Reproductive BioMedicine Online (2010) **20**, 75–82

www.sciencedirect.com
www.rbmonline.com

ARTICLE

# Development of a real-time PCR method for rapid sexing of human preimplantation embryos

**CD Martinhago [a,b,c], LD Vagnini [a], CG Petersen [d], AL Mauri [d], RL Baruffi [d], RM de Oliveira [c], JG Franco Jr [d,e,*]**

[a] *CPDP, Paulista Centre of Diagnosis and Research, Ribeirão Preto, Brazil;* [b] *Doctoral Postgraduate Program of Gynecology, Obstetrics and Mastology, Botucatu Medical School/UNESP, Botucatu, Brazil;* [c] *RDO Medical Diagnosis, São Paulo, Brazil;* [d] *CRH, Centre for Human Reproduction Professor Franco Junior, Ribeirão Preto, Brazil;* [e] *Department of Gynecology and Obstetrics, Botucatu Medical School/UNESP, Botucatu, SP, Brazil*
[*] Corresponding author. *E-mail address:* franco@crh.com.br (JG Franco).

Dr Ciro Martinhago graduated in Medicine in 1999 and completed his residency in Medical Genetics at São Paulo University from 2001–2004. He is board certified in clinical genetics (Brazilian Society of Clinical Genetics) and recently received his PhD degree for work on molecular biology in preimplantation genetics diagnosis. Currently, he is the head of the molecular and genetics division of a private laboratory and is involved in three projects to develop an approach for non-invasive prenatal diagnosis. He has many publications in international scientific medical journals of medical genetics and human reproduction.

**Abstract** Genes on the X chromosome are known to be responsible for more than 200 hereditary diseases. After IVF, the simple selection of embryo sex before uterine transfer can prevent the occurrence of affected offspring among couples at risk for these genetic disorders. The aim of this investigation was to develop a rapid method of preimplantation genetic diagnosis (PGD) using real-time polymerase chain reaction (PCR) for the sexing of human embryos, and to compare it to the fluorescence in-situ hybridization technique, considered to be the gold standard. After biopsies were obtained from 40 surplus non-viable embryos for transfer, a total of 98 blastomeres were analysed. It was possible to analyse 24 embryos (60%) by both techniques, generating a total of 70 blastomeres (35 per technique), while 28 blastomeres from 16 embryos (40%) were analysed only by real-time PCR. A rapid and safe method was developed in the present study for the sexual diagnosis of a single human cell (blastomere and buccal cell) using the emerging technology of real-time PCR.



**KEYWORDS:** fluorescence in-situ hybridization (FISH), preimplantation genetic diagnosis (PGD), real-time quantitative PCR, sexing human embryos, single cells, TaqMan MGB probes

## Introduction

Today it is known that genes on the X chromosome are responsible for at least 250 hereditary diseases (Online Mendelian Inheritance in Man, 2006) with most of them following the recessive form of inheritance and being inherited in 50% of children born from mothers carrying a mutated allele. In addition, one may consider conditions related to infertility that are linked to chromosome Y, as is the case for microdeletions (Stouffs et al., 2005). Thus, in both the

1472-6483/$ - see front matter 
doi:10.1016/j.rbmo.2009.10.008

above situations the simple selection of embryo sex can prevent the recurrence of the disease. Gender selection of embryos after PGD for other independent medical reasons is a highly controversial and hotly debated topic, and it has been discussed by some authors (Heng, 2006). The only method that can diagnose a genetic disease before pregnancy is established is preimplantation genetic diagnosis (PGD). In 1990, Handyside was responsible for the first pregnancy in which the embryos were selected by sex before being transferred to the maternal uterus (Handyside et al., 1990). After cases of diagnostic errors due to failure of amplification, polymerase chain reaction (PCR)-based methods were significantly reduced in favour of the more accurate in-situ hybridization technique for sex chromosome analysis (Staessen et al., 1999). Fluorescent in-situ hybridization (FISH) with probes for chromosomes X and Y and an autosomal probe is currently the PGD technique most frequently used for embryo sexing (Sermon et al., 2007), since its main advantage is to detect aneuploidies of sex chromosomes. However, poor blastomere fixation and the lack of a rigorous control in signal counting may reduce the reliability of the FISH technique (Munné et al., 1998). In addition, the FISH technique may produce errors in from 1.2 to 10% of cases (Magli et al., 2001; Staessen et al., 1999).

Real-time PCR uses fluorescent reporter dyes to combine the amplification and detection steps of the PCR assay in a single tube format (Higuchi et al., 1992). Among the various systems for real-time PCR, the one most frequently employed is TaqMan, which uses a labelled oligonucleotide (probe) with a fluorescent molecule (fluorophore) at the 5′ end and an intramolecular quencher probe at the 3′ end, in addition to the pair of oligo-primers used in standard PCR (Heid et al., 1996).

