Article

PGD for X-linked and gender-dependent disorders using a robust, flexible single-tube PCR protocol

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Abstract

X-linked genetic diseases include a wide range of disorders such as the dystrophinopathies. Additionally in some rare genetic diseases, severity of expression is gender dependent. Prevention of such disorders usually involves prenatal diagnosis and termination of affected pregnancies, while preimplantation genetic diagnosis (PGD) represents a specialized alternative that avoids pregnancy termination. To preclude the rejection of unaffected male embryos that cannot be differentiated from those affected when using fluorescence in-situ hybridization, a flexible protocol based on multiplex fluorescence polymerase chain reaction (PCR) was standardized and validated for gender determination in single cells, which can potentially incorporate any disease-specific locus. The final panel of nine loci included four loci on the Y chromosome, two on the X chromosome plus up to three microsatellite markers to either support the gender diagnosis or to further monitor extraneous contamination. The protocol, standardized on single lymphocytes, established a PCR efficiency of >93% for all loci with maximum allele dropout rates of 4%. Microsatellite analysis excluded external contamination and confirmed biallelic inheritance. Proof of principle for the simplicity and flexibility of the assay was demonstrated through its application to clinical PGD cycles for lipoid congenital adrenal hyperplasia, which presents a more severe clinical course in males, and Duchenne muscular dystrophy.

Keywords: *Duchenne muscular dystrophy, lipoid congenital adrenal hyperplasia, multiplex fluorescent PCR, preimplantation genetic diagnosis, X-linked diseases*

Introduction

Prenatal diagnosis and termination of affected pregnancies is the current approach for controlling severe genetic disorders especially since curative therapy is not as yet feasible. Preimplantation genetic diagnosis (PGD), developed in the late 1980s, represents a diagnostic procedure that avoids termination of an affected pregnancy by transferring genetically healthy IVF embryos. Clinical application of PGD is the method chosen by many couples at risk for transmitting inherited diseases, especially when they also have an unsuccessful reproductive history (Kanavakis and Traeger-Synodinos, 2002; Sermon *et al*., 2004; Ogilvie *et al*., 2005).

X-linked disorders include a wide range of genetic diseases such as dystrophinopathies and haemophilias. In addition there are some rare monogenic diseases in which severity is gender dependent, including lipoid congenital adrenal hyperplasia (LCAH). Most of the reported clinical PGD cycles for X–linked disorders involve the use of fluorescence in-situ hybridization

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(FISH) (Staessen *et al*., 1999), which allows direct analysis of the sex chromosomes for gender identification, but the method risks the exclusion of healthy male embryos. A more appropriate approach would be one allowing simultaneous investigation of both embryonic gender and single-gene defects. Using multiplex fluorescent PCR, several loci in a single cell can be simultaneously analysed (Wells and Sherlock, 1998; Findlay *et al*., 1999, 2001; Sermon, 2002).

This study sets out to design an accurate, robust, rapid, and flexible PCR protocol for analysing single-cell samples, applicable to PGD diagnosis for the wide spectrum of severe X-linked and gender-dependent diseases. This involved the optimization of a multiplex PCR for the amplification of nine different loci, including four loci on the Y chromosome, four on the X-chromosome and one on an autosomal chromosome for additional monitoring of contamination, whilst addressing the inherent problems of single-cell PCR (Findlay *et al*., 1995, 1998). Proof of principle for the simplicity and flexibility of the basic single-tube multiplex PCR-based protocol was demonstrated by the fact that it was easily modified to detect both embryonic gender and single-gene defects in either an X-linked disease, Duchenne muscular dystrophy (DMD), and a gender-dependent disease (LCAH).

DMD (OMIM accession number 310200), an X-linked recessive disease, is one of the most common causes of muscular dystrophy in childhood with an incidence of approximately 1 in 3500 males, for which PGD has more commonly been applied (Goossens *et al*., 2008). The disease causes early muscle degeneration and cardiac insufficiency in hemizygous boys (Boland *et al*., 1996) and is rapidly progressive, with most affected children becoming wheelchair-bound by the age of 12. Death usually occurs in the third decade as a result of respiratory failure and cardiomyopathy. DMD is caused by mutations in the dystrophin gene located at Xp21 locus. It is one of the largest human genes spanning 2.3 Mb and composed of 79 exons. It has been demonstrated that 75.8% of detectable deletions (representing 48.8% of Greek patients) present deletions in the 3′ end of the gene and between exons 44–53 (Florentin *et al*., 1995). Thus the optimized gender-specific assay was modified to incorporate analysis for the DMD hot-spot exon deletions and was subsequently applied in a clinical PGD cycle.

