

ARTICLE

# PGD for hereditary breast and ovarian cancer: the route to universal tests for *BRCA1* and *BRCA2* mutation carriers

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Preimplantation Genetic Diagnosis (PGD) is a method of testing *in vitro* embryos as an alternative to prenatal diagnosis with possible termination of pregnancy in case of an affected child. Recently, PGD for hereditary breast and ovarian cancer caused by *BRCA1* and *BRCA2* mutations has found its way in specialized labs. We describe the route to universal single-cell PGD tests for carriers of *BRCA1/2* mutations. Originally, mutation-specific protocols with one or two markers were set up and changed when new couples were not informative. This route of changing protocols was finalized after 2 years with universal tests for both *BRCA1* and *BRCA2* mutation carriers based on haplotyping of, respectively, 6 (*BRCA1*) and 8 (*BRCA2*) microsatellite markers in a multiplex PCR. Using all protocols, 30 couples had a total of 47 PGD cycles performed. Eight cycles were cancelled upon IVF treatment due to hypostimulation. Of the remaining 39 cycles, a total of 261 embryos were biopsied and a genetic diagnosis was obtained in 244 (93%). In 34 of the 39 cycles (84.6%), an embryo transfer was possible and resulted in 8 pregnancies leading to a fetal heart beat per oocyte retrieval of 20.5% and a fetal heart beat per embryonic transfer of 23.5%. The preparation time and costs for set-up and validation of tests are minimized. The informativity of microsatellite markers used in the universal PGD-PCR tests is based on CEPH and deCODE pedigrees, making the tests applicable in 90% of couples coming from these populations.

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## INTRODUCTION

In the Netherlands, the lifetime risk for women to develop breast cancer is one in eight, leading to a diagnosis of breast cancer in about 12 000 women each year. Breast cancer is thereby the most prevalent form of cancer in women in the Netherlands. In about 5–10% of the total hereditary breast and ovarian cancer (HBOC) patients, the mode of inheritance is autosomal dominant with incomplete penetrance.<sup>1</sup> The majority of this heritability is explained by mutations in genes *BRCA1* (MIM:113705, Genbank:U14680) and *BRCA2* (MIM:600185, Genbank:U43746), located on chromosomes 17q21 and 13q12.3, respectively. Both BRCA proteins act as tumor-suppressor genes and have, in this role, been shown to function in transcriptional regulation, DNA repair, DNA recombination and cell-cycle checkpoint control, thereby explaining how heterozygous loss of these genes can contribute to cancer initiation and progression.<sup>2</sup> Since the discovery of the two *BRCA* genes in 1994<sup>3</sup> and 1995,<sup>4</sup> >1500 different mutations for the *BRCA1* gene and >1200 mutations for the *BRCA2* gene have been identified in patients worldwide (Human Gene Mutation Database: www.hgmd.cf.ac.uk). Several founder

mutations have been identified in specific ethnic populations as the Ashkenazi Jews<sup>5</sup> or in specific regions/countries, such as Norway,<sup>6</sup> Poland<sup>7</sup> and China.<sup>8</sup> Even though in the Netherlands there have been some regional founder mutations,<sup>9</sup> the majority of mutations detected in *BRCA1* and *BRCA2* genes represent private mutations.

Preimplantation Genetic Diagnosis (PGD) was introduced into the clinic at the Maastricht University Medical Center (MUMC+) in 1995.<sup>10</sup> The introduction of PGD for HBOC resulted in lots of debate and discussion due to the late onset, incomplete penetrance and availability of preventive and therapeutic options. PGD for HBOC is now permitted in some countries as the UK,<sup>11</sup> Israel,<sup>12</sup> Belgium<sup>13</sup> and the Netherlands. Due to the variety of mutations carried by the different PGD applicants, mainly mutation specific tests or tests with few markers are described. As we experienced the continuing need for protocol adjustments, we aimed at designing universal PGD protocols for *BRCA1/2* mutation carriers. The methodology applied in these universal protocols is based on genetic linkage, using highly informative microsatellite markers in the close vicinity of the *BRCA1* and *BRCA2* genes that are likely inherited together during meiosis.

