Combined PGD and PGS: Enrichment of PGD genes during whole genome amplification

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Aim

The aim of this study was to determine the feasibility of synchronous whole genome amplification and gene specific amplification by PCR for combined PGD for monogenic disorders and PGS for aneuploidy utilizing aCGH or NGS.

Background

Microarray-based comparative genomic hybridization (aCGH) presents an opportunity to determine the genetic composition of uncharacterised human genomes.

The resolution and specific characteristics of the microarray dictate the genetic information that can be derived. Some arrays are able to detect copy number variations, deletions and duplications. In the preimplantation setting, the disadvantage in using high resolution arrays is that they frequently detect genetic alterations of unknown clinical consequence.

Reproductive Health Science Ltd (RHS) has developed a proprietary low resolution array specifically for the detection of whole chromosomal aneuploidy (PGS). In parallel with development of the array, RHS has optimised a single cell whole genome amplification (WGA) method using advanced DOP-PCR (Figure 1).

The ability to screen PGD embryos for aneuploidy from one biopsy is technically challenging as the method used to whole genome amplify the limited starting material is usually optimised to the downstream platform. However, by multiplexing WGA primers and gene specific PCR primers, a method has been developed for aneuploidy detection by aCGH and also for single gene disorder diagnosis using higher resolution molecular assays.

There have been limited reports of combined PGD+PGS, including by NGS where the WGA products were further amplified for the PGD gene and the products pooled (Wells et al, 2014) and qPCR where additional primers were added to the PCR (Treff et al, 2013, Zimmerman et al 2016).

Advanced DOP-PCR provides a unique WGA approach not represented in any other commercially available kits. The use of latest generation polymerases increases the accuracy of amplification and the PCR approach is ideally suited to multiplexing with PGD primers.

Methods

WGA for PGS+PGD

Single cells sorted from 47,XY,+15 or 48,XXY,+21 aneuploid cell lines (Coriell Institute for Medical Research, USA) were subjected to advanced DOP-PCR with the inclusion of sequence specific PCR primers in the same reaction. The primers were designed to amplify either D4S43 (chr4: 2,351,567-2,351,849, marker for Huntington's disease); F-AAA TTT TTT GCC AAT AAA GATA and R-TCT CAG CAA GCT ATG AGT AGGT or sex-determining region Y (chrY: 2,655,203-2,655,456 SRY) ; F-CAT GAA CGC ATT CAT CGT GTG GTC R-CTG CGG GAA GCA AAC TGC AAT TCTT. Single cell controls underwent WGA in the absence of additional sequence specific PCR primers.

EmbryoCellect™ Microarray CGH

To determine the chromosomal complement of the test cell, an aliquot of the WGA DNA was labelled with Cy3-like fluorescent dye, then mixed with WGA amplified male reference gDNA that had been labelled with a Cy5-like fluorescent dye, and competitively hybridized to the RHS microarray according to manufacturer's instructions. The arrays were scanned using a GenePix 4000B microarray reader (Axon Instruments, USA) and analyzed using GenePix 6.0 software (Axon Instruments). The ratio of test to reference dye intensity after normalization was used to determine the ploidy status of each chromosome.

PCR

Enrichment of the specific target sequence during the WGA was determined using semi-quantitative gene specific PCRs and agarose gel electrophoresis. Gene specific PCR was performed on single cells and gDNA that had not undergone WGA using the primers sets listed above and KAPA 2G Robust kit (Kapa Biosystems, South Africa).