LCAH (Bhangoo *et al*., 2006), an autosomal recessive disease, is associated with mutations in the steroidogenic acute regulatory (*StAR*) gene. Although the *StAR* gene is located on chromosome 8p11.2 (OMIM 600617) (Stocco , 2002; Manna and Stocco, 2005), the phenotype of homozygotes for StAR mutations is more severe in males than in females. All XY children carrying a StAR mutation are undervirilized and usually raised as girls, often involving removal of the nonfunctional testes to prevent long-term neoplastic risk. On the other hand, females (XX) with LCAH usually require oestrogen replacement at or after puberty, although to date, ovulation and pregnancy has not been reported, even in cases that have been diagnosed early and treated optimally with glucocorticoid replacement to suppress ACTH-induced lipoid damage to the ovaries. A request for a PGD cycle for LCAH was made by an affected female who had a spouse carrying a StAR mutation. To this end, appropriate modifications were made to the PCR-based sexing protocol to allow the detection of both embryonic gender and disease-causative mutations.

Materials and methods

Cell samples for method optimization

For this study, 553 single lymphocytes and 20 single blastomeres were used. Single lymphocytes are amongst the cells commonly chosen as a model for establishing new PCR-based PGD protocols due to their ready accessibility from fresh whole-blood samples (Thornhill *et al*., 2005). All cell types were isolated or biopsied as previously described (Vrettou *et al*., 1999; Kokkali *et al*., 2005, 2007).

The multiplex PCR protocol for gender determination was optimized using initially single lymphocytes (294, including 184 of male and 110 of female origin). Validity of the method was subsequently evaluated in 20 single blastomeres from different spare embryos.

Prior to performing the PGD cycle for DMD, the multiplex PCR protocol for gender determination and deletion status for DMD was optimized using 199 lymphocytes including 157 single lymphocytes from healthy male controls, five single lymphocytes from healthy female controls and 37 single lymphocytes from affected individuals with known deletions.

Prior to performing the protocol for the PGD cycle for LCAH, the multiplex PCR protocol was tested in 60 single lymphocytes, 30 from each of the prospective parents.

Cell lysis

In all trials and the clinical cycle, cell lysis was achieved based on proteinase K as previously described (Traeger-Synodinos *et al*., 1997; Vrettou *et al*., 1999).

Design and optimization of genotype analysis

Gender determination

From amongst 11 loci initially tested (data not shown), nine were finally selected, including four loci distributed throughout the Y-chromosome (SRY, Amel Y, SY127, SY149 AZF region), two X-specific loci (Amel X, AR exon 6), two X-chromosome microsatellite markers (STR 45, and CAG repeat of AR exon 1) and one autosomal microsatellite marker (D13S314), the latter to support additional monitoring of contamination (**Table 1**).

PCR primer sets were selected from previously published data using criteria which assured compatibility of annealing temperatures and distinct amplicon sizes for each locus due to the limitation that the in-house automatic sequencer has only a single laser (Visible Genetics OpenGene System automatic DNA sequencer with Gene Objects software; Visible Genetics, High Wycombe, UK) (**Table 1**). One primer of each pair was fluorescently labelled to facilitate analysis of PCR products on the automatic sequencer.

For gender determination, initial experiments on 91 single lymphocyte samples from a male individual were set up

to investigate the preferable conditions for the multiplex amplification of all nine loci. PCR reactions were carried out in 50 µl volumes containing the premixed solution of buffer, dNTP and *Taq* polymerase (Multiplex PCR Kit, no. 206143; Qiagen, Germany) and all primers (**Table 1**). The optimal PCR cycling conditions were 15 min initial denaturation step at 95°C, followed by 40 cycles of 95°C for 1 min, 58°C for 1 min (annealing temperature) and 72°C for 1 min, and a 10 min final extension step at 72°C. Successful amplification was indicated by the presence of correctly sized PCR products on the automatic DNA sequencer.

Following optimization for the multiplex amplification of all loci, the PCR efficiency and allele dropout (ADO) rates were evaluated by genotyping a further 203 single lymphocytes (93 of male and 110 of female origin). The method was subsequently applied in 20 single blastomeres derived from different spare embryos.

DMD protocol

For application to DMD PGD cycles involving deletions of the hotspot exons 44–53, the PCR method was modified to allow detection of both embryonic gender and deletions in the dystrophin gene The loci selected included four from the multiplex gender assay (SRY, Amel Y, SY149 and Amel X), an X-chromosome microsatellite marker (STR 49) and two exons of the dystrophin gene: either exons 45 and 47 (set A) or exons 50 and 51 (set B), allowing application of a common annealing temperature (58°C) (**Table 1**). The X-chromosome microsatellite marker STR 45 was replaced by STR 49 to avoid overlapping amplicon sizes in the panel.

