SIMULTANEOUS APPLICATION OF CHROMOSOMAL MICROARRAY ANALYSIS AND POLYMERASE CHAIN REACTION GENETIC DISEASE DETECTION

by

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Fairfax, VA

Simultaneous Application of Chromosomal Microarray Analysis and Polymerase Chain Reaction Genetic Disease Detection

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Bachelor of Science
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DEDICATION

This work is dedicated to my wonderful and supportive wife Kim, my son Nathan, and our three dogs.

ACKNOWLEDGEMENTS

I would like to thank the Genetics & IVF Institute for the opportunity and financial support to conduct my research in partnership with GMU. Thanks to the staff in the PGD laboratory that helped me with data collection and analysis. Drs. Liotta and Mueller were of invaluable help. Special thanks to Dr. Mariani for all your ideas, guidance, and support. Finally, thanks go out to all who supported me during this journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

SIMULTANEOUS APPLICATION OF CHROMOSOMAL MICROARRAY ANALYSIS AND POLYMERASE CHAIN REACTION GENETIC DISEASE DETECTION

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George Mason University, 2014

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This thesis demonstrates the successful integration and application of chromosomal microarray analysis (CMA) and mutation-specific polymerase chain reaction (PCR) within 24 hours for the detection of unaffected euploid embryos from couples at risk for genetic disease undergoing *in vitro* fertilization treatment. Currently, array comparative genomic hybridization (aCGH) for detecting aneuploidy and PCR, and/or sequencing, for mutation status detection are performed at two different time points, thus, not appropriate for delivering results within a time frame suitable for a fresh embryo transfer. The techniques presented here have been optimized for analysis and reporting within a 24-hr time frame, allowing clinicians to offer their patients the option of fresh embryo transfer.

Whole genome amplification (WGA) of trophectoderm embryo biopsy was performed and aliquots of WGA product were divided for CMA and for laboratory-

developed fluorescent PCR coupled with capillary electrophoresis for genetic disease diagnosis. The combined protocol requires no modification of the CMA protocol and no pre-amplification steps were required prior to WGA. The WGA products were subjected to single round PCR testing for mutational and linked marker detection, except for mutations involving triplet repeats where nested PCR was necessary.

Although current techniques are available for detecting aneuploidy and genetic mutation status, such as aCGH or SNP arrays for aneuploidy and PCR or sequencing for gene mutation, these methods cannot be combined for analysis within a 24-hour time frame. The successful integration and application of these important techniques provides a comprehensive diagnostic approach that can be offered to couples at risk for single-gene disorders wishing to receive a fresh embryo transfer. This new approach allows laboratories currently equipped to perform aCGH and PCR to utilize their existing setup for a novel comprehensive diagnostic protocol without additional equipment acquisition.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) is a diagnostic procedure that is offered to patients undergoing *in vitro* fertilization (IVF) treatment and is used as a selective tool within the area of assisted reproductive technology for the determination of normal and abnormal embryos prior to transfer to the uterus *(Braude et al., 2002).* This technology was initially implemented in the clinical setting for the detection of embryos affected and unaffected with a familial genetic disease from couples with reproductive risks *(Braude et al., 2002; Handyside et al, 1990).* The application of PGD, however, now expands beyond the screening of genetic disease abnormalities, to include detection of chromosomal abnormalities, also referred to as preimplantation genetic screening (PGS) *(Van Assche et al.,1999; Rubio et al., 2003; Wilton, 2005; Treff et al., 2010).* Several embryo biopsy techniques are available for obtaining the specimen required for PGD, including polar bodies biopsy from oocytes, blastomere biopsy from cleavage-stage embryos, and trophectoderm (TE) biopsy from blastocyst-stage embryos *(Dayal and Athanasiadis, 2013).*

The idea of polymerase chain reaction (PCR) has evolved since its conception in the early 1980s to be a powerful method implemented within the research community as well as in the clinical setting. Fluorescent PCR and capillary electrophoresis (CE), where the PCR products are fluorescently labeled, separated, and detected by a laser within a

light-sensitive scanner, has been developed and adopted as a reliable technique used in PGD for detection of desired single-gene mutations *(Findlay and Quirke, 1996)*. The ability to distinguish wild-type alleles and mutations associated with a particular genetic disease is not new. In 1985, Saiki and colleagues interrogated fetal DNA obtained from amniocentesis or chorionic villus sampling with PCR and restriction site analysis techniques for the presence of wild-type and sickle-cell alleles from β-globin genomic sequences found in sickle cell anemia *(Saiki et al., 1985).* In the case of couples undergoing IVF treatment with reproductive risks for passing on an undesirable genetic mutation, PCR tests can be developed to target the specific mutation(s). PGD utilizing these PCR tests can be used adjunct with IVF treatment plans to screen for embryos free of the genetic disease in question.

PGD for the detection of chromosomal anomalies have been suggested to reduce the risk of pregnancy loss in women with advanced maternal age *(Munne et al., 2005)*. Aneuploidy detection can be performed by using a variety of methods, including fluorescent *in situ* hybridization (FISH), single-nucleotide polymorphism (SNP), conventional metaphase comparative genomic hybridization (CGH), and microarray-CGH (aCGH) *(Pinkel et al., 1988; Conn et al., 1998; Munne et al., 1998, 2000; Treff et al., 2010; Alfarawati et al., 2011)*. PGD using FISH has limitations, as FISH-based techniques do not allow for a comprehensive cytogenetic assessment. FISH probes are available for detection of only a subset of chromosomes, including chromosomes 13, 15, 16, 18, 21, 22, X, and Y *(Vysis FISH probes – Abbott Molecular)*. The FISH-based method has been traditionally viewed as a principle technique for PGD. In recent years,

however, chromosomal microarray analysis (CMA) techniques are becoming more common and accepted as reliable resources for a comprehensive detection of cytogenetic abnormalities. CMA allows for aneuploidy detection of all 23 pairs of chromosomes. It has been suggested that the future direction of PGD should involve comprehensive embryo testing, to include not only the screening for chromosomal abnormalities, but also the detection of genetic disease mutations *(Hens et al., 2013).*

The ultimate goal for any couple undergoing IVF treatment is to get pregnant. The topic of performing a fresh embryo transfer versus freezing an embryo for transferring at a later time is an ongoing debate, however, evidence have shown that performing a fresh embryo transfer could increase the pregnancy rate *(Check et al., 1995, 2012; Borini et al., 2006).* The decision for performing fresh or frozen embryo transfer is relied partly by the clinicians' preference within an IVF center and partly by the technical limitations of generating PGD results within a timeframe required for performing a fresh embryo transfer. Currently, methods for determining both single-gene mutations and cytogenetic profiles are available, however, in order to perform both for a given biopsied sample; a frozen embryo transfer must be performed due to the time limitations of each method. PGD techniques for successfully combining these two protocols must be optimized for completion within 24 hours if clinicians want to offer their patients the option of receiving a fresh embryo transfer.

It is the purpose of this thesis to demonstrate the successful integration and clinical application of CMA and fluorescent PCR to determine both chromosomal aberrations and single-gene mutation status within a 24-hour time frame. By

implementing this integrated method, PGD laboratories that are equipped to perform PCR and aCGH can use their existing setup to offer a novel comprehensive diagnostic assay within 24 hours; a valuable and cost effective option that could be offered to IVF patients desiring a fresh embryo transfer.