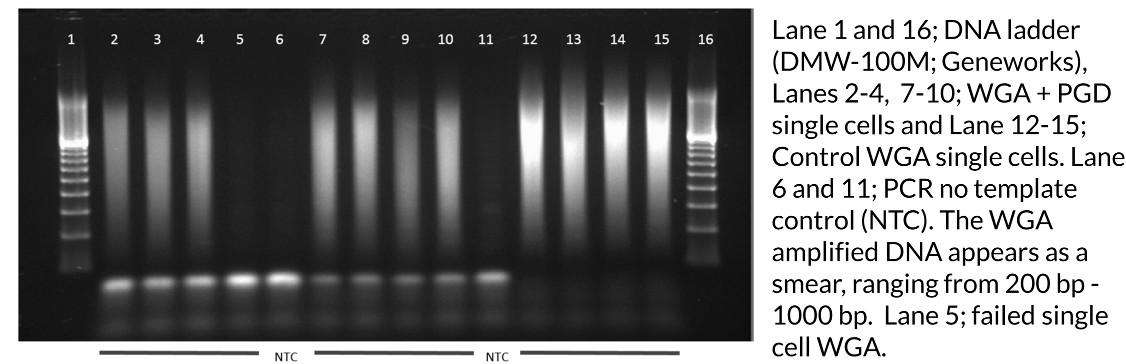
NGS

Sample libraries were quantified during library generation using NeoPrep technology, Bioanalyzer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Due to variation observed in DNA concentrations (Table 1), dilutions for library pooling were based on Qubit quantification. TruSeq NeoPrep libraries of 550bp were prepared from 5ng of WGA single cell and unamplified gDNA samples. The gDNA was fragmented by Covaris according to the protocol. The quality of the libraries was assessed using a Bioanalyzer (Agilent Technologies). Paired-end dual index 2_75bp sequencing was performed following the Illumina standard workflow on a MiSeq (Illumina) using 150 cycles v3 kit. The sequencing data was bioinformatically interrogated using BaseSpace apps (Illumina). Data was aligned to hg19 with Isaac Whole Genome Sequencing v2 (Illumina, Inc; default settings) and run metrics generated using FastQC (BaseSpace Labs; default setting). Sequence alignment and gene coverage were visualized using IGV v2.3 (The Broad Institute of MIT and Harvard). The number of mapped reads per chromosome was compared to an internally generated reference using SAMtools (MIT).

Results

WGA for PGS+PGD

The amplification process resulted in whole genome amplification of the DNA template. WGA amplification in the presence of gene specific primers was slightly weaker than control WGA (Figure 1).



Lane 1 and 16; DNA ladder (DMW-100M; Geneworks), Lanes 2-4, 7-10; WGA + PGD single cells and Lane 12-15; Control WGA single cells. Lane 6 and 11; PCR no template control (NTC). The WGA amplified DNA appears as a smear, ranging from 200 bp - 1000 bp. Lane 5; failed single cell WGA.

FIGURE 1: Gel electrophoresis of DOP-PCR WGA single cell DNA.

EmbryoCellect™ Microarray CGH

Correct aneuploidy diagnoses were achieved for all single cells carrying either trisomy 15 or 21 despite the addition of sequence-specific primers (n=19). In addition, correct diagnoses from cell lines with abnormal sex chromosome numbers such as XXY were obtained (Figure 2).

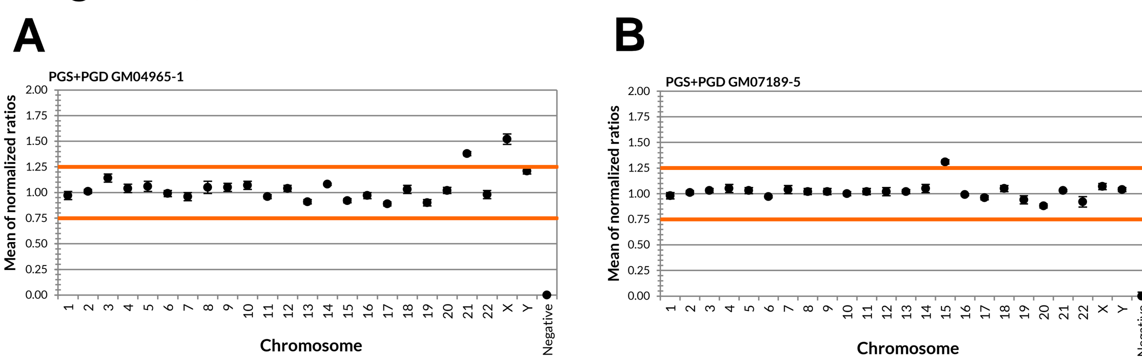


FIGURE 2: EmbryoCellect™ microarray CGH results. Examples of single cell PGS results following multiplex WGA with the addition of D4S43 and SRY primers. Array A) Trisomy 21; arr(21)x3,(X)x2,(Y)x1 and B) Trisomy 15; arr(15)x3,(X)x1,(Y)x1.