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Using this methodology, the need to incorporate the specific familial mutation is omitted; however, at least two informative family members (meiosis) are needed to definitely determine the 'risk' haplotype. These universal protocols reduce the patients' waiting time as well as set-up and validation costs.

## PATIENTS AND METHODS

### PGD couples and counseling

Thirty couples applied for a PGD procedure for HBOC between 2009 and 2011. Verbal and written information regarding the procedure, including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the risks and complications of IVF, PGD, the risk of misdiagnosis in PGD, the success rate of the treatment and the health of children born after PGD, was provided by a clinical geneticist or PGD physician and/or a gynecologist. The safety of ovarian stimulation for IVF treatment in *BRCA*-positive women was discussed and counselors explained that current knowledge does not suggest a significant increased risk for breast cancer in these women.<sup>14</sup> Fifteen couples were *BRCA1* and 15 couples were *BRCA2*. In 60% (9/15) of both *BRCA1* and *BRCA2* couples, the female carrier was the index case (Table 1). The average age of the women at the start of the first cycle was 30.5 years for *BRCA1* and 30.7 years for *BRCA2*. Three of the 17 women had had breast cancer before starting with the PGD procedure; all others had undergone pre-symptomatic testing because of an affected relative.

### Design of the PGD protocols

Microsatellite markers in or in the near vicinity of the *BRCA1* and *BRCA2* gene loci were explored using free accessible databases (c.q. NCBI; <http://www.ncbi.nlm.nih.gov>, UCSC; <http://genome.ucsc.edu>). This exploration of markers provided a list of approximately 25–30 microsatellite markers flanking the *BRCA1* and *BRCA2* loci (10–15 on either side of the locus). An overview of the developed protocols is summarized in Figure 1. Positions of markers relative to the *BRCA1/2* loci are depicted in Figure 2. During the process of protocol set-up and validation, the final selection of markers for the universal protocols were made based on heterozygosity/informativity of the markers, redundancy with others markers, competition with other markers in the multiplex PCR and percentages of allelic drop-out (ADO). For the universal *BRCA1* protocol, six informative markers were selected: three proximal, one intragenic and two distal to the *BRCA1* locus (Figure 2, universal markers underlined). The genetic distance between the outer markers is 2.1 cM, according to Genethon and 2.14 cM according to Marshfield genetic maps (deCODE not available). The average heterozygosity of markers in the universal protocol is 0.77. For the *BRCA2* protocol, eight informative markers were selected: four proximal and four distal to the *BRCA2* locus (Figure 2). The genetic distance between the outer markers is 4.65 cM according to deCODE, 5.4 cM according to Genethon and 3.36 cM according to Marshfield genetic maps. The average heterozygosity of the markers in the universal protocol is 0.74. For a detailed description of the primers used, the fluorescent labels and PCR product lengths, see Table 2. For non-informative PGD couples or couples for whom the risk haplotype could not be established by at least two meioses, a mutation-specific protocol was designed by detection of the private mutation combined with at least one informative marker. In these tests, single substitution mutations were detected using the difference in fragment length in case of base pair deletions/insertions or using the double allele amplification refractory mutation systems technique.<sup>15</sup>

### Validation of PGD protocols

The primers for the described microsatellite markers were developed using the free web program 'primer3 Input' (<http://frodo.wi.mit.edu/primer3/>). Criteria for primer design were primer size (20–30 nucleotides), GC content (40–60%) and  $T_m$  of the primers (60–70 °C, with maximum difference of 4 °C), primers preferably ending 3' with a guanine or cytosine and PCR product lengths <300 bp. Fluorescent labels were designed in such a way that PCR products with the same label did not overlap. All primers were mixed in one multiplex PCR following the ESHRE guidelines.<sup>16</sup> After optimization of the single-cell multiplex PCR, all protocols were validated by testing at least 50 single

leucocytes heterozygous for each marker in at least three separate experiments to assess amplification efficiency and ADO rate. Additionally 15–20 PCR blanks were analyzed to determine contamination. Only amplification efficiency rates >90% and ADO rates <10% were acceptable for implementation in the single-cell protocol.