With the advent of a new probe technology called TaqMan minor groove binder (MGB), it was possible to develop smaller probes, but with the same annealing temperature. In addition, these probes do not have a fluorescent signal at the 3′ end to which the quencher is coupled, permitting the use of one more filter in addition to that used by the real-time machine. With these characteristics it is possible to design primers and probes for the amplification of very small genome regions (70 base pairs; bp), which cause this detection system to be more sensitive and to permit the use of several probes at the same time (multiplex) (Kutyavin et al., 2000). Thus, the TaqMan MGB system permits an increase in sensitivity without losing specificity, and can be very useful for systems that require the detection of various regions in the same reaction (multiplex).

The main objective of the present study was to develop a rapid and safe method based on real-time PCR for human blastomere sexing and to compare it with the FISH technique, considered to be the gold standard.

## Materials and methods

### Blastomere isolation

After the couples involved gave written informed consent, non-viable embryos unfit for transfer were selected for analysis. A blastomere biopsy was performed in 2- to 8-cell embryos and at least two blastomeres were removed after opening the zona pellucida with a diode laser (Fertilase™ system; Medical Technologies Montreux, Lausanne, Switzerland).

After removal, one of the blastomeres was transferred to a slide and fixed for the beginning of the hybridization technique (FISH), and the other was transferred to a microtube for PCR. For PCR analysis, each blastomere was washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS) and then directly transferred to a 0.2 ml PCR tube containing 5 μl PBS or 3 μl lysis solution, depending on the protocol used. A control sample was prepared using a similar volume of washing solution and also placed in a PCR tube under the same conditions as described above to be used as the control of contamination. The PCR tubes were then frozen at −20°C for at least 3 h.

### Preparation and isolation of a single buccal cell

Two individuals of different sex were selected at random as donors of buccal cells. Buccal mucosal cells were obtained with a smear using a cytology brush. After the procedure, the brush was shaken in a 1.5 ml PCR microtube containing 0.5 ml 0.9% NaCl. The tube was then centrifuged a 3000 g for 5 min and the supernatant was removed and the washing was repeated two more times. After washing, the material left at the bottom of the tube was resuspended in 0.2 ml calcium- and magnesium-free sterile PBS. Immediately after this procedure, the cells were isolated manually using a capillary under a stereoscope and transferred individually to a 0.2 ml PCR tube containing 5 μl of saline solution (calcium- and magnesium-free PBS) or directly added to 3 μl of lysis solution depending on the protocol used (50 and 25 μl respectively). The cells were then frozen at −20°C until the time of use as positive male and female control, or for the standardization of the PGD reactions. All material used was sterile and the procedure was carried out under a laminar flow hood.

### Real-time PCR

The reactions were performed with a real-time PCR machine (7500 Real Time PCR System; Applied Biosystems, Foster City, CA, USA) using the TaqMan system for the detection of the amplification product. In order to obtain better results, TaqMan MGB, probes were used, which have MGB linked to their 3′ extremity. On this basis, the probe does not use the fluorescence of a filter of the machine that it would use with the fluorophores of the quencher, which would permit the possibility of using it with another probe in a multiplex reaction. In addition, the TaqMan MGB probes are more specific even when used in duplex to distinguish the difference of only one nucleotide in single nucleotide polymorphism analysis, in which two probes are used, one with the normal allele and the other with the mutated allele, and the same pair of primers (Kutyavin et al., 2000). Thus, standard TaqMan probes must be designed in a larger size in order to reach the same annealing temperature as that of a TaqMan MGB probe. Additionally, MGB probes also have high specificity even in regions rich in A—T nucleotides (Walburger et al., 2001).

## Design of primers and TaqMan MGB probes

The choice of the genomic region, as well as the design of primers and probes, is fundamental for the success and efficiency of a multiplex reaction. Two genomic regions were chosen to perform duplex PCR. One is shared by both sexes (β-globin) and the other is specific for a region of chromosome Y called *TSPY* (DYS-14). The β-globin region chosen has four genomic copies in a diploid cell (β- and δ-globin), serves as an internal control of amplification and prevents a false-negative result due to PCR inhibitors. *TSPY* is a region which has 20—30 copies on chromosome Y, different from other regions such as SRY, which has only one copy. This increases the sensitivity of the reaction, since if one of these copies should suffer DNA damage, other copies are available for amplification, whereas in regions with a single copy, amplification may not occur if DNA is damaged.

The primers and probes were designed using the Primer Express 2.0 program (Applied Biosystems) under the conditions suggested by the manufacturer. For real-time PCR using the TaqMan MGB system, small amplicons (50—150 bp) typically yield more consistent results and robust signals. The designed oligonucleotides were submitted to basic local alignment search tool (BLAST) analysis and in-silico PCR for the determination of their specificity. BLAST and in-silico PCR are biotechnology tools available free of charge on the internet (http://www.ncbi.nlm.nih.gov/blast/; http://genome.ucsc.edu/cgi-bin/hgPcr both accessed 13 October 2009), which are used to check the similarity of DNA regions in order to determine their specificity. The primers and probes used and information about their products are presented in **Table 1**.