Clinical PGD cycles

PGD cycle for DMD

A clinical cycle was offered to a couple who requested PGD for DMD. Molecular analysis of the dystrophin gene(OMIM 310200, see Online Mendelian Inheritance in Man, www.ncbi.nlm.nih.gov/omim) revealed a heterozygous deletion in the female carrier (exons 45–52 deleted). Following ovarian stimulation, 12 oocytes were retrieved of which seven were fertilized by intracytoplasmic sperm injection (ICSI). Cleavage-stage biopsies, using a non-contact laser (ZILOS-tk; Hamilton Thorne Biosciences, Beverly, MA, USA), were performed on six embryos, which had reached >6 cell stage (Kokkali *et al.*, 2007). Ovarian stimulation, oocyte recovery and ICSI procedures were carried out using standard protocols (Kokkali *et al.*, 2007). Six single blastomeres were transported to the Department of Medical Genetics for genetic analysis.

PGD cycle for LCAH

A clinical cycle was offered to a couple who requested PGD for the gender influenced disease, LCAH. Molecular analysis of the *StAR* gene revealed an 11-bp deletion in exon 6, in the homozygous state in the prospective mother (c.834del11 bp/834del11 bp) and in the heterozygous state in the prospective father (c.834del11 bp/Normal) (Sertedaki *et al.*, 2008). The couple requested PGD analysis for gender as well as the disease-causing mutation such that if no embryos heterozygous for the StAR mutation were available, they could select to transfer a homozygous female embryo. To facilitate co-analysis of the disease-related mutation and embryo gender, the multiplex reaction was modified by excluding the primers for the AR exon 1 CAG locus, while introducing a primer set specific for the detection of the *StAR* gene deletion.

Before entering a programme of IVF, the patient had several unsuccessful attempts to conceive. Based on the molecular data indicating an early defect of steroidogenesis, not only in the adrenal but in the ovary as well, the IVF protocol was modified as described (Kokkali *et al.*, 2007; Sertedaki *et al.*, 2008).

Following ovarian stimulation, 21 oocytes were retrieved from the patient, of which 11 were at the metaphase II (MII) stage of maturation and were fertilized by ICSI. Eight oocytes were normally fertilized and were cultured until day 3 post insemination. Cleavage-stage biopsies, using a non-contact laser (ZILOS-tk), were performed on seven embryos, which had reached >6 cell stage (Kokkali *et al.*, 2007). Seven single blastomeres were transported to the Department of Medical Genetics for genetic analysis.

Precautions against contamination

In all experiments and PGD cycles, precautions against contamination were most stringent. Manipulation of cells and PCR set-up were carried out in separate UV-treated laminar flow hoods. PCR set-up employed dedicated PCR pipettes and pipette tips with filters. In the multiplex PCR, negative (blank) controls included two tubes containing IVF medium, two tubes with cell lysis mixture (prepared alongside cell biopsies in the IVF unit) and one tube containing the PCR mixture alone (prepared in the genetics laboratory during PCR set-up). To exclude contamination within the system, all negative controls were analysed in the Visible Genetics OpenGene System (Vrettou *et al.*, 2004; Kokkali *et al.*, 2007).

For the clinical PGD cycles, simultaneous study of all embryonic DNA as well as parental DNA samples by microsatellite analysis excluded external contamination and confirmed biallelic inheritance of parental alleles.

Results

Design and optimization of genotype analysis

Gender determination

Based on analysis of 91/294 single lymphocytes (male origin), the optimal concentration of magnesium chloride was determined to be 3 mmol/l and the primer concentrations are summarized in **Table 1**. For each locus, PCR efficiency and ADO rates were evaluated in 203 lymphocytes (**Table 2**) and 20 single blastomeres (data not shown) revealing similar values for both cell types. A typical multi-site analysis is illustrated in **Figure 1**.

Table 1. Primer list.