### IVF/ICSI/PGD procedure

Controlled ovarian hyperstimulation was performed as described earlier.<sup>17</sup> Oocytes were retrieved under ultrasound guidance. After 5 h of maturation, MII oocytes were fertilized by means of ICSI<sup>18</sup> followed by embryo culturing.<sup>19</sup> Embryo morphology grade was used as one of the parameters to assess the embryo quality. On the morning of day 3 post fertilization, blastomeres were biopsied from cleavage stage embryos. From each cleavage-stage embryo, one (4–7 cells) or two blastomeres (>8 cell stage) were biopsied according to the ESHRE PGD guidelines.<sup>20</sup> Of 4–7 cell cleavage-stage embryos, only once cell was biopsied as this has been a general policy in our IVF centre for >15 years. Rationale for biopsying two cells is to increase the number of conclusive genetic results, in case of (partly) PCR failure. Biopsied blastomeres were rinsed three times in washing buffer (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline with 1% polyvinylpyrrolidone (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.1 mg/ml Phenol Red (Sigma-Aldrich Chemie BV)) and 2 µl washing buffer was transferred into 0.2-ml PCR tubes. Blank samples, containing 2 µl of the last washing droplet, were collected for each blastomere to monitor contamination. Single blastomere and blank samples were stored at least for 20 min at –20 °C, until the PCR was performed.

### PCR and genescan analysis

Single cells (blastomeres and leukocytes) were lysed by incubation at 65 °C for 10 min. in lysisbuffer (50 mM DTT and 200 mM NaOH) before amplification. Multiplex PCR for the polymorphic markers and allele-specific PCR for a private mutation contained 1 × QIAGEN Multiplex PCR Master Mix (QIAGEN, Venlo, the Netherlands), 20 mmol/l Tricine (Sigma) and primers (Applied Biosystems, Warrington, UK) in a final volume of 25 µl. The primer concentrations of the individual sets in the multiplexed PCRs varied between 100 nM and 1 mM per primer pair. All PCR reactions were performed with an initial activation step of 15 min denaturation at 95 °C. The denaturation–annealing–elongation cycles for the *BRCA1* multiplex PCR were 42 cycles of 10 s at 95 °C—90 s at 62 °C—60 s at 72 °C and for the *BRCA2* multiplex 10 initial cycles of 10 s at 95 °C—60 s at 63 °C—60 s at 72 °C followed by 32 cycles of 10 s at 95 °C—45 s at 63 °C—60 s at 72 °C. The PCR products were diluted (10 × *BRCA1* PCR, 20 × *BRCA2* PCR) and separated based on fragment length using capillary electrophoresis on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Fragment lengths were analyzed using the GeneMapper software provided by the manufacturer. For an example of genescan analysis and the corresponding haplotypes, see Figure 3.

### Quality assessment

After genetic diagnosis of embryos and embryo transfer of one or two healthy embryo(s) into the uterus and cryopreservation of the remaining unaffected, good quality, embryos, the remaining embryos were collected for quality assessment purposes after informed consent of the couple involved. Usually, the total embryo is selected as a whole to confirm the genetic diagnosis made during the PGD cycle. In a minority of cases, embryos were split into single blastomeres and analyzed separately. Of the 30 PGD couples, the untransferred embryos of 16 couples (8 *BRCA1*, 8 *BRCA2*) were collected to determine accuracy of genetic diagnosis/misdiagnosis rate.

