Preimplantation Genetic Diagnosis Using Fluorescent Polymerase Chain Reaction: Results and Future Developments

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Purpose: Fluorescent polymerase chain reaction (PCR) is a multipurpose technique that can be used for diagnosing sex, single-gene defects, and trisomies as well as determining DNA fingerprints from single cells. However, its effectiveness must be assessed before clinical preimplantation genetic diagnosis (PGD) application.

Methods: Single and multiplex fluorescent PCR was applied to single cells and blastomeres.

Results: Fluorescent PCR can be used to diagnose sex from blastomeres and has been successfully applied in a clinical PGD sexing program resulting in a confirmed pregnancy. A further major advantage of fluorescent PCR is the ability to multiplex, providing multiple diagnoses and DNA fingerprints with a high reliability (~75% for trisomy, 86% for DNA fingerprint) and good accuracy (70–80%). Allele dropout in multiplex PCR is ~20% per allele and does not appear to be associated with the fragment size.

Conclusions: Fluorescent PCR is a powerful technique for PGD, and the effects of allele dropout must be considered, particularly in multiplex PCR.

KEY WORDS: single-cell PCR; fluorescent PCR; preimplantation genetic diagnosis; sexing.

INTRODUCTION

Although several techniques have been used for single-cell genetic diagnoses, particularly preimplantation genetic diagnosis (PGD), polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) have been used most often. In general, FISH is used for single-cell sexing and detecting trisomy status, whereas PCR is used to detect a wide range of disorders, particularly single-gene defects.

There are three main reasons why PCR has generally been of limited value in analyzing small numbers of cells:

1. Low reliability (or high amplification failure) due to poor sensitivity of many PCRs (particularly multiplex reactions). This results either in such a small amount of signal that it cannot normally be detected or in total failure of amplification.
2. Low accuracy (and thus misdiagnosis) due to selective amplification failure (allelic dropout) of one or more signals.
3. Misdiagnosis due to a failure to detect contamination.

The reasons for the relatively poor reliability of single cells are unclear and are likely to be numerous. They may include problems with sample preparation, e.g., failure to transfer the cell, degradation or loss of the target sequence, and/or problems associated with the PCR.

Perhaps a more serious clinical problem in single-cell genetic diagnosis is allele dropout in heterozygous individuals. This is where one allele may fail to amplify while the other allele amplifies successfully (1). Allele dropout can result in misdiagnosis and is the most probable reason for at least three reported cases of misdiagnosis in clinical PGD programs. Allele dropout is perhaps the most important problem in single-cell PCR analysis and has been reviewed previously (2–5).

One method that could be used to minimize the effects of allele dropout is to include several different primer sets within the same PCR (multiplex PCR). Multiplex PCR contains several different primers so that each will amplify independently and sufficiently allowing simultaneous diagnoses to be performed. This can facilitate the diagnosis either of a specific genetic disease or of multiple diseases since it provides information for multiple different loci at the same time. One example of multiplex PCR in a non-single-cell

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PCR is the amplification of nine regions of the Duchenne muscular dystrophy (DMD) gene in a single reaction (6), resulting in a single assay that can detect 81% of all the DMD deletions (7).

Unfortunately, multiplex PCR is a very sophisticated technique that requires very exacting reaction conditions to allow each probe to amplify equally, particularly as the number of primers within the PCR increases. Careful design of the primers and PCR conditions is absolutely critical so that non-specific amplification and spurious alleles are avoided. The primers must also generate different-sized products to facilitate analysis by avoiding overlap. However even in multiplex PCR, allele dropout is well known; for example, even in large numbers of cells (~100 cells), allele dropout occurs in more than 20% of samples (8). At levels below 500 ng, reliability was much reduced, to less than 50%, due to frequent allele dropout and the intensity of the signals being too low to interpret correctly (8,9).

It is only in recent years that PCR modifications, such as fluorescent PCR and Genescan analysis, have allowed multiplex PCR of three or more primer sets at the single-cell level (10–20). This is potentially a very powerful technique, allowing as many as nine chromosomes to be investigated simultaneously (12) or providing confirmation of diagnosis (16,21,22).

Although fluorescent PCR techniques have been shown to be highly reliable and accurate in single-cell genetic diagnoses (2), including single blastomeres for cystic fibrosis (CF) diagnosis (13), and compare very well with alternative techniques (18), they have yet to be applied to clinical PGD.

Fluorescent PCR was therefore investigated to evaluate its potential for application to PGD in two ways.

