

## Preimplantation Diagnosis of Thalassemias

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**Purpose:** Preimplantation genetic diagnosis (PGD) is an important option for couples at risk of having children with  $\beta$ -globin mutations to avoid selective abortions of affected fetuses following prenatal diagnosis.

**Methods:** We performed PGD for thalassemia in 12 clinical cycles (IVS1-110, and IVS-745 mutations) using biopsy of the first and second polar bodies (PBs) extruded from oocytes during maturation and fertilization, coupled with nested polymerase chain reaction analysis and restriction digestion.

**Results:** A total of 118 oocytes was obtained, of which 78 had results for both the first and the second PBs. This resulted in the selection and transfer of 30 unaffected embryos (2.5 embryos per cycle). To avoid a possible misdiagnosis due to allele dropout (ADO), we have also introduced simultaneous detection of two highly polymorphic linked markers, a short tandem repeat immediately at the 5' end of the globin gene and HUMTH01 which is a syntenic short tandem repeat. The application of multiplex polymerase chain reaction of the  $\beta$ -globin gene and linked polymorphic markers enabled detection of ADO in five first PBs, thus avoiding the transfer of potentially affected embryos resulting from their corresponding oocytes.

**Conclusions:** Confirmation studies of the embryos resulting from the oocytes predicted to contain an affected gene confirmed the diagnosis in 98% of the cases, thus demonstrating the accuracy and reliability of PB PGD of thalassemia mutations. The application of PB analysis in six patients resulted in two ongoing pregnancies with a thalassemia-free fetus already confirmed in both of them by prenatal diagnosis.

**KEY WORDS:** preimplantation genetic diagnosis; multiplex PCR; thalassemia; polymorphic markers;  $\beta$ -globin gene; allele dropout.

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## INTRODUCTION

Preimplantation genetic diagnosis (PGD) is an option for couples at risk for producing offspring with thalassemia, to avoid termination of an affected fetus following prenatal diagnosis. Some of these couples have the unfortunate experience of undergoing two or more terminations of wanted pregnancies before they have a healthy child. PGD has already been applied to more than 700 couples at risk of having children with single gene and chromosomal disorders resulting in the birth of more than a hundred unaffected children (1). Although PGD for thalassemia and sickle cell disease was performed for only a few cases, it could be used more extensively if it were available in the Mediterranean region and other areas of the world where thalassemias are most prevalent. For example, in Cyprus, where almost no new thalassemia cases have been observed in the past few years, more than a quarter of couples experience two or more pregnancy terminations following prenatal diagnosis (2).

This paper presents the first clinical experience of PGD of thalassemias, demonstrating the accuracy and reliability of the polar body (PB) PGD of  $\beta$ -globin gene mutations.

## MATERIALS AND METHODS

PGD of  $\beta$ -thalassemias was performed in 12 clinical cycles (8 cycles for IVS1-110 and 4 for IVSII-745), using biopsy of the first (I) and second (II) PBs. Oocytes were obtained after a standard in vitro fertilization (IVF) stimulation procedure, and their IPBs were removed and used for polymerase chain reaction (PCR) analysis as described elsewhere (3). Second PBs were removed following fertilization in the same way as IPBs. To avoid possible contamination by

sperm DNA in the follow-up PCR analysis of IIPBs, assisted fertilization by intracytoplasmic sperm injection (ICSI) was applied.

DNA analysis of IPBs and IIPBs was performed using nested PCR to amplify the affected sequence from IPB and IIPB followed by restriction digestion (4). Depending on the thalassemia mutation studied, different primer systems were designed (Table I, Figs. 1 and 2). Because of the possibility of preferential amplification or allele dropout (ADO) in single-cell PCR analysis (4,8,9), simultaneous detection of two highly polymorphic markers, short tandem repeats

(STRs) 5' to the  $\beta$ -globin gene and HUMTH01 (10,11), were performed. The 5' locus is tightly linked to  $\beta$ -globin gene. To identify possible contamination of the IPB and IIPB DNA samples by maternal follicular cells or sperm DNA, two systems for STR loci on chromosome 21 and VWF gene on chromosome 12 were also tested (12,13). The conditions of PCR analysis are presented in Table II.