## Control of contamination

The preparation of lysis solution and of the PCR reagents was carried out under a laminar flow hood dedicated only to this activity, using material destined only for this manipulation. All the material (pipettes and surface on which the reactions were performed) was used only for this project and was always manipulated with gloves free of talcum powder. Before each reaction, decontamination was performed routinely with 70% alcohol or with 10% chlorine solution. The person who performed the reactions wore a disposable mask, cap and gown. The operator changed gloves every time contact was made with a possibly contaminated environment. At the end of the analysis of each reaction, all the material was discarded, with care taken not to contaminate previously amplified material (in the case of PCR).

After the biopsy, the blastomeres were washed with PBS in order to prevent contamination of any cell residue. For each blastomere analysed, the same PBS volume as used for washing was used to determine if there was contamination with exogenous material during the embryo biopsy. In addition, two blank samples were used in each reaction to determine if there was any contamination in the formulation of the PCR mix.

The reactions were carried out using the Taqman Universal PCR Master Mix (Applied Biosystems, PN 4304437). The use of a ready mix reduces the chances of contamination that might occur during formulation. In addition, the Master Mix contains dUTP and *AmpErase UNG*, which are the products of a strategy against possible contamination originating from previous reactions. Thus the amplified DNA will contain this nucleotide unit in its strands. Each reaction was started at a temperature of 50°C for 2 min so that the enzyme *AmpErase UNG* could digest the possible contamination of these products which might eventually be incorporated into the current reaction.

## Optimization of the real-time PCR protocol

For the optimization of the protocol of duplex reaction, the primers and probes were first optimized in separate reactions (singleplex). After the ideal concentration of each region was determined separately, the efficiency of amplification was checked and was found to be 100% for both regions. An efficiency of 100% is considered to be present when two copies of the initial products are produced after each PCR cycle, and in a 10-fold diluted DNA it must present a mean of 3.3 cycles from one to the other. Once the concentration of the reagents and the efficiency of each reaction are determined, duplex standardization is started. Duplex standardization was carried out with different concentrations of primers and probes for each region in the same reaction, using a sample of male DNA at a concentration equivalent to a genomic copy (~6.6 pg of DNA). For real-time PCR, the associated concentrations that generate the lowest threshold cycle ($C_t$) are considered, followed by the analysis of the concentrations that provide a more robust signal, i.e. the concentrations that generate a $\Delta R_n$ (baseline-corrected normalized fluorescence) with greater fluorescence. Using these methods, a concentration of primers was chosen for the two 400 nmol/l regions, whereas the concentrations for the probes were 150 nmol/l for *TSPY* and 250 nmol/l for the β-globin region, since the latter required a higher concentration to reach the same $\Delta R_n$ levels as the former. In addition to these criteria, the rate of success of amplification and the lowest $C_t$ variance

**Table 1** Primers and probes used in the project.

| *Region* | *Primers* | $T_m$ (°C) | *MGB probe* | *Fragment length (bp)* |
|---|---|---|---|---|
| β-Globin | F — 5′-TGCTGTTATGGGCAACCCTAA-3′<br>R — 5′-GAGCCAGGCCATCACTAAAGG-3′ | 62.6<br>63.2 | (VIC)TGAAGGCTCATGGCAAG | 74 |
| TSPY | F — 5′-AGAGCGTCCCTGGCTTCTG-3′<br>R — 5′-GAGAGCACCTCTCCACTAGAAAGG-3′ | 63.0<br>62.1 | (FAM)TCCTTCTCAGTGTTTCTT | 77 |

bp = base pairs; F = forward; FAM = carboxy fluorescein; MGB = minor groove binder; R = reverse; $T_m$ = melting temperature.

between replicates were used to choose the definitive concentrations of primers and probes for each region. Once the concentrations of the primers and probes were considered for each region for the duplex procedure, several reactions were carried out with male DNA equivalent to a genomic copy in order to determine the regularity of the method applied. It was then observed that the difference in $C_t$ between the two regions was of approximately four $C_t$, i.e. the DYS-14 region amplified ~4 $C_t$s before β-globin because it presented the largest number of initial copies. This reflects the difference in copies between the two regions, and demonstrates the extreme sensitivity of the technique for relative quantification.

The last standardization step concerned the condition for the execution of PGD; however, instead of a blastomere, a single buccal cell was used, which was also used to test the condition of the cell lysis solution and the specificity of the technique by employing female and male cells.

