

Real-time PCR using molecular beacons for accurate detection of the Y chromosome in single human blastomeres

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We describe a highly accurate method for determining the sex of human embryos via real-time polymerase chain reaction (PCR) amplification of highly-conserved, moderately-repeated sequences within the *TSPY* genes on the Y chromosome and the *U2* genes on chromosome 17. Individual male lymphocytes, female lymphocytes, and blastomeres from donated cleavage-stage embryos were lysed prior to PCR using an optimized buffer containing proteinase K. Molecular beacons, a new type of fluorescent probe, were used to detect and quantify accumulating amplicons during each cycle of PCR carried out in closed tubes. The present work is part of an ongoing study to construct and implement a new, convenient and reliable system of preimplantation genetic diagnosis (PGD).

Key words: fluorescent PCR/preimplantation genetic diagnosis/quantitative PCR/sexing human cells/Y chromosome detection

Introduction

Advances in molecular genetics are rapidly generating the tools needed to identify the causes of many inherited human diseases. Preimplantation genetic diagnosis (PGD) seeks to apply those tools to the detection of both chromosomal abnormalities and disease-causing genes in single cells biopsied from human embryos. The resulting information can be used to decide which embryos should be transferred to the uterus to establish a pregnancy. Thus, PGD offers couples who want to avoid having a child afflicted by a severe disease of genetic aetiology an alternative to prenatal diagnosis and termination of an ongoing pregnancy. In recent years, many births have been reported following uterine transfer of embryos tested by PGD (Verlinsky and Kuliev, 1998; ESHRE PGD Consortium Steering Committee, 1999). Despite these favourable outcomes, PGD remains a difficult and moderately reliable technology and is currently only used on an experimental basis at a few selected IVF clinics around the world.

Genes on the X chromosome are responsible for at least 155 inherited diseases (McKusick, 1998), most of which follow a recessive mode of inheritance and are therefore expressed in 50% of the sons born to mothers who carry one abnormal allele. Additional conditions related to infertility are linked to the Y chromosome (Lahn and Page, 1997). PGD by means of fluorescent in-situ hybridization (FISH) with probes to the X and Y chromosomes has been used in several cases involving X-linked diseases (Griffin *et al.*, 1994) and has the potential advantage of also detecting sex chromosomes aneuploidies. However, the efficiency and accuracy of this technique are highly dependent on the technical skills and experience of the laboratory involved. Poor fixation and lack of stringent scoring criteria can reduce the reliability of FISH (Munné *et al.*, 1998).

In addition, FISH cannot be extended to detection of allelic differences within single copy genes, which are the cause of most inherited diseases.

Polymerase chain reaction (PCR) amplification of one or more DNA sequences in single cells offers an alternative to FISH. Conventional methods of PCR involve repeated cycles of sequence amplification followed by some form of end-product analysis. This type of analysis was the first method used to identify the sex of embryos for couples known to be at risk for transmitting X-linked diseases (Handyside *et al.*, 1990). The test involved amplification of a highly reiterated sequence on the Y chromosome in single biopsied cells. Embryos that did not generate the Y-specific product were diagnosed as female and were transferred to the uterus, thereby avoiding the birth of potentially afflicted males. Several girls were born using this approach, but the reported misdiagnosis and transfer of a male embryo, presumably due to PCR failure, illustrated one of the risks of this strategy (Hardy and Handyside, 1992). Subsequent protocols reduced this risk by co-amplification of reiterated sequences on the X chromosome as an internal control for cell lysis and overall PCR efficiency (Kontogianni *et al.*, 1991, 1996; Strom *et al.*, 1991; Grifo *et al.*, 1992). Nevertheless, high rates of misdiagnosis (3–8%) and amplification failures (7–20%) lead many investigators to abandon this approach in favour of FISH or other PCR strategies.

In an effort to increase the specificity of PCR, investigators turned to amplification of single copy genes on the Y chromosome, a strategy that included the use of nested PCR primers when starting with a single cell. Using this approach male and female blastomeres have been distinguished via amplification of the *SRY* gene (Cui *et al.*, 1994), the amelogenin gene

(Levinson *et al.*, 1992), and the *ZFY* gene (Chong *et al.*, 1993). Homologous but non-identical copies of both the *amelogenin* and *ZFY* genes are also located on the X chromosome and can serve as internal controls for successful amplification using the same sets of primers. Y chromosome-specific sequences are distinguished from their X chromosome analogues using gel electrophoresis. Nevertheless, the nested PCR strategy requires more cycles of amplification, increased sample handling, and some means of distinguishing the PCR products. These additional steps increase the time required to complete the assay, as well as the risk of contaminating either the sample or the laboratory with released amplicons.

Further improvements in the amplification of single-copy genes for sex chromosome identification were achieved through the use of fluorescently-labelled primers (Findlay *et al.*, 1995a,b; Liu *et al.*, 1995; Sherlock *et al.*, 1998). The increased sensitivity of this technique provides greater accuracy compared to other conventional PCR methods (Findlay *et al.*, 1998) and reduces the number of amplification cycles required, thereby eliminating the need for nested PCR. However, the overall time requirement of the assay remains high, since the amplicons must be separated using capillary electrophoresis. Furthermore, PCR analysis of single copy genes is plagued by the problem of allele drop-out, i.e. the selective failure to amplify one of the target sequences present in the starting cell (for review, see Lissens and Sermon, 1997). Improved protocols for cell lysis (Gitlin *et al.*, 1996; El-Hashemite and Delhanty, 1997), DNA denaturation prior to PCR (Ray *et al.*, 1996), and the use of fluorescently-labelled primers (Findlay *et al.*, 1995a) have decreased the rates of allele drop-out, but this phenomenon continues to be a problem.