MATERIALS AND METHODS

Embryologists at the Genetics & IVF Institute (GIVF) performed TE biopsies to provide the PGD laboratory with samples for testing. An embryo biopsy worksheet was provided for all samples subjected for TE biopsy with information including embryo quality and number of cells taken during biopsy. All patients were properly consented and given all applicable information regarding the IVF and PGD procedures. TE biopsies were taken from day 5 and/or day 6 of embryo development. The TE biopsies were subjected to whole genome amplification (WGA) via the Sureplex DNA amplification system according to the manufacturer's instructions *(BlueGnome, Sureplex)*, where a portion of the WGA product is used for the CMA PGD procedure and the remaining portion is stored at -20° C as extra. In efforts for developing a suitable method for combining the CMA and PCR protocols, the excess WGA DNA samples were used for investigating the ability of laboratory-developed primer sets to successfully amplify target regions from WGA DNA. Twenty excess WGA DNA samples, 10 biopsies containing 2 cells and 10 biopsies containing 8 cells, were used for determining the effects of PCR amplicon length and biopsy cell number on adequate of target amplification. Upon completion of the genetic disease detection assay development and compatibility assessment between PCR primer sets and WGA DNA, the integrated CMA and genetic disease detection PCR protocol was utilized for clinical testing of 42 embryos

from various cases requiring both CMA and genetic disease diagnosis. Analyses of CMA data and genetic disease PCR data were completed within 24 hours of embryo biopsy for cases requiring a fresh embryo transfer (Figure 1).

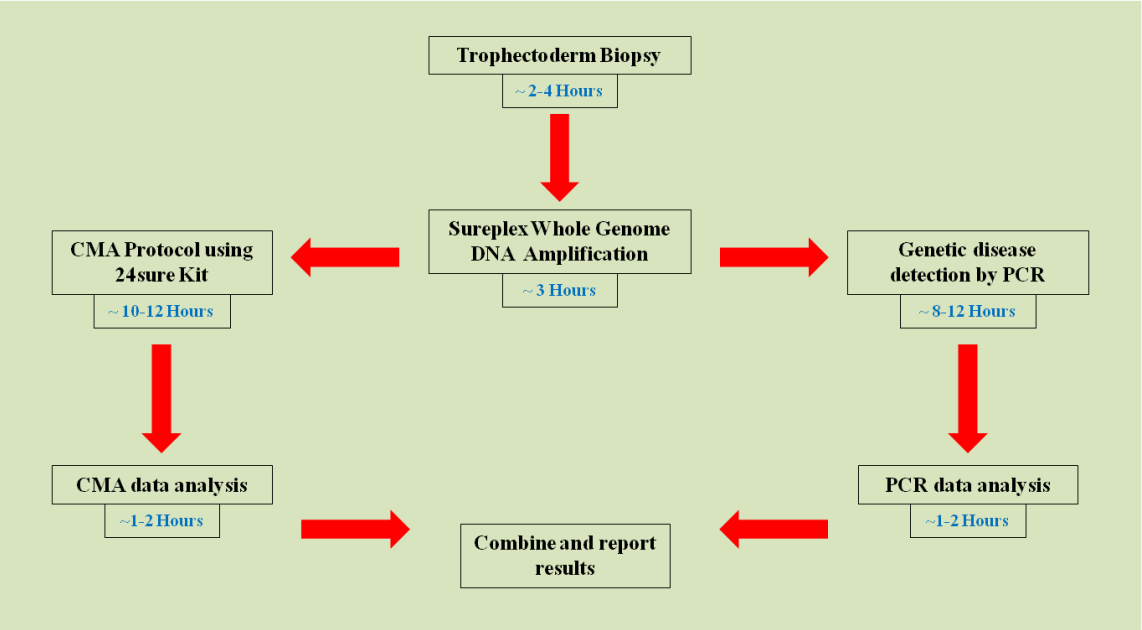

Figure 1. Flow chart for combing CMA and PCR steps with corresponding time required for each step.

Genetic disease test development

All patients enrolled in the PGD program undergo extensive genetic counseling regarding the risks and benefits of PGD, as well as other reproductive options such as post-conception prenatal testing, use of a gamete donor, or adoptive options. Prior to initiation of IVF, informed consents for PGD and related topics are recorded, including the option to use patient results and laboratory-generated materials, i.e. abnormal embryos and/or amplified DNA, for research or publication purposes provided that all

patient identifiers are removed. Records of confirmed DNA mutation analysis on the proband, or the at-risk couple, are obtained and reviewed prior to assay development. Once the mutation was identified and determination that an assay can be developed to target the mutation of interest, whole blood and/or buccal swab samples from all individuals required for the workup were obtained for the test development phase. DNA purification from the patient samples was performed by using QIAGEN silica-membrane-based QIAamp DNA Blood Mini Kit according to the manufacturer's instructions *(QIAGEN-Blood DNA mini kit)*. The purified DNA was used for mutational and short tandem repeats (STR) markers test development with linkage determination.

Primer design was performed using the Primer3Plus online software application *(Rozen, 2000)* and Molecular Biology Insights's Oligo v6.61 Primer Analysis software. Reference DNA nucleotide sequences for target genes and STR markers were obtained from online reference databases *(NCBI GenBank; UCSC Genome Browser)*. The STR markers were used for haplotyping, monitoring allele drop-out (ADO) and recombination, and screening for contamination by external DNA sources. Laboratory-developed PCR primer sequences were sent to Eurofins MWG Operon for synthesis and fluorescent dyes modifications *(Eurofins Scientific)*. Diluted patient genomic DNA was used to determine whether the custom-designed primers are successful in detecting the mutation of interest and whether the STR markers were informative loci – yielding different allele sizes for the couple undergoing PGD. Excess Sureplex whole genome amplified DNA from previously tested TE biopsies were used to determine compatibility between Sureplex generated DNA products and the laboratory-developed PCR primers.

CMA and genetic disease detection

The Sureplex DNA amplification system generates about 2 to 5 micrograms of double stranded DNA from single cells with 70% to 90% genome coverage, yielding DNA fragment size averaging 500-600 base pairs (bp) *(Bluegnome, Sureplex)*. Successful amplification was determined by 12% polyacrylamide gel electrophoresis of DNA products, coupled with SYBR® Green staining and transilluminator visualization. The final product from Sureplex WGA samples was divided for simultaneous CMA and genetic disease testing by PCR. The CMA portion was performed by using BlueGnome© 24sure v3 arrays according to the manufacturer's instructions (BlueGnome, 24sure). Scanner-generated TIFF images were imported into the BlueFuse© Multi Software for analysis. For genetic disease diagnosis, a 5-µL aliquot containing approximately 300 ng of Sureplex© amplified DNA was subjected to PCR using laboratory-developed PCR primer sets for all single-gene mutation tests. PCR conditions and primer concentrations varied for some STR markers and mutation tests depending on the nature of the primers. All PCR reactions were performed using the Bio-Rad C1000 Thermal Cycler and were analyzed by capillary electrophoresis (CE) using the Applied Biosystems 3130xl Genetic Analyzer. Visual fragment length analyses were done with Applied Biosystems GeneMapper Software v4.1. Results for both CMA and genetic disease status were obtained within 24 hours, from the time of embryo biopsy, for cases requiring a fresh embryo transfer.