## Standardization of real-time PCR in single cells

The first step consisted of standardizing the single cell technique in order to establish the best final reaction volume and the best lysis method. Each reaction involved, in addition to the blastomeres, a male and a female control which consisted of a cell of each sex, as well as two blank controls denoted no template control (NTC). Thirty-one buccal cells and nine blastomeres from five embryos were tested with the 50 μl volume. In this protocol, the cell was transferred to a tube containing 5 μl of PBS, and then it was added 10 μl of the lysis solution [17 mmol/l sodium dodecyl sulphate (SDS, Sigma, USA) and 125 mg/ml proteinase K (Qiagen, Hilden, GmbH, Germany)] (Piyamongkol et al., 2003) and the tubes were frozen for 3 h. The tube was then thawed at room temperature for 10 min and placed in a block of the thermocycler (PCR) at 37°C for 60 min for the action of proteinase K, and at 96°C for 15 min for inactivation of the enzyme. Immediately after this procedure the tubes were placed under a laminar flow, the PCR reagents (mix, primers and probe) were added and the material was returned to the thermocycler for analysis. The controls with the buccal cells were submitted to cell lysis together with the blastomeres in all reactions as a control for the lysis procedure and later also for the analysis of the results for the blastomeres. In view of the fact that the buccal cell and the blastomere were being submitted to lysis simultaneously and analysed in the same reaction, the same technical efficiency was expected for both, and consequently the parameters for comparison were as close to the ideal as possible. The rate of successful amplification was 87.1% for the buccal cells (27 of 31 detected) and 55.6% for the blastomeres (five of nine).

In view of the poor results obtained with 50 μl, tests with a volume of 25 μl and using another lysis procedure were carried out in the second phase of the experiment. After isolation of the blastomere or of the buccal cell, the tube containing the single cell with 3 μl of the lysis solution only, without PBS, was frozen for at least 3 h. Following thawing, the lysis parameters were the same in both protocols (50 or 25 μl).

Real-time PCR was performed immediately after the lysis procedure with the following parameters for cycling: initial incubation at 50°C for 2 min to permit the AmpErase uracil *N*-glycosylase activity to break down any possible contamination product originating from previous reactions. The first denaturation step was 10 min at 95°C, followed by 45 PCR cycles in two steps, i.e. denaturation at 95°C for 15 s, followed by annealing at 60°C for 60 s. Continuous fluorescence was monitored in the annealing step. For greater safety, positive controls (a female and male buccal cell) were used in each reaction in addition to two blanks (all the reagents without DNA). The threshold was fixed at 0.10 in all reactions in order to standardize the results ($C_t$). The mean duration of a 45 cycle reaction was 1 h and 40 min.

For the total of PCR volume, half was of Taqman Universal PCR Master Mix (Applied Biosystems, PN 4304437), and then added to the primers of β-globin at a concentration cited before. Sterile pure water was used to complete the final volume of each reaction.

With a volume of 25 μl 19 blastomeres from 11 embryos were analysed, nine of them females and two males. The cycling conditions and reagent concentrations were the same as used for the 50 μl volume. In this phase it was possible to determine the sex in the blastomeres in 100% of cases (19/19), as was also the case for the buccal cells. Unspecific amplification was detected in only one of the reactions, which generated a signal for *TSPY* that was positive for one of the two NTC ($C_t$: 40.50), but did not influence the result of the samples because it presented a 'high' $C_t$. The other NTC in the same reaction did not present a signal; in addition, the result for the samples was female sex. However, possible contamination should never be ignored, and this result was considered as contamination, because it might lead to misdiagnosis.

The amplification rate for buccal cells and for blastomeres is presented in **Table 2**.

After the excellent results obtained in this phase, the reaction was considered to be optimized and its results were compared with those obtained by the FISH technique.

## Comparison between FISH and real-time PCR for embryo sexing

During the phase of direct comparison of the two techniques, all non-viable embryos with at least two intact blastomeres and with a nucleus viewed were biopsied. After biopsy, one blastomere was fixed on a slide and the other was placed in the PCR microtube containing the lysis solution. Each blastomere was numbered in a different manner in the two techniques so that the study could be carried out in a blind fashion. After analysis of the blastomeres by each technique, the result was handed over to an embryologist, who filed the data during the project. At the end of the analysis of all cases, the results were compared and analysed statistically.

## Fluorescence in-situ hybridization

The biopsied blastomeres were washed in Earle's medium supplemented with 0.5% human albumin under an inverted microscope and fixed individually on slides at a site previously marked with a circle behind the slide, to which 1—2 μl of diffusion solution (0.01 mol/l HCl and 0.1% Tween) was added (Coonen et al., 1994). After this procedure, the

**Table 2** Summary of human sexing in a single cell by real-time polymerase chain reaction (PCR).

| *Sample* | *PCR volume (μl)* | *Total no. of cells* | *Sex* | | *Amplification rate (%)* |
|---|---|---|---|---|---|
| | | | *Female* | *Male* | |
| Buccal cell | 50 | 31 | 14 | 13 | 87.1 |
| | 25 | 33 | 17 | 16 | 100.0 |
| Blastomere | 50 | 9 | 0 | 5 | 55.5 |
| | 25 | 54 | 35 | 10 | 83.3 |

slides were left at room temperature for 30 min and then transferred to the FISH laboratory.