Real-time PCR is a recent technological innovation which allows the rate of amplicon accumulation to be measured by detection of fluorescently-tagged probes at each cycle of the reaction. Accurate quantitative analysis of the number of target molecules present at the outset is accomplished by determining the PCR cycle at which the fluorescent signal is first detected (Heid *et al.*, 1996; Tyagi *et al.*, 1998). A particularly promising adjunct to real-time PCR involves the use of molecular beacons, a new class of fluorescent probes invented by Tyagi and Kramer (1996). A molecular beacon is a single-stranded oligonucleotide 25–35 bases long in which the last 5–8 bases on the 3' and 5' ends are complementary (Figure 1B). Thus, a beacon forms a hairpin structure at ambient temperatures. The double-helical stem of the hairpin brings a fluorophore attached to the 5' end of the beacon very close to a quencher attached to the 3' end of the beacon. The beacon does not fluoresce in this conformation. If the beacon is heated or allowed to hybridize to a target oligonucleotide which is complementary to the sequence within the single-strand loop of the beacon, the fluorophore and the quencher are separated and the resulting conformation fluoresces. Thus, when fluorescent readings are acquired at the annealing temperature in a series of PCR cycles the signal strength increases in proportion to amplicon accumulation (Figure 1). Molecular beacons with different loop sequences can be constructed with different fluorophores in order to monitor increases in different

amplicons in multiplex reactions (Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998).

This paper describes the use of molecular beacons and real-time PCR to optimize the conditions required for detection of chromosomal markers in single human lymphocytes and blastomeres. The highly-conserved *TSPY* genes, repeated 27–40 times, were selected for detection of the human Y chromosome (Zhang *et al.*, 1992; Manz *et al.*, 1993). The *U2* genes, present on chromosome 17 in 20–40 identical or nearly-identical copies per diploid cell, served as an internal control for successful amplification (Van Arsdell and Weiner, 1984; Westin *et al.*, 1984; Pavelitz *et al.*, 1995). The fact that *TSPY* and *U2* are both moderately-repeated genes eliminated the possibility of allele drop-out, since the PCR product is obtained from both sequences even if several copies of these gene targets fail to initiate amplification.

Our goal was to utilize real-time PCR with molecular beacons for simultaneous quantitative detection of the Y chromosome and chromosome 17 present in single blastomeres. We anticipated that the kinetics of amplicon accumulation would be affected by genetic variations within donated embryos, including differences in cell cycle, ploidy, mosaicism, anucleate cells, and apoptosis. The presence of such natural variations would make it difficult to optimize the conditions of our basic assay. For this reason, we first analysed the rate and extent of *TSPY* and *U2* amplification initiated with single male and female lymphocytes. Lymphocytes are readily available and are virtually all diploid and arrested in the cell cycle prior to DNA duplication. This homogeneity of the lymphocyte population permitted us to optimize the conditions needed to reliably obtain robust *TSPY* and *U2* signals. Analysis of blastomeres under the same conditions demonstrated that real-time PCR with molecular beacons can accurately diagnose the gender of embryos. Assays of both lymphocytes and blastomeres are rapid (<3 h) and conveniently carried out in closed tubes, thereby greatly reducing the chances of laboratory contamination. We foresee that these new technologies will soon be available for PGD.

Materials and methods

Preparation, handling, and lysis of lymphocytes

Blood from single male and female donors was drawn directly into tubes containing EDTA to prevent clotting. Whole blood (3 ml) was layered over 3 ml of Histopaque-1077 (Sigma, St Louis, MO, USA) and centrifuged at 400 g for 30 min. Most of the plasma was discarded and the layer of mononuclear leukocytes (predominantly lymphocytes) was collected and washed three times with DPBS lacking calcium or magnesium (PBS; Sigma, cat. no. D-8537). The cells were resuspended in 70% PBS, 30% glycerol and chilled on ice. Aliquots were placed in screw-cap, 0.5 ml centrifuge tubes, and frozen in liquid nitrogen.

For transfer of individual lymphocytes, an aliquot of the cell suspension was thawed and 1 µl was added to 3 ml of PBS in a Costar ultra-low-attachment culture plate (Fisher Scientific, Pittsburgh, PA, USA; cat. no. 07-200-601). A single cell was aspirated into a finely-drawn glass pipette while viewing at ×100 magnification with an Olympus IX70 microscope. The pipette contents were expelled directly into 10 µl of Lysis Buffer (Hamilton Thorne Research Inc, Beverly, MA, USA) in a 0.2 ml MicroAmp optical PCR tube (PE