Nucleotide substitutions mutations

Detection of single base substitutions was achieved by generating an unlabeled amplicon spanning the mutation sites, followed by ABI SNaPshot® single-base extension

of a fluorescently labeled chain terminating dideoxinucleotide to the 3'-end of the extension primer, placed adjacent to, but not including, the mutation site. This approach yielded fluorescently labeled single-base extension products corresponding to the normal and/or mutated nucleotide. The color of the resulting product indicated which nucleotide was incorporated based on the dye attached to the nucleotide (Table 1).

Deletion, duplication, and larger genomic mutations

Detection of small deletions or duplications was accomplished by using fluorescently labeled primers to generate amplicons spanning the genetic alteration, followed by fragment size analysis by CE. Similarly, larger genomic alterations, such as expanded trinucleotide repeat mutations found in Huntington disease, were detected by fluorescent-PCR fragment size analysis using PCR strategies specifically designed for GC-rich regions as described by Stern et al. *(2002)*.

RESULTS

All PCR protocols were successfully adapted and compatible for use with WGA products from TE biopsy samples. No modification of the CMA protocol was necessary, including no pre-amplification steps prior to Sureplex DNA amplification. Sureplex WGA products from TE biopsies from various cases underwent CMA testing using 24sure v3 CGH arrays and in parallel, single round PCR for mutational and linked marker detection, except for mutations involving triplet repeats where nested PCR was necessary (Table 2). The results of marker alleles and patient-specific mutational PCR profiles as analyzed by CE were consistent between lymphocyte-derived DNA from a proband and/or the parents and related TE WGA DNA products in all cases (Table 3).

Table 1. Dye assignment from ABI PRISM ® SNaPshot Multiplex Kit *(Life Technologies)*

ddNTP	Dye Label	Color of Peak
A	dR6G	Green
C	dTAMRA ™	Black
G	dR110	Blue
T (U)	dROX ™	Red

Table 2. Single-gene mutation and detection methodology

Specific mutation	Mutation class	Detection method	PCR amplicon length
COL2A1 Exon 41 c.2680G>T	Single base substitution	Single base extension	91 bp
CLCN5 Exon 2 c.82C>T	Single base substitution	Single base extension	119 bp
PEX6 Exon 8 c.1802G>A and Exon 13 c.2434C>T	Single base substitution	Single base extension	121 bp
CFTR Exon 10 ΔF508	Deletion	Fragment length detection	149 bp / 152 bp
ATP7A Exon 4 c.1020-1024dupGGGGC	Duplication	Fragment length detection	115 bp / 120 bp
HTT Exon 1 CAG repeats expansion	Trinucleotide expansion	Fragment length detection	130 bp - 220 bp

Table 3. Gene flanking STR marker sizing. Red bolded allele size is linked to corresponding gene mutation.

Gene Name		Flanking STR Marker	
		D12S1627	
COL2A1	Maternal	85 / 93	
	Paternal	**79** / 89	
	Embryo 1	**79** / -	
	Embryo 2	**79** / 93	
	Embryo 3	89 / 93	
	Embryo 4	89 / 93	
		PEX6SetC	**PEX6SetD**
PEX6	Maternal	**169** / 177	**180** / 182
	Paternal	**169** / 177	**178** / 192
	Embryo 1	**169**	**178 / 180**
	Embryo 2	**169** / 177	**178** / 182
	Embryo 3	177 / **169**	192 / **180**
	Embryo 4	**169** / 177	**178** / 182
	Embryo 5	**169**	**178 / 180**
	Embryo 6	177 / 169	192 / 180
		ATP7AsetA	**ATP7AsetD**
ATP7A	Maternal	114 / **122**	183 / **185**
	Paternal	114	183
	Embryo 1	114	183
	Embryo 2	**122**	**185**
	Embryo 3	**122**	**185**
	Embryo 4	- / -	183 / **185**
	Embryo 5	**122**	**185**
	Embryo 6	114 / **122**	183 / **185**
	Embryo 7	**122**	-
	Embryo 8	114 / -	183 / **185**
	Embryo 9	114	183
	Embryo 10	**122**	-
	Embryo 11	**122**	-
		CLCN5SetG	**CLCN5SetD**
CLCN5	Maternal	**186** / 196	**107** / 109
	Paternal	173	109
	Embryo 1	173 / **186**	109 / **107**
	Embryo 2	173 / 196	109 / 109
	Embryo 3	173 / 196	109 / 109
	Embryo 4	173 / **186**	109 / **107**
	Embryo 5	173 / 196	109
	Embryo 6	**186**	-
	Embryo 7	196	109
	Embryo 8	196	109
	Embryo 9	173 / **186**	109 / **107**
		IntraB	**3CFTRset4**
CFTR	Maternal	**163** / 165	**183** / 183
	Paternal	**163** / 169	**183** / 181
	Embryo 1	169 / 165	183 / 181
	Embryo 2	169 / 165	183 / 181
	Embryo 3	**163**	**183**
	Embryo 4	165 / 169	181 / 183
	Embryo 5	**163**	**183**
	Embryo 6	169 / **163**	181 / **183**
	Embryo 7	169 / 165	181 / 183
		D4S412	**D4S2285**
HTT	Maternal	164 / **158**	181 / **169**
	Paternal	166 / 168	169 / 169
	Embryo 1	166 / 164	169 / 181
	Embryo 2	166 / **158**	169 / **169**
	Embryo 3	168 / **158**	169 / **169**
	Embryo 4	168 / 166	169 / 181
	Embryo 5	168 / 166	169 / 181

Amplicon length and biopsy cell number

It is essential to test the ability of Sureplex WGA products to serve as an adequate template to support target-specific PCR. In this context, it was discovered that two important variables must be considered; amplicon length and the number of cells in the TE biopsy sample. Given that the DNA isolated from TE biopsy samples undergoes a fragmentation step during the Sureplex WGA to yield a population of molecules 500-600 bp in length, it was necessary to evaluate the ability of laboratory-developed PCR primer sets to successfully amplify targeted regions. The other variable influencing data quality was the number of cells taken at the time of TE biopsy, ranging from 1-2 cells up to 8-10 cells per biopsy, with an average of 4-5 cells per TE biopsy. PCR primers selection for the mutation site and flanking STR markers was designed to generate final product sizes ranging from 90 to 200 bp in majority of the cases, well below the 500-600 bp product produced by the Sureplex DNA amplification system (Table 2).

