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Validity of real time PCR in preimplantation gender determination for single human blastomere

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- Whole genome amplification
- Real time PCR.

Abstract

Preimplantation genetic diagnosis (PGD) offers couples who need to avoid having a child affected with a severe genetic disease, an alternative to prenatal diagnosis and termination of an existing pregnancy. One of the main indications of PGD is sex selection to avoid sex-linked genetic disorders. The main objective of the present study was to investigate the feasibility of whole genome amplification (WGA) and real time PCR for sexing of single human blastomere and to confirm the results by sequencing. Forty non-viable embryos were selected for analysis. WGA technique was employed on single blastomeres that were biopsied from embryos and on buccal mucosal cells obtained from five men and five women as positive and negative controls respectively. Whole genomic DNA amplification efficiency from a single human blastomere and from optimized buccal cells was 100 % and the level of amplification reached hundreds fold. The obtained genomic DNAs were then subjected to PCR amplification of the SRY, DYS14 and DAZ genes for gender determination. DAZ sequences correctly identified the gender of all male and female embryos with 100% sensitivity and specificity. However, the obtained sensitivity and specificity for SRY sequences were 92.6% and 100% respectively and the obtained sensitivity and specificity for DYS14 sequences were 100% and 93.8% respectively. The results of the present study prove the feasibility of WGA PCR assay for the detection of specific DNA fragments from single cells and the real-time PCR assay of the multicopy DAZ sequence in single human blastomeres detected correctly the embryo's gender. This will pave their use in preimplantation gender determination genetic diagnosis.

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INTRODUCTION

Preimplantation genetic diagnosis (PGD) is a strategy used to avoid or decrease transmitting genetic defects to the offspring of patients with known genetic disorders. The main indications of PGD included inherited chromosomal abnormalities, single gene disorders, and sex selection [1]. Sex selection is an option for couples who need to avoid transmitting sex-linked genetic diseases to their children. It additionally may appeal to parents who have children of one sex and need a child of the other sex, which is sometimes called "family balancing." Also, sex selection can be used during infertility treatment [2].

The PGD techniques include IVF, embryo biopsy, molecular diagnostic testing of the single blastomere. Embryos diagnosed as disease free are then transferred to the uterus for establishing an unaffected pregnancy [3]. Handyside was the first one who transferred the sex-selected embryos to the maternal uterus in 1990 [4]. The obvious PGD benefit is the ability of selection of embryos free from the disorder of concern for implantation and thus preventing conceiving an affected pregnancy that oblige termination [3].

At least 250 hereditary diseases are caused by X-chromosome genes abnormalities [5]. Most of them show recessive inheritance and are inherited in 50% of offspring of mothers who carry a mutated allele. Other conditions linked to the Y chromosome are related to infertility [6], as in case for microdeletions [7].

Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are the most common methods used in PGD in many IVF laboratories [8]. PGD by means of FISH with the Y and X-chromosomes probes has been used in several conditions that involve X-linked diseases [10] and also has the possible advantage of identifying sex chromosomes' aneuploidies. However, the accuracy and efficiency of this technique are largely dependent on the individual technical skills and the experience of the involved laboratory. Poor blastomere fixation and lack of strict scoring criteria can decrease the FISH reliability [11]. In addition, FISH can't be used in identification of allelic differences in the single copy genes that cause most of inherited diseases. Furthermore, errors of FISH technique can occur in 1.2 - 10% of cases [12, 13]. On the other hand, real-time PCR assays are very effective in detection of multiple copy Y chromosomal sequences. It is rapid and has the advantage of performing the amplification and detection procedures in the same tube, thus greatly reducing the chances of laboratory contamination [9].

Taking into consideration that one human diploid cell contains only about 7 pg DNA and that modern methodologies of genomics need hundreds of nanograms of DNA, the genome(s) of the biopsied cell(s) should be firstly amplified thousands of times in order to permit genome-wide analysis [14]. Whole genome amplification (WGA) is considered a technique that amplifies the entire genome extracted from single cells up to microgram levels, thus providing a large quantity of DNA sufficient for many PCR amplifications, permitting diagnosis confirmation or other genes analysis. This overcomes the limits of low DNA quantity of the individual cells' genome. Moreover, WGA eliminates the need of optimizing multiplex

PCR reactions to a single cell level and thus simplifying the diagnostic procedure [15,16].