1. Sexing of single blastomeres. Reliability and allele dropout rates were investigated. These techniques were then applied to a clinical PGD program.

2. Multiplex PCR in single cells. Reliability and allele dropout were investigated in two multiplex PCRs. The first multiplex (multiple trisomy) contained six primer sets to diagnose sex and trisomies 21, 18, and 13 and allow confirmation of trisomies 18 and 13. The second multiplex (DNA fingerprint) contained seven primer sets, one to diagnose sex and the other six to determine a DNA fingerprint of the single cell. Reliability and allele dropout rates were investigated, as well as relationships between reliability, accuracy, and allele dropout and locus size.

MATERIALS AND METHODS

It is difficult to design experiments to test parameters for amplification failure and allele dropout since very large numbers of cells are required to achieve statistical validity, particularly when high reliability or low allele dropout rates are observed. For example, more than 4000 samples ($\alpha=5\%$, $\beta=20\%$) are required to compare two methods which have allele dropout rates of 5% and 8%. Multiplex PCR is even more difficult, as experiments must also be performed on different primer sets and at different primer concentrations since allele dropout may be affected by the size of the allele.

Three experiments on human embryos were performed.

1. Blastomeres were disaggregated from grade 2 and 3 embryos and sexed using fluorescent PCR and the amelogenin primers. The purpose of this experiment was to determine the reliability and accuracy of blastomere sexing using fluorescent PCR. This was done by comparing the results with cells from the same embryo, which should be identical. The majority result was deemed the true result.

2. Blastomeres were biopsied from grade 1 and 2 human embryos. The blastomeres were immediately analyzed and the biopsied embryo was put back into culture to develop further. The following day the zona pellucida was removed from the embryo and the embryo disaggregated. The object of this experiment was, first, to determine if the embryos survived biopsy and, second, to add to the results from the previous experiment.

3. A further study using 101 embryos was undertaken to investigate whether blastomere biopsy might be detrimental to embryo development. Rather than disaggregating the biopsied embryo immediately after biopsy, the embryo was left in culture for 4 days, allowing it to develop further.

Two experiments using multiplex PCR were performed.

1. Blastomeres were analyzed using the multiplex DNA fingerprint PCR. The object of this experiment was to determine the reliability of the multiplex PCR as a whole, as well as assessing the reliability and allele dropout of individual alleles within a single PCR. This was done by comparing the results with blastomeres from the same embryo, which should provide identical results. This experiment also allowed blastomere variation such as aneuploidy within the embryo to be investigated.

2. Buccal cells were analyzed using the multiplex multiple trisomy PCR. Again, the object of this experiment was to determine the reliability of the multiplex PCR as a whole, as well as assessing the reliability and allele dropout of individual alleles within a single PCR. This was done by comparing the results with cells from the same individual, which should provide identical results.

Cell Preparation

Buccal Cells. Buccal cells for the multiplex PCR were collected from a variety of individuals and were collected, processed, and isolated as described previously (10). In brief, samples were centrifuged (at 7.5g) for 5 min to obtain a cell pellet, then washed three times in phosphate-buffered saline (PBS). Using inverted microscopes (a Nikon Diaphot with Hoffman optics or a Leica DMIL with phase and relief contrast optics), cells were then aspirated into a thinly pulled, autoclaved, glass 230-mm pipette. Cells were washed several times through 1- to 2- μ l PBS microdrops to ensure that only a single cell was in the pipette and were immediately transferred in approximately 1–2 μ l PBS to a sterile, irradiated 0.2-ml Eppendorf tube on ice, and frozen at -20°C until use.

Blastomeres. Human blastomeres were isolated either by disaggregation or by embryo biopsy from embryo surplus to in vitro fertilization requirements. This work was approved by the Local Ethics Committee of the United Leeds Teaching Hospitals Trust and the Human Fertilization and Embryology Authority (HFEA).

PCR

Sexing in Blastomeres. PCRs were undertaken using amelogenin primers following the protocols described previously (2). DNA analysis was conducted in a fashion identical to that reported previously (19), except that samples were not coloaded and ROX 350 was used as the internal standard.

Multiplex PCR. Six (multiple trisomies) or seven (DNA fingerprint) primer sets were multiplexed together in a single PCR to maximize the information obtained from a single PCR. This maximizes the number of diagnoses that can be obtained from a single cell, i.e., up to eight different chromosomes, as well as investigating possible effects of primer and reagent competition.