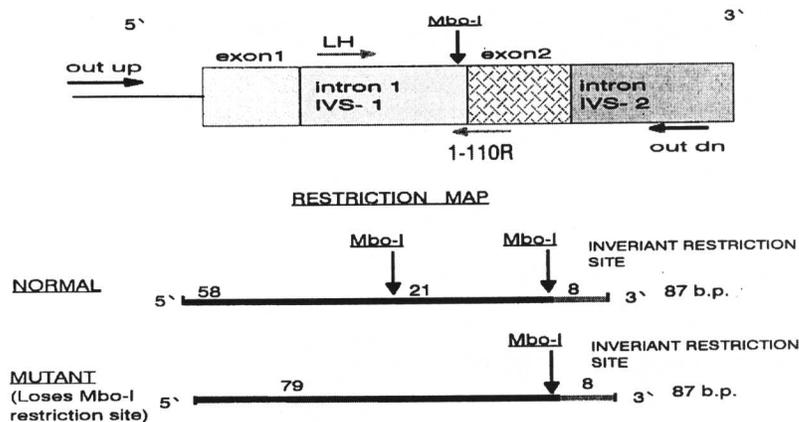
Following nested amplification, PCR products were digested with either *Mbo*I for IVSI110 or *Rsa*I for IVSII 745 and analysis was carried out on 6% polyacrylamide gels. Gels were photographed as described

Table I. Primer Sequences Used

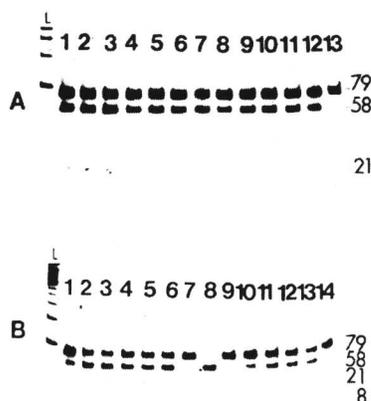
Loci	Primers (5'-3')	Size of product (bp)	Affected restriction endonuclease site	Ref.
Globin				
IVSI - 110				
Outside <sup>a</sup>				
C	ggccaatctactcccaggag	597		(5)
D	acatcaagggtcccatagac		Gains MboI site with mutation-specific LH primer	
Inside				
LH	atgtggagacagagaagactcttgggtt	87		(6)
IVSI- 110R	ccaggatcctaagggtgggaaaatagat			
IVSI-6				
Inside				
N1	cagggcagagccatctattgctta	363		(7)
N2	gcatcaggagtggacagatcccca		Gains SfaNI Site (also polymorphic SfaNI site at codon 2)	
IVSI-1				
Inside				
N1	cagggcagagccatctattgctta	363		(7)
N2	gcatcaggagtggacagatcccca		Loses BspMI site	
Nonsense codon 39				
Inside				
LH	atgtggagacagagaagactcttgggtt	148		(6,7)
N2	gcatcaggagtggacagatcccca		Gains Mael site	
IVSII - 745				
Outside				
THAL 1	ctaactctttcttcagg	454		— <sup>b</sup>
THAL 2	aacctttaatagaattgg			
Inside				
THAL 3	ataacagtataattctgg	362		— <sup>b</sup>
THAL 4	aaagcgaacttagtgatac		Gains RsaI Site	
5' globin STR				
Outside				
H-1	cctgatgagggttgagacag	302-310		— <sup>b</sup>
H-2	ctgccctacctggaaac			
Inside				
H-3	atagaggatccagtttctttg	102-110		— <sup>b</sup>
H-4	agctctaactctgaaactacg			
HumTHO1 STR				
Outside				
THO 6	ttcccaggctctagcagc	222-240		— <sup>b</sup>
THO 7	agctcccattatccagc			
Inside				
THO 4	agggtatctggctctgg	115-131		— <sup>b</sup>
THO 5	ctccgagtcaggtcac			

<sup>a</sup> Same outside primers were used to amplify IVSI-1, IVSI-6, and codon 39 mutations.