In this study, we employed a new isothermal WGA method by utilizing multiple displacement amplification (MDA) technology. In MDA technology, primers first anneal to a denatured single-cell DNA template in a random manner. Accordingly, a DNA polymerase that has strong strand displacement capacity makes several copies of the genome in an isothermal reaction adjusted at 30°C [17]. When the 3' end of the extending DNA fragment reaches the 5' end of the neighboring primed chain, it will lead to displacement of this neighboring strand, thus releasing single-stranded DNA suitable for new primer annealing and DNA synthesis [17]. The bacteriophage φ29 DNA polymerase is the most common enzyme utilized for MDA. It can generate long nucleotide chains over 10 kb in size and possess a strong proofreading capacity, thus guaranteeing correct copying of nucleotide during WGA [18]. The products of WGA using MDA technology often cover most of the human genome and the amplification is reported to be uniform [19].

This study investigates the use of real-time PCR in identification of Y-chromosome markers in single human blastomeres. The genomic regions on the Y-chromosomes chosen to be amplified were the highly conserved DYS14 gene, which has 20–30 copies on the Y chromosome [20], deleted in azoospermia (DAZ) gene, which also contains a variable number of copies on the Y chromosome [21] and the single copy sex-determining region Y (SRY) gene that is specific for the Y chromosome [22]. The β-globin region that is shared by both sexes was chosen to serve as an endogenous control of amplification to avoid false-negative results caused by PCR inhibitors [20].

Recently, direct sequencing was used in PGD. It is accurate, reliable and by confining the sequence analysis (post-PCR) to a smaller region of interest, the time needed to get results is greatly decreased [9].

Investigation of the feasibility of WGA and real time PCR for single human blastomere sexing was the main aim of the present study to be confirmed by sequencing.

MATERIALS AND METHODS

- **Blastomere isolation**

After approval of this study by the Ethics Committee of Benha Faculty of medicine and after obtaining written informed consent from the involved couples, 40 non-viable unwanted embryos were selected for analysis. When embryos reached the eight-cell stage, a blastomere biopsy was performed. One blastomere containing a nucleus from each embryo was gently aspirated following zona pellucida opening by a diode laser.

After removal, the blastomere was then transferred to a microtube for PCR. Calcium- and magnesium-free phosphate-buffered saline (PBS) was used to wash each blastomere twice. After that, each blastomere was transferred to a 0.2ml PCR tube containing 5µl PBS. Another PCR tube containing 5µl PBS was used as a negative control for detection of contamination. Then the PCR tubes were stored at -80°C until further processing.

- **Preparation and isolation of a single buccal cell**

Five men and five women were selected as donors of buccal cells to be used as positive and negative controls respectively. A sterile cytology brush was used to obtain a smear of buccal mucosal cells. Shaking of the brush was done in a 1.5ml eppendorf PCR microtube containing 0.5ml 0.9% NaCl to be followed by centrifugation of the tube at 5000 rpm for 5 min. Aspiration of the supernatant and repetition of washing two more times with subsequent resuspension of the precipitate in 0.2ml calcium- and magnesium-free sterile PBS were done. Manual isolation of the cells was done immediately, utilizing a capillary under a stereoscope and individually transferred to a 0.2ml PCR tube containing 5µl calcium-and magnesium-free PBS. Storage of the cells was then carried out at -80°C for later processing.

- **Blastomere genomic DNA isolation and amplification**

Blastomeres were lysed and genomic DNA was amplified using REPLI-g Mini Kits (Qiagen, USA) according to the manufacturer's protocol. In brief, the cell sample was lysed and the DNA was denatured after mixing with denaturation buffer, centrifugation and incubation for 10 min at 65°C. After cessation of denaturation by adding stop solution, addition of master mix consisting of REPLI-g sc DNA Polymerase with reaction buffer was done. The reaction of isothermal amplification proceeded for 8 hours at 30°C. REPLI-g sc DNA Polymerase was then inactivated at 65°C for 3 min. The previous two steps were preprogrammed in a T100 thermal cycler, Bio-Rad, Singapore. The quality and the quantity of the amplified DNA were measured by the NanoDrop2000 spectrophotometer (Thermofisher scientific, USA). For PCR analysis, an aliquot of REPLI-g amplified DNA was diluted in nuclease free water 1:5.