The seven primer sets used for DNA fingerprinting single cells have been described previously (14). The

Table I. Sexing Disaggregated Blastomeres^a

Blastomeres	Reliability	Accuracy (within-embryo)
Male	88% (49/56)	100% (49/49)
Female	90% (38/42)	95% (36/38)

^a Reliability and accuracy of sexing disaggregated blastomeres using fluorescent PCR; includes some previously published data (13). Both reliability and accuracy are high. Two female blastomeres were misdiagnosed as male, probably as a result of male contamination. Allele dropout was not observed.

primers used were amelogenin (23) for sex determination, HUMVWFA31/A (VWA) (24), HUMTHO1 (THO) (24), D21S11 (D21) (24,25), HUMFIBRA (FGA) (26), D18S51 (D18) (27), and D8S1179 (28).

The six primer sets used for diagnosing multiple trisomies in single cells have been described previously (17,22). The primers used were amelogenin (23) for sex determination, D21S11 (D21) (24,25), D18S51 (D18) (27), D13S631 (20), D18S851, and D13S258.

DNA analysis for all multiplex PCR was conducted in a fashion identical to that reported previously (19), except that samples were not coloaded and TAMRA 350 was used as the internal standard.

RESULTS

In all cases, reliability was defined as the number of times an informative signal was obtained, and accuracy as the number of times the signal matched those from other blastomeres from the same embryo or known samples.

Sexing in Blastomeres

Blastomeres from both disaggregated embryos and biopsied embryos were analyzed. Results from disaggregated embryos are shown in Table I, and those from biopsied embryos in Table II. Reliability and accuracy in both groups are high, although both reliability and

Table II. Sexing Biopsied Blastomeres^a

Blastomeres	Reliability	Accuracy (within-embryo)
Male	100% (55/55)	100% (55/55)
Female	100% (48/48)	100% (48/48)

^a Reliability and accuracy of sexing biopsied blastomeres using fluorescent PCR. Reliability and accuracy are high. Allele dropout was, again, not observed.

Table III. Summary of the Reliability and Accuracy of Sexing Human Blastomeres^a

Sex	Reliability	Accuracy
Male	94% (104/111) (49/56 disaggregated + 55/55 biopsy data)	100% (104/104) (49/49 disaggregated data + 55/55 biopsy data)
Female	96% (86/90) (38/42 disaggregated + 48/48 biopsy data)	98% (84/86) (36/38 disaggregated data + 48/48 biopsy data)
Total	95% (190/201)	99% (188/190)

^a Reliability and accuracy of sexing blastomeres using fluorescent PCR. Reliability and accuracy are high. Allele dropout was not observed.

accuracy are higher in the biopsied group. The relatively low reliability and accuracy in disaggregated cells may reflect the fact that these embryos were usually of poorer grade than those used for embryo biopsy. Allele dropout was not observed in any blastomeres.

The results of sexing human blastomeres are summarized in Table III, which shows that overall reliability for blastomere sexing was 95% (190/201) and overall accuracy was 99% (188/190).

The results outlined in Table IV provide supporting evidence demonstrating that the act of removing a blastomere from a human embryo does not decrease its developmental potential, at least in vitro. In fact the biopsied group had a higher blastocyst rate, even though the embryo quality was similar in both biopsied and control groups, although this is not significant due to the small sample numbers. The biopsied group also had many more embryos progressing beyond the four- to eight-cell block (72%; 36/50) than the control group (50%; 26/52) even though the embryo quality was similar.

Multiplexing

The results in Table V show the results obtained from performing multiple trisomy multiplex in single buccal cells. Overall reliability was 74% from 628 alleles, with an overall allele dropout rate of 30%. Reliability rates per allele ranged from 68% to 84%, while allele dropout rates varied from 27% to 33%.

Neither reliability nor allele dropout rates appear to be greatly influenced by the size of the PCR fragment (Fig. 1). The trend bars in Fig. 1 show that the reliability increases only very slightly and the allele dropout rate decreases very slightly as the fragment size increases.

The results in Table VI show the results obtained from performing multiplex PCR to determine DNA fingerprint in single buccal cells. Overall reliability was 86% from 606 alleles, with an overall allele dropout rate of 17%. Reliability rates per allele ranged from 77% to 90%, while allele dropout rates varied from 11% to 29%. As with the trisomy multiplex, neither reliability nor allele dropout rates appear to be greatly influenced by the size of the PCR fragment (Fig. 2). The decrease in allele dropout rates in the DNA fingerprint multiplex appears somewhat steeper than that in the trisomy multiplex, however, this may be due to a single very large (29%) result.

