic paralysis. *Reprod Biomed Online* 2010;21:206-11.
26. Altarescu G, Brooks B, Eldar-Geva T, Margalioth EJ, Singer A, Levy-Lahad E, *et al.* Polar body-based preimplantation genetic diagnosis for N-acetylglutamate synthase deficiency. *Fetal Diagn Ther* 2008;24:170-6.
27. Wilton L, Thornhill A, Traeger-Synodinos J, Sermon KD, Harper JC. The causes of misdiagnosis and adverse outcomes in PGD. *Hum Reprod* 2009;24:1221-8.
28. Harper JC, Sengupta SB. Preimplantation genetic diagnosis: State of the art 2011. *Hum Genet* 2012;131:175-86.
29. Hussey ND, Davis T, Hall JR, Barry MF, Draper R, Norman RJ, *et al.* Preimplantation genetic diagnosis for beta-thalassaemia using sequencing of single cell PCR products to detect mutations and polymorphic loci. *Mol Hum Reprod* 2002;8:1136-43.
30. Shen X, Xu Y, Zhong Y, Zhou C, Zeng Y, Zhuang G, *et al.* Preimplantation genetic diagnosis for  $\alpha$ - and  $\beta$ -double thalassaemia. *J Assist Reprod Genet* 2011;28:957-64.
31. Langmore JP. Rubicon Genomics, Inc. *Pharmacogenomics* 2002;3:557-60.
32. Fiorentino F, Magli MC, Podini D, Ferraretti AP, Nuccitelli A, Vitale N, *et al.* The minisequencing method: An alternative strategy for preimplantation genetic diagnosis of single gene disorders. *Mol Hum Reprod* 2003;9:399-410.
33. Vanneste E, Melotte C, Voet T, Robberecht C, Debrock S, Pexsters A, *et al.* PGD for a complex chromosomal rearrangement by array comparative genomic hybridization. *Hum Reprod* 2011;26:941-9.
34. Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara N, Shaw MA, *et al.* Karyomapping: A universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet* 2010;47:651-8.
35. De Rycke M, Van de Velde H, Sermon K, Lissens W, De Vos A, Vandervorst M, *et al.* Preimplantation genetic diagnosis for sickle-cell anemia and for beta-thalassaemia. *Prenat Diagn* 2001;21:214-22.
36. Figueira RC, Setti AS, Cortezzi SS, Martinhago CD, Braga DP, Iaconelli A, Jr. *et al.* Preimplantation diagnosis for beta-thalassaemia combined with HLA matching: First "savior sibling" is born after embryo selection in Brazil. *J Assist Reprod Genet* 2012;29:1305-9.
37. Van de Velde H, De Rycke M, De Man C, De Hauwere K, Fiorentino F, Kahraman S, *et al.* The experience of two European preimplantation genetic diagnosis centres on human leukocyte antigen typing. *Hum Reprod* 2009;24:732-40.