- **Quantitative real time PCR**

Primers with specific sequences for SRY, DYS14 [23], and DAZ genes [24] as specific genetic markers for the Y-chromosome and β-globin gene as an internal positive control were used [25] (to ensure sufficient genomic DNA after extraction). Specificity of the chosen primers was assessed by basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). It is a bio-technology tool utilized for determining the specificity of DNA regions by checking their similarity with other regions. The primers sequences are listed in Table 1.

Table (1): Primers sequences of the genes involved in the study

Gene	Forward primer	Reverse primer
β-globin	GTGCACCTGAC TCCTGAGGAGA	CCTTGATACCAAC CTGCCCAG
SRY	TGGCGATTAAG TCAAATTCGC	CCCCCTAGTACCC TGACAATGTATT
DYS14	GGGCCAATGTT GTATCCTTCTC	GCCCATCGGTCAC TTACACTTC
DAZ	TACCTCCAAAG CACCAGAGC	AATCTACCCATTC CCGAACC

PCR was carried out as a singleplex for each primer set. The amplification reaction contained 400ng of DNA, 0.6µl (10mM) of forward primer and 0.6µl (10mM) of reverse primer (for DYS14, DAZ and β-globin genes), 0.4µl (10mM) of forward primer and 0.4µl (10mM) of reverse primer for SRY gene, 10µl of QuantiTect® SYBR® Green PCR (Qiagen, Germany) and RNase-free water up to 20µl as a final volume. Each PCR reaction included a male control consisting of a male buccal mucosal cell and a

female control consisting of a female buccal mucosal cell together with no template control. The PCR conditions were performed in Rotor-Gene Q (Qiagen, Germany) according to the following program: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 s, annealing at 49 °C, 52°C, 51°C and 55°C for 30 s for SRY, DYS14, DAZ and β-globin genes respectively and extension at 72 °C for 30 s. Melting curve analysis using Rotor-Gene Q system software was performed to assess specificity of the amplification products.

- **Sequencing analysis**

PCR amplification: It was performed using the same previously used DAZ primers for 205 bp amplicon on T 100 thermal cycler, Bio-Rad-Singapore, using MyTaq^{TM} Red Mix supplied by Bioline, UK. The PCR mix contained 10ul of Taq PCR master mix 2x, 0.6µl of each primer, 400ng of DNA and nuclease free water to reach a final volume of 20ul. The following program was applied: initial denaturation at 95ºC for 1 min, 45 cycles of denaturation at 95 ºC for 15 s, annealing at 51°C for 15 s and extension at 72 ºC for 30 s then hold at 4 ºC. PCR amplification products were separated on 2% agarose gel containing 0.3µg/ml of ethidium bromide. The DNA bands of DAZ were visualized using UV transilluminator (254nm) and photographed (Figure 1).

DNA purification and sequencing: DNA purification was carried out using **QIAquick PCR Purification kit, QIAGEN** according to the manufacturer's instructions**.** After purification, direct sequencing of each amplified fragments was done in a forward direction on **3500 Genetic Analyzer, ThermoFisher Scientific** with the BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems, USA) and Big-Dye X-terminator Purification Kit, Applied Biosystems (Figure 2). The DNA sequences obtained were aligned using NCBI BLAST analysis (Figure 3).

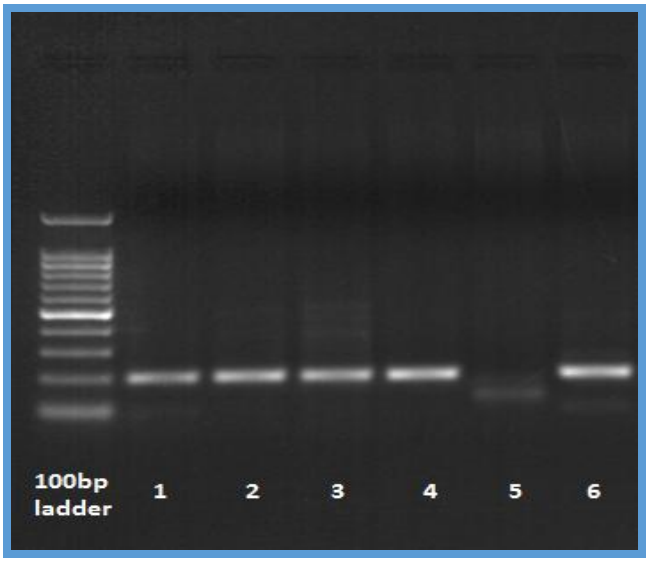

Figure 1: Agarose gel electrophoresis of amplification products of DAZ. Lanes 1,2,3,4 and 6 show the 205bp DAZ amplicon. Lane 5 shows the absence of the 205bp DAZ amplicon.

