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The "Spanning Protocol": A new DNA extraction method for efficient single-cell genetic diagnosis

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***Purpose*:** We evaluated methods of preparation of DNA from single cells for amplification and preimplantation genetic diagnosis (PGD), including our "spanning protocol."
***Methods*:** Dystrophin gene exons 45 and 51 were amplified by nested polymerase chain reaction (PCR) from a single lymphocyte or blastomere. Amplification efficiencies were compared between DNA extraction by (A) lysis in distilled water with freeze-thawing and boiling; (B) two-step lysis involving potassium hydroxide and dithiothreitol; and (C) the spanning protocol, using *N*-lauroylsarcosine.
***Results*:** With method A, amplification efficiency was 66/120 (55%) and false-positive such as amplification failure or allele drop out was 42/120 (35%); with B, 96/120 (80%) and 21/120 (17.5%); and with C, 111/120 (92%) and 5/120 (4.2%), using single blastomeres and unaffected lymphocytes from male. Occurrence of false-negative such as contamination of another DNA with method A was 4/120 (3.3%); with B, 10/120 (8.3%); and with C, 2/120 (1.7%) from using single lymphocytes from affected males.
***Conclusion*:** The spanning protocol was most efficient for extracting DNA from a single cell and should be particularly useful for preimplantation genetic diagnosis.

KEY WORDS: DNA extraction; nested polymerase chain reaction; preimplantation genetic diagnosis.

INTRODUCTION

Some genetic abnormalities now can be detected in preimplantation embryos (1,2). Preimplantation genetic diagnosis (PGD) is a technique for lessening the likelihood of having offspring, affected by genetic disease for couples who are at risk (2). PGD is an alternative to conventional prenatal diagnostic techniques, where a positive test is an indication for termination of an established pregnancy. PGD is an application of the techniques of in vitro fertilization (IVF) and those of molecular biology (1). Improved microfertilization techniques, particularly intracytoplasmic sperm injection (ICSI), have reduced the risk of contamination (1) from spermatozoa trapped in the zona pellucida (3). After ICSI, single blastomeres can be obtained from an embryo at the four- or eight-cell stage (4). Seventeen centers worldwide have reported successful PGD for gender determination or for chromosomal disorders (5). The most frequent form of PGD so far has been gender determination in X-linked recessive disorders (6,7), which number approximately 300 diseases (8). While X-linked diseases are rare overall, couples having a gene defect face a 25% risk of having an affected child (approximately 50% of the boys). On average, one-half daughters will be of normal phenotype but

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will be heterozygous for the gene, the other half will be both phenotypically normal and homozygous for the normal gene. Gender determination can be used for all X-linked diseases but requires discarding all male embryos (1). Identification of sex chromosomes in embryos from patients carrying X-linked genetic diseases (9,10) is only an indirect method for identifying embryos that might be affected (1)

PGD allows couples to greatly reduce the risk of establishing a pregnancy with a fetus involved by a specific genetic disease. However, some centers have reported misdiagnosis of a biopsied cell leading to transfer of affected embryos to the uterus (2,11). These misdiagnoses were the result of contamination with another cells or the result of either amplification failure or failure to amplify both alleles correctly (allele dropout or ADO) (104). With PGD, preclinical sensitivity needs to extend to a single-cell level (6,7). In preparation for clinical applications of PGD, DNA extraction method is very important, so our laboratory analyzed genomic DNA from single lymphocytes and single blastomeres.

Until recently, embryonic cells obtained by biopsy of embryos simply have been lysed in distilled water and subjected to freeze-thawing (8). Since this DNA extraction method was not efficient and culminated in reduced amplication efficiency, another technique of two-step lysis protocol was developed while this DNA extraction method improved amplication efficiency, this method needed many exact manipulations, resulting in increased rate of false-negatives.