Twenty excess WGA DNA, 10 samples containing 2 cells and 10 samples containing 8 cells, from previously tested TE biopsies were analyzed using two PCR primer sets for a highly polymorphic locus located on chromosome 18 (D18S51). The two primer sets, short and long, yielded amplicon sizes of 130 bp and 328 bp, respectively (Figure 2). Sureplex WGA DNA from two groups of TE biopsies, 10 samples containing 2 cells and 10 samples containing 8 cells, were analyzed in parallel reactions for both the short and long PCR primer sets. The short PCR primer set detected two alleles for both groups of cell number sample. The long PCR primer set detected two alleles in nine out of the ten 8-cell biopsy samples. For the 2-cell biopsy samples, the long PCR primer set detected 2 alleles for 6 samples, 1 allele for 3 samples, and failed to

detect anything for one sample. An example of the CE profiles for one 2-cell sample and one 8-cell sample analyzed using the short and long PCR primer sets are represented in Figure 3 A, B, C, and D. The relative fluorescence unit (RFU) for each allele peak for both amplicon lengths and from both cell number sample types were assessed and recorded for comparison purposes. In summary, the RFU's from the short amplicon primer pair were consistently superior compared to those of the long amplicon primer pair for both cell number sample type (Table 4).

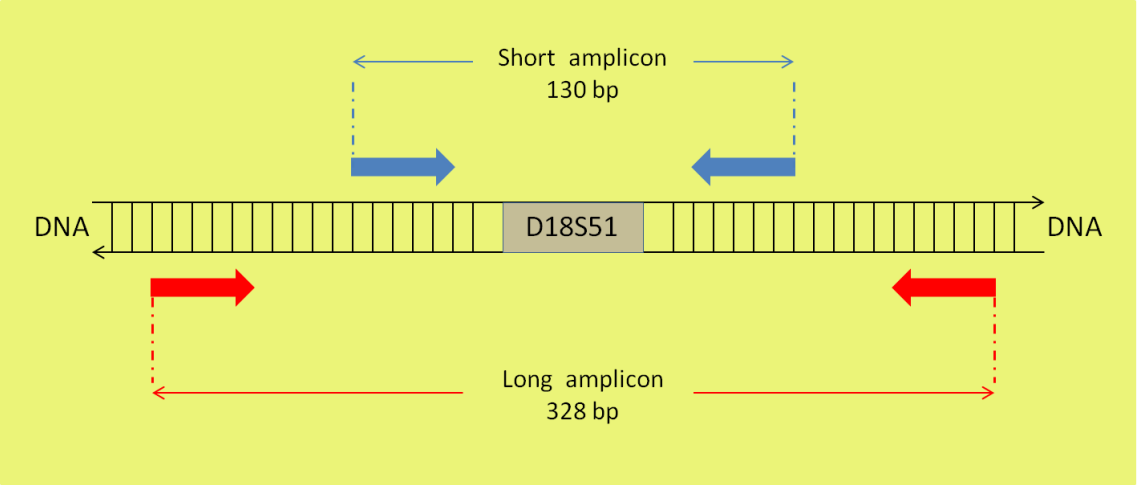

Figure 2. Schematic representation of the design for investigating the impact of PCR amplicon length on successfully generating products from WGA DNA.

Table 4. Amplicon length and biopsy cell number assessment. Each sample was analyzed with both the short and the long PCR primer pairs, yielding short and long amplicons, respectively. The number of alleles detected was counted for each sample for both the short and long amplicons. The relative fluorescence units (RFU) for each allele peak were recorded, ranging from 0 to 8242 RFU.

Sample Number	Cell Number	Short Amplicon Alleles detected	Short Amplicon Allele 1 RFU	Short Amplicon Allele 2 RFU	Long Amplicon Alleles detected	Long Amplicon Allele 1 RFU	Long Amplicon Allele 2 RFU
1	2	2	7842	6730	0	0	0
2	2	2	7611	7459	2	656	714
3	2	2	6979	6979	1	0	5203
4	2	2	7022	7763	1	0	415
5	2	2	6612	6311	1	0	954
6	2	2	6897	6324	1	0	922
7	2	2	7605	6778	2	446	593
8	2	2	7153	7236	2	2430	3716
9	2	2	7557	7153	2	270	521
10	2	2	7560	7536	2	349	919
11	8	2	7630	7958	2	3600	1215
12	8	2	7245	7676	2	873	840
13	8	2	6715	7056	2	1860	1704
14	8	2	7806	7490	2	4099	4253
15	8	2	7653	8242	1	0	3074
16	8	2	6810	7531	2	1858	653
17	8	2	8170	8088	2	3387	1794
18	8	2	7262	7825	2	967	446
19	8	2	7269	7822	2	690	612
20	8	2	7425	7908	2	1849	870

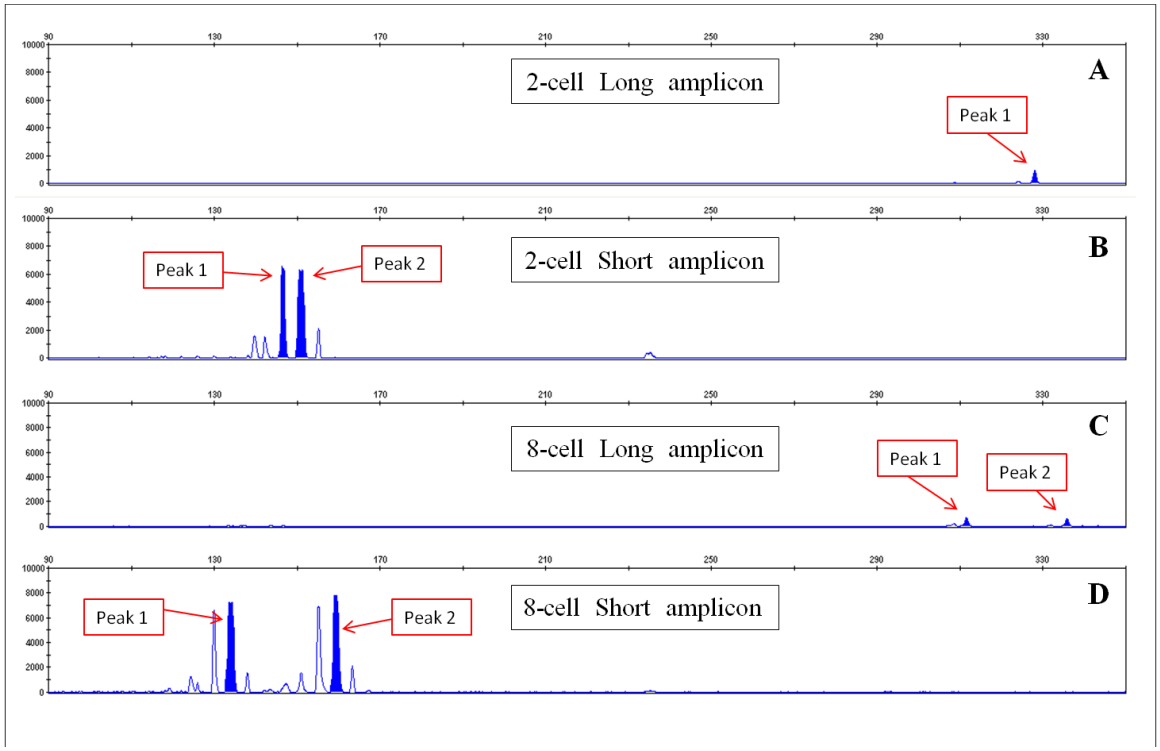

Figure 3. CE profile for one 2-cell and one 8-cell sample tested with primer sets designed to generate a long (A, C) amplicon and a short (B, D) amplicon.