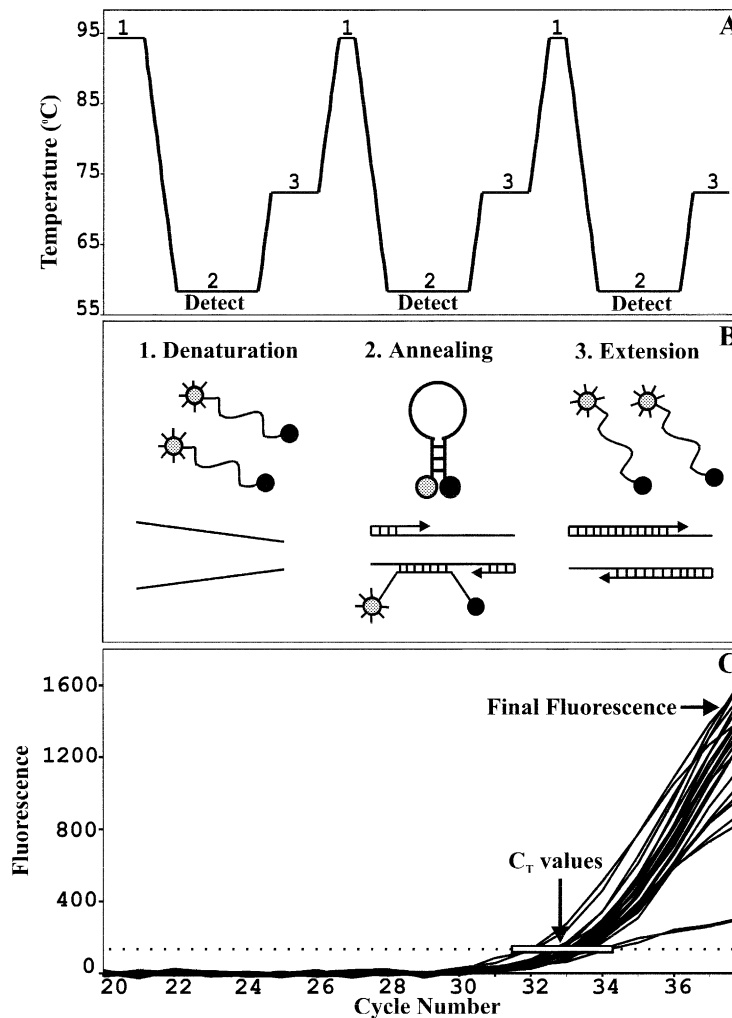


Figure 1. Graphical representation of molecular beacons, primers, and amplicons during polymerase chain reaction (PCR). (A) Cycle profile used for amplification of TSPY and U2. (B) Conformational changes that occur in molecular beacons and target DNA molecules during PCR. During high-temperature denaturation, step 1, all DNA is single stranded and the molecular beacon is linear. At the lower annealing temperature, step 2, molecular beacons hybridize to available complementary strands and fluoresce. Molecular beacon molecules not bound to the amplicon assume a hairpin structure that is non-fluorescent. Fluorescent readings are taken during this step at each cycle to quantify the amount of accumulating amplicon. Beacon and amplicon dissociate during amplicon extension, step 3 and polymerization of the new amplicon strand is completed. Filled circles represent quenching moiety on 3' end of the beacon. Open circles represent fluorescent moiety on 5' end of the beacon. (C) Amplification plot of fluorescence readings of *U2* genes in individual female lymphocytes after background subtraction. The detection threshold is shown as a dotted line. The threshold cycle (C_T) for each sample is determined as the point at which the fluorescence plot crosses this line. Final fluorescence is measured at cycle 38.

Applied Biosystems, Foster City, CA, USA). The tube was kept on ice until the transfer of all cells was complete. Samples were then removed from ice and placed in a thermal cycler block that had been preheated to 50°C. A heated cover was positioned over the samples to prevent condensation and the samples were incubated at 50°C for 60 min, then 95°C for 10 min.

Blastomere isolation and lysis

Non-viable embryos deemed unsuitable for transfer to patients were obtained for experimental analysis following written patient consent and Internal Review Board approval at The Institute for Reproductive Medicine and Science of Saint Barnabas. Embryos on day 3 or day 4 post-insemination (4–12-cell stage) were treated briefly in acidified Tyrode's solution to remove the zona pellucida, then rinsed 3–5 times in PBS containing 0.1% polyvinylpyrrolidone (PVP-40; Sigma) and incubated for ~30 min in that solution. Embryos were disaggregated into individual blastomeres by repeated aspiration into a narrow

diameter plastic pipette with bore size 0.16 mm (Drummond Scientific Company). A small number of blastomeres was obtained by biopsy rather than disaggregation. In all cases, each blastomere was rinsed twice in PBS containing 0.1% PVP-40, once in PBS containing 0.01% PVP-40, then transferred directly into 10 μ l of Lysis Buffer in a 0.2 ml MicroAmp optical PCR tube. Control samples were prepared by transferring a similar volume of final wash buffer (<1 μ l) to the lysis buffer. All tubes were kept on ice until they could be placed in a thermal cycler for the lysis incubation as described above.

Molecular beacons and primers

Primers and molecular beacons were designed with the aid of Oligo 5.0 software (National Biosciences Inc, Plymouth, MN, USA). Desalted primers were purchased from Life Technologies (Gaithersburg, MD, USA). Theoretical folding structures of the amplified sequences as determined by oligonucleotide nearest-neighbour thermodynamics

(SantaLucia, 1998) were examined by submitting the sequence for analysis at the following internet site: <http://mfold.wustl.edu>. Molecular beacons were designed according to previously described methods (Tyagi and Kramer, 1996; as detailed on the internet site: <http://molecular-beacons.org>). The guidelines included the following parameters: (i) amplicon regions that could form stable hairpins were avoided as possible targets, as were sequences with strong complementarity with any of the primers; (ii) the melting temperature (T_m) of the hybridized loop sequence was 5–10°C higher than the T_m of the primers; and (iii) the oligonucleotide folding program predicted a hairpin as the only stable structure for the molecular beacon in the absence of target at the PCR annealing temperature. The predicted T_m for that hairpin structure was ~10°C above the annealing temperature. Molecular beacons were purchased from Research Genetics Inc (Huntsville, AL, USA).

For TSPY amplification, the upper primer sequence was: 5' ATACAGGGCTTCTCATTCCA 3' and the lower primer sequence was: 5' GTTAGATCCTGCGAAGTTGTG 3'. These primers amplify a 133 bp segment of TSPY exon 4 and were based on sequences from clone Y-231 (Zhang *et al.*, 1992). The TSPY molecular beacon sequence was: 5'CGCGCTTTGTGGTGTCTGCGGCGATAGGCA-GCGCG 3' with the fluorophore TET covalently attached to the 5' end and the quencher DABCYL covalently attached to the 3' end.

Amplification of the U2 small nuclear RNA gene (Pavelitz *et al.*, 1995) generated a 175 bp sequence with upper primer, 5' AAGAAAT-CAGCCCGAGAGT 3', and lower primer, 5' CTTGATCTTAGCCA-AAAGGT 3'. The lower primer contains a mismatch to its target at the 3' end in order to reduce possible dimerization with the TSPY primers, and to reduce efficiency of priming such that the TSPY amplification is preferentially amplified under competitive conditions. The U2 molecular beacon sequence was 5' CTGGCCTGTCTCGTCC-ACAGCGCTATTGAGGCCAG 3' with the fluorophore FAM covalently attached to the 5' end and the quencher DABCYL covalently attached to the 3' end. Electrophoresis through a 3% agarose gel was used following a single PCR with multiplexed primer pairs to confirm the production of amplicons with the expected sizes for U2 and TSPY.