PCR primers	*Sequence*	*Size (bp)*	*Locus*	*Concentration (µmol/l)*	*Reference/ GenBank accession*
Amel F	5′–CCC TGG GCT CTG TAA AGA ATA GTG–3′	115 (X), 121 (Y)	Xp22.2	0.3/0.3	Ray *et al.* (2001)
Amel R[a]	5′-AGG CCA ACC ATC AGA GCT TAA ACT–3′		Yp11.2	0.4/0.4[b]	
SY149 F	5′–TGT CAC ACT GCC CTA ATC CT–3′	132	Yq	0.3/0.3	G73322
SY149 R[a]	5′–TGG TCA TGA CAA AAG ACG AA–3′			0.4/0.4[b]	S86117
D13S314 F[a]	5′–GAG TGG AGG AGG AGA AAA GA–3′	137–155	13q14.3	0.56/0.56	Thomas *et al.* (1994)
D13S314 R	5′–GTG TGA CTG GAT GGA TGT GA–3′				
STR 45 F[a]	5′–GAG GCT ATA ATT CTT TAA CTT TGG C–3′	156–184	Xp21.1	0.6/0.6	Clemens *et al.* (1991)
STR 45 R	5′–CTC TTT CCC TCT TTA TTC ATG TTA C–3′				
AR-1 F[a]	5′–ACC AGG TAG CCT GTG GGG CCT CTA CGA TGG GC–3′	228–288	Xq11.2-q12	0.3/0.3	M35844
AR-1 R	5′–CCA GAG CGT GCG CGA AGT GAT CCA GAA CCC GG–3′				
AR-6 F	5′–CAA TCA GAG ACA TTC CCT CTG G–3′	267	Xq11.2-q12	0.3/0.3	AF039825
AR-6 R[a]	5′–AGT GGT CCT CTC TGA ATC TC–3′				
SY127 F	5′–GGC TCA CAA ACG AAA AGA AA–3′	274	Yq11.2	0.4/0.4	G11998
SY127 R[a]	5′–CTG CAG GCA GTA ATA AGG GA–3′				
SRY F	5′–CAG TGT GAA ACG GGA GAA AAC AG–3′	336	Yp11.31	0.6/0.6	L08063
SRY R[a]	5′–ACT TCG CTG CAG AGT ACC GAA G–3′			1/1[b]	
STR 49 F[a]	5′–CGT TTA CCA GCT CAA AAT CTC AAC–3′	226–238	Xp21.1	0.8/0.8	Clemens *et al.* (1991)
STR 49 R	5′–CAT ATG ATA CGA TTC GTG TTT TGC–3′				
Exon 45 F[a]	5′–CTT TCT TTG CCA GTA CAA CTG CAT GTG–3′	307	Xp21.1	0.8/0.8	Abbs *et al.* (1991)
Exon 45 R	5′–CAT TCC TAT TAG ATC TGT CGC CCT AC–3′				
Exon 47 F[a]	5′–CGT TGT TGC ATT TGT CTG TTT CAG TTA C–3′	181	Xp21.1	0.4/0.4	Abbs *et al.* (1991)
Exon 47 R	5′–GTC TAA CCT TTA TCC ACT GGA GAT TTG–3′				
Exon 50 F[a]	5′–CAC CAA ATG GAT TAA GAT GTT CAT GAA T–3′	271	Xp21.1	0.4/0.4	Abbs *et al.* (1991)
Exon 50 R	5′–TCT CTC TCA CCC AGT CAT CAC TTC ATA G–3′				
Exon 51 F[a]	5′–GAA ATT GGC TCT TTA GCT TGT GTT TC–3′	388	Xp21.1	0.8/0.8	Abbs *et al.* (1991)
Exon 51 R	5′–GGA GAG TAA AGT GAT TGG TGG AAA ATC–3′				
StAR F	5′–TAA GCA GCC AGT GCA AGA GA–3′	223	8p11.2	0.6/0.6	Sertedaki *et al.* (2008)
StAR R[a]	5′–GGA ATG GGA AGA GCC TGT TT–3′				

[a]Cy5.5 fluorescent label at the 5′ end.
[b]Concentrations of primers when used for the preimplantation genetic diagnosis protocol for Duchenne muscular dystrophy.

Table 2. Gender determination protocol: polymerase chain reaction efficiency and allele dropout in 203 lymphocytes.

Locus	*PCR efficiency in 203 lymphocytes (%)*	*ADO in 203 lymphocytes (%)*
Amel X	98.0	1
Amel Y	98.0	1
SY149	98.9	NA[a]
D13S314	93.4	0
STR 45	95.6	0
AR-1	99.5	4
AR-6	94.3	NA[a]
SY127	96.7	NA[a]
SRY	94.6	NA[a]

ADO = allele dropout; NA = not applicable; PCR = polymerase chain reaction.
[a]These loci are represented by one allele and their unsuccessful amplification should not be considered as ADO but as PCR inefficiency.

Table 3. Duchenne muscular dystrophy protocol: polymerase chain reaction efficiency and allele dropout in 199 lymphocytes.

Locus	*PCR efficiency in 199 lymphocytes (%)*	*ADO in 199 lymphocytes (%)*
Amel X	98.5	0
Amel Y	98.5	0
SY149	100	NA[a]
SRY	94.6	NA[a]
STR 49	90.4	0
Exon 45	90	NA[a]
Exon 47	100	NA[a]
Exon 50	90	NA[a]
Exon 51	83.7	NA[a]

ADO = allele dropout; NA = not applicable; PCR = polymerase chain reaction.
[a]These loci are represented by one allele and their unsuccessful amplification should not be considered as ADO but as PCR inefficiency.