PCR

Semi-quantitative gene specific PCR and agarose gel electrophoresis confirmed enrichment of the target sequence in comparison to the control single cell WGA DNA (Figure 3).

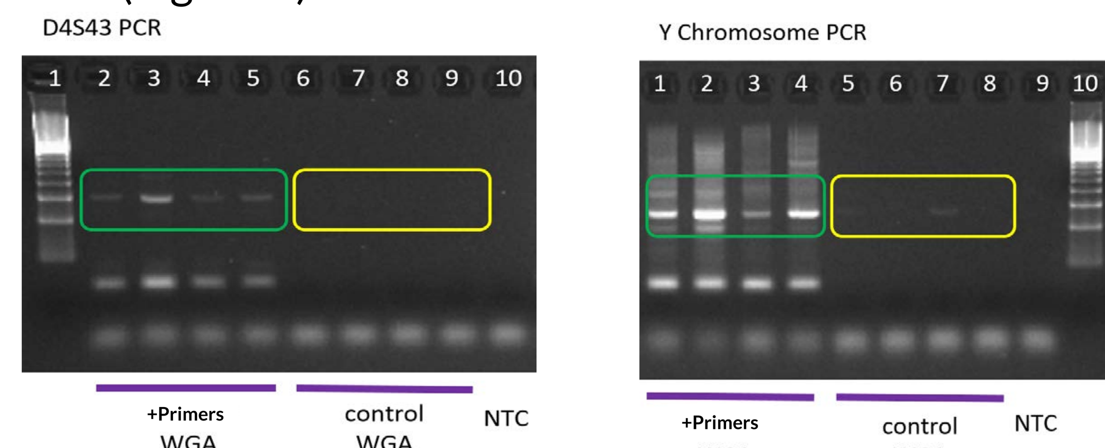


FIGURE 3: Sequence specific PCR. Examples of enrichment of targeted DNA during WGA following the addition of sequence specific primers. D4S43 amplicon size; 283bp and SRY amplicon size; 254bp.
Lane marker; DNA ladder (DMW-100M; Geneworks),
NTC is no template control for PCR.

NGS

The average size of the fragments prepared for the MiSeq NGS run was ~1207bp. The total number of reads generated was 29,080,788, the Passed Filter total read count was 10,498,046 of which 98.44% were identified.
Correct aneuploidy diagnoses were achieved for all single cells carrying either trisomy 15 or 21 regardless of the addition of sequence-specific primer sets (see examples Figure 4). In addition, correct diagnoses from cell lines with abnormal sex chromosome numbers such as XXY were obtained (Figure 4).
Enrichment of the targeted sequence in the NGS data is evident (Figure 5). However, these results are expected to be impacted by the variation in total reads sequenced per sample. Fragments show enrichment of the 3' and 5' ends of the desired PGD amplicon, corresponding to the 75bp fragment size sequenced.

TABLE 1 Comparison of quantification methods for library preparation and library normalization for pool. The final reads per sample following MiSeq sequencing are also presented.

Sample	Qubit (WGA)	NeoPrep		Bioanalyzer		Qubit	MiSeq data
	ng/ul	Quantification (nM)	Norm (nM)	size (bp)	conc pg/uL	ng/ul	Total mapped reads
1: D+Y	28.30	38.80	10.00	1334	268	3.80	373,457
2: D	28.10	47.81	10.13	1150	19	3.42	252,363
3: D	26.85	21.15	9.31	1527	71	3.05	178,120
4: D+Y	23.85	31.83	10.04	1269	126	3.18	162,520
5: D+Y	24.00	34.22	9.84	1165	164	3.05	220,029
6: control	34.50	37.20	9.88	1208	171	2.83	242,022
7: control	31.60	29.19	9.85	979	48	3.65	325,229
9: D+Y	41.85	too high		1248	730	1.18	2,240,000
10: control	41.90	41.62	10.73	-	-	3.51	260,562
11: control	35.25	too high		-	-	1.34	2,144,824
12: control	35.10	27.79	10.30	-	-	3.39	267,595
15: control	25.05	too high		-	-	1.07	2,537,136
16: control	58.00	37.36	9.92	919	95	2.57	278,849