The slides were rinsed with PBS and dehydrated in ethanol. After drying, they were treated with 0.01 mol/l HCl containing 50 μl 10% pepsin at 37°C for 5 min, rinsed with purified water and PBS, immersed in Carnoy fixative at 4°C for 10 min, and rinsed again.

After a new dehydration step, the slides were left at room temperature, and then the probe solution (Multivision PGT Probe Panel; Vysis Inc., Downers Grove, IL, USA, Ref. 32—131080) was added to a previously marked site. The coverslip was placed on the solution and the slide was denatured at 75°C on a heating plate for 3 min. The probes were hybridized in a humidified chamber overnight at 37°C.

After the washing procedure and the addition of antifade solution (Vectashield, Burlingame, CA, USA), the slides were ready for analysis.

## Statistical analysis

Concordance values were compared by the Mann—Whitney test.

## Ethical aspects

The couples who kindly donated the non-viable embryos for research signed a term of consent in agreement with resolution 196/96 CNS-MS after being fully informed about, and agreeing with, the research project. The project was evaluated and approved by the Research Ethics Committee of the Faculty of Medicine of Botucatu, São Paulo, and was designed according to the requirements of this entity.

## Results

A total of 98 blastomeres from 40 embryos non-viable for transfer were analysed in the present study. Twenty-six were sexed as females, 12 as males and two could not be diagnosed. Of this total, it was possible to analyse 24 embryos (60%) by both techniques, generating a total of 70 blastomeres (35 per technique). On the other hand, 16 embryos (40%) from which 28 blastomeres were removed were analysed only by real-time PCR (**Table 3**).

During the phase of comparison of the two methods, 17 of the 24 embryos were sexed as females, six as males, and one could not be diagnosed. For the 35 blastomeres analysed by each technique, real-time PCR was able to diagnose the sex in 26, generating a rate of 74.3%. In contrast, using the FISH technique it was possible to analyse

**Table 3** Summary of embryo analysis.

| | *No. of embryos (blastomeres)* |
|---|---|
| Total | 40 (98) |
| Analysed only by real-time PCR | 16 (28) |
| Comparison between methods | 24 (70)[a] |
| Females | 26 |
| Males | 12 |
| Result could not be obtained | 2 |

[a] Thirty-five blastomeres per technique; PCR = polymerase chain reaction.

28 of the 35 blastomeres, leading to a rate of 80.0%. In two of the nine cases in which the diagnosis by real-time PCR was not possible, the diagnosis was also impossible by FISH.

The general mean $C_t$ value was 36.79 for β-globin and 34.42 for *TSPY*, and the mean value for $\Delta R_n$ was 0.96 and 1.26, respectively. The difference between the $C_t$ of the two regions was 3.99 and the difference in $\Delta R_n$ was 0.72. In one of the blastomeres (embryo 21) it was not possible to diagnose the sex by real-time PCR, with the reaction showing amplification of both regions, but with a lower $C_t$ for β-globin, in contrast to the general rate, which amplified, on average, four cycles earlier for the *TSPY* region. The result of FISH for this same embryo (embryo 21) was male sex, but the results remained undefined because the amplification of the two regions did not establish the 'rule' cited above. Also, it was not possible to diagnose the sex in one of the blastomeres analysed by FISH (embryo 5) because it presented only two signals for chromosome 18.

Using FISH it was possible to determine that 33.3% of the 24 embryos (8/24) were normal for the chromosomes studied, 58.3% (14/24) were abnormal and 8.3% (2/24) could not be analysed.

The data regarding all real-time PCR procedures and the mean $C_t$ and $\Delta R_n$ values are presented in **Table 4** and **Figure 1**.

## Discussion

Because it is a test involving a high level of responsibility and complexity, PGD can be routinely employed only after exhaustive standardization of the method used. For this reason, the best possible conditions were chosen for the development of the real-time PCR method and the results were

**Table 4** Mean threshold cycle ($C_t$) and fluorescence $\Delta R_n$.

| *Cell type* | *Total* | *Signal* | $C_t$ *(SD)* | $\Delta R_n$ *(SD)* |
|---|---|---|---|---|
| Female buccal | 17 | β-Globin | 37.34[a] (±1.18) | 0.94 (±0.25) |
| Male buccal | 15 | β-Globin | 36.76[b] (±1.35) | 0.72 (±0.23) |
| | | *TSPY* | 33.47[c] (±1.40) | 1.17 (±0.44) |
| Female blastomere | 38 | β-Globin | 37.27[a] (±2.08) | 1.09 (±0.40) |
| Male blastomere | 9 | β-Globin | 36.59[b] (±1.00) | 0.83 (±0.15) |
| | | *TSPY* | 33.57[c] (±2.98) | 1.28 (±0.38) |

*Note:* second and third phase (25 μl)[a,b,c] are showing no $C_t$ significant difference between different single cells in the same polymerase chain reaction volume (25 μl). $\Delta R_n$ = baseline-corrected normalized fluorescence.