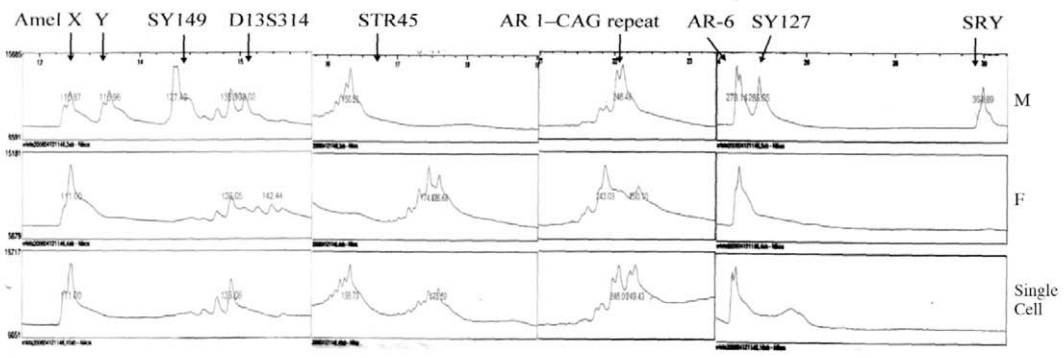

Figure 1. Typical multi-site analysis for the nine loci. Comparative analysis of parental DNA and 'single-cell' DNA allows monitoring of biallelic inheritance. (**M**) Profile in a genomic DNA sample from a male individual; (**F**) profile in a genomic DNA sample from a female individual; (**Single cell**) profile of a female descendant with normal biallelic inheritance.

Table 4. Results of the preimplantation genetic diagnosis cycle for Duchenne muscular dystrophy (DMD) (deletion of exons 45–52 had been detected in the family).

	Amel X	*Amel Y*	*SY149*	*Exon 47*	*STR 49[a]*	*Exon 45*	*SRY*	*Diagnosis gender/ DMD phenotype*
Mother	+	–	–	+	1	+	–	Female/Carrier
Father	+	+	+	+	2	+	+	Male/Normal
Single cell								
1	+	+	+	–	–	–	+	Male/Affected
2	+	+	+	–	–	–	+	Male/Affected
3	+	+	+	–	–	–	+	Male/Affected
4	–	–	–	–	–	–	–	Not informative
5	+	–	–	+	2	+	–	Female/Carrier
6	+	–	–	–	–	–	–	Female/Not informative

+ = amplification; – = no amplification.
[a]Classification of alleles is arbitrary.

Table 5. Results of the preimplantation genetic diagnosis cycle for lipoid congenital adrenal hyperplasia (LCAH).

	Amel X	*Amel Y*	*SY149*	*D13S314*[a]	*STR 45*[a]	*StAR c.834del11 bp*	*StAR normal*	*AR exon 6*	*SY127*	*SRY*	*Diagnosis gender/LCAH phenotype*
Mother	+	–	–	2, 3	1, 3	+	–	+	–	–	Female/Affected
Father	+	+	+	1, 3	2	+	+	+	+	+	Male/Non-affected carrier
Single cell											
1	+	–	–	–	–	–	–	–	–	–	Not informative
2	+	–	–	–	2, 3	–	–	+	–	–	Female/Not informative
3	–	–	–	–	–	–	–	–	–	–	Not informative
4	+	+	+	3, 3	3	+	–	+	+	+	Male/Affected
5	+	+	+	1, 3	3	+	+	+	+	–	Male/Non-affected carrier
6	+	+	+	1, 2	1	+	–	+	+	+	Male/Affected
7	+	–	–	2, 3	2, 3	+	+	+	–	–	Female/Non-affected carrier

+ = amplification; – = no amplification.
[a]Classification of alleles is arbitrary.

Work-up for the PGD cycles

PCR efficiency and ADO rates were estimated for both the group of 199 lymphocytes analysed with the DMD protocol (**Table 3**) and the 60 lymphocytes analysed for the work-up of the LCAH PGD. For the latter case, the rates assessed were comparable to those observed when applying the gender determination protocol (**Table 2**) while StAR amplification showed a 94% PCR efficiency and 0% ADO.

No PCR products were observed in any of the negative controls (blanks) in all samples tested, indicating that contamination from external sources or PCR carry-over was not a problem with the stringent precautions applied.

Clinical PGD cycles

Results for the DMD PGD cycle

Out of the six single-blastomere samples from the six embryos fertilized by ICSI, four gave PCR products at two or more loci (**Table 4**). Results in five embryos established gender and/or deletion status for exons 45 and 47 in the dystrophin gene: three embryos were affected males, one embryo lacked all but Amel X sequence and one embryo was normal female (i.e. unaffected carrier) (**Table 4**). The unaffected female carrier embryo (number 5 in **Table 4**) was transferred on the evening of the fifth post-ICSI day, according to the embryo-transfer strategy by the IVF centre, but a pregnancy was not achieved.