<sup>b</sup> Original design.



### Genotyping Single Cells for the IVS1-110 Thalassemia Mutation Using MboI Restriction Digestion



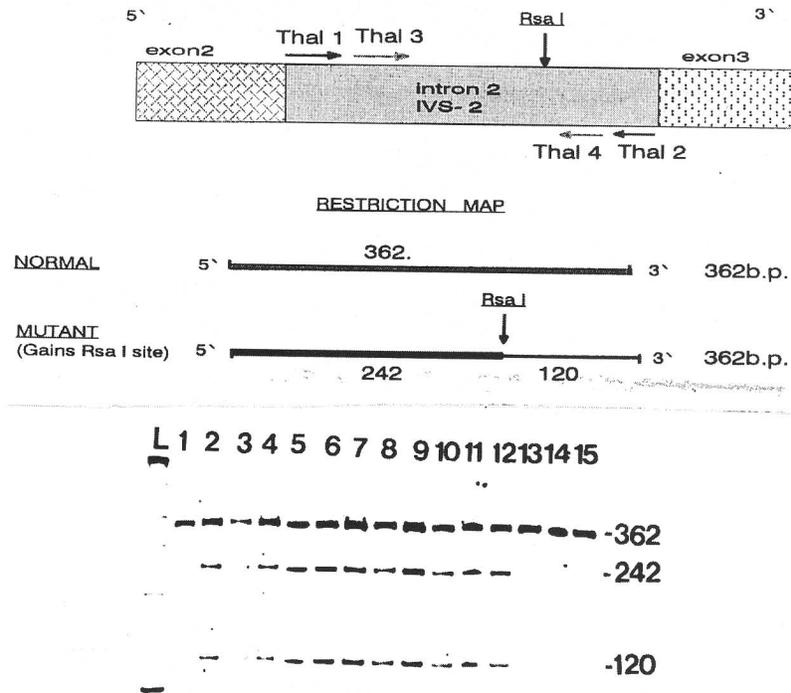
**Fig. 1.** Scheme for nested PCR diagnosis for thalassemia IVS1-110 (g->a) transversion. Top: Diagram of a nested PCR scheme and a restriction map (primer sequences are given in Table I). Bottom: Acrylamide gel analysis of *Mbo*I restriction digestion of heterozygous single fibroblasts (A) and dissociated single blastomeres from one heterozygous embryo (B). 79, undigested product (mutant allele); 58, 58-bp restriction fragment (unaffected allele); 21, 21 bp restriction fragment (unaffected allele); 8, 8-bp invariant restriction fragment (not visible on these photographs) which is used as an internal control for completeness of restriction digestion. Fibroblast 13 demonstrates ADO of normal allele. Blastomeres 7, 9, and 14 demonstrate ADO of normal allele. Blastomere 8 demonstrates ADO of mutant allele.

previously (4). Statistical analyses were performed using chi-square analysis in the computer program Epistat.

The embryos resulting from the oocytes whose IPB and IIPB analysis predicted the presence of unaffected maternal allele were transferred back to woman to achieve a pregnancy, while affected embryos were used for confirmation of the IPB and IIPB diagnosis.

### RESULTS AND DISCUSSION

Before clinical application, the efficiency of amplification by the above multiplex system and ADO rates were studied using single fibroblasts, isolated from patients' skin cultures. The results of amplification efficiency and ADO rates in single fibroblasts obtained from individuals heterozygous for different  $\beta$ -globin



**Fig. 2.** Scheme of a nested PCR diagnosis for thalassemia IVS 2-745 (c->g transversion). Top: Diagram of a nested PCR scheme and a restriction map (primer sequences are given in Table I). Bottom: Acrylamide gel analysis of *Rsa*I restriction digestion of dissociated single blastomeres from one heterozygous embryo. 362, 362-bp undigested (unaffected allele); 242, 242-bp restriction fragment (mutant allele); 120, 120-bp restriction fragment (mutant allele). Blastomeres 1, 13, and 15 demonstrate ADO of mutant allele. Blastomere 3 demonstrates preferential amplification of the unaffected allele.