PCR conditions

In the initial lymphocyte series, 15 µl of concentrated PCR reagent mixture was added to each tube containing a lysed cell (or no-cell control) giving final concentrations of 50 mmol/l Tris, pH 8.3, 3.5 mmol/l MgCl₂, 0.4 mmol/l each dNTP, 0.3 µmol/l each primer, 0.3 µmol/l each molecular beacon, and 0.5 units of *Taq* polymerase (Promega, Madison, WI, USA) per 25 µl reaction. *Taq* polymerase was preincubated with TaqStart antibody (Clontech, Palo Alto, CA, USA) for 5 min at room temperature before it was added to the PCR mixture to inhibit polymerase activity until the first denaturation step (hotstart PCR). The PCR reagent mixture was altered to 100 mmol/l Tris and 1 unit of *Taq* polymerase in later experiments, including all those with blastomere samples.

Amplification and fluorescence detection was carried out using an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). The cycling profile for TSPY and U2 amplification included an initial denaturation at 95°C for 3 min, followed by 38 cycles of 95°C for 10 s, 58°C for 45 s, and 72°C for 10 s, with fluorescence readings taken during the 58°C step. The PCR takes ~90 min to complete.

Contamination control

Preparation of Lysis Buffer and PCR reagent mix was carried out in a room restricted to those activities, using dedicated pipettors and supplies. All pipetting was done within PCR enclosure hoods (Labconco or similar with plexiglass front panels) using aerosol-resistant pipette tips. Hood surfaces, pipettors, and supplies were

treated with UV light between uses and were touched only with gloved hands. Surfaces were treated approximately once per week with 10% chlorine bleach. Investigators wore disposable surgical masks and caps, gloves with extended cuffs, and lab coats that remained in the PCR preparation room.

Single lymphocytes were aspirated while viewing through an inverted microscope on the open bench and were then expelled into sample tubes opened in an adjacent PCR enclosure. Each tube was recapped immediately. All manipulations of embryos and blastomeres were carried out within a laminar flow hood. Following the lysis incubation, samples were returned to the PCR enclosure where PCR reagent mixture was added and tubes were resealed with new caps.

Following PCR, sample tubes were either sealed within a bag for disposal, or were taken to a separate laboratory for electrophoretic analysis. Electrophoresis equipment and supplies were never brought into the PCR laboratories. Investigators wore disposable lab coats when handling PCR products and were not allowed to participate in PCR preparation later the same day.

Statistical analysis

The utility and efficiency values obtained for blastomeres from embryos with different levels of fragmentation were compared using two-sample contingency tests for homogeneity of binomial proportions. Blastomere concordance values were compared using Fisher's exact test.

Results

Designing a real-time PCR assay for the detection of specific chromosomes

TSPY and *U2* were chosen as targets for chromosome-specific multiplex amplification, because both genes are moderately-repeated and each set of repeats is highly-conserved. We reasoned that all copies of each gene could be amplified and detected with a single pair of primers and a molecular beacon specific to that gene. In addition, the repeated nature of each gene would reduce the impact of small variations in initiating amplification and would enhance the chances of detecting amplicons from a single cell without use of nested primers.

Figure 1C illustrates several real-time PCR analyses of the *U2* genes in individual female lymphocytes. Amplicon accumulation is first detected when the fluorescence of the hybridized beacon exceeds a threshold value set at ~10 SD above background, a point called the threshold cycle (C_T) of the reaction. The C_T value reflects both the number of copies of the target gene available at the start of the reaction and the overall rate of PCR gene amplification. Assays with the lowest and least variable C_T values are the most efficient for cell lysis and amplification.

Once a reaction reaches its C_T , it usually continues to accumulate amplicons until it approaches the end of the exponential phase of amplification. We routinely terminate assays for *TSPY* and *U2* at cycle 38, before amplicon accumulation plateaus. At cycle 38, *TSPY* signals from single male lymphocytes, as well as *U2* signals from single female lymphocytes typically reach fluorescent intensities of several hundred units. However, individual reactions exit exponential amplification at different times and a small fraction of reactions slow down dramatically, even though they exhibit C_T values similar to those of other samples (Figure 1C). For this reason