Consequently, we developed a new DNA extraction method with *N*-lauroylsarcosine salt solution that was one of a detergent, and it was named the "Spanning protocol." This DNA extraction method is efficient and does not need so many exact manipulations, culminating in decreased rate of false-negatives. Diagnostic results with single blastomeres and lymphocytes show that our spanning protocol decreases not only the incidence of amplification failure but also the rate of false-negatives. Although failures of amplification from blastomeres have not been eliminated, the spanning protocol improved the amplification efficiency and resulted in more consistent amplifications. This cell-lysis method, therefore, also is believed to provide consistent amplification of other unique or repeating sequences for preimplantation diagnosis from single cells.

MATERIALS AND METHODS

Embryos and Lymphocytes Source

This study was conducted on 30 couples on whom were performed intracytoplasmic sperm injection to ensure high fertilization rates because of male infertility with oligospermia ($<1 \times 106\ \mathrm{ml}^{-1}$), 20 normal males, and one patient of Duchenne muscular dystrophy (DMD). All embryos and all lymphocytes used in this study were obtained from volunteers after informed consent. All embryos in this study were 'spare' embryos that were dispensable for these couples, because they already had been pregnant. Only monospermic embryos, embryos developing from dipronucleated zygotes, were used for this study to avoid the attachment of spermatozoa to the zona pellucida, which would interfere with the genetic analysis after embryo biopsy. These embryos were obtained from patients undergoing IVF treatment for infertility at Keio University Hospital.

Lymphocytes were obtained from 20 normal males and one patient with DMD after informed consent. Patients found to have deletions from exon 44 to 47 have been followed several years in the Keio University Hospital and have been diagnosed as DMD on the basis of grossly raised serum creatine kinase activity, pseudohypertrophy of the calf muscles, electromyographic abnormalities characteristic of myopathy, and muscle biopsy findings consistent with muscular dystrophy.

The research procedure was approved by the Research Ethics Committee of Keio University School of Medicine, and was in accordance with the Guidelines of the Japan Society of Obstetrics and Gynecology.

IVF Protocols

After pituitary-gonadal suppression with a gonadotropin releasing hormone agonist (Busereline; Hoechst, London, UK), patients were superovulated with human menopausal gonadotrophin (HMG; Pergonal, Serono Laboratories, Welwyn Garden City, UK). A dose of 10,000 IU human chorionic gonadotrophin (HCG; Pregnyl, Organon Laboratories, Cambridge, UK) was given 34 h before egg collection. Oocytes were collected and preincubated for 6 h and ICSI was performed to ensure high fertilization rates in these patients (day 0). ICSI was performed with an inverted microscope (Nikon Corporation, Tokyo) at 400× magnification using the Hoffman Modulation Contrast System

(Modulation Optics Inc., Greenvale, NY) equipped with two coarse positioning manipulators and two three-dimensional hydraulic remote-control micromanipulators (Narishige, Tokyo). The microinjection was performed as previously described. Fifteen hours later, the oocytes were checked for the development of two pronuclei, to confirm normal fertilization. Embryos were then cultured under a gas phase of 5% CO_2, 5% O_2, and 90% N_2. On day 2, each fertilized embryo was examined and two or three embryos were selected on the basis of their morphology for embryo transfer, on day 2 or 3. The surplus embryos were freezed and conserved.

Cell Collection

Blastomere. We used cryopreserved embryos in the present study. After obtaining informed consent of patients, the surplus embryos were allowed to study. These embryos were biopsied at a 4–8 stage following ICSI, and one blastomere was analyzed by nested polymerase chain reaction (PCR) with multiple primers. We made an opening in the zona pellucida using partial zona dissection (PZD). The same micropipette then was rinsed and filled with medium for injection deep to the zona to force one cell through the dissection opening. Blastomeres can be displaced though the opening by flow of medium from a micropipette inserted though the zona pellucida (Fig. 1). After careful examination for interphase nuclei by light microscopy (9), each blastomere was washed three times in PCR buffer (Sigma, St. Louis, MO, made without the calcium supplement) or HEPES-buffered medium supplemented with bovine serum albumin (BSA; Sigma, St. Louis, MO) and prepared for DNA amplification. Embryo gender had been determined independently by biopsy and dual fluorecescence in situ hybridization (FISH) with X- and Y-specific probes (12–14).