Transition and transversion mutations

Three examples of PGD for substitution mutations are presented here to demonstrate the simultaneous application of CMA and genetic disease detection for clinical application: dominant Stickler syndrome Type 1 (a disorder of type II collagen), X-linked recessive Dent disease (X-linked hypercalciuric nephrolithiasis), and recessive Zellweger syndrome spectrum (a group of peroxisome biogenesis diseases) (Table 5).

Stickler syndrome involved a G>T transversion in exon 41 at nucleotide position c.2680 of the *COL2A1* gene. A primer set spanning the mutation site generating a 100-bp amplicon was used as template for the single-base extension reaction. Extension of a sequencing primer corresponding to the 'top' strand of the amplicon incorporated

Guanine, a dR110- labeled base, for the normal gene copy and Thymine, a dROX™-labeled base, for the mutated gene copy. Four embryos were tested for chromosomal abnormalities and the *COL2A1* mutation; of which 2 were affected and 2 were unaffected. Of the 4 embryos tested, 1 had chromosomal anomalies (Figure 4).

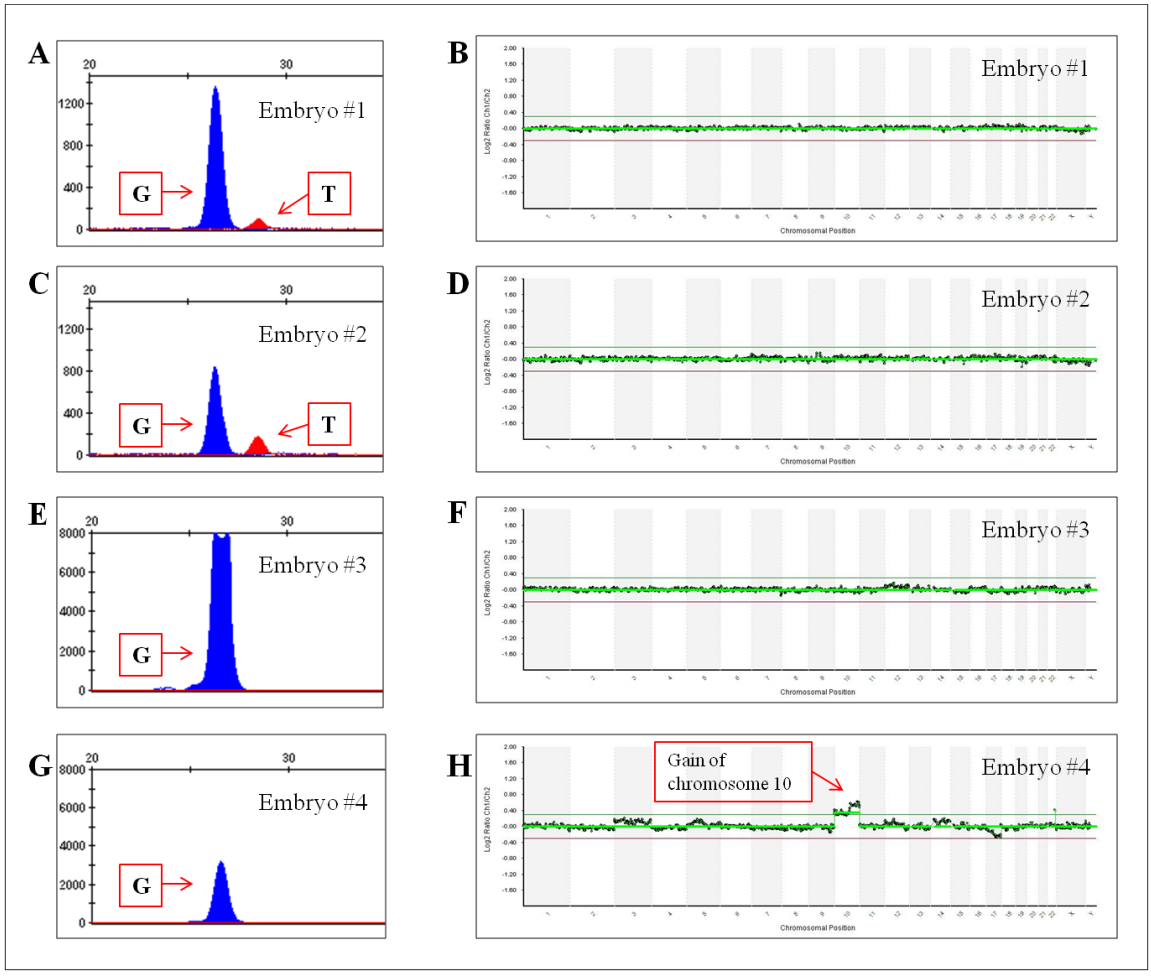

Figure 4. *COL2A1* Exon 41 c.2680G>T single base extension CE profiles (A, C, E, G) and corresponding CMA profiles (B, D, F, H) for embryos 1 through 4.

Dent disease involved a single base mutation within the *CLCN5* gene that resulted in the replacement of an Arginine codon (CGA) with a stop codon (TGA). The PCR assay utilized a 119-bp amplicon spanning the nonsense C>T transition at nucleotide position c.82 in exon 2 of the *CLCN5* gene. The single-base extension of a sequencing primer corresponding to the 'top' strand of the gene produced a dTAMRA™-labeled Cytosine from the normal sequence and a dROX™-labeled Thymine from the mutation. A total of 9 embryos were tested, resulting in 3 carriers, 3 non-carriers, 2 unaffected embryos, and 1 affected embryo (Table 5).

The third substitution mutation case involved a couple who were compound heterozygotes for recessive Zellweger syndrome spectrum, with each parent carrying a different mutation in the *PEX6* gene. The affected paternal allele had a G>A missense transition in exon 8 at position c.1802 and the affected maternal allele had a C>T missense transition in exon 13 at position c.2434. Out of the 6 embryos tested, 4 were carriers and 2 were affected with both mutations. Three of the 6 embryos were chromosomally abnormal (Table 5).

Deletion and duplication mutations

The strategy for testing small deletions and duplications involved detecting fragment length differences of fluorescently labeled amplicons spanning the mutation site. A case involving the ΔF508 3-bp deletion in exon 10 of the *CFTR* gene found in cystic fibrosis is presented here. Due to the autosomal recessive inheritance nature of the disease, a carrier profile would show the normal fragment (152-bp) and the fragment bearing the 3-bp deletion (149-bp). Seven embryos were tested, 2 of which were

homozygous for the ΔF508 mutation, 1 was a carrier, and 4 were non-carriers. Two of the 7 embryos tested were aneuploid (Figure 5).