## RESULTS

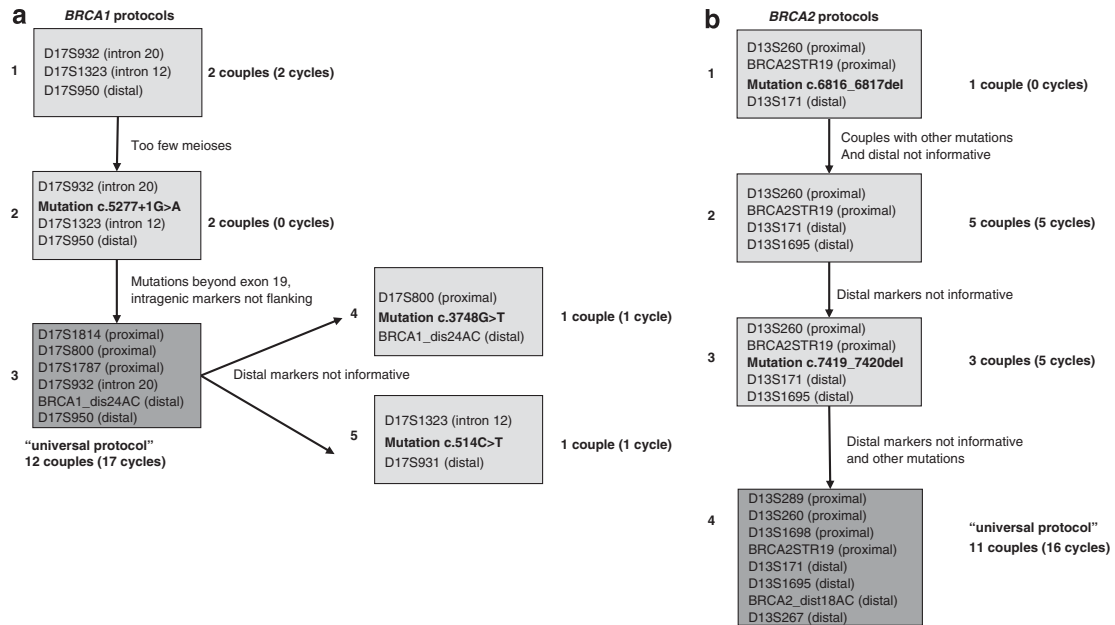
### PGD protocols

Five protocols for *BRCA1* and four protocols for *BRCA2* have been developed and validated at the single-cell level (Figures 1a and b). For *BRCA1*, we started with the first protocol of three markers of which two were intragenic and one distal (protocol 1, Figure 1a). The next couple had too few family members and the risk haplotype could not

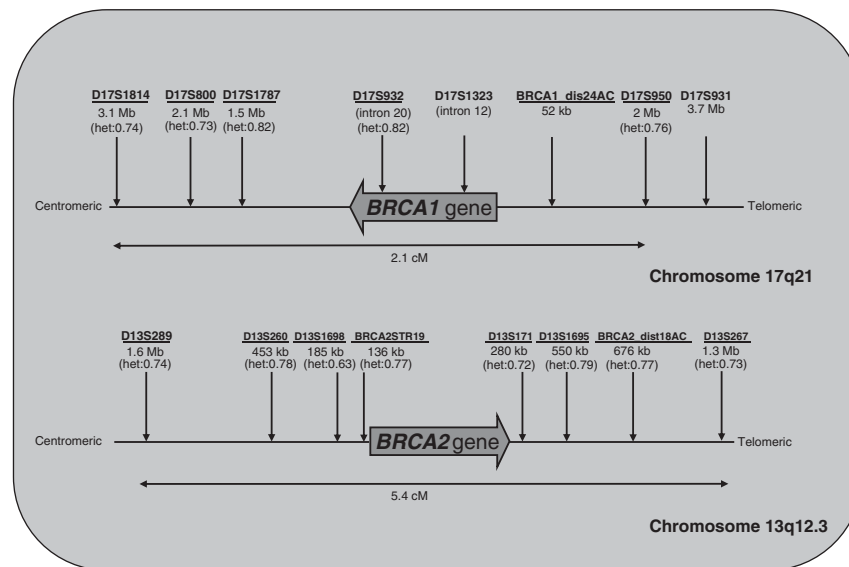
**Table 1 PGD cycle information per couple**