Lymphocyte. Lymphocytes also were isolated under a dissecting microscope, and then serially rinsed through three micropipetted drops of PCR buffer to remove any extraneous cells. All manipulations were carried out on a microscope stage heated to maintain the temperature at 37°C. Pipette movements were controlled using Narashige hydraulic micromanipulators. The work was carried out in a clean area located in the IVF room, specifically allocated to single-cell DNA analysis. Samples containing more than 10 pg of DNA were never brought into this area. Surgical caps and masks were worn, and surgical gloves were changed frequently to avoid contamination. Each cell then was placed in a PCR reaction tube (Gene-Amp, Perkin-Elmer, Norwalk, CT) and 180 samples were evaluated after DNA preparation by each of the following methods.

Preparation of DNA from Single Cell

Liquid Nitrogen Method (Freeze–Thraw Lysis Protocol). Cells were placed in 10 μl of highly purified sterile water, frozen and thawed twice using liquid nitrogen, and then boiled at 100°C for 10 min.

Potassium Hydroxide Method (Two-Step Lysis Protocol). Cells were placed in 5 μl of a solution of 200 mM potassium hydroxide and 50 mM dithiothreitol, centrifuged, and heated at 65°C for 10 min. Then, 5 μl of a neutralizing solution (900 mM

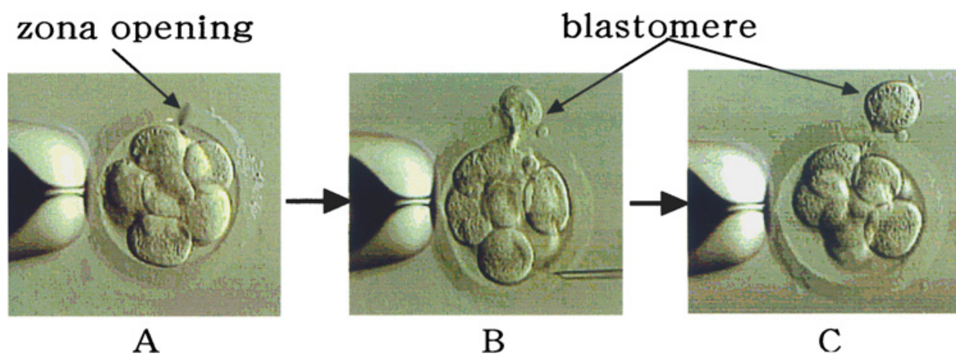

Fig. 1. Embryo biopsy procedure. Late on day 3, we made an opening in the zona pellucida using partial zona dissection. The same micropipette was rinsed and filled with medium for injection deep to the zona to force one cell through an opening. Blastomeres thus were displaced though the zona opening by flow of medium from the micropipette. (A) An embryo is immobilized on a holding pipette, with a hole being made by a beveled micropipette. (B) Beginning of partial removal of a single blastomere. (C) Completion of removal of the blastomere from the embryo.

Table I. Composition of Solutions for the Spanning Protocol

Lysis buffer	
H_2O	2 μl
250 ng/μl Polyadenylic acid	2 μl
10 mM EDTA	2 μl
250 mM 1-Dithiothreitol solution	2 μl
0.5% *N*-lauroylsarcosine salt solution	2 μl
Reaction mixture	
PCR buffer	10 μl
2.5 mM dNTP	8 μl
Primer for exon 45 (foward)	5 μl
Primer for exon 45 (reverse)	5 μl
Primer for exon 51 (foward)	5 μl
Primer for exon 51 (reverse)	5 μl
H_2O	43.5 μl
Taq polymerase	0.5 μl

Note. Firstly, a single lymphocyte or a single blastomere was placed in PCR tubes containing 10 μl of DNA lysis buffer. Secondly, 8 μl of $MgCl_2$ was added to 10 μl of lysis buffer containing a single cell. Then, 82 μl of reaction mixture was added. PCRs were performed in 100 μl of reaction buffer containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.0 mM Mg^{2+}, 200 μM each of dNTP and 1.25 μ Taq polymerase. Two consecutive PCRs with nested primers were developed to detect sequences in exons 45 and 51 of the dystrophin gene. For each primer 25 pmol was used.