Results for the LCAH PGD cycle

Out of seven single-blastomere samples from the seven embryos biopsied, five gave PCR products at two or more loci (**Table 5**). Results in five embryos established gender and/or status for the StAR mutation: two embryos were male, and homozygous for the StAR mutation, one embryo was male and heterozygous for the StAR mutation and one embryo was female and heterozygous for the StAR mutation (**Table 5**).

Two unaffected embryos (numbers 5 and 7 in **Table 5**) were transferred on the fifth post-ICSI day, according to the strategy of the IVF centre, and a singleton pregnancy was achieved. CVS prenatal diagnosis was subsequently performed in week 12 of gestation and confirmed a female fetus heterozygous for the StAR mutation. The LCAH patient, gave birth to a healthy female representing, as far as is known, the first pregnancy among patients with *StAR* gene mutations (Sertedaki *et al.*, 2008).

Discussion

When confronting X-linked and gender-dependent diseases, PGD embryo sexing is an accepted approach that helps to avoid initiation of an affected pregnancy; it can be achieved with either FISH or PCR-based analysis or a combination of both (Smith *et al.*, 1997). Although the first PGD cycle for sexing was performed with PCR using a single locus on the Y-chromosome (Handyside *et al.*, 1990) the technical pitfalls of single-cell PCR analysis made PCR-based sexing methods less reliable than those based on FISH. Thus to date, the majority of reported cycles for PGD-sexing are based on FISH analysis (Goossens *et al.*, 2008). However, with FISH diagnosis being limited to the chromosomal level, unaffected male embryos will be rejected. Although there are some reports describing the sequential analysis of single biopsied cells by FISH followed by PCR to diagnose the single-gene defect, it is technically extremely difficult to achieve such diagnoses (Smith *et al.*, 1997).

With the advent of improved DNA polymerases and overall a greater experience with PCR-based technologies, there have been a few recent reports of PCR-based methods for X-linked genetic disorders such as DMD, combining gender

determination and analysis of specific dystrophin gene mutations (Ray *et al.*, 2001; Girardet *et al.*, 2003; Malcov *et al.*, 2005). They included, however, only a single locus on each of X- and Y-chromosomes for embryo sexing, reducing the potential robustness of the protocol. The analysis of multiple sites on both X- and Y-chromosomes, along with various microsatellite markers described here, is potentially much more robust, with additional flexibility to introduce primer sets appropriate for the analysis of disease-specific genotypes. The successful co-amplification of as much as 10 different loci provides the possibility to maintain two or three Y-specific sites and one or two X-specific sites and still have the option to incorporate five or more disease-linked loci, e.g. the specific gene locus and linked microsatellites in the reaction.

In the trials for the gender-specific assay, PCR efficiency for all loci was above 93% and for the microsatellite markers, ADO ranged from 0–4%, all well within acceptable limits for single-cell PCR according to the European Society for Human Reproduction and Embryology PGD Consortium best practice guidelines (Thornhill *et al.*, 2005). When the protocol was applied on single blastomeres, the ADO rates and PCR efficiency were comparable to those reached for lymphocytes. It should be noted that these blastomeres originated from high-quality spare embryos that underwent PGD for cystic fibrosis or thalassaemia and were rejected for implantation as affected. Conversely, during the LCAH PGD cycle, the low PCR efficiency observed as amplification failure in two of the seven embryos is possibly due to their poor quality since they arrested in culture (**Table 5**).

For the DMD-specific assay, PCR efficiency for most of the dystrophin gene exons included was slightly lower (**Table 3**), most notably for exon 51. The latter may be due to the fact that the amplicon for this exon is ~400 bp, which may reduce the amplification efficiency in single-cell PCR (Sermon *et al.*, 1996; Vrettou *et al.*, 1999). Overall the inclusion of more than one locus for each of the sex chromosomes supports the correct diagnosis of embryonic sex. The theoretical possibility of misdiagnosing a male embryo as female, in the unlikely event that all of the Y loci fail to amplify, is precluded by the analysis of the X-linked polymorphic sequence variations, which are highly likely to be informative (rate of heterozygosity 0.89% for STR 45 and 0.93% for STR 49).

This study proposes that the minimal requirements to ensure accurate diagnosis should be based on the observation of at least three Y-loci and one X-locus for male embryos, or two X-loci as well as normal biallelic inheritance for X-linked microsatellites for female embryos.

The flexibility of the PCR-based PGD sexing protocol was demonstrated by its application in two clinical PGD cycles, one for LCAH and the other for DMD. With respect to the cycle for LCAH, this is probably one of the first cases in which a pregnancy has been achieved in a female affected with LCAH.