BRCA1 couples	Protocol according to Figure 1	Female age (years)	Carrier	Mutation	Amino-acid change	Exon	PGD cycle number	Total no. of embryos'	Not affected	Affected	Aberrant	No result	Transfer	Pregnant	Genotype embryos	
															Genotype embryos	Genotype embryos
1	Uni	31	Male	IVS13 + 4123ins6081	p.Glu1250X	11	1	3	3	0	0	0	2	No		
2	Uni	34	Male	c.3748G>T			2	6	2	4	0	0	2	No		
3	Uni	30	Female	c.68_69del	p.Glu23ValfsX17	2	1	13	5	7	1(h)	0	2	No		
4	Uni	34	Female	c.5266dup#	p.Glu1756ProfsX74	20	2	11	4	3	4(nc)	1	2x FET (1 embryo)	No		
5	Uni	28	Female	c.191G>A	p.Cys64Tyr	5	1	8	3	3	1(h)+1(nc)	0	2 (FET)	Yes		
6	Uni	28	Female	c.2197_2201del	p.Glu733ThrfsX5	11	1	7	0	3	1(h)	0	1	No		
7	Uni	26	Female	c.2338C>T	p.Gln780X	11	1	6	2	3	2(h)	0	2	Yes		
8	Uni	27	Female	c.2080del	p.Ser694AlafsX7	11	2	4	0	3	2(h)+1(nc)+1(r)	0	0	No		
9	Uni	28	Female	c.2685_2686del	p.Pro897LysfsX7	11	2	8	5	3	1(h)	0	1	No		
10	Uni	34	Male	c.2685_2686del	p.Pro897LysfsX7	11	1	9	4	4	3(h)	1	2 (FET)	No		
11	Uni	36	Female	c.1319del#	p.Leu440X	11	1	6	1	2	1(h)	0	1	Yes, miscarriage		
12	Uni	29	Male	c.3748G>T*	p.Glu1250X*	11	1	5	2	4	1(h)	0	2	No		
13	Uni	33	Male	c.514C>T**	p.Gln172X**	8	1	6	1	2	0	0	1	Yes		
14	Uni	31	Female	c.843_846del	p.Ser282TyrfsX15	11	1	2	1	0	1(h)	0	0	No		
15	Uni	29	Male	c.5277 + 1G>A			1	10	3	3	1(h)	3	1	No		
<b>BRCA2 couples</b>																
1	Uni	37	Female	c.7419_7420del***	p.Cys2473X***	14	1	5	2	2	0	1	2	No		
2	Uni	30	Male	c.7419_7420del***	p.Cys2473X***	14	1	2	2	0	0	0	1	No		
3	Uni	31	Male	c.7419_7420del***	p.Cys2473X***	14	2	4	Cancel > foll	2	2	0	2	Yes		
4	Uni	29	Female	c.7618-2A>G			1 (second child)	14	5	7	1(h)+1(t)	0	1	Yes, after FET		
5	Uni	31	Female	c.582G>A	p.Trp194X	7	2	12	7	3	1(h)	1	1	Yes		
6	Uni	28	Male	c.5213_5216del	p.Thr1738IlefsX2	11	1	3	Cancel < foll	1	1(h)	0	1	No		
7	Uni	25	Female	c.7976 + 3del2			2	7	1	7	0	1	1	No		
8	Uni	35	Male	c.1310_1313del	p.Lys437IlefsX22	10	1	4	4	1	0	1	2	No		
9	Uni	29	Female	c.3847_3848del	p.Val1283LysfsX2	11	1	2	0	1	1(h)	0	0	No		
10	Uni	33	Female	c.462_463del	p.Asp156X	5	3	7	3	3	1(h)	1	1	Yes, sp.abortion		
11	Uni	25	Male	c.4533del	p.Glu1511AspfsX32	11	2	5	Cancel < foll	1	1(nc)	0	1	Yes, sp.abortion		
12	Uni	33	Female	c.6275_6276del	p.Leu2092ProfsX7	11	2	6	2	4	0	0	1	No		
13	Uni	29	Female	c.9672dup	p.Tyr3225IlefsX30	27	1	5	3	3	1(nc)	0	1	No		
14	Uni	29	Male	c.462_463del	p.Asp156X		1	2	1	0	0	1	1	No		
15	Uni	37	Female	c.6275_6276del	p.Leu2092ProfsX7	11	2	6	Cancel < foll	2	0	1	2	No		

sp.abortion = spontaneous abortion, uni = universal protocol, \*/\*\*/\*\* = mutation-specific protocols, see Table 2 for details, # = thawed cycles, + = second child, h = haploid, t = triplod, nc = non-conclusive, r = recombination, cu = cycle cancel due to hypo-stimulation, co = cycle cancel due to hyper-stimulation, FET = frozen embryo transfer.



**Figure 1** PGD protocols. Time-line overview of the developed single-cell PGD-PCR protocols for *BRCA1* and *BRCA2* mutation carriers. (a) Developed protocols for *BRCA1*, (b) developed protocols for *BRCA2*.