Tris–HCl at pH 8.3; 300 mM potassium chloride; 200 mM HCl) was added (12).

***N*-Lauroylsarcosine Salt Solution Method (Spanning Protocol).** First, single lymphocytes or single blastomere removed by micromanipulation was placed in a PCR tube containing 10 μl of DNA lysis buffer composed of H_2O (2 μl), 250 ng/μl polyadenylic acid (2 μl), 10 mM EDTA (2 μl), 250 mM dithiothreitol solution (2 μl), and 0.5% *N*-lauroylsarcosine salt solution (2 μl). Each lymphocyte and blastomere had its own blank, which was taken from the droplet used for washing the lymphocyte or blastomere. PCR tubes containing lymphocytes, blastomeres, or blanks were stored at −20°C until the PCR assays (Table I).

DNA Analysis by Nested PCR. First, 8 μl of 25 mM-$MgCl_2$ was added to 10 μl of sample containing a single lymphocyte, a single blastomere, genomic DNA, or blank at 0°C. Then, 82 μl of reaction mixture was added. PCRs were performed in 100 μl of reaction buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM Mg^{2+}, 200 μM of each dNTP, and 1.25 μ Taq polymerase (Perkin Elmer Cetus, Norwalk) (Table I). Two consecutive PCRs with nested primers were developed to detect sequences in exons 45 and 51 of the dystrophin gene. Twenty-five picomoles of each primer were used (Table II).

The primers were designed from a published exon 45, 51, and intron sequence (Chamberlain *et al.*, 1988; EMBL/Genebank) and amplified the entire exon. Oligonucleotides were synthesized on an Applied Biosystem DNA Synthesizer (Applied Biosystem, ABI, Warrington). Full-length oligomers were purified on 20% denaturing polyacrylamide gels, eluted in 3 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, ethanol precipitated and washed extensively with 70% ethanol to remove excess NaCl. Primers were resuspended in water at 0.4 μg/μl and stored at −20°C.

Table II. Primer Sequences

Exon 45		
Inner	F	5′-GACATGGGGCTTCATTTTTG-3′
	R	5′-GATCTGTCGCCCTACCTCTT-3′
Outer	F	5′-GTACAACTGCATGTGGTAGC-3′
	R	5′-CATTCCTATTAGATCTGTCG-3′
Exon 51		
Inner	F	5′-GACTCTTTAGCTTGTGTTTC-3′
	R	5′-CCACCAATCACTTTACTCTC-3′
Outer	F	5′-GCATGAGAATGAGCAAAATCG-3′
	R	5′-CTAGACCATTTCCCACCAGT-3′

Note. F, forward; R, reverse.

Following 5 min of incubation at 94°C, 30 cycles were carried out with denaturing for 30 s at 94°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C, with a final extension step of 10 min at 72°C in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk). For the second PCR, 99 μl of the reaction buffer described above and 1 μl of the first PCR product were mixed. Thirty cycles of 30 s at 94°C, 1 min at 60°C, and 2 min at 72°C were followed by a final extension step of 10 min at 72°C. The temperature cycling conditions were modified from those used previously (6) by increasing the annealing temperature to 60°C to improve the specificity. Two picograms of DNA that was extracted from lymphocytes of normal male and diluted or sterile distilled water was used as controls for each experiment. After these two rounds of amplification, 20 μl of each amplification product was analyzed by 2.0% agarose gel electrophoresis in Tris–EDTA buffer at 200 mV for 30 min in a Hoefer minigel apparatus (Amerham Biosciences, Tokyo). Ethidium bromide (Amerham Biosciences, Tokyo) was incorporated directly into the gel for visualization of amplification procucts using ultraviolet light at 312 nm. This assay that co-amplifies exon 45 and exon 51 sequences of the dystrophin gene, yields a 380-bp band in patient and two bands of 154 and 380 bp in normal male. If some non-specific bands were expressed in the gel, we defined it "unclarified." The work was carried out in a clean area specifically allocated to single-cell DNA analysis.