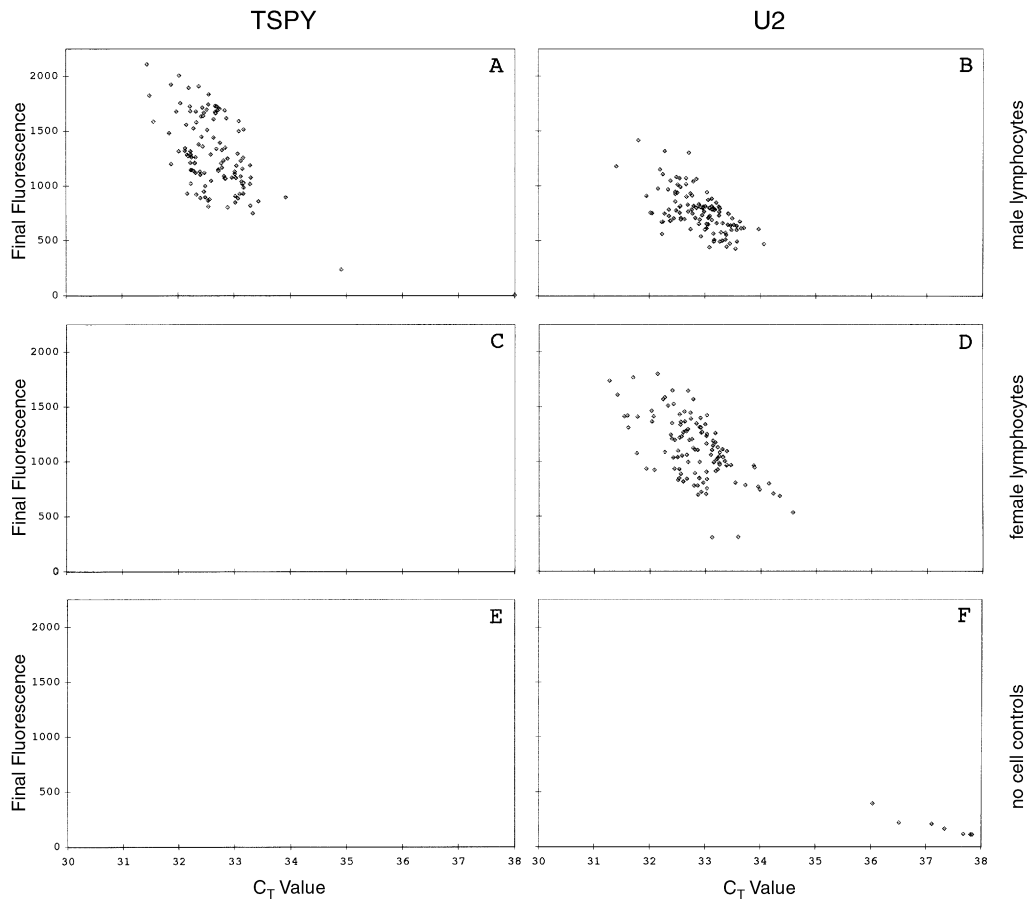


Figure 2. Scatter diagrams of threshold cycle (C_T) and final fluorescence values for an initial series of lymphocyte samples. (A) TSPY signals and (B) U2 signals from male lymphocytes, (C) TSPY signals and (D) U2 signals from female lymphocytes, (E) TSPY signals and (F) U2 signals in no-cell controls.

we employ both the C_T value and the cycle 38 fluorescence intensity (final fluorescence) as measures of the robustness of each reaction.

Optimization of the assay using male and female lymphocytes

Under standardized PCR conditions the initiation of amplification depends on the availability of the target genome. Several methods for lysing single lymphocytes prior to PCR amplification were compared using C_T values as a measure of the relative accessibility of target genes. Lysis Buffer from HTR yielded the lowest mean C_T values with the smallest standard deviation compared to other proteinase K-based lysis methods, alkaline lysis, and freeze-thaw in water (Pierce *et al.*, 1999).

Using Lysis Buffer we analysed 120 reactions prepared with single male lymphocytes and 120 reactions prepared with single female lymphocytes to determine the reliability of the assay. Among the 240 reactions, eight (3.3%) showed no U2 or TSPY signals at all, probably because cells were not successfully transferred into those reaction tubes. Of the 232 reactions that did have signals, all had at least one with a C_T value of <35. A total of 114 of these reactions contained a male lymphocyte and 113 generated a TSPY signal (Figure 2A). The single remaining sample lacked a TSPY signal, but did generate a strong U2 signal. The samples with TSPY

signals also had U2 signals with similar C_T values (Figure 2B), except for one sample which had the lowest TSPY fluorescence intensity and which lacked a U2 signal. As expected, all of the 118 female cell reactions that generated a U2 signal lacked a TSPY signal (Figure 2C, D). The means and SD for the C_T and final fluorescence values for all reactions with signals are presented in Table I.

An additional set of 72 no-cell control reactions was also tested to screen for possible TSPY or U2 contamination within the laboratory. No TSPY signals were observed in any of these reactions, while seven reactions exhibited U2 signals with C_T values >36 and fluorescent intensities far weaker than any of the robust signals observed in reactions containing a lymphocyte (Figure 2E,F).

Despite the high percentages of robust reactions in Figure 2, a close examination of the data suggested that PCR conditions were not yet fully optimized. In particular, comparison of the data in Figures 2B and 2D revealed that the average U2 signal obtained from male lymphocytes had lower final fluorescence intensity than that obtained from female lymphocytes, although the mean C_T values of the two data sets were comparable (Table I). This observation suggested that simultaneous amplification of TSPY in the male samples might partially inhibit U2 amplification during the final few PCR cycles. In contrast, amplification of U2 did not suppress TSPY

Table I. Mean threshold cycle (C_T) and cycle 38 fluorescence for TSPY and U2. Values given as means \pm SD

Cell type	Signal	C_T	Final fluorescence
First experimental series:			
male lymphocytes	TSPY	32.62 \pm 0.49	1294 \pm 331
	U2	32.90 \pm 0.48	768 \pm 191
female lymphocytes	U2	32.83 \pm 0.59	1116 \pm 287
Second experimental series:			
male lymphocytes	TSPY	33.13 \pm 0.52	2684 \pm 445
	U2	33.17 \pm 0.36	1690 \pm 379
female lymphocytes	U2	33.17 \pm 0.45	2137 \pm 442
blastomeres with both signals	TSPY	33.32 \pm 1.62	2571 \pm 1060
	U2	32.34 \pm 1.30	2033 \pm 735
blastomeres with one signal	U2	33.02 \pm 1.71	2390 \pm 1056

The first and second experimental series were carried out using different beacon preparations and under different polymerase chain reaction (PCR) conditions.

amplification, even when a single male cell was tested in the presence of 100 female genomes (data not shown). In order to minimize effects of *TSPY* on *U2* we increased the concentrations of *Taq* polymerase and Tris buffer. In fact, this adjustment increased the final fluorescence intensity of all signals and brought the *U2* fluorescence in male cell samples closer to that in female cell samples (Table I and Figure 3).