Control of contamination

All manipulations of buccal cells and blastomeres in addition to embryos were performed inside a biologically safety cabinet. To avoid contamination of the blastomeres with cellular DNA from sperm or maternal cumulus cells, washing of the blastomeres with PBS after the biopsy was done.

The preparation of the lysis buffer and PCR reagents was performed within a biologically safety cabinet, using material destined only for this manipulation. All pipetting was done utilizing sterile pipette tips with filters. Safety cabinet surfaces, pipettes, and supplies were subjected to UV light in between uses and were touched with

gloved hands only. Before each reaction, decontamination was performed routinely with 10% chlorine. The laboratory staff wore disposable gloves, caps, surgical masks and lab coats that kept inside the lab. Gloves were changed when contact with possible contaminated environment occurred. In addition, one blank sample was used in each run to detect possible contamination. After each reaction analysis, disposal of all materials used was done, taking care not to contaminate formerly amplified materials.

RESULTS

Results of the current study showed that amplification of DNA material either from blastomere of unwanted embryos or from buccal cells were successfully performed using WGA and the obtained DNA was included for subsequent sex determination by PCR. The DNA concentrations, as measured by the NanoDrop2000, are presented in Table (2) which demonstrates that the amplification level thus reached hundreds folds. In all tested samples, β globin gene was amplified indicating the presence of enough DNA in the samples extracted. Comparing of the obtained results with the results of sequencing indicates that from 24 male embryos, 22 embryos were correctly diagnosed and only in two cases, negative results were obtained by using the SRY gene for analysis. Moreover, all the 16 female embryos were negative for SRY. In addition, by using DYS14 sequences, 15 out of 16 female embryos were correctly diagnosed and only one embryo gave a false positive result, while all the 24 male embryos were correctly diagnosed. On using the DAZ sequence, gender of all male and female embryos was identified correctly (Table 3). It is to be noticed that there were no cut off values determined for the cycle threshold (Ct) of the amplification curves of both SRY and DAZ genes, for discrimination of positive and negative results. This is since both SRY and DAZ genes gave either clear positive or negative amplification curves for the true positive and negative cases respectively, and the two false negative cases for SRY gene showed clear negative amplification curves. Also no cut off value determined for the Ct of the amplification curves of DYS14 gene since this gene gave either clear positive or negative amplification curves for the true positive and negative cases respectively and the Ct of the false positive DYS14 case was 17.2, which was within the range of the Cts of the true positive DYS14 cases (range; 15.84-24.32).

Sensitivity, Specificity, positive predictive value (PPV) and negative predictive value (NPV) of SRY, DYS14 and DAZ sequences in gender determination are presented in Table (4) and Figure (4).

DISCUSSION

In this study, WGA and real-time PCR amplification protocols were found to be accurate for detection of the Y chromosome in single human blastomeric cells. The present study revealed that WGA using the REPLI-g Mini Kit (Qiagen, USA) is very successful in amplifying DNA extracted from single cells as indicated by the high concentration of DNA measured by the NanoDrop and by the positive PCR amplification of β-globin gene. Genomic DNA amplification was obtained from all tested samples so that the general DNA amplification rate of a single human