**Table II.** Optimal Conditions for Nested PCR of Single Cells

	Round I PCR	Round II PCR		
		Separate reactions		
	Multiplex	IVSI-110	IVSI-1 <sup>a</sup>	IVSII-745
MgCl <sub>2</sub>	1.5 mM	1.5 mM	1.5 mM	1.5 mM
Glycerol	10%			
dNTPs (4)	100 μM (each)	50 μM (each)	50 μM (each)	50 μM (each)
PCR buffer		50 mM KCl, 10 mM Tris-HCl, pH 9.0	50 mM KCl, 10 mM Tris-HCl, pH 9.0	50 mM KCl, 10 mM Tris-HCl, pH 9.0
Outside primers	0.2 μM (each)			
Inside primers		0.4 μM (each)	0.4 μM (each)	0.04 μM (each)
Lysis buffer + Neutral buffer (14)	5 μl + 5 μl			
Taq polymerase	1.25 U	1 U	1 U	1 U
Cycles	28	25	25	25
Annealing temp.	45°C	55°C	60°C	45°C
Denaturing temp.	95°C	95°C	95°C	95°C

<sup>a</sup> Conditions for the second-round PCR for mutations IVSI-6, codon 39, 5' globin STR, and HumTHOI were the same as for IVSI-1.

**Table III.** Allele Dropout Rate in Heterozygote Single Fibroblasts with Different Thalassemia Mutations

Mutation	Total No. of cells	No. amplified	ADO total
IVS I-1	39	35 (89.7%)	2 (5.71%)
IVSI-6	91	85 (93.4%)	9 (10.58%)
IVSI-110	289	276 (95.5%)	28 (10.1%)
IVSII-745	50	47 (94%)	3 (6.38%)
Codon 39	45	41 (91.1%)	2 (4.87%)
Total	514	484 (94.1%)	44 (8.5%)

mutations are presented in Table III. Of 514 single fibroblasts, 484 amplified (94.1%), with the amplification rates varying from a low of 89.7% for IVSI-1 to 95.5% for IVSI-110. These differences were not statistically significant ( $P=0.526$ ). Overall, ADO was observed in 44 fibroblasts (8.5%), with a low of 4.9% for codon 39 mutation and a high of 10.6% for IVSI-6. These differences were not statistically significant ( $P = 0.665$ ). More data will be needed to investigate

if the ADO rates depend on the type of  $\beta$ -globin mutation.

The results of genotyping 111 oocytes by PBs for  $\beta$ -globin gene mutations, including IVSII-745 and IVSI-110, and the polymorphic markers, are presented in Table IV. The majority of the oocytes studied by IPB were heterozygous for the mutant genes (87 of 118), as well as for polymorphic markers (65 of 86). The genotype of the gametes resulting from these heterozygotes may be evaluated only by testing of their IIPBs. Of 89 oocytes studied by IIPB, 78 were tested by both IPB and IIPB. The resulting genotypes from these oocytes are presented in Table V and demonstrate that analysis of the IIPB increases considerably the number of oocytes with established diagnosis as compared to IPB analysis alone. The number of unaffected oocytes after IIPB testing increased from 6 to 31, by adding 25 additional unaffected hemizygote oocytes which had heterozygote IPB. Consistent results were obtained for polymorphic markers. This demonstrates that analysis of oocytes by sequential IPB and IIPB removal allows accurate genotyping for the majority of aspirated oocytes (76 from 78 oocytes for the  $\beta$ -globin gene and 60 of 62 for polymorphic markers). In a