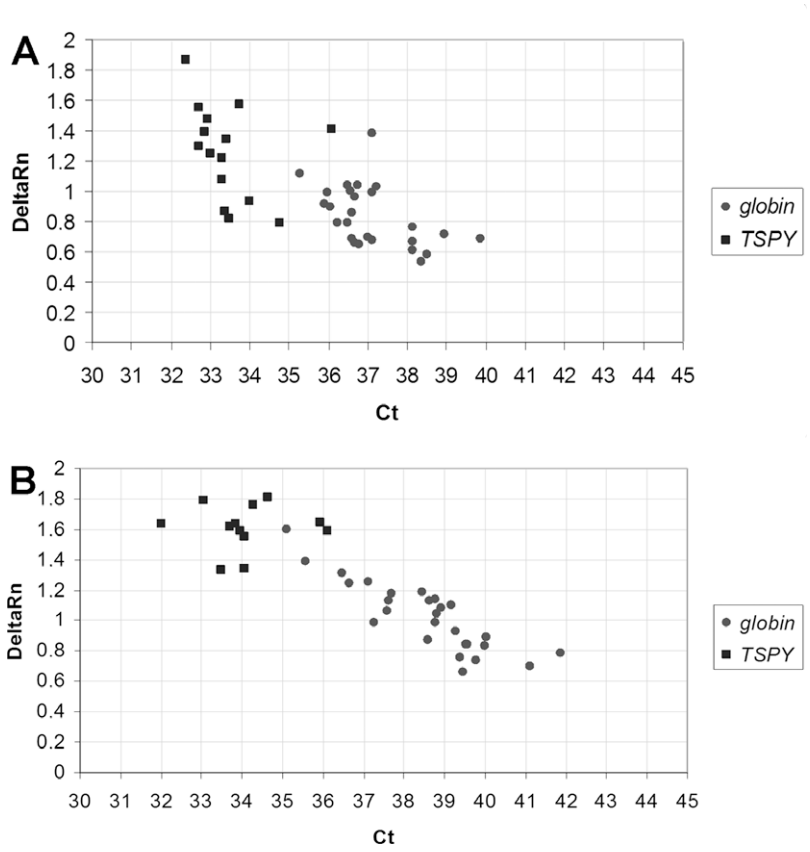


**Figure. 1** Scatter diagrams of threshold cycle ($C_t$) and the baseline-corrected normalized fluorescence ($\Delta R_n$) from single cells (buccal cell and blastomere). Comparison of TSPY and β-globin signals on real-time polymerase chain reaction reaction with 25 μl (**A**), and 50 μl (**B**).

compared with those obtained with FISH, the gold standard technique for embryo sexing. The choice of the best cell lysis method and of the best genomic regions for the design of primers and probes is of fundamental importance in order to obtain the best possible results. In addition, extreme care regarding possible contamination with exogenous DNA is a crucial point if consistent and reliable results are to be obtained.

The *TSPY* (DYS-14) and β-globin regions were chosen as targets because they are specific for chromosome Y and chromosome 11, respectively. The *TSPY* region is a moderately repetitive and conserved region of chromosome Y. The repetitive nature of the region may reduce the impact of small variations at the beginning of amplification, and consequently may increase the chance of success of the technique in a single cell. On this basis, it increases the sensitivity of the technique compared with the detection of only one copy, which is what occurs for the *SRY* region, another specific region of chromosome Y. Additionally, the detection of only one initial copy may generate allele drop-out (ADO), an event that would seldom occur with *TSPY*, which has 20–30 copies per genome. Indeed, the

*TSPY* region has been reported for the sexing of embryos using real-time PCR with fine outcomes (Pierce et al., 2000), but in this case molecular beacon probes were used. The region of the β-globin gene was chosen for three main reasons. First, this is a region that has been used in the study laboratory for a long time for other experiments with a high rate of successful amplification, even using small amounts of DNA (Martinhago et al., 2006). Second, this is a region located on chromosome 11, which seldom presents aneuploidy (Milunsky, 1998), thus representing an excellent control that reduces the possibility of wide variations in $C_t$. Finally, the primers were designed in order to amplify a fragment of the delta and β-globin region that has four copies, and not simply one or two, which would reduce the sensitivity of the technique.

The lysis solution based on proteinase K was chosen because of its high rates of successful amplification and also because, according to some authors (Pierce et al., 1999; Piyamongkol et al., 2003), it currently represents the best method of cell lysis against ADO. Other lysis protocols that yielded successful amplification much below the expected rate were also tested. The cell lysis solution was prepared at 3 month intervals in 30 μl aliquots that were immediately frozen at −20°C. Any thawed aliquot that was not used was discarded because, once thawed, proteinase K may lose its activity. Thus a new aliquot was used for each reaction.