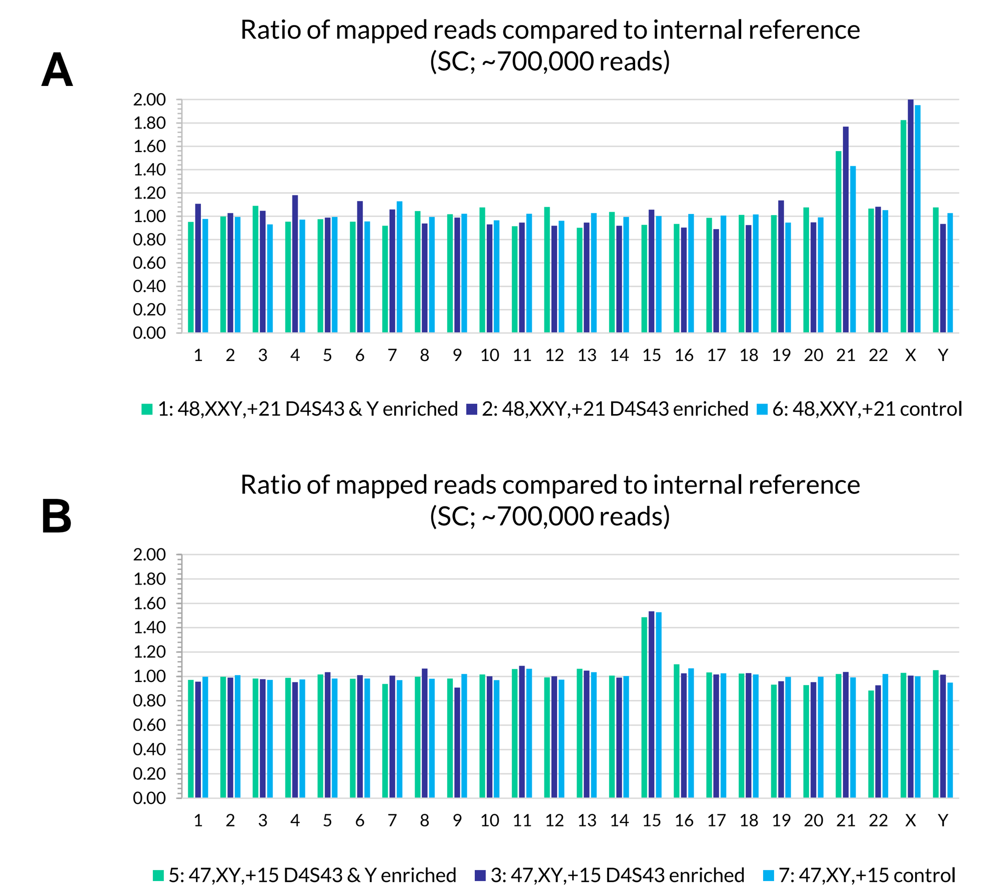
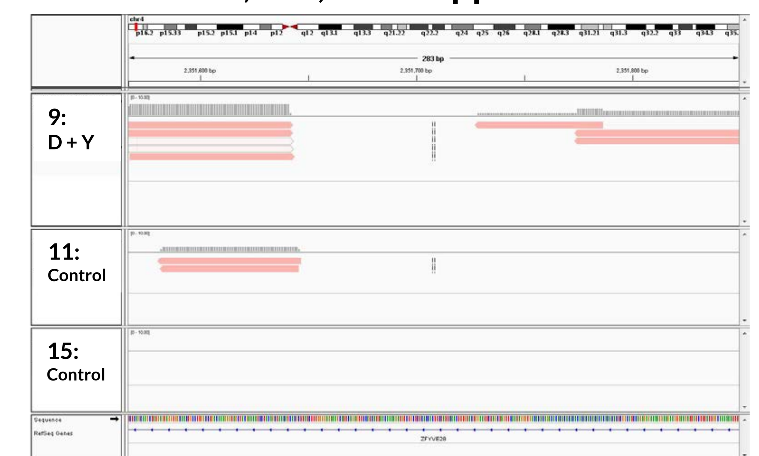
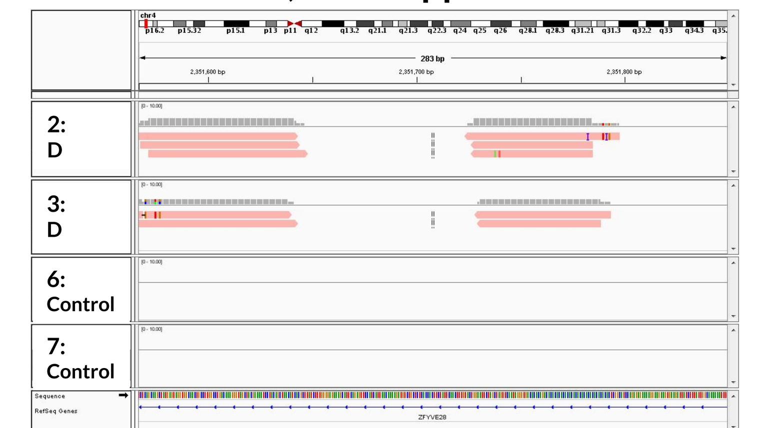