**Table IV.** Results of Two-Step Polar Body Analysis of Oocytes and the Follow-up of the Diagnosis in the Resulting Embryos

Locus	Total oocytes studied/ amplified	Oocytes with IPB data		Oocytes with IIPB data	Oocytes with IPB and IIPB data		Resulting embryos	
		Heterozygote	Homozygote		Total	ADO	Studied	Confirmed
IVSII-745	54	43	11	49	38	1	25	25
IVSI-110	64	44	20	40	40	4	26	25
Total	118	87	31	89	78	5	51	50 (98.0%)
5' Globin STR	76 <sup>a</sup>	57	19	61	54	2	39	39
HumTHO1	10 <sup>a</sup>	8	2	10	8	1	5	5
Total	86	65	21	71	62	3	44	44

<sup>a</sup> These numbers do not correspond to the numbers of oocytes, because STRs and mutations were studied simultaneously in the same oocytes.

**Table V.** Results of Two-Step Polar Body Analysis in Predicting the Genotype of Oocytes Following the First and Second Meiotic Divisions

Locus	Total oocytes analyzed by IPB + IIPB	Homozygote oocytes after IPB PCR		Hemizygote oocytes after IIPB PCR		Total No. of oocytes with predicted genotype	
		Normal (A1)	Mutant (A2)	Normal (A1)	Mutant (A2)	Normal (A1)	Mutant (A2)
IVSII-745	38	1	8	11	16	12	24
IVSI-110	40	5	7	14	14	19	21
Total	78	6	15	25	30	31	45
5' Globin STR	54 <sup>a</sup>	11	2	20	19	31	21
HumTHO1	8 <sup>a</sup>	1	1	3	3	4	4
Total	62 <sup>a</sup>	12	3	23	22	35	25

<sup>a</sup> These numbers do not correspond to the numbers of oocytes, because STRs and mutations were studied simultaneously in the same oocytes.

**Table VI.** Allele Dropout in Blastomeres from Embryos Heterozygous for Different  $\beta$ -Globin Mutations

Mutation	No. of embryos	No. of blastomeres amplified	Both alleles amplified	A1 amplified	A2 amplified	ADO
IVS II-745	20	85	74	80	79	11
IVS I-110	17	76	58	68	66	18
Total	37	161	132	148 (91.9%)	145 (90.0%)	30 (18.6%)
5' Globin STR	10 <sup>a</sup>	51 <sup>a</sup>	41	45	47	10

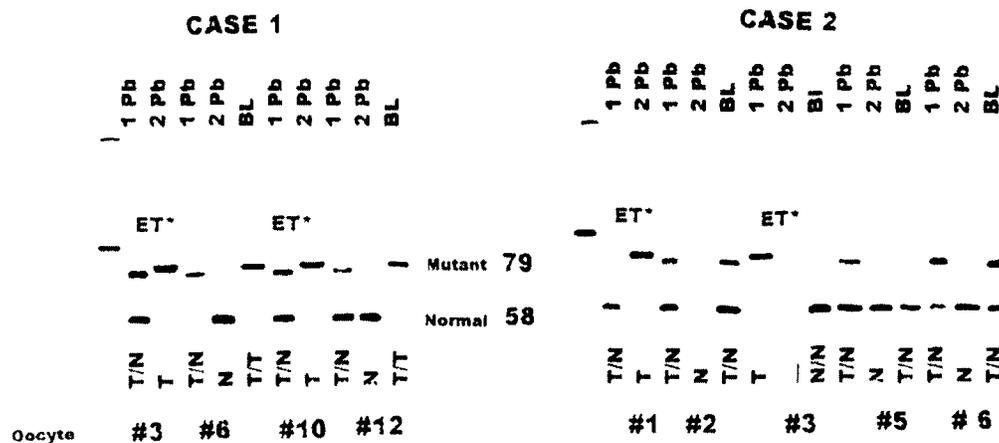
<sup>a</sup> These numbers do not correspond to the numbers of embryos or blastomeres, because STRs and mutations were studied simultaneously in the same blastomeres.

small number of oocytes the genotype of the resulting gamete could not be predicted (in two oocytes each for thalassemia and the polymorphic markers) because of ADO, which could have led to misdiagnosis without use of the sequential analysis of both PBs.