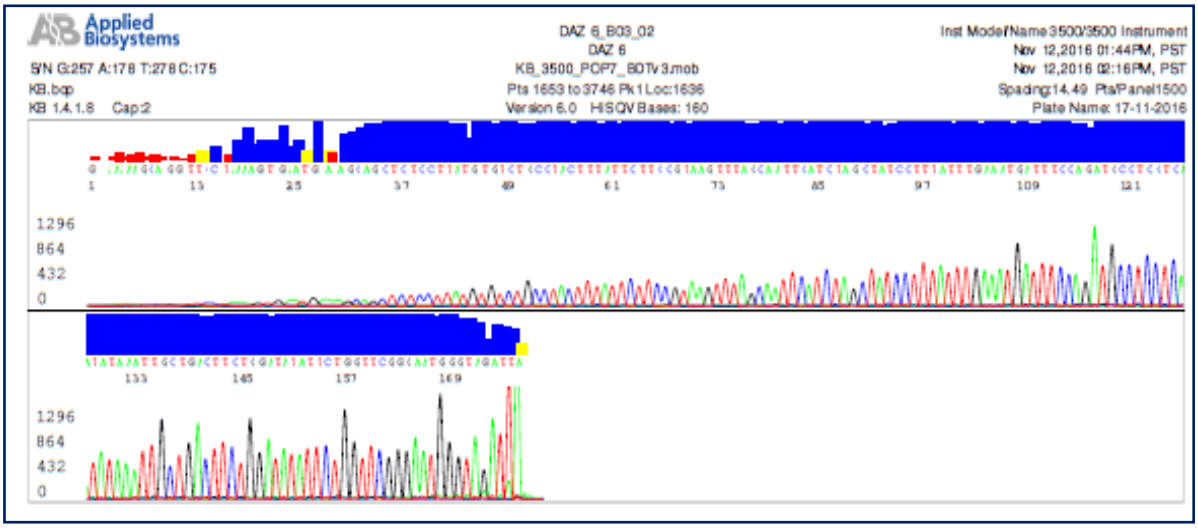

Figure 2: Sequence of the amplified DAZ fragment.

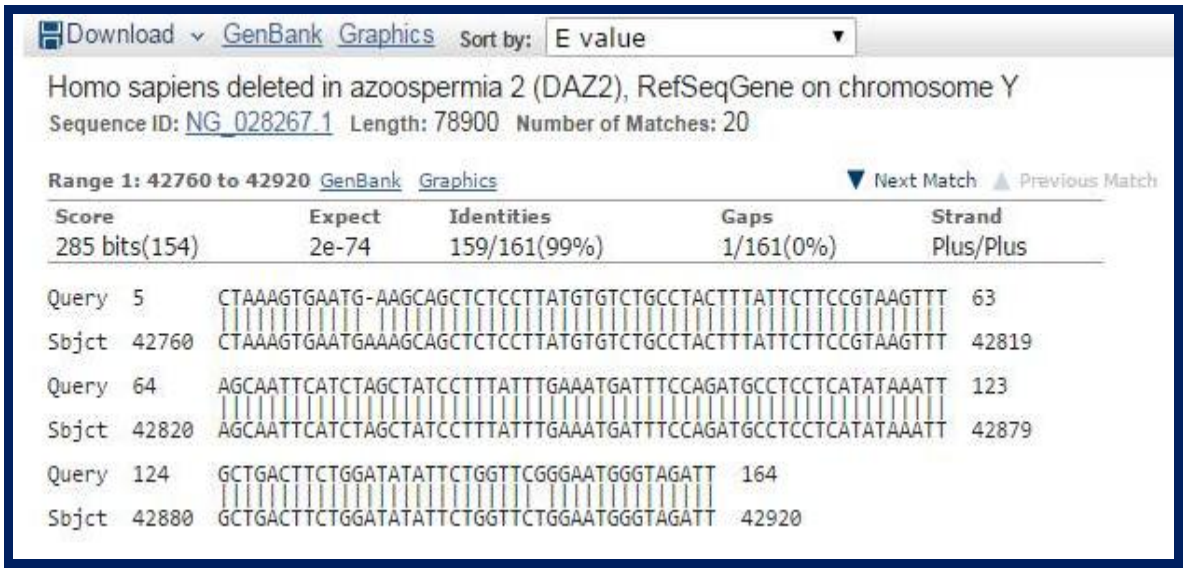

Figure 3: Matching (Alignment) of the obtained sequence of DAZ with that on the gene bank, showing 99% similarity. The DNA sequences obtained were aligned using NCBI BLAST analysis.