The main reason for the choice of 25 μl as the final reaction volume was the fact that the protocol in which it was used provided more uniform and consistent results, in addition to involving a lower rate of contamination than the protocol that used a final volume of 50 μl. Regarding contamination, in the protocol with a greater volume, it was obligatory to open the microtubes before the cell lysis step in order to add the proteinase K solution. In addition, saline solution was used inside the tubes to deposit the cell, this being one more source of contamination. In contrast, in the 25 μl volume the embryologist placed the blastomere directly in the lysis solution, with the microtube having to be opened only to add the PCR reagents. The reasons for choosing a ready mix for the execution of real-time PCR were that, first, their use avoids possible sources of contamination during mix formulation, and, second, the mix is already optimized for use, with no need for previous optimization of the reagents, a very expensive process.

There is only one study in the literature which used real-time PCR as a technique for human embryo sexing (Pierce et al., 2000), although it used another method for the detection of the amplified product (molecular beacons), and another region (U2 genes) as control. However, although the number of blastomeres analysed in the present study was smaller, the same general amplification rate was obtained, i.e. 83.33% (45/54), as compared with 83.78% (155/185) in the cited study. In addition, the general rate of amplification of a single optimized buccal cell (100%, 33/33) was similar to that reported by the cited investigators for lymphocytes, i.e. 99.07% (107/108). In the report by Hartshorn, real-time PCR was used for sexing embryos with a multiplex Xist/Sry real-time RT-PCR assay. The authors discussed different pattern of Xist gene expression and DNA methylation in female mouse embryos (Hartshorn et al., 2003).

In view of the data related to real-time PCR in a single cell, there was no significant difference in $C_t$ between the buccal cells and the blastomeres with a volume of 25 μl. However, the variance of $C_t$ among the blastomeres was greater than in the buccal cells, regardless of the final reaction volume, as can be seen from **Table 4**. This was probably due to the adverse conditions that may be encountered in each blastomere analysed, since the DNA of these cells originates from different samples and may be in different conditions of haploidy (3*n*, 2*n* or *n*) or aneuploidy (trisomies and monosomies). In addition, since these are embryos that stopped their cell division, their blastomeres may be in a condition of apoptosis which may result in poor or absent amplification. More consistent results and a better amplification rate would certainly be obtained if normally cleaved blastomeres were analysed. In contrast, buccal cells from the same individual present high genomic similarity and therefore can generate more uniform results. Furthermore, there is the possibility of choosing the best cells, which were well delimited and contained a visible nucleus during the procedure of buccal cell isolation, a fact that is not always possible for blastomeres. Another important fact is that the mean of the $C_t$ resulting from the volume of 25 μl was less than 50 μl regardless of cell type. This indicates an increase in the sensitivity of the reaction with the smaller volume, except for *TSPY* in male buccal cells. In addition, the standard deviation of the $C_t$ was lower in all reactions of 25 μl, with the sole exception of male buccal cells (**Figure 1**).

When real-time PCR was compared with FISH for embryo sexing in blastomeres, the real-time PCR index of 74.3% was lower than the 80.00% index of FISH. However, there was no discrepancy in embryo diagnosis between the two techniques, which showed absolute specificity. Nevertheless, if the errors in blastomere fixation are not considered, or if the cases in which the nucleus was not seen on the slide before analysis are taken into account ($n = 5$), the index for FISH increased to 96.67% of cases (29/30). The data showed that in spite of the results obtained with real-time PCR, FISH is still a better technique for sexing embryos.

Since the real-time PCR machine used has the ability to detect probes of five different wavelengths, and in the sexing reactions only three were used, one for FAM, one for VIC and the third as the passive reference contained in the mastermix (ROX), there is the possibility of incorporating at least one or two more probes with their respective primers. With the incorporation of the detection of an additional genomic region, such as in chromosome 21 (Down syndrome), it may be possible to differentiate cases of trisomy 21 (Down syndrome) by relative quantitation between two regions (Tsujie et al., 2006; Zimmermann et al., 2006). Thus, a critical region of chromosome 21 (*DSCR1*) was used as a test with a NED-labelled probe whose wavelength is different from VIC (chromosome 11) and relative quantitation was performed in the DNA of an individual with Down syndrome compared with the DNA of a normal individual. The preliminary results are encouraging since it was possible to clearly differentiate the additional copy of the DNA carrying 21 trisomy (data not shown). One would expect to be able to standardize the reaction for small amounts of DNA, such as in a single cell, within a short time and consequently to diagnose blastomeres in terms of sex and the presence of trisomy 21.

Another possibility for the future study of embryos using the two techniques is the comparison of the expression of

some genes that may be involved in the mechanism of aneuploidy. Normal and aneuploid embryos are diagnosed by the FISH technique and gene expression is compared by real-time PCR (Gal et al., 2006; Rinaudo et al., 2006).