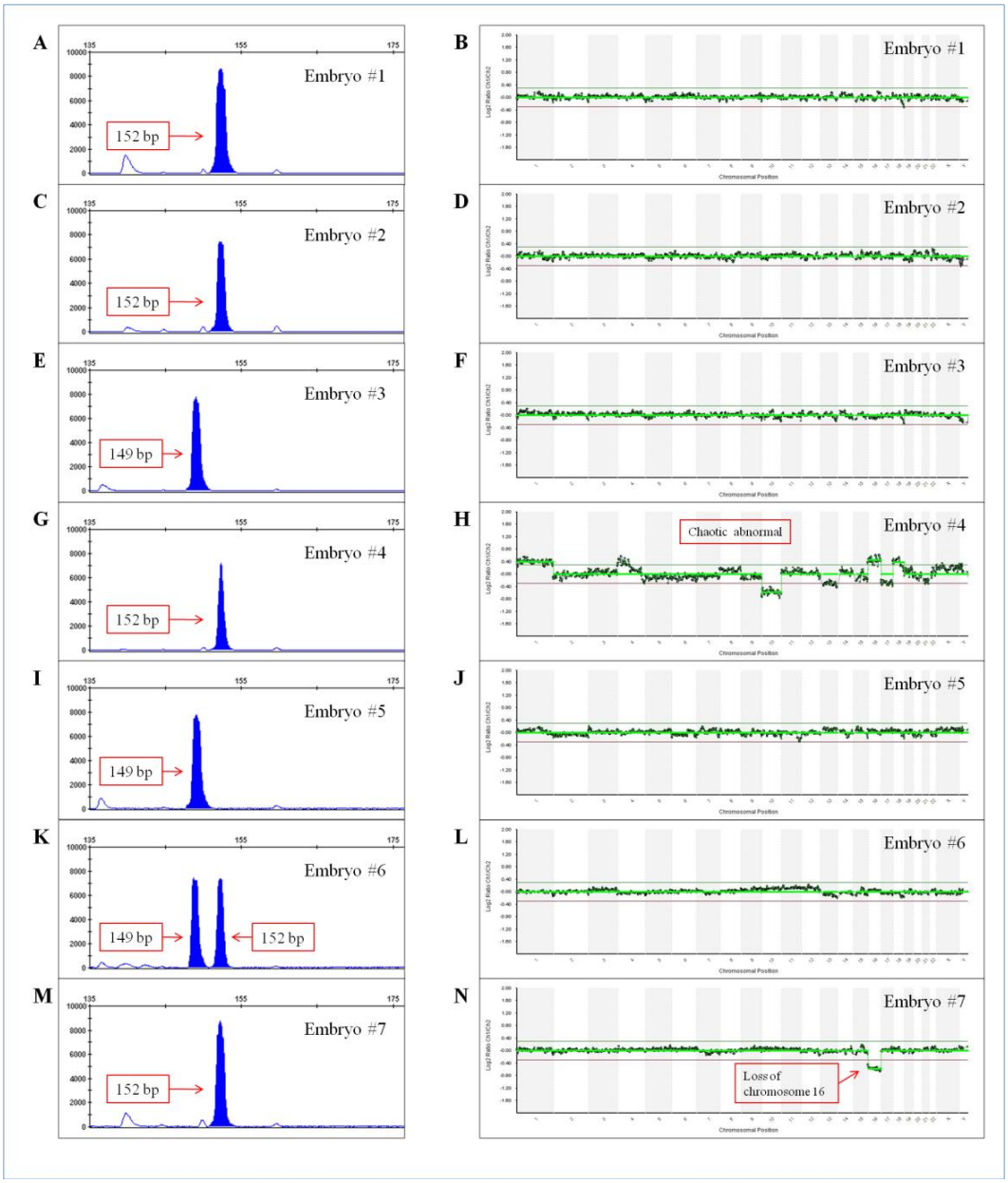

Figure 5. *CFTR* ΔF508 deletion mutation CE profiles (A, C, E, G, I, K, M) and corresponding CMA profiles (B, D, F, H, J, L, N) for embryos 1 through 7.

For duplication (or insertion) mutations, a PGD test for recessive Menkes disease (a disorder of copper metabolism) was designed to detect a 5-bp duplication in exon 4 (c.1020_1024dupGGGGC) of the *ATP7A* gene, resulting in a frameshift and premature termination of protein synthesis. Genetic testing determined that both parents were heterozygous carriers for the duplication. Using WGA DNA as template, an amplicon spanning the duplication yielded a 120-bp fragment containing the 5-bp duplication compared to the 115-bp fragment generated from the normal gene. Eleven embryos were tested, 2 of which were either unaffected or non-carrier for Menkes disease, 3 were carriers, and 6 were affected. Of these 5 potentially transferable embryos only 2 were chromosomally normal (Table 5).

Trinucleotide expansion mutations

Molecular PGD for Huntington disease is an important example of mutational testing involving expansion of trinucleotide sequence motifs within or flanking genes. Using PCR primer sets spanning the region of expansion, normal verses expanded allelic variants can be differentiated by CE fragment size analysis. For the *HTT* gene, the CAG trinucleotide expansion in exon 1 is a GC-rich region presenting a difficult template for Taq polymerase read-through leading to a relatively high rate of ADO using standard PCR conditions. The nested PCR protocol presented here was designed to enhance polymerase processivity for highly expanded alleles leading to a significant reduction in ADO *(Stern et al., 2002)*. A PGD test was designed to detect normal allele sizes as well as the abnormally expanded repeats. The two paternal *HTT* alleles were 18 repeats (139-bp) and 19 repeats (142-bp), the larger of which was the same size as the normal maternal

allele. The abnormal maternal *HTT* allele was 48 repeats. Examination of the mutational data showed that 3 of 5 embryos available for testing were unaffected (Figure 6). Of the 3 unaffected embryos, one contained two normal 19-repeat (142-bp) alleles, one from each parent as verified by *HTT*-linked STR analysis (Table 3). The other two unaffected embryos contained the paternal 18-repeat allele and maternal 19-repeat allele. All 3 unaffected embryos were chromosomally normal (Figure 7).