Table (2): DNA concentration (ng/µl) of single blastomeres and single buccal cells before and after whole genome amplification

DNA quantity / Cell type	Before WGA	After WGA	P value
	Mean±SD (Range)		
Blastomeres	7.15±1.9 (4.7 to 10.8)	2312.2±196 (1970 to 2582)	0.001
Buccal cells	10.63±1.6 (8.3 to 13.2)	2363.4±177.9 (1998 to 2564.5)	0.001

WGA; Whole Genome Amplification, $p<0.05$ significant

Table (4): Sensitivity, specificity, PPV, NPV of SRY, DYS14 and DAZ genes in prediction of male gender

Gene	Sens.%	Spec.%	PPV%	NPV%	AUC	P
SRY	92.6%	100%	100%	88.9%	0.958	<0.001
DYS14	100%	93.8%	96%	100%	0.969	<0.001
DAZ	100%	100%	100%	100%	1.0	<0.001

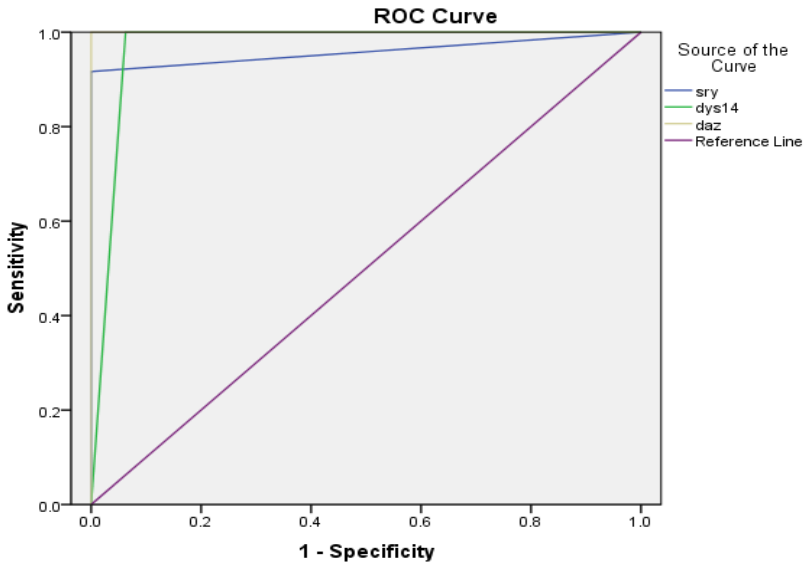

Figure 4: AUC for the SRY, DYS14 and DAZ genes in prediction of male gender

Table (5): Results of the determined Gender by combination of the results of the three genes

	SRY	DYS14	DAZ	Number of embryos with this result	Gender
Real time PCR result	Positive	Positive	Positive	22	Male
	Negative	Negative	Negative	15	Female
	Negative	Positive	Negative	1	Female (DYS 14 result considered false positive)
	Negative	Positive	Positive	2	Male (SRY result considered false negative)

blastomere was (100%, 40/40) similar to that of single optimized buccal cell (100%, 10/10). Our results were higher than that obtained by Pierce et al., 2000 [26] and Martinhago et al., 2010 [20]. Pierce et al., 2000 [26] reported that the general amplification rate of a single human blastomere was 83.78% (155/185) and the general amplification rate of a single lymphocyte was 99.07% (107/108). Pierce et al., 2000 [26] used molecular beacons as a method for the detection of the amplified product and used U2 genes as a control. In addition, Martinhago et al., 2010 [20] performed real time PCR for embryo sexing using DYS14 gene as a marker for Y chromosome and β-

globin as a control and revealed that the general amplification rate of a single human blastomere was 83.33% (45/54) and the general amplification rate of a single optimized buccal cell was (100%, 33/33).

Decreased efficiency of amplification can be caused by numerous problems encountered between sample collection and the PCR procedure itself. Operator problems, for example, cell loss during the process of cell transfer to the tube or spontaneous cell lysis before the cell entering the tube contribute to amplification failure or decreased amplification efficiency. In fact, blastomeres from low quality embryos yield lower amplification efficiencies than their high quality counterparts, underlining the importance of blastomere selection during embryo biopsy. After successful transfer of a high quality nucleated cell, the cell lysis protocol utilized also influences the amplification success [9]. Therefore, REPLI-g Mini Kit was chosen for this study, which is designed for efficient genomic DNA amplification.

