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Original Article

# Single-cell high resolution melting analysis: A novel, generic, pre-implantation genetic diagnosis (PGD) method applied to cystic fibrosis (HRMA CF-PGD)☆,☆☆

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**Abstract**

*Background:* Institutions offering CF-PGD face the challenge of developing and optimizing single cell genotyping protocols that should cover for the extremely heterogeneous CF mutation spectrum. Here we report the development and successful clinical application of a generic CF-PGD protocol to facilitate direct detection of any *CFTR* nucleotide variation(s) by HRMA and simultaneous confirmation of diagnosis through haplotype analysis.

*Methods:* A multiplex PCR was optimized supporting co-amplification of any *CFTR* exon-region, along with 6 closely linked STRs. Single cell genotypes were established through HRM analysis following melting of the 2nd round PCR products and were confirmed by STR haplotype analysis of the 1st PCR products. The protocol was validated pre-clinically, by testing 208 single lymphocytes, isolated from whole blood samples from 4 validation family trios. Fifteen PGD cycles were performed and 103 embryos were biopsied.

*Results:* In 15 clinical PGD cycles, genotypes were achieved in 88/93 (94.6%) embryo biopsy samples, of which 57/88 (64.8%) were deemed genetically suitable for embryo transfer. Amplification failed at all loci for 10/103 blastomeres biopsied from poor quality embryos. Six clinical pregnancies were achieved (2 twin, 4 singletons). PGD genotypes were confirmed following conventional amniocentesis or chorionic villus sampling in all achieved pregnancies.

*Conclusions:* The single cell HRMA CF-PGD protocol described herein is a flexible, generic, low cost and robust genotyping method, which facilitates the analysis of any *CFTR* genotype combination. Single-cell HRMA can be beneficial to other clinical settings, for example the detection of single nucleotide variants in single cells derived from clinical tumor samples.



*Keywords:* Cystic fibrosis; High resolution melting analysis; Single-cell genotyping; Preimplantation genetic diagnosis

☆ Gene Symbol: CFTR, Approved name: cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7).

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## 1. Introduction

Cystic fibrosis (CF) (MIM#219700) is the most common autosomal recessive, severe genetic disease in Caucasians and it is characterized by an extremely heterogeneous mutation spectrum (>1900 mutations, Cystic Fibrosis Mutation Database available at http://www.genet.sickkids.on.ca/) [1,2], the majority being point mutations or small deletions (1–84 bp). According to published data by the European Society of Human Reproduction Embryology (ESHRE) PGD Consortium, to date the largest number of preimplantation genetic diagnosis (PGD) cycles among single gene disorders, has been performed for CF [3]. Institutions offering CF-PGD face the challenge of developing and optimizing single cell genotyping protocols that cover a potentially wide range of CF-causing mutations, following the best practice guidelines for PGD of cystic fibrosis [4]. PCR based-PGD methods entail the amplification and subsequent analysis of the target sequence(s) in a single nucleus (6.6 pg) and thus have to overcome the inherent risks of single cell PCR [PCR failure and allelic drop out (ADO)]. Currently used, CF-PGD methods aim at the simultaneous amplification of a panel of polymorphic STRs, to detect the mutant and the wild allele through linkage analysis, and detection of the most common mutation worldwide, p.Phe508del [5–8]. However, these approaches are inadequate when relatives or offspring are not available for segregation studies prior to the clinical PGD cycle and also in cases when the couple referred for CF PGD does not carry the p.Phe508del mutation. This is especially relevant in countries with extensive genetic heterogeneity, such as Greece (frequency of p.Phe508del ~53%) as well as other Southern European countries [9,10]. To facilitate clinical CF-PGD in those cases, the laboratory has to develop a "private" method. Novel "private" method development increases the cost and waiting time for the clinical implementation leading to a significant rise of the couple's financial and emotional burden.

High resolution melting analysis (HRMA) is simple, low cost, and has rapid turnaround times and a robust performance in a variety of settings. These characteristics render it a particularly attractive method for detection of nucleotide variations in clinical research and genetic diagnosis [11,12]. Briefly, in the simplest setting, HRMA involves a closed-tube PCR–melting curve analysis assay facilitated by ready mixed reagents including a saturation dye. PCR products are subsequently "melted," producing melting curves that correspond to wild type amplicons and amplicons bearing sequence variants. The majority of HRMA relevant published reports involve gene-scanning methods for the diagnosis of inherited genetic diseases [12]. Montgomery and co-workers, first reported a robust, accurate and fast *CFTR* gene scanning and genotyping method by HRMA [13]. Recently, our group published data relevant to the clinical implementation of a novel multiplex HRMA CF prenatal diagnosis protocol, demonstrating the dynamic of HRMA in prenatal diagnosis [14].

Here we report the development, optimization and successful clinical application of a single-cell HRMA method for CF-PGD. The reported assay is a novel, flexible, generic PCR PGD protocol, which facilitates direct detection of any *CFTR* nucleotide variation(s) by high resolution melting analysis, and simultaneous confirmation of diagnosis through STR-haplotype analysis.

## 2. Materials and methods

### 2.1. Validation study samples

The study samples, which were used in the development, optimization and validation of the method, are termed validation cases. The validation cases involve 4 family trios (4 CF carrier couples, 2 affected fetuses and 2 affected offspring) (Table 1). The couples requested to be considered for CF PGD by the Department of Medical Genetics (DMG) (Medical School — University of Athens), following the termination of an affected pregnancy or after the birth of an affected child. The University of Athens Ethics Committee approved the study and couples provided the DMG with their informed consent prior to participating in the study.

Whole blood samples were collected in EDTA tubes from the 4 CF carrier couples and the 2 affected offspring. Two DNA samples from amniocytes corresponding to affected terminated fetuses were also included in the study.

Whole blood samples were used to extract DNA, with Qiagen's Biorobot (M48) following the manufacturer's instructions (Qiagen Gmbh, Germany) and also to isolate single lymphocytes for PGD protocol optimization. For this purpose 208 single lymphocytes were isolated from the buffy coat following micromanipulations as previously described (Table 1) [15,16].

### 2.2. Validation cases: family trio work-up

A family trio was considered suitable for the development of the PGD method when alleles at the microsatellite loci included in the method were informative to facilitate segregation analysis. To establish trio informativity, a multiplex PCR aiming to co-amplify six microsatellite markers: D7S486, D7S677, IVS1-CA, IVS8-CA, IVS11-CA, and D7S2460 (Supplemental Table 1) was performed using approximately 40 ng of genomic DNA as template. Primers used to amplify the markers were labeled with 6-FAM, HEX or TAMRA for subsequent fragment analysis in an ABI3500 Genetic Analyzer sequencer (Applied Biosystems, Life Technologies Corporation, USA) (Supplementary Table 1). Allele sizes were analyzed by the GeneMarker Software v.1.95 (SoftGenetics LLC, PA, USA).

### 2.3. Multiplex PCR design

Each *CFTR* exon region of interest was amplified using published primers from Montgomery and co-workers [13] (Supplementary Table 2). The previously published primers were selected for the development of a CF PGD genotyping strategy for the following reasons: i) the amplicon sizes are relatively small (range 146–322 bp), hence minimizing any competitive amplification effect when included in a multiplex