Optimized gene detection and gender diagnosis in lymphocytes

In order to establish objective quantitative criteria for diagnosing the presence of the Y chromosome in single human cells, 54 samples of single male lymphocytes and 54 samples of single female lymphocytes were tested under the fully-optimized conditions in parallel with blastomere samples (see below) and the results were plotted in terms of C_T value and final fluorescence (Figure 3A–D). One male lymphocyte sample showed no *TSPY* or *U2* signal, presumably because the cell was not successfully transferred into that reaction tube. The C_T and final fluorescence values for the remaining 107 lymphocyte samples (Table I) were used to define a robust reaction as one that yields a C_T value not >3 SD above the mean and a final fluorescence not less than 3 SD below the mean. Thus, a robust *TSPY* signal has a C_T of <34.7 and final fluorescence of at least 1349 units, and a robust *U2* signal has a C_T of <34.5 and final fluorescence of at least 811 units. These limits are indicated by the dashed lines in Figure 3. All 53 signal-positive male lymphocyte samples yielded both robust *TSPY* and robust *U2* signals. All 54 female lymphocytes yielded robust signals for only *U2*. Two female lymphocyte samples showed low-level fluorescence for *TSPY*, in the final cycles ($C_T > 37$) which was easily distinguished from robust *TSPY* signals in samples of male lymphocytes. None of 16 control samples without a lymphocyte yielded either signal.

Table II summarizes the lymphocyte data from the diagnostic perspective. The term diagnostic utility refers to the percentage of samples that generate any detectable signal. Failure to obtain any detectable signal is most likely due to failure to transfer the cell into the tube, or transfer of a cell with degraded DNA (e.g. due to apoptosis). The diagnostic utility of the lymphocyte tests was 99.1%, since only one of the 108 reactions did not yield a detectable signal. Diagnostic utility

is distinct from diagnostic efficiency, which is the percentage of samples in which the detected signals are strong enough to be scored as robust signals. We reasoned that only samples having robust signals should be used to diagnose gender, since weak or delayed signals could be caused by low levels of a contaminant or by suboptimal PCR. In accord with these standards the two female lymphocyte samples containing weak *TSPY* signals were scored as undiagnosable. Thus, the overall diagnostic efficiency of this assay applied to lymphocytes was 98.1% (105 out of 107). Diagnostic accuracy is the percentage of samples correctly scored for gender based on robust signals. Among the 105 samples that displayed only robust signals, all male lymphocytes scored positive for both *TSPY* and *U2*, while all female lymphocytes scored positive for *U2* only. Therefore, the diagnostic accuracy for this set of lymphocytes was 100%.

Gene detection and gender diagnosis in blastomeres

The above tests using lymphocytes served as a basis of comparison for evaluating the real-time PCR assay applied to single human blastomeres. As discussed in the Introduction, blastomeres differ from lymphocytes in many fundamental aspects that might alter the kinetics of amplification and complicate diagnostic evaluation. Accordingly, 47 non-viable embryos deemed unsuitable for clinical use were analysed in accordance with Institutional Review Board approvals and patient consent. The embryos were scored according to their level of fragmentation and were disaggregated into individual blastomeres which were tested for *TSPY* and *U2* sequences exactly as described for lymphocytes. The resulting C_T and final fluorescence values were used to assess the robustness of the PCR signals based on lymphocyte-generated criteria and to calculate the diagnostic utility, efficiency, and accuracy.

The mean C_T and final fluorescence values for *TSPY* and *U2* signals obtained from blastomeres were similar to those of lymphocytes, although the SD were considerably greater for blastomeres (Table I). The higher variability among blastomere measurements is also apparent in Figure 3. Some of the increased variability was consistent with some blastomeres having completed DNA replication prior to dissection and, therefore, having twice the DNA per cell when compared with a quiescent lymphocyte. Doubling the amount of DNA per