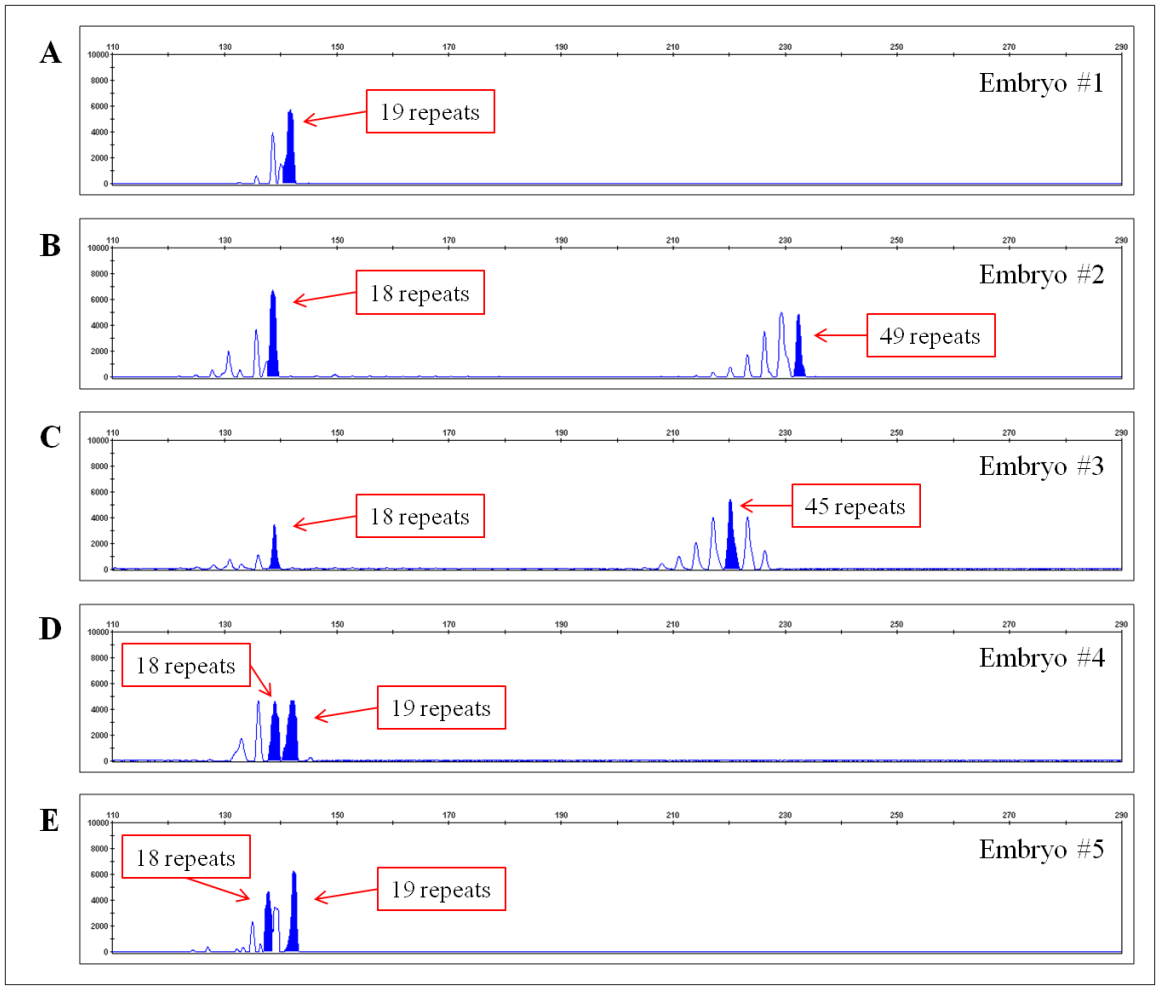

Figure 6. *HTT* CAG trinucleotide repeats CE profiles for embryos 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E).

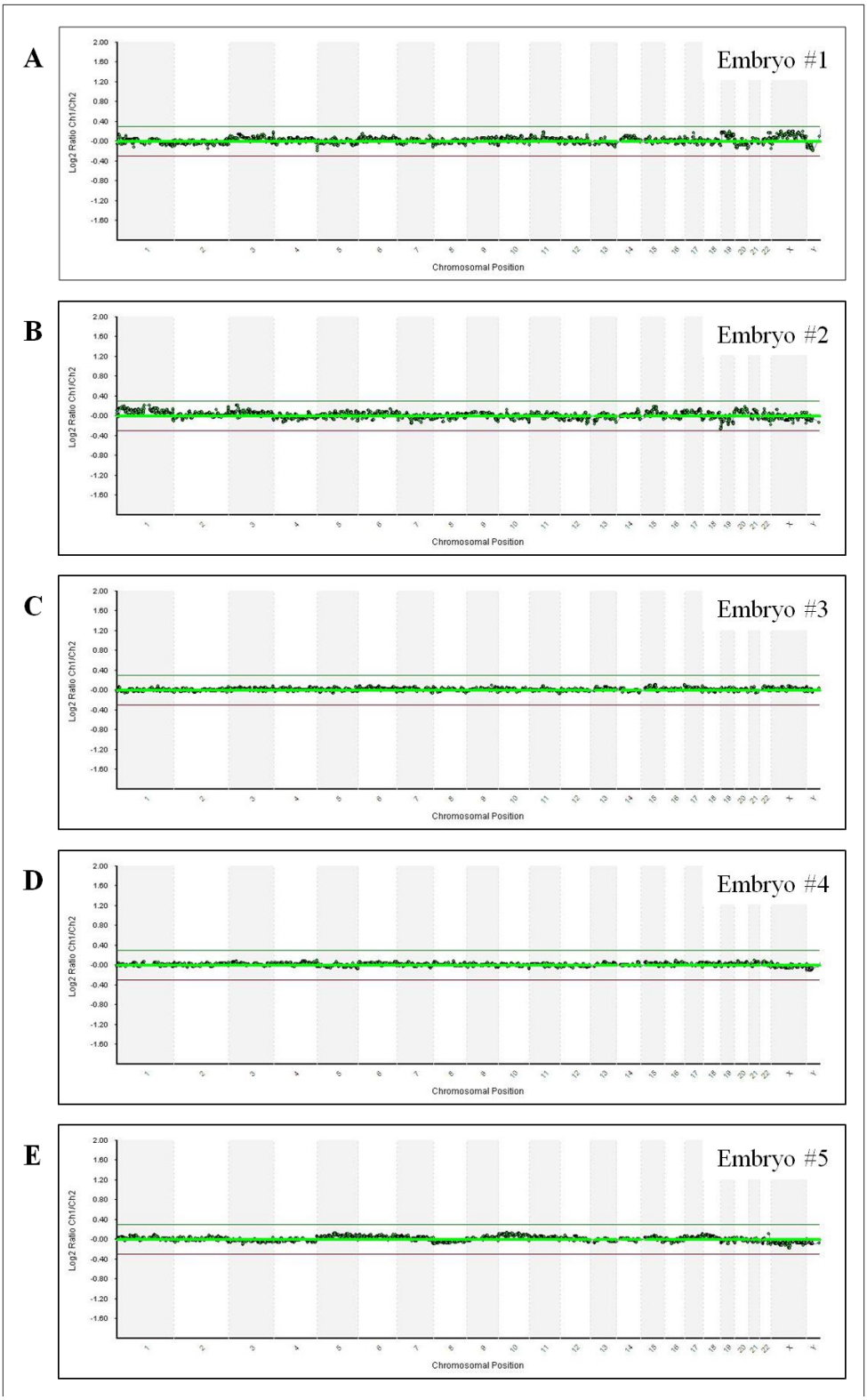

Figure 7. CMA profiles for *HTT* CAG expansion embryos 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E).

In summary, it was found that of the 42 embryos tested in these cycles, 27 were either non-carriers / unaffected (16/27) or recessive carriers (11/27) of a genetic mutation. Of these 27 potentially transferable embryos, 13 were available for transfer after assessment of both genetic disease status and chromosomal abnormalities (Table 5).

Table 5. Results summary for combined CMA and genetic disease detection cases.