This kit depends on multiple displacement amplification (MDA)-based genome amplification. As compared with other WGA methods such as degenerate oligonucleotide primed (DOP) and primer extension preamplification (PEP), MDA has been shown by Burtt (2011) [19] and Spits et al. (2006) [18] to generate DNA with a higher molecular weight and shows better genome coverage. The whole procedure takes about 16 hours and generates appreciable amounts of DNA from only single blastomere that is efficient for many downstream applications as sequencing, short tandem repeat (STR) analysis, locus-specific PCR or array comparative genomic hybridization (array-CGH) [15].

The choice of the genomic region, as well as the primer design are essential for PCR efficiency and success. DYS14, DAZ and SRY regions were selected as targets as they are specific for the Y chromosome. Moreover, the repetitive nature of DYS14 and DAZ regions increase the chance of success of the technique in a single cell compared with the detection of only one initial copy (SRY region) which may generate allele drop-out (ADO), an event that would seldom occur with multicopy genes [20]. Furthermore, these primers were used in our previous study [27], which proved high efficiency in detecting male bearing pregnancies from maternal cffDNA especially when these genes are used in combination.

The region of the β-globin gene was selected for three reasons. First, this region is located on and specific for chromosome 11, that rarely presents aneuploidy [28], hence it represents a magnificent control that decreases the probability of broad Ct variations. Second, this region has been utilized in the laboratories for a long time for other experiments revealing a high rate of successful amplification, even utilizing small DNA amounts. Finally, the β-globin region selected possess four genomic copies in a diploid cell (delta and β-globin) and the primers were designed to amplify a fragment of the delta and β-globin region and not simply one or two to avoid reducing the technique sensitivity [20].

In this study, two embryos gave false negative results using SRY sequence, after confirmation by sequencing and comparing this result with that obtained by DYS14 and DAZ sequences, which

gave positive results for these two embryos. This agrees with our previous study [27] on cffDNA that reported that positive DYS14 and DAZ gene results correctly diagnosed a male fetus with 100% sensitivity and specificity in spite of the fact that about 60% of cffDNA is fragmented [29]. As previously stated, these false negative results using SRY sequence is due to the fact that it is a single copy gene, while DYS14 and DAZ are multicopy genes [22].

In addition, one embryo gave false positive result using DYS14 sequences despite being female as confirmed by sequencing and by the results of the other two genes. As previously mentioned, the DYS14 sequence has considerable homology to sequences other than the Y chromosome [30]. Therefore, female embryos could be falsely classified as males by the DYS14 gene. However, in our previous study on cffDNA [27], using real time PCR, when DYS14 gave positive result and at the same time, SRY and DAZ gave negative results, the case was considered male, however in that study, a cut off value was applied which increased the specificity of the DYS14 to 100%, therefore, the case was considered male.

In this study, combinations of the three Y-chromosome sequences increased the accuracy of the test, a finding consistent with our previous study [29]. However, the most interesting finding in this study is that embryo sexing by DAZ gene using real time PCR yielded the highest sensitivity and specificity (100%) which may place this test in competition with FISH technique, which is the gold standard for human blastomere sexing.

The accuracy of this real-time PCR assay of the multicopy DAZ sequence was higher than the other methods reported for gender determination in single cells. A previous study compared different methods and reported accuracy rates of 97% accuracy for PCR with fluorescent primers, 89% for conventional PCR and 96% for FISH [31]. However, Martinhago et al., 2010 [20] reported accuracy rates of 74.3% for real time PCR and 80% for FISH.

In addition to the efficiency and accuracy of this assay, it permits sexing within 4 hours after WGA. Consequently, the whole procedure presented in this study is suitable for gender determination in PGD for couples at risk for sex-linked genetic diseases and for non–medical sex selection. Moreover, the obtained DNA could be agreeable to other molecular analyses such as array CGH and single nucleotide polymorphism (SNP) analysis, and may allow the identification of a wider spectrum of specific inherited disorders [15].

In conclusion, WGA is effective in obtaining sizable amounts of DNA from single human blastomeres. Moreover, the obtained DNA can be successfully utilized for rapid, safe and reliable real-time PCR-based gender determination for preimplantation human embryos and this will pave the way for pre-implantation genetic diagnosis of different inherited disorders.

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