Direct Sequencing of the Amplified Products. Each PCR product was loaded on a 6% polyacrylamide sequencing gel and electrophoresed over a distance of 24 cm at 30 W for 5 h, and 154- and 380-bp fragments were purified and directly sequenced by the ABI Prism Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Data were collected and analyzed using GENESCAN software (Applied Biosystem, ABI, Warrington). All the analysis from DNA extraction to DNA sequense was completed in less than 11 h.

Data Analysis

Data on efficiency, false-positive rate and false-negative rate between the different methods of DNA preparation were analyzed by means of the Chi-square test using Yate's correction for continuity, two-tailed at 5% significance.

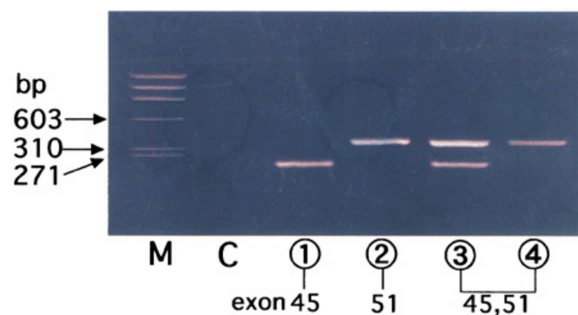

Fig. 2. Multiplex polymerase chain reaction. Amplification products are from unaffected lymphocytes, affected lymphocytes, and appropriate controls. DNA sequences unique to the dystrophin gene were amplified simuitaneously from single cells, and amplification products were applied to an agarose gel for electrophoresis. The gel then was stained with ethidium bromide and exposed to ultraviolet light. Lane M is a size marker; lane C, a water control; lane 1, DNA amplified from an unaffected single male lymphocyte (single-nested PCR for exon 45); lane 2, an unaffected single male lymphocyte (single-nested PCR for exon 51); lane 3, an unaffected single male lymphocyte (multiplex-nested PCR for exons 45 and 51); and lane 4, an affected single male lymphocyte (multiplex-nested PCR for exons 45 and 51).

RESULTS

Multiplex-Nested PCR

We developed a multiplex-nested PCR assay from a single cell. In a PCR with a single set of primers, it is difficult to determine whether the result is right or misdiagnosis, if the band is not expressed. The failure of amplification or the occurrence of sampling error will diagnose an unaffected as an affected. To avoid such misdiagnosis, we designed exon 45 and exon 51 multiplex primer pairs that can work well together in one reaction tube. In addition, we have developed a multiplex-nested PCR assay, in which sequences specific to both the exons 45 and 51 are simultaneously amplified for accurate deletion diagnosis of single cells.

A total of 180 blastomeres disaggregated from 30 normal-appearing human embryos, 180 lymphocytes from 20 normal males, and 180 lymphocytes from one patient of DMD were prepared for PCR amplification at exons 45 and 51 sequences of the dystrophin gene. More than 60 blastomeres were analyzed for each experiment. A representative gel is shown in Fig. 2. Lanes 1 and 2 are the result of single-nested PCR for exons 45 and 51 with unaffected single male lymphocyte. We confirmed each a single band at 260 and 350 bp. Lane 3 is the result of multiplex-nested PCR for exon 45 and 51 with unaffected single male lymphocyte, and lane 4 is the result of multiplex-nested PCR for exons 45 and 51 with affected single male lymphocyte. Electrophoresis of the amplified products after 30 cycles of multiplex-nested PCR showed that normal male-derived amplified fragments were 260 and 350 bp, and patient-derived amplified fragments with deletions from exons 44 to 47 was 350 bp. Thus, a sample with two bands came from a normal male, whereas a sample with a single band came from patient with DMD.