**Figure 2** *BRCA1* and *BRCA2* loci and markers. Schematic overview of the *BRCA1* and *BRCA2* locus on chromosomes 17 and 13, respectively. The large gene arrows indicate the direction of the loci with respect to chromosome orientation. Marker names and their genetic (cM) and physical distances (Mb) to the *BRCA* loci are indicated in the figure. Genetic distance is based on the Genethon genetic map. Heterozygosity of marker alleles (if known) are indicated between brackets. Underlined markers are the markers used in the described universal tests.

be determined with certainty. Therefore the mutation was included (protocol 2, Figure 1a). Thereafter many more couples applied with mutations beyond exon 19, leading to non-flanking markers. To avoid repetitive redesign of the protocol, it was adapted to the universal 6 marker protocol (protocol 3, universal, Figure 1a). For two couples thereafter the distal markers of the universal protocol were not informative and mutation-specific protocols were developed (protocols 4 and 5, Figure 1a).

For *BRCA2*, a first protocol with a mutation and three markers, two proximal, one distal, was set up (protocol 1, Figure 1b). Thereafter couples with different mutations applied and in many couples the one distal marker was not informative. The protocol

was changed to four markers (protocol 2, Figure 1b). As for some additional couples the two distal markers were not informative, the specific mutation was built in the existing protocol (protocol 3, Figure 1b). In the last phase, four additional markers were added to increase informativity (protocol 4, universal, Figure 1b).

The universal PGD protocols were developed approximately 2 years after continuously changing protocols and were thereafter applied to all new PGD couples. A detailed description of the validation of these protocols is depicted in Table 3. Also for couples who had a first PGD cycle performed with an old protocol, the next cycles were adapted to the novel universal protocols (see Table 1 for details). Since their introduction, 12 of the 14 *BRCA1* couple haplotypes (86%) were

**Table 2** *BRCA1* and *BRCA2* markers, primers and labels for universal PGD tests

Protocol	Marker	Primer sequence (5' → 3')	Tag	Size (bp)
<i>BRCA1</i> (six markers)				
	D17S932	Fw—ACACGGATGGCCTTTAGAAAGTGGTC Rev—AACACACAGACTTGCTCTACTGCCAT	VIC	145–157
	D17S1814	Fw—ATGCTCCCAATGACGGTGATG Rev—AGCTGGAGGTTGGCTTGTGGAT	NED	150–166
	D17S950	Fw—CATAACAGCACTTGCCCCATGT Rev—ACAACAGCACACGCCCTGCAC	FAM	169–187
	D17S1787	Fw—TGCAAGACCCTTACGCTTTGTC Rev—CTTGGTGGTTCCTTCGTCCTTG	NED	186–198
	BRCA1_dist24AC	Fw—TGCAGAACAATTGTAGCAGCACACAG Rev—GTGGTCAGAACAATGCAAATTGAAGC	VIC	205–235
	D17S800	Fw—ACATCACCCAGGGAGGTGAGTTC Rev—AAGTGGGAGGAGCCATGAATGA	NED	266–276
<i>BRCA2</i> (eight markers)				
	D13S1695	Fw—TGTTCTAATGCCTGGGTATCATCC Rev—CAGGTGATCTGAGACTCAATAGCTTAACA	VIC	98–122
	D13S267	Fw—TCCTCCCATCCACCTTTCTCC Rev—CAGGTCCCACCATAAGCACAAGC	FAM	133–147
	D13S171	Fw—AAGGGAAGGAGAAAGGGAGGTG Rev—GCATTGACCTTAGGGCCATCCA	NED	150–166
	D13S289	Fw—GGTTGAGCGCATTGAAAACAG Rev—CACCTTCATCACCTTGATATGG	FAM	163–177
	BRCA2_dist18AC	Fw—GCCGCCTTTCAGTAAGCACAG Rev—AATGGGAACCAATTCAGCAAGG	PET	180–210
	D13S260	Fw—GGATCTGCTGCAATGCCAAA Rev—TCTCCAGATATAAGGACCTGGCTATG	VIC	210–224
	D13S1698	Fw—TGGGATTACAGGCTTGAGCCACA Rev—TCTGACACAGCTGTTTGTCTATTACC	FAM	215–235
	BRCA2STR19	Fw—GAATTTGTTCCAGGTGAGAATTGC Rev—ATGGGGTGCCTATGGCCTGAA	NED	235–259

Fw = forward, Rev = reverse, Tag = fluorescent dyes tagged to 5' end of forward primers.

informative and all (100%) *BRCA2* family haplotypes were informative. These couples were ready to be scheduled for the PGD procedure within 1–2 months. For two PGD couples, the *BRCA1* universal protocol was not applicable, because the markers at the distal side were not informative.