**Table VII.** Preimplantation Diagnosis of Thalassemia

Mutation	Patient/cycle	Total No. of oocytes	No. of oocytes with I + II PB	No. of normal embryos transferred	No. of embryo transfers (ET)
IVS II-745	1/4	66	38	12	4
IVS I-110	5/8	64	40	18	7
Total	6/12	130	78	30	11

The results of the follow-up study in the embryos resulting from the oocytes predicted to be affected by both IPB and IIPB (Table IV) show that the predicted genotypes of the embryos were confirmed in 50 of 51 cases for mutant alleles, as well as in all cases for polymorphic markers. The study of 37 embryos heterozygous for IVS I-110  $\beta$ -globin mutation and predicted to be affected demonstrated a statistically significantly higher ADO rate in blastomeres (18.6%) than in fibroblasts (10.1%;  $P = 0.001$ ) (Tables III and VI). Of 161 blastomeres obtained from these 37 embryos, both alleles amplified in 132 (81.4%) of 161 blastomeres analyzed for thalassemia mutations and in 41 (81.9%) of 51 blastomeres studied for polymorphic markers, demonstrating a greater risk of misdiagnosis in PGD



**Fig. 3.** Preimplantation genetic analysis of polar bodies and blastomeres for the IVS1-110 thalassemia mutation (*Mbo*I restriction digestion). Left: Case 1. Right: Case 2. #, oocyte sequential number; 1 Pb, first polar body; 2 Pb, second polar body; BL, blastomere; ET\*, embryo transfer; T, thalassemia allele; N, normal allele. 79, 79-bp undigested thalassemia allele; 58 = 58-bp restriction fragment (normal allele). In case 1, embryos 3 and 10, resulting from oocytes with a heterozygous polar body and a hemizygous abnormal second polar body, have been transferred. In case 2, embryo 1, resulting from oocyte with a heterozygous first polar body (signal for mutant allele was very weak and not seen here) and a hemizygous abnormal second polar body, have been transferred. Embryo 3 in this case was also transferred based on the results of the first polar body, containing abnormal allele, and the results of blastomere biopsy, showing the presence of only normal allele (the data from correspondingly second polar body were not available).

based on blastomeres. We have also noted a higher ADO rate in blastomeres than in IPBs, but this difference did not reach statistical significance. Applying a simultaneous amplification of linked polymorphic markers is of great value to decrease the undetected ADO rate and the risk for misdiagnosis, especially when the parents are carriers of different  $\beta$ -globin gene mutations.

Of 78 oocytes studied by both IPB and IIPB, 30 embryos predicted to carry the unaffected maternal allele were identified and transferred in 11 cycles, resulting in two ongoing pregnancies, both of which have already been confirmed to be free of the maternal  $\beta$ -globin mutation (Table VII).

The results of PGD in cases of IVSI-110 are demonstrated in Fig. 3, showing the details of the PCR analysis in the identification of IVSI-110 free embryos for transfer. Embryos 3 and 10 in case 1, and 1 in case 2 resulted from oocytes whose IPBs were heterozygous and IIPBs hemizygous affected. The follow-up genotyping of 51 embryos resulting from oocytes tested by both IPB and IIPB analysis confirmed the predicted PB diagnoses in all but one of them (Table IV), demonstrating the accuracy of the IPB and IIPB analysis for PGD of  $\beta$ -thalassemias.

This demonstrates the accuracy and feasibility of the technique of the sequential PB analysis for the preimplantation diagnosis of Mendelian disorders, with increased accuracy of diagnosis resulting from the use of linked markers. Because of the increased ADO rate observed in blastomeres with respect to PBs, we cannot endorse blastomere analysis alone for PGD of Mendelian disorders when both parents have different mutations or in dominant disorders.

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