In this blinded prospective study, it has been shown that rapid and reliable real-time PCR-based gender identification of preimplantation human embryos using a single blastomere can be performed within ~4 h after biopsy. The method developed here proved to be rapid and safe for the detection of gender in a single human cell (buccal cell and blastomere) by an emerging technology called real-time PCR. In addition, comparison of real-time PCR and FISH showed absolute agreement for the diagnosis of embryo sex between the two techniques. However, at present FISH is still the best method for sexing embryos, especially because it can detect numerical chromosome aberrations.

## References


Coonen, E., Dumoulin, J.M.C., Ramaekers, F.C.S., Hopman, A.H.N., 1994. Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescent in situ hybridisation. Hum. Reprod. 9, 533–537.

Gal, A.B., Carnwath, J.W., Dinnyes, A., et al., 2006. Comparison of real-time polymerase chain reaction and end-point polymerase chain reaction for the analysis of gene expression in preimplantation embryos. Reprod. Fertil. Dev. 18, 365–371.

Hartshorn, C., Rice, J.E., Wangh, L.J., 2003. Differential pattern of Xist RNA accumulation in single blastomeres isolated from 8-cell stage mouse embryos following laser zona drilling. Mol. Reprod. Dev. 1, 41–51.

Handyside, A.H., Kontogianni, E.H., Hardy, K., Winston, R.M., 1990. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 344, 768–770.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Heng, B.C., 2006. Gender selection of embryos after PGD for other independent medical reasons — a viewpoint from Singapore. Reprod. BioMed. Online 12, 392–393.

Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology 10, 413–417.

Kutyavin, I.V., Afonina, I.A., Mills, A., et al., 2000. 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28, 655–661.

Magli, M.C., Sandalinas, M., Escudero, T., et al., 2001. Double locus analysis of chromosome 21 for preimplantation genetic diagnosis of aneuploidy. Prenat. Diagn. 21, 1080–1085.

Martinhago, C.D., de Oliveira, R.M., Tomitao Canas, M. do C, et al., 2006. Accuracy of fetal gender determination in maternal plasma at 5 and 6 weeks of pregnancy. Prenat. Diagn. 26, 1219–1223.

Milunsky, A., 1998. Genetic Disorders and the Fetus, 4th edn. The Johns Hopkins University Press, Baltimore, USA, p. 184.

Munné, S., Marquez, C., Magli, C., et al., 1998. Scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18 and 21. Mol. Hum. Reprod. 4, 863–870.

Online Mendelian Inheritance in Man 2006 http://www.ncbi.nlm.nih.gov/omim.

Pierce, K.E., Rice, J.E., Sanchez, J.A., et al., 2000. Real-time PCR using molecular beacons for accurate detection of the Y chromosome in single human blastomeres. Mol. Hum. Reprod. 6, 1155–1164.

Pierce, K.E., Rice, J.E., Sanchez, J.A., Wangh, L.J., 1999. Comparison of cell lysis conditions for single cell PCR using molecular beacons to monitor reactions in real time. Fertil. Steril. 72s, 169.

Piyamongkol, W., Bermudez, M.G., Harper, J.C., Wells, D., 2003. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. Mol. Hum. Reprod. 9, 411–420.

Rinaudo, P.F., Giritharan, G., Talbi, S., et al., 2006. Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil. Steril. 86s, 1252–1265.

Sermon, K.D., Michiels, A., Harton, G., et al., 2007. ESHRE PGD Consortium data collection VI: cycles from January to December 2003 with pregnancy follow-up to October 2004. Hum. Reprod. 22, 323–336.

Staessen, C., Van Assche, E., Joris, H., et al., 1999. Clinical experience of sex determination by fluorescent in-situ hybridization for preimplantation genetic diagnosis. Mol. Hum. Reprod. 5, 382–389.

Stouffs, K., Lissens, W., Tournaye, H., et al., 2005. The choice and outcome of the fertility treatment of 38 couples in whom the male partner has a Yq microdeletion. Hum. Reprod. 20, 1887–1896.

Tsujie, T., Takemura, M., Kimura, T., et al., 2006. Rapid detection of trisomy 21 by gene dosage analysis using quantitative real-time polymerase chain reaction. J. Obstet. Gynaecol. Res. 32, 368–372.

Walburger, D.K., Afonina, I.A., Wydro, R., 2001. An improved real time PCR method for simultaneous detection of C282Y and H63D mutations in the HFE gene associated with hereditary hemochromatosis. Mutat. Res. 432, 69–78.

Zimmermann, B., Levett, L., Holzgreve, W., Hahn, S., 2006. Use of real-time polymerase chain reaction for the detection of fetal aneuploidies. Methods Mol. Biol. 336, 83–100.


*Declaration: The authors report no financial or commercial conflicts of interest.*

Received 29 January 2009; refereed 17 February 2009; accepted 14 September 2009.