### Clinical cycles

The results of the individual clinical cycles are summarized in Table 1. Thirty couples underwent a total of 47 cycles. Two of these 47 cycles concerned PGD analyses of embryos cryopreserved before biopsy in another IVF centre. Of the remaining 45 cycles, 8 cycles were cancelled before oocyte retrieval due to hyper-stimulation (2) or hypo-stimulation (6). In the 18 *BRCA1* cycles, a total of 134 embryos were biopsied and a genetic diagnosis was obtained in 93% (125/134); in the 21 *BRCA2* cycles, a total of 127 embryos were biopsied and a genetic diagnosis was obtained in 93% (118/127). Of the embryos with a genetic diagnosis for *BRCA1*, 44.4% carried the mutation, 35.7% were unaffected; for *BRCA2*, 46.6% carried the mutation and 42.4% were unaffected. All remaining embryos were genetically abnormal or inconclusive.

In 34 of the 39 cycles (15 *BRCA1*/19 *BRCA2*, 87.2%) an embryo transfer was possible and resulted in 10 pregnancies of which 8 were with a fetal heart beat (4 *BRCA1*/4 *BRCA2*), leading to a fetal heart beat per oocyte retrieval of 20.5% and a fetal heart beat per embryo

transfer of 23.5%. For the *BRCA1* cycles, four pregnancies were obtained (see Table 1): Couple 3 delivered a healthy girl at 41<sup>+1</sup> weeks with a birth weight of 3625 g, Couple 6 delivered a healthy twin, a boy and a girl (two embryos transferred) with a birth weight of 3220 and 2660 g respectively. Couple 10 had an early miscarriage at 5 weeks. Couple 11 has an ongoing (single) pregnancy. For *BRCA2* PGD couples, four pregnancies were obtained: Couple 3 delivered a healthy girl with a birth weight of 3120 g in August 2010 and is currently pregnant with a second child after a cryo-embryo transfer from the first cycle (at term 14 October 2012). Couple 4 delivered a healthy girl at 40<sup>+4</sup> weeks with a birth weight of 2620 g. The pregnancy of couple 15 is still ongoing. So far, none of the couples opted for prenatal diagnosis to confirm the determined unaffected genetic status in the PGD cycle.

### Quality assessment

A total of 73 embryos were collected after PGD for re-analysis. In all, 13, 45 and 15 embryos were genotyped as unaffected, affected or aberrant during the PGD cycles. As the unaffected morphological good embryos are used for embryo transfer, the number of affected embryos succeeds the number of unaffected embryos in the re-analysis procedure. Of the 13 unaffected embryos, 11 showed conclusive genotype results after re-analysis. Two of 11 embryos gave one parental haplotype (monosomy), the non-risk marker haplotype



**Table 3 Validation of universal protocols**

Gene	Marker	No. of	No. of	ADO	PCR	Positive
		single				
		cells		per	(%) per	per
		tested		locus	locus	locus
BRCA1	D17S1814	57	54	1.8	96.5	0/13
	D17S800	57	55	1.8	96.5	0/13
	D17S1787	57	55	1.8	96.5	0/13
	D17S932	57	55	1.8	96.5	0/13
	BRCA1_dis24AC	57	55	0.0	96.5	1/13
	D17S950	57	54	0.0	96.5	0/13
BRCA2	D13S289	106	104	1.9	98.1	0/24
	D13S260	106	69	5.8	98.1	0/24
	D13S1698	106	104	5.8	98.1	0/24
	BRCA2STR19	106	103	1.9	97.2	0/24
	D13S171	106	35	2.9	98.1	0/24
	D13S1695	106	34	0.0	97.2	1/24
	BRCA2_dist18AC	106	70	0.0	97.2	0/24
	D13S267	106	69	0.0	98.1	0/24