FIGURE 4. A) 48,XXY,+21 and B) 47,XY,+15 single cell ratios of mapped reads per chromosome normalized to a bioinformatically generated internal single cell reference. Each bar represents a single cell following MiSeq sequencing.

D4S43 Enrichment ~2,300,000 mapped reads



D4S43 Enrichment ~250,000 mapped reads



SRY Enrichment ~2,300,000 mapped reads

SRY Enrichment ~290,000 mapped reads

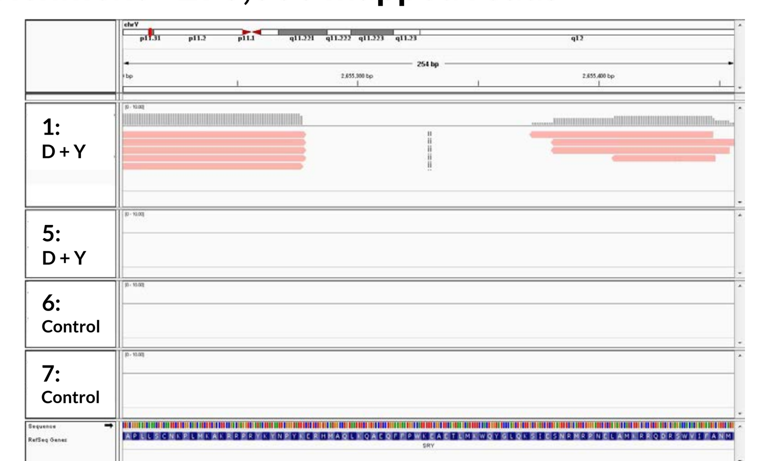


FIGURE 5 Visualization of D4S43 amplicon size; 283bp and SRY amplicon size; 254bp in data generated from sequencing of single cell WGA DNA in IGV. Data grouped into high or low MiSeq reads per sample.

Conclusions

- These results confirm the ability to concurrently amplify one or more targeted genes from a single cell during WGA as part of a standard microarray or NGS PGS protocol.
- Combined PGS and PGD provides an attractive new technology for improving IVF outcomes for PGD cases.
- This methodology is compatible with existing PGD-PCR primer sets.

Provisional Patent pending

Treff et al Fertil Steril 2013; 99(5):1377-1383
Wells et al 2014 J Med Genet 2014;51:553–562
Zimmerman et al Fertil Steril 2016;105(2):286-94

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