Similar preimplantation diagnostic procedures for single-gene disorders using multiple loci analysis include whole genome amplification (WGA) methods such as the multiple displacement amplification (MDA) (Renwick *et al.*, 2006; Ren *et al.*, 2007). Although MDA gives the most complete coverage and unbiased amplification of any WGA method reported to date, it is still not widely applied because it presents high ADO incidence (0–60%) and is time consuming (8–16 h for MDA completion) (Handyside *et al.*, 2004; Hellani *et al.*, 2004, 2005; Burlet *et al.*, 2006; Lledo *et al.*, 2006; Renwick *et al.*, 2006; Spits *et al.*, 2006; Ren *et al.*, 2007). Moreover, when WGA is applied, the need to avoid misdiagnosis due to high ADO rates becomes more critical. Therefore, additional sites need to be co-analysed which, together with the introduction of one more step for the WGA process, multiplies the turnaround time, the total cost of the test and the risk of carry-over contamination. On the other hand, in the case where more than one monogenic disorders need to be excluded (e.g. cystic fibrosis and thalassaemia), a WGA procedure may be the only choice for PGD. This study considered that it was unlikely to increase the robustness of the test and would additionally delay the whole procedure.

Overall the multiplex PCR-based sexing method appears to be very robust and flexible overcoming the need for a whole genome amplification step when a single X-linked or gender-dependent monogenic disorder is studied. Additionally, compared with FISH based methods for embryo sexing, it is inexpensive and rapid requiring less than 5 h for completion. The protocol offers a solid basis for extending sexing assays to additionally include X-linked specific disease-causing loci.

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References

Abbs S, Yau SC, Clark S *et al.* 1991 A convenient multiplex PCR system for the detection of dystrophin gene deletions: a comparative analysis with cDNA hybridisation shows mistypings by both methods. *Journal of Medical Genetics* **28**, 304–311.

Bhangoo A, Anhalt H, Ten S, King SR 2006 Phenotypic variations in lipoid congenital adrenal hyperplasia. *Pediatric Endocrinology Reviews* **3**, 258–271.

Boland BJ, Silbert PL, Groover RV *et al.* 1996 Skeletal, cardiac and smooth muscle failure in Duchenne muscular dystrophy. *Pediatric Neurology* **14**, 7–12.

Burlet P, Frydman N, Gigarel N *et al.* 2006 Multiple displacement amplification improves PGD for fragile X syndrome. *Molecular Human Reproduction* **12**, 647–652.

Clemens PR, Fenwick RG, Chamberlain JS *et al.* 1991 Detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. *American Journal of Human Genetics* **49**, 951–960.

Findlay I, Matthews P, Mulcahy BK, Mitchelson K 2001 Using MF-PCR to diagnose multiple defects from single cells: implications for PGD. *Molecular and Cellular Endocrinology* **183**, S5-S12.

Findlay I, Matthews P, Quirke P 1999 Preimplantation genetic diagnosis using fluorescent polymerase reaction: results and future developments. *Journal of Assisted Reproduction and Genetics* **16**, 199–206.

Findlay I, Matthews P, Quirke P 1998 Multiple genetic diagnosis from single cells using multiplex PCR: reliability and allele dropout.