protocols with some informative markers for first couples. These protocols were not applicable to all couples and thus were adapted by either replacing the mutation-specific primers or adding/replacing markers. Also in the literature the same laborious strategy is visible; Spits *et al.*<sup>13</sup> have described a protocol for *BRCA1* using two markers, flanking the *BRCA1* locus. These markers are both intragenic, making the protocol suitable only if both markers are informative and the mutation itself is located between the two intragenic markers. Jasper *et al.*<sup>23</sup> have included one specific mutation in *BRCA1* combined with two proximal markers but used two rounds of PCR. Sagi *et al.*<sup>12</sup> have described one *BRCA1* and two *BRCA2* founder-mutation-specific protocols, including three or four markers flanking the *BRCA* loci, applied to 10 PGD couples from Israel, and finally Ramon *et al.*<sup>24</sup> have described one mutation-specific *BRCA1* protocol with two markers. These protocols are not universal as they are mutation specific and/or include only a very small number of markers limiting informativity and using intragenic markers creates the limitation that the mutation must be located between the markers. Additionally, it takes a lot of time to design and optimize multiplex single-cell PCRs to maximize PCR efficiency and minimize ADO percentages for each specific mutation. To circumvent this costly and laborious procedure, other strategies have already been illustrated. Pre-implantation genetic 'haplotyping' has been described as a more universal strategy for several inherited monogenic disorders.<sup>25</sup> However, this strategy uses an extra step of multiple displacement amplification (MDA) of the single cell. This additional step is needed to allow a combination of several multiplexed PCRs in the following step, which would otherwise not be feasible on a single blastomere. This principle is excellent in the sense that it is applicable to all familiar monogenic diseases and using several multiplexed PCRs will optimize informativity of markers and detection of recombinations. One major drawback of MDA is, however, the high percentage of ADO, which needs to be compensated with a higher number of markers tested. A second drawback can be the extra time needed (16 h) to perform the MDA, which may put pressure on the timing of embryonic transfer if transfer is performed on day 4 post fertilization (which is the case in our centre). Therefore, for centres that transfer at day 5, the time needed to do MDA is not an issue. We developed an 'off-the-shelf' and fast test for *BRCA1* and *BRCA2* mutation carriers

applicable to nearly all couples; we designed one multiplexed PCR test, including as much as possible flanking informative markers, working on a single biopsied blastomere.

For the *BRCA1* locus, we were able to multiplex six highly polymorphic markers in one PCR. The maximal distance of the two outer markers is 2.1 cm. Three markers are located in the proximal region, two in the distal region and one is located intragenic (intron 20). For the *BRCA2* locus, we were able to multiplex eight highly polymorphic markers in one PCR, with a maximal distance between the outer markers of 4.65 cm. All eight markers are flanking the *BRCA2* locus, four proximally and four distally. As mentioned earlier, we started out with approximately 20–30 markers per locus in the early stages of development. Taking several criteria (heterozygosity, ADO percentage, competition, etc) as well as single-cell PCR limitations into account, the final number as well as the specific location of markers chosen in the universal protocols are not unique; other numbers or combinations of markers would be possible as long as they meet the same validation criteria and are informative in the majority of new couples.

Eighty-six percent of the *BRCA1* carriers and all *BRCA2* mutation carriers could so far be analyzed with the universal tests. If the pedigrees of the 'old protocol' cycles are reassessed and the universal protocols would have been used, 13/15 *BRCA1* couples (87%) and 14/15 (93%) were informative. Only two *BRCA1* PGD couples were not informative at the distal side and one *BRCA2* couple would not have been informative at the distal side. Preparation time is limited to 1–2 months, let alone the cost reduction saved by using the 'off-the-shelf' tests.

The robustness of the multiplex *BRCA* PCR tests are in accordance with the latest edition of the ESHRE PGD consortium guidelines,<sup>16</sup> with a PCR efficiency >90% and ADO rates <10%.

## CONCLUSIONS

In summary, we present an universal 'off-the-shelf' PGD tests for *BRCA1* and *BRCA2* mutation carriers that are robust, easy and quick to implement for a wide range of families choosing PGD to avoid transmission of a *BRCA1/2* mutation to future offspring.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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