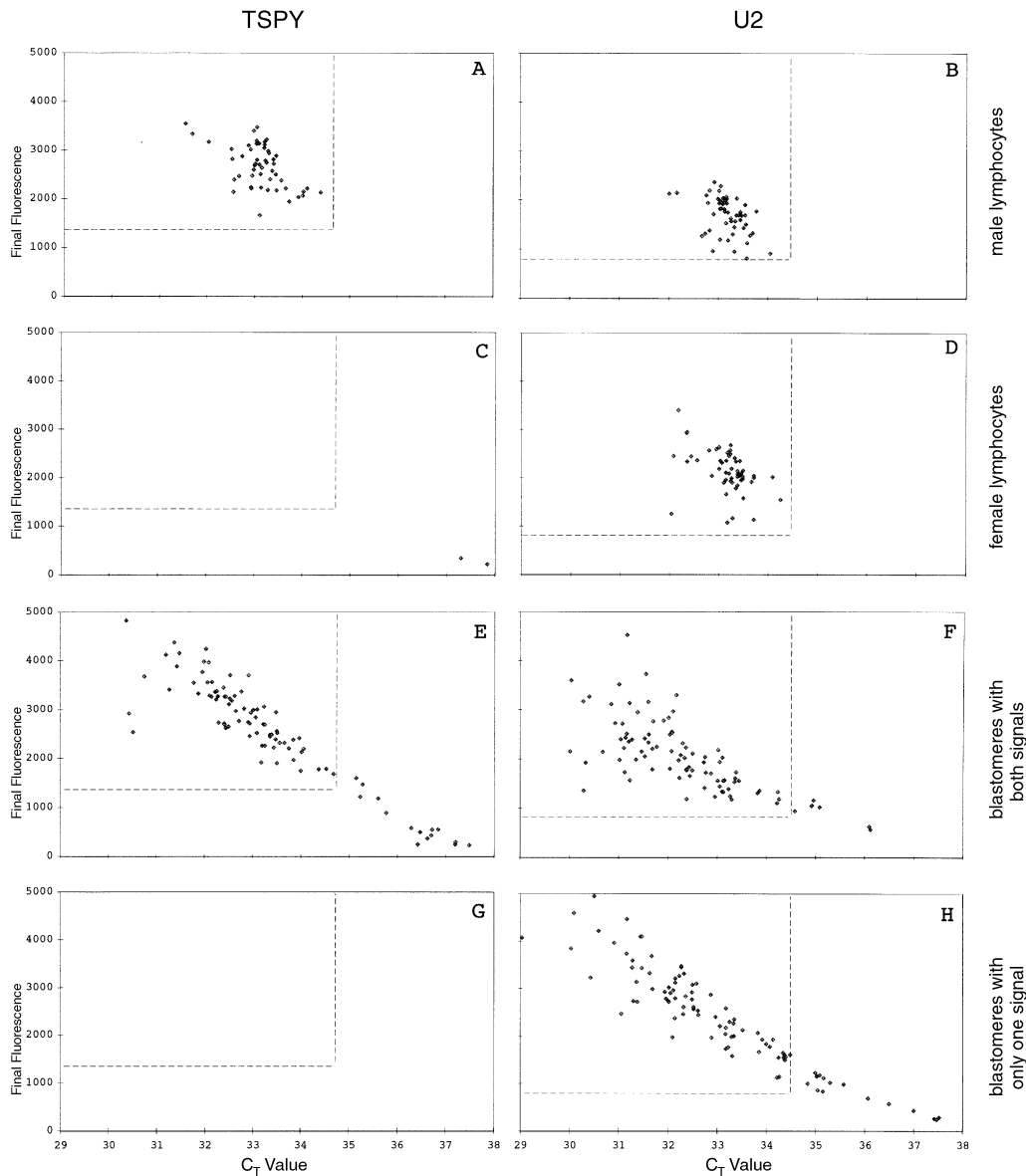


Figure 3. Scatter diagrams of threshold cycle (C_T) and final fluorescence values from blastomeres and control lymphocytes assayed in parallel. (A) TSPY signals and (B) U2 signals from male lymphocytes, (C) TSPY signals and (D) U2 signals from female lymphocytes, (E) TSPY signals and (F) U2 signals from blastomeres generating both signals, (G) TSPY signals and (H) U2 signals from blastomeres generating only one of those signals. None of the blastomeres generated TSPY signals without U2 signals, thus the absence of sample points in (G). All no-cell controls in this series lacked signals and are not depicted. Robust signals used for gender diagnosis are those within the area bounded by the broken lines. PCR conditions and molecular beacon lots differ from those used for samples shown in Figure 2 (see text).

cell is expected to decrease C_T values by about one cycle. Additional causes of increased signal variability for blastomeres are discussed below.

The diagnostic utility for blastomere assays was considerably lower than that of lymphocyte assays. Overall, 185 of 248 (74%) blastomere samples generated at least one signal (Table II). However, when these data are analysed on the basis of embryo fragmentation, signals were detected in 42 of 70 (60%) blastomeres from highly-fragmented embryos, 46 of 62 (74%) blastomeres from embryos with moderate fragmentation, and 97 of 116 (84%) blastomeres from embryos with low-level fragmentation. The difference in diagnostic utility between blastomeres from embryos with low and high fragmentation

was statistically significant ($P < 0.001$), suggesting that embryo quality, rather than experimental technique was a primary cause for amplification failures. Abnormally developing embryos frequently have anucleate blastomeres, which could account for the absence of signals. In an experiment designed to test this hypothesis, blastomeres with an observed nucleus were assayed for TSPY and U2. Signals were detected in 29 of 31 (93.5%) of these blastomeres; this is significantly different ($P < 0.01$) from the overall rate of 156 of 217 (71.9%) in the remaining blastomeres that were not examined for the presence of a nucleus.

The diagnostic efficiency of blastomere assays was also lower than that of lymphocytes, but did not vary significantly

Table II. Evaluation of real-time polymerase chain reaction (PCR) for gender diagnosis

Sample	Total	Any signal	(diagnostic utility) ^a	Robust signal	(diagnostic efficiency) ^a	TSPY+ U2+	TSPY- U2+	(diagnostic accuracy) ^a
All lymphocytes	108	107	(99.1)	105	(98.1)			
male lymphocytes	54	53	(98.1)	53	(100)	53	0	(100)
female lymphocytes	54	54	(100)	52	(96.3)	0	52	(100)
All blastomeres	248	185	(74.3)	155	(83.8)			
low fragmentation (0–15%)	116	97	(83.6) ^b	80	(82.5)	27	53	
moderate fragmentation (20–30%)	62	46	(74.2)	41	(89.1)	30	11	
high fragmentation (35–75%)	70	42	(60.0) ^b	34	(81.0)	19	15	
No-cell controls	47 ^c	0						

^aDiagnostic utility, efficiency, and accuracy are shown as percentages defined in the text.

^bSignificant difference ($P < 0.001$).

^cIncludes 31 controls with blastomere wash buffer and 16 controls prepared in parallel with lymphocyte samples.

Table III. Diagnostic concordance among blastomeres from the same embryo

Fragmentation level	Total no. of embryos ^a	Diagnosed blastomeres	Concordant blastomeres ^b	Diagnostic accuracy (%)
Low (0–15%)	20	78	78	100 ^c
Moderate (20–30%)	9	39	38	97.4
High (35–75%)	11	33	29	87.9 ^c

^aEmbryos with more than one blastomere displaying robust signal(s).

^bNumber of blastomeres with signals consistent with embryo diagnosis.

^cSignificant difference ($P < 0.05$).

with the degree of embryo fragmentation. Overall, 155 of 185 (84%) blastomere samples with signals exhibited only robust signals (Figure 2E–H, Table II). In accord with our strict criteria, diagnosis was not made for samples with non-robust signals.