Prenatal Diagnosis **18**, 1413–1421.
Findlay I, Ray P, Quirke P *et al*. 1995 Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation diagnosis of sex and cystic fibrosis. *Molecular Human Reproduction* **1**, 1609–1618.
Florentin L, Mavrou A, Kekou K, Metaxotou A 1995 Detections patterns of Duchenne and Becker muscular dystrophies in Greece. *Journal of Medical Genetics* **32**, 48–51.
Girardet A, Hamamah S, Dechaud H *et al*. 2003 Specific detection of deleted and non-deleted dystrophin exons together with gender assignment in preimplantation genetic diagnosis of Duchenne muscular dystrophy. *Molecular Human Reproduction* **9**, 421–427.
Goossens V, Harton G, Moutou C *et al*. 2008 ESHRE PGD Consortium data collection VIII: cycles from January to December 2005 with pregnancy follow-up to October 2006. *Human Reproduction* **23**, 2629–2645.
Handyside AH, Robinson MD, Simpson RJ *et al*. 2004 Isothermal whole genome amplification from single and small numbers of cells: a new era for preimplantation genetic diagnosis of inherited disease. *Molecular Human Reproduction* **10**, 767–772.
Handyside AH, Kontogianni EH, Hardy K, Winston RM 1990 Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* **344**, 768–770.
Hellani A, Coskun S, Tbakhi A *et al*. 2005 Clinical application of multiple displacement amplification in preimplantation genetic diagnosis. *Reproductive BioMedicine Online* **10**, 376–380.
Hellani A, Coskun S, Benkhalifa M *et al*. 2004 Multiple displacement amplification on single cell and possible PGD applications. *Molecular Human Reproduction* **10**, 847–852.
Kanavakis E, Traeger-Synodinos J 2002 Preimplantation genetic diagnosis in clinical practice. *Journal of Medical Genetics* **39**, 6–11.
Kokkali G, Traeger-Synodinos J, Vrettou C *et al*. 2007 Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study. *Human Reproduction* **22**, 1443–1449.
Kokkali G, Vrettou C, Traeger-Synodinos J *et al*. 2005 Birth of healthy infant following trophectoderm biopsy from blastocysts for PGD of beta-thalassaemia major. *Human Reproduction* **20**, 1855–1859.
Lledo B, Ten J, Galan FM *et al*. 2006 Preimplantation genetic diagnosis of Marfan syndrome using multiple displacement amplification. *Fertility and Sterility* **86**, 949–955.
Malcov M, Ben-Yosef D, Schwartz T *et al*. 2005 Preimplantation genetic diagnosis (PGD) for Duchenne muscular dystrophy (DMD) by triplex-nested PCR. *Prenatal Diagnosis* **25**, 1200–1205.
Manna PR, Stocco DM 2005 Regulation of the steroidogenic acute regulatory protein expression: functional and physiological consequences. *Current Drug Targets. Immune, Endocrine and Metabolic Disorders* **5**, 93–108.
Ogilvie MC, Braude RP, Scriven NP 2005 Preimplantation genetic diagnosis – an overview. *Journal of Histochemistry and Cytochemistry* **53**, 255–260.
Ray PF, Vekemans M, Munnich A 2001 Single cell multiplex PCR amplification of five dystrophin gene exons combined with gender determination. *Molecular Human Reproduction* **7**, 489–494.
Ren Z, Zhou C, Xu Y *et al*. 2007 Mutation and haplotype analysis for Duchenne muscular dystrophy by single cell multiple displacement amplification. *Molecular Human Reproduction* **13**, 431–436.
Renwick PJ, Trussler J, Ostad-Saffari E *et al*. 2006. Proof of principle and first cases using preimplantation genetic haplotyping – a paradigm shift for embryo diagnosis. *Reproductive BioMedicine Online* **13**, 110–119.
Sermon K 2002 Current concepts in preimplantation genetic diagnosis (PGD): a molecular biologist's view. *Human Reproduction* **8**, 11–20.
Sermon K, Van Steirteghem A, Liebaers I 2004 Preimplantation genetic diagnosis. *Lancet* **363**, 1633–1641.
Sermon K, Lissens W, Joris H *et al*. 1996 Adaptation of the primer extension preamplification (PEP) reaction for preimplantation genetic diagnosis: single blastomere analysis using short PEP protocols. *Molecular Human Reproduction* **2**, 209–212.
Sertedaki A, Pantos K, Vrettou C *et al*. 2008 Conception and pregnancy outcome in a patient with 11bp deletion of the steroidogenic acute regulatory protein (*StAR*) gene. *Fertility and Sterility* **91**, 934.
Smith TA, Wall MB, Muggleton-Harris AL 1997 Archival fixed and analysed preimplantation human embryonic cells: a DNA resource for retrospective PCR analysis at the cellular level. *Molecular Human Reproduction* **3**, 149–155.
Spits C, le Caignec C, de Rycke M *et al*. 2006 Optimization and evaluation of single-cell whole genome multiple displacement amplification. *Human Mutation* **27**, 496–503.
Staessen C, Van Assche E, Joris H *et al*. 1999 Clinical experience of sex determination by fluorescent in situ hybridization for preimplantation genetic diagnosis. *Molecular Human Reproduction* **5**, 382–389.
Stocco DM 2002 Clinical disorders associated with abnormal cholesterol transport: mutations in the steroidogenic acute regulatory protein. *Molecular and Cellular Endocrinology* **31**, 19–25.
Thomas GR, Bull PC, Roberts EA *et al*. 1994 Haplotype studies in Wilson disease. *American Journal of Human Genetics* **54**, 71–78.
Thornhill AR, deDie-Smulders CE, Geraedts JP 2005 ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)'. *Human Reproduction* **20**, 35–48.
Traeger-Synodinos J, Palmer G, Tzetis M 1997 Preliminary studies on the feasibility of preimplantation diagnosis for beta-thalassemia. *Medizinische Genetic* **9** (Suppl.), 60.
Vrettou C, Traeger-Synodinos J, Tzetis M 2004 Real time PCR for single cell genotyping in sickle cell and thalassemia syndromes as a rapid, accurate, reliable and widely applicable protocol for preimplantation genetic diagnosis. *Human Mutation* **23**, 513–521.
Vrettou C, Palmer G, Kanavakis E *et al*. 1999 A widely applicable strategy for single cell genotyping of β-thalassemia mutations using DGGE analysis: application to preimplantation genetic diagnosis. *Prenatal Diagnosis* **19**, 1209–1216.
Wells D, Sherlock JK 1998 Strategies for preimplantation genetic diagnosis of single gene disorders by DNA amplification. *Prenatal Diagnosis* **18**, 1389–1401.

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