Efficiency and False-Positive With Unaffected Single Cell

Results of amplification using single unaffected male blastomeres, single unaffected male lymphocytes, unaffected male DNA at a dilution equivalent to the amount in a diploid cell (2 pg), and control blanks are presented in Table I.

Overall, PCR amplification of exon 45 was detected in 140 of 180 unaffected male blastomeres (78%) including 36 of 60 following the freeze–thaw lysis protocol (60%), 48 of 60 following the two-step lysis protocol (80%), and 56 of 60 following the spanning protocol (93%). Amplification of exon 45 was detected in 133 of 180 unaffected single lymphocytes from males (74%), 30 of 60 following the standard lysis protocol (50%), 48 of 60 following the

two-step lysis protocol (80%), and 55 of 60 following the spanning protocol (92%). In medium-containing blanks amplification was observed in 5 of 180 samples in the freeze–thraw lysis protocol (2.8%), 15 of 180 in the two-step lysis protocol (8.3%), and 4 of 180 in the spanning protocol (2.2%). Of 360 samples, 19 (5.3%) could not be analyzed because of non-specific amplification.

There were no significant differences in efficiencies between the three methods with unaffected male DNA (NS) at a dilution equivalent to the amount in a diploid cell (2 pg). However, there were significant differences in efficiencies between the freeze–thaw lysis protocol and the spanning protocol with a single blastomere ($p < 0.001$) and a single lymphocyte ($p < 0.005$). In addition, there were significant differences in efficiencies between the two-step lysis protocol, and the spanning protocol with a single blastomere ($p < 0.05$) and a single lymphocyte ($p < 0.05$), implying that DNA extraction was the most important in amplification of DNA from a single cell.

Efficiency and False-Negative with Affected Single Cell

Results of amplification using single affected male lymphocytes, affected male DNA at a dilution equivalent to the amount in a diploid cell (2 pg), and control blanks are presented in Table II. The efficiency of the DNA amplification from single affected lymphocytes was 56/60 (93%) and false-negative was 2/60 (3.3%) following the standard freeze–thaw lysis protocol, 53/60 (88%) and 6/60 (10%) following the two-step lysis protocol, and 57/60 (95%) and 1/60 (1.7%) following the spanning protocol. There were no significant differences in efficiencies between the three methods with single affected lymphocytes. Although there were no significant differences in false-negative rate between the three methods, the spanning protocol had a tendency to decrease false-negative rate compared with the two-step lysis protocol.

Direct Sequence

To verify the specificity of each PCR product, we directly sequenced the 260- and 350-bp fragments and compared their sequences with those previously reported. We ascertained that the 260-bp fragment was derived from exon 45 and the 350-bp fragment was derived from exon 51 (data not shown).

DISCUSSION

Sample preparation is critical for DNA analysis, particularly with cleavage-stage blastomeres (4). We have observed that some of these cells are remarkably resistant to lysis in distilled water. Furthermore, boiling prior to PCR to inactivate endogenous proteinases and nucleases also fixes the DNA in situ (3), rendering it inaccessible to the primers and thus preventing success in the critical first round of amplification (1,14). This may partly explain the exceptionally low amplification efficiency when blastomeres were simply freeze-thawed in medium. After lysis in distilled water and freeze-thawing (2), weak amplification of exon 45 was achieved from male blastomeres (60%) with double rounds of PCR for 30 cycles. Nevertheless, amplification failed with 18 of 60 male blastomeres. Use of a two-step lysis protocol involving an initial incubation in potassium hydroxide and dithiothreitol followed by a neutralization step significantly improves amplification efficiencies for a number of unique sequences from single amniotic cells (3), which otherwise are notoriously resistant to amplification because of their tightly condensed nuclei and other physical characteristics. With blastomeres, efficiency of amplification of exon 45 was improved, but amplification failure occurred with 11 of 60 single lymphocytes from males and 10 of 60 blastomeres. Use of the spanning protocol significantly improved amplification efficiency with single blastomeres and single lymphocytes, compared with the conventional standard freeze–thaw lysis protocol. In addition, amplification failure was eliminated in the spanning protocol.