	Genetic Disease Status			**CMA Status**		**Total Tested**	**Total Available for Transfer**
Genetic Disease Mutation	Non-carrier or Unaffected	Carrier	Affected	Abnormal	Normal		
COL2A1 Exon 41 c.2680G>T	2	N/A*	2	1	3	4	1
CLCN5 Exon 2 c.82C>T	5	3	1	4	5	9	3
PEX6 Exon 8 c.1802G>A and Exon 13 c.2434C>T	0	4	2	3	3	6	1
CFTR Exon 10 ΔF508	4	1	2	2	5	7	3
ATP7A Exon 4 c.1020-1024dupGGGGC	2	3	6	5	6	11	2
HTT Exon 1 CAG repeats expansion	3	N/A*	2	2	3	5	3
* Autosomal dominant inheritance pattern will only yield affected or unaffected segregants							

DISCUSSION

The development of protocols for the detection of genetic disease and aneuploidy has been on parallel but separate tracts over the past 20 years *(Munne et al., 1993; Vidal et al., 1998; Wells et al., 1999; Goshen et al., 1996).* The introduction of genome-wide CMA and WGA allows for the combination of microarray platforms with PCR-based genetic disease detection protocols to be feasible, since both methods can use amplified DNA as starting material. In 2006 and 2007, studies showed advancement in microarray technology for PGD, reviewed by Treff and Scott *(2012)* and Vanneste *et al. (2012).* One approach utilized aCGH for CMA, in which a sample of WGA DNA from an embryo biopsy competes for hybridization to immobilized bacterial artificial chromosome or oligonucleotide probes with DNA from a karyotypically normal reference *(Le Caignec et al., 2006; Fiegler et al., 2007).* Duo-color fluorescent labeling is used to assess the comparative signal intensities for each sample to the surface-bound probes to quantify gains or losses of signal for individual chromosomes from the sample relative to the reference. Besides array-based methods, an alternative approach has been validated using fluorescent-based quantitative real time PCR (qPCR) to determine comprehensive chromosomal copy number *(Treff and Scott, 2013).* With this protocol, primers for genetic disease analysis can be added and thus genetic disease detection and CMA testing can be combined.

SNP arrays have also been used for genetic disease detection by haplotyping *(Treff et al., 2008; Kearns et al., 2007).* The SNP-based approach used genome-wide genotypic data from parental samples to generate haplotype maps and recombination patterns in DNA from biopsied blastomeres *(Rabinowitz et al., 2009; Schoolcraft et al., 2011).* In this manner it is possible to identify the segregation of affected and unaffected parental haplotypes in each embryo and also to make predictions of chromosomal aneuploidy, however, results cannot be obtained within the time frame required for a fresh embryo transfer. *(Handyside et al., 2010).* A case study published in 2011 reported on a protocol using WGA on DNA from TE samples for SNP analysis for aneuploidy, and in a different laboratory, PCR-based haplotyping was performed on the same amplified DNA for mutation detection *(Brezina et al., 2011).* This demonstrated the feasibility of testing for both aneuploidy and single gene mutations, however, the results cannot be obtained in time for a fresh embryo transfer since the biopsy samples were sent to separate laboratories for testing. The concept of combined microarray and PCR strategies on the same biopsy material has merit *(Hens et al., 2013).* Embryo biopsy is already being performed in these cases for genetic disease testing or CMA. Therefore, the integration of CMA and genetic disease detection to a PGD assay does not add any additional risk to the developing embryo. As opposed to SNP-based methods that rely on tracking the inheritance of affected haplotypes using parental genotypes as a guide, the PCR-based method provides direct data on the presence or absence of mutation in each embryo, including carrier status in some cases.

The successful combination of CMA and PCR for genetic disease detection demonstrated in this study is in part the result of selecting primer sets that generate amplicons compatible with WGA product as template. Greater cell number biopsy samples also contribute to the generation of CMA and single-gene mutation data. The results demonstrated that when using shorter amplicons and a higher cell number TE biopsy sample, the occurrence of ADO was significantly reduced or eliminated and the overall fluorescent intensities were superior (Table 4). This approach increases amplification efficiency and reduces ADO compared to cleavage stage single cell biopsy or lower cell number TE biopsy.

There are technical limitations that could impede the PCR-based single gene mutation detection. First, the quality of the starting template is vital to the success of the PCR reaction. The WGA product used for both CMA and PCR is produced from a commercially available kit, such as the SurePlex Amplification System used in this protocol, which provide only up to 90% genome coverage. Careful considerations must be made when designing primers for a specific target, as primer efficiencies could be negatively affected by insufficient genomic availability. Second, repetitive genomic sequences could be inadequately represented from WGA. Primer design and selection to target trinucleotide repeats, specifically CG-rich regions, should be performed carefully to ensure successful target amplification. Nested PCR should improve the target detection rate for these regions. The nested PCR protocol used in the study to detect the CAG trinucleotide repeats expansion in the *HTT* mutation was able to accurately determine the repeats number from SurePlex-generated WGA DNA. Third, mosaicism seen in CMA

could be difficult to interpret due to unclear levels of normal and abnormal cell population within a sample. Interpretation guidelines for mosaic samples should be clearly established in order to adequately deal with this situation *(Novik et al., 2014).*

Even though there are limitations, the integrated protocol described here ultimately provides valuable data for both genetic mutation and chromosomal abnormalities within 24 hours after embryo biopsy. This cost effective and efficient protocol allows clinicians to offer their patients the option for receiving a fresh embryo transfer, if they so choose. The comprehensive PGD method also permits optimal utilization of elective single embryo transfer for those patients interested in reducing the risk of multiple pregnancies. Since significant levels of embryonic aneuploidy can be expected in otherwise fertile couples bearing single-gene disorders, testing for the genetic defect alone in PGD does not adequately address concerns of aneuploidy, thus potential susceptibility to pregnancy failure and miscarriage *(Munné et al., 2007; Franasiak et al., 2013).* The data present here show that 52% of the embryos suitable for transfer based on mutational analysis alone were chromosomally abnormal (Table 5). This limitation has been successfully addressed by demonstrating that PCR-based genetic disease detection strategies can be successfully combined with standard CMA to enhance the probability of a healthy live birth. When taking into account both CMA and single-gene mutation data, 31% of the total embryos tested were available for transfer.

The availability of various reference human sequence information databases allows for accurate location of causative mutations and selection of STR markers from high density genome maps. Development and validation of PCR strategies for single-

gene mutations are now straightforward, inexpensive, and extremely accurate. In looking forward, there has been much interest in the potential use of Next Generation Sequencing (NGS) technologies for mutation identification in PGD cases for SGD *(Treff et al., 2013).* This technology holds much promise; however, more extensive clinical validation and cost analyses must be completed before this technique can be used clinically. At the current time, PGD evaluation will not likely employ extensive whole genome sequencing, but will likely target the condition for which the pregnancy is at-risk, as well as several other significant clinical disorders where sequence changes have been identified. For the purpose of avoiding the transmission of deleterious gene-specific regions, sequence-based information from linked, informative polymorphic features (SNP and STR) can be used to identify and track parental haplotypes in biopsied embryos. Sequencing approaches will depend on the continued improvement of unbiased amplification strategies allowing for adequate genomic coverage and read depth of informative regions of interest.

Although methods such as NGS and other emerging technologies may be applicable in the future of PGD testing, the combination of CMA and PCR for genetic disease detection presented here can serve as a cost effective and highly reliable methodology at the current time. Laboratories that are currently setup for CMA and PCR analysis can implement this combined protocol without additional equipment acquisition. Until NGS or other data-rich diagnostic methods are established for embryo diagnosis, integration of proven technologies already in use can serve as an adequate technique until a single methodology is available for a comprehensive preimplantation diagnosis.

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