Diagnostic accuracy of blastomere assays

In contrast to lymphocytes, the diagnostic accuracy of single blastomere assays cannot be determined directly because gender is not known in advance. All blastomeres from the same embryo, however, can be expected to have the same chromosomal composition, unless the embryo is chromosomally mosaic. Thus, in order to establish the diagnostic accuracy of the TSPY/U2 assay for sexing embryos, we examined the concordance of gender diagnosis among multiple blastomeres recovered from single embryos. Diagnostic accuracy, like diagnostic utility, improved with embryo quality (Table III). For embryos with high levels of fragmentation, 29 of 33 blastomeres from 11 embryos generated a diagnosis consistent with that obtained from the other blastomeres of the same embryo. Thus, if each blastomere is viewed as a separate test of an embryo, an accurate diagnosis was obtained in 87.9% of those cases. In contrast, all but one of 39 blastomeres from embryos with moderate fragmentation were concordant with others from the same embryo, yielding a 97.4% diagnostic accuracy. Each of the five embryos with non-concordant blastomeres contained only one cell diagnosed as female, while additional cells were diagnosed as male. These results are consistent with the possibility of non-disjunction and are unlikely to reflect contamination with male DNA. Finally, all 78 blastomeres from embryos with low fragmentation were concordant, yielding a diagnostic accuracy

of 100% for that group. The difference in percentage concordance between the low and high fragmentation groups was statistically significant ($P < 0.01$).

Discussion

We have developed an accurate method for detecting the Y chromosome in single human cells based on optimized lysis and real-time PCR amplification protocols. Real-time technology adds a quantitative dimension to PCR analysis, thereby providing objective criteria on which to base gender diagnoses of blastomeres. It also enables the identification of reactions that are atypical in comparison with optimally prepared single cells. It is logical and prudent to eliminate such samples from clinical evaluation, since they are subject to higher rates of misdiagnosis.

Co-amplification and molecular beacon detection of conserved sequences within the multiple copies of the *TSPY* and *U2* genes resulted in correct identification of gender in all 105 diagnosable reactions from single lymphocytes. Even in a series conducted prior to final optimization of PCR conditions, only a single false-negative (a U2 signal without a TSPY signal) was observed among 114 male lymphocyte samples and no false positives were obtained among 118 female lymphocyte samples. Thus, we estimate that the assay is at least 99.7% accurate when employed to detect the Y chromosome in single lymphocytes.

The accuracy of this real-time PCR assay is higher than other reported methods for gender identification in single cells. Findlay *et al.* (1998) compared different methods and obtained accuracy rates of 97% for PCR with fluorescent primers, 96% for FISH, and 89% for conventional PCR. Other reports based

on conventional PCR for single copy genes vary in their accuracy, primarily because of different rates of allele drop-out (Lissens and Sermon, 1997), a problem circumvented via the use of multi-copy *TSPY* and *U2* genes. The highly conserved nature of the *TSPY* sequences avoids the problems associated with amplifying heterogeneous, highly reiterated α satellite sequences (Kontogianni *et al.*, 1991).

We have introduced the term 'diagnostic utility' to refer to the percentage of cells that produce a detectable signal. The absence of any signal can reflect failure to transfer the cell, absence of a nucleus, or extensive degradation of the DNA within the cell. Our results demonstrate that diagnostic utility for blastomeres is directly correlated with embryo quality. In one experiment employing only blastomeres with a visible nucleus, signals were detected in 94% of the samples. It is likely that the diagnostic utility for blastomeres biopsied from good-quality embryos used in clinical PGD cases will exceed that level.

In order to ensure the highest level of accuracy for the real-time PCR assay, diagnosis was made only for samples displaying robust signals meeting two objective quantitative criteria established with lymphocytes. The percentage of samples achieving these criteria is referred to as the diagnostic efficiency of the assay. Diagnostic efficiency was high for lymphocytes, as almost all samples produced only robust signals. Although the diagnostic efficiency for blastomeres, 84%, was lower than that for lymphocytes, this value may increase when good-quality embryos are used in clinical cases. Moreover, unlike other assays used for PGD, the failure to obtain a diagnosable signal may provide useful information about the quality of the genetic material of that embryo. For example, some embryos had multiple blastomeres that generated only weak signals, a possible indication that these embryos are in early stages of apoptosis or necrosis. Similarly, blastomeres that generated signals with very low C_T values (<31) may be polyploid or aneuploid. Thus, quantitative analysis of the signal in the real-time PCR assay may identify embryos unsuitable for transfer.

Diagnostic concordance among blastomeres from the same embryo demonstrates the accuracy of Y chromosome detection using the real-time PCR assay. All 78 blastomeres from embryos with low-level fragmentation provided diagnoses that were concordant with other blastomeres from the same embryo. The few discordant results among blastomeres from embryos with higher levels of fragmentation were likely due to mitotic non-disjunction. This increase in the percentage of mixed diagnoses from embryos with high fragmentation is consistent with the increased frequency of mosaicism detected in such embryos (Munné and Cohen, 1998). Additional studies are underway to test this conclusion by carrying out FISH and real-time PCR on different blastomeres from the same embryos.

Mosaicism in human embryos is a potential problem for any PGD technique that depends on analysis of only one or two cells. However, even though the present study utilized embryos unsuitable for transfer, only one out of 117 blastomeres (0.9%) did not provide an accurate diagnosis for embryos with up to 30% fragmentation. Potential errors due to mosaicism might be reduced further by using embryos with good morpho-

logy and by testing two cells when feasible. Moreover, a high percentage of mosaic embryos are incompatible with implantation (Munné and Cohen, 1998) and would not result in a pregnancy with a genetically-affected fetus. We conclude that mosaicism will have minimal effect on PGD outcomes when real-time PCR with molecular beacons is used for gender analysis of embryos with good morphology.

The present study is part of our ongoing efforts to develop and implement a new, convenient, and reliable system of PGD. Real-time PCR with molecular beacons will only realize its full potential in PGD when it is applied to simultaneous diagnosis of multiple alleles of one or more disease-causing genes. Such multiplex assays will involve the use of several molecular beacons with different fluorophores designed to distinguish normal and disease-causing alleles. This is possible because even a single nucleotide mismatch between a beacon and its target oligonucleotide can prevent binding and fluorescence (Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998). This high degree of specificity is unmatched by other types of fluorescent probes or primer strategies. Experiments along these lines are underway in our laboratory.

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