Possible reasons for amplification failure from single cells are varied (9,12). These include problems with sample preparation such as failure to transfer the cell (10,15), inefficient cell lysis (3), inadequate separation of DNA from associated proteins in chromatin (14), and degradation or loss of the target sequence (8,9). Subsequently, there are other problems associated with PCR, such as flawed oligonucleotide primer design (16). In addition, in blastomeres isolated from human cleavage-stage embryos, abnormal nuclei are relatively prevalent (4). Sex chromosome mosaicism also has been reported (14). In this study, transfer of each blastomere into distilled water or lysis buffer was confirmed by direct observation. In the PCR with single primer, an unaffected sample is diagnosed as an affected sample when the failure of amplification or the sampling error would have occured. To avoid such misdiagnosis, we designed exon 45

and exon 51 multiplex primer pairs that could work well together in one reaction tube, and developed a multiplex-nested PCR assay, in which sequences specific to both the exons 45 and 51 were simultaneously amplified for accurate deletion diagnosis of single cells. Our method could reduce misdiagnosis by using multi-primers. The low efficiency of DNA extraction probably leads to the cause of low diagnostic efficiency. Optimal extraction of DNA has important implications for PGD. Thus, the development of the spanning protocol represents a significant step forward.

The melting of cell membrane, diffusion of DNA, and effective binding of primers to DNA are important processes to amplify DNA from a single cell with PCR. The freeze–thraw lysis protocol and the two-step lysis protocol have an ability to melt cell membrane, but do not have an ability to diffuse DNA, while the spanning protocol has both the abilities. We evaluated methods of preparing DNA from a single cell or amplification and preimplantation genetic diagnosis, including our "spanning protocol" using *N*-lauroylsarcosine, that was one of a detergent. Generally detergents inhibit the PCR reaction, whereas low concentrations of *N*-lauroylsarcosine do not inhibit the PCR reaction. There was no significant difference in efficiencies between the three methods with unaffected male DNA at a dilution equivalent to the amount in a diploid cell (2 pg).

The amplification efficiency was greater in the spanning protocol compared with the freeze–thraw lysis protocol and two-step lysis protocol implying that DNA extraction was the most important in amplification of DNA from a single cell. Furthermore, the spanning protocol had a tendency to decrease false-negative rate compared with the two-step lysis protocol. In consequence, the spanning protocol was more effective for extracting DNA from a single cell than the freeze–thraw lysis protocol and the two-step lysis protocol.

Amplification of contaminating DNA cause serious errors when attempting specific diagnosis of single-gene defects (1,10). Therefore, both stringent precautions against contamination and use of control blanks are essential to prevent misdiagnosis (3,4). Use of the spanning protocol not only reduced amplification failures for exon 45 of the dystrophin to 5%, but also reduced the false-negative rate to 1.7%. However amplification from at least two blastomeres is desirable to reduce the risk of misdiagnosis even further (11,17). Two blastomeres can be removed from a cleavage-stage embryo without adversely affecting development to the blastcyst stage (16).

Rather than causing misdiagnosis, amplification failures simply would preclude analysis for the particular embryo. Nevertheless, high efficiency remains important to correctly assess as many biopsied embryos as possible. Pregnancy rates following preimplantation diagnosis are significantly higher if two unaffected embryos rather than one are transferred (5). Use of the spanning protocol with cleavage-stage blastomeres has given consistently good amplification efficiencies and improved diagnostic accuracy.

In conclusion, multiplex-nested PCR is effective for reducing misdiagnosis with a single cell. Furthermore, the use of spanning protcol that has both ability to melt cell membrane and to diffuse DNA, not only reduced amplification failure, but also reduced the false-negative rate. The spanning protocol is likely to improve efficiency of amplification of unique sequences for specific diagnosis of single-gene defects.

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