DISCUSSION

These results demonstrate that sexing blastomeres using fluorescent PCR is both reliable (95%) and accurate (99%). The increased reliability and accuracy from biopsied blastomeres (which were better quality) are particularly promising since these blastomeres are more representative of those obtained during clinical PGD. These results are also very similar to the reliability (89%) and accuracy (96%) rates obtained from

Table IV. Rate of Blastocyst Development After Embryo Biopsy^a

Number	Fate	Grade (mean)	Destroyed	Cleavage failure	Cleavage	No. of blastocysts	Blastocyst rate
51	Biopsied	2.294	1	14	36	12	23.53%
52	Controls	2.289	0	26	26	9	17.31%

^a Embryo biopsy is not detrimental to embryo development to the blastocyst stage. Only one embryo (2%) was destroyed during the embryo biopsy procedure. As a result of the results shown in Tables III and IV, a clinical PGD program was established. This resulted in a confirmed pregnancy after four in vitro fertilization cycles (unpublished results).

Table V. Multiplex PCR (Multiple Trisomies): Buccal Cells

Primer	No. of heterozygous alleles	Amplified alleles	Allele dropout	Reliability	Allele dropout
Amel	72	49	14	68%	29%
D13S631	139	106	35	76%	33%
D18S851	112	84	28	75%	33%
D21S11	139	99	28	71%	28%
D13S58	67	56	17	84%	30%
D18S51	99	71	19	72%	27%
Total	628	465	141	74%	30%

blastomeres in preliminary results (13) as well as the 96% reliability and 96% accuracy obtained from buccal cells (2). These preliminary results also demonstrate that human blastomeres can also be reliably (up to 91%) and accurately diagnosed for CF using fluorescent PCR, and this applies to both homozygote and heterozygote blastomeres (Table VII).

Although the accuracy rates of 99% for sex (this study) and 100% for CF (2,13) appear very encouraging, this may be due simply to the small number of blastomeres analyzed in each group. Although allelic dropout was not observed in human blastomeres, this, again, may have been due to the small number of blastomeres analyzed.

Although the data are insufficient for statistical analysis, they suggest that reliability appears to be linked to the quality of the embryo since both reliability and accuracy were lower when poorer-quality embryos were used. These results are in general agreement with those of other workers (29), who also showed a general decrease in reliability with a decrease in embryo quality.

One further embryo, which is not included in the CF data above, as it was disaggregated at the morula stage rather than the four- to eight-cell stage, produced inconsistent results and appeared to be a mosaic. This embryo was disaggregated into 14 blastomeres and

yielded of 4 CF carriers, 5 CF unaffected blastomeres, and 5 failed amplifications. Although it is possible that the CF unaffected signals may be due to allelic dropout from CF carrier blastomeres, this possibility is remote for two reasons: first, the very high incidence of allelic dropout in this embryo (27%; 4/14) compared to other cell types and, second, the nonrandom nature of the results since all the signals obtained were all CF unaffected. It is therefore more likely that this embryo was a mosaic of CF unaffected blastomeres and CF carrier blastomeres or, alternatively, that some blastomeres were anucleate. It is uncertain, however, whether this is a result of the late stage of disaggregation or of other factors. This agrees with previous studies using FISH suggesting that some embryos appear to be mosaics, at least as far as the sex chromosomes and chromosomes 21 and 18 (30–32). Mosaic embryos may also account for cases of misdiagnosis, previously attributed to allelic dropout.

In another embryo (not included in the CF data above), no signal could be obtained from any of its four blastomeres. However, the possibility of all the individual blastomeres failing to amplify by chance alone, or being anucleate, is remote. It is likely that, even though the embryo appeared morphologically normal, there may be some unknown mechanism within the embryo itself interfering with the PCR

Fig. 1. Reliability, accuracy, and allele dropout linked to allele size in a single-cell multiplex (trisomy) PCR. **Fig. 2.** Reliability, accuracy, and allele dropout linked to allele size in single-cell multiplex (DNA fingerprint) PCR.

Table VI. Multiplex PCR (DNA Fingerprinting): Blastomeres

Primer	No. of heterozygous alleles	Amplified alleles	Allele dropout	Reliability	Allele dropout
Amelo	62	52	10	84%	19%
VWA	114	103	11	90%	11%
THO	88	68	20	77%	29%
D8	80	70	10	88%	14%
FGA	118	99	19	84%	19%
D21S11	50	45	5	90%	11%
D18S51	94	82	12	87%	15%
Totals	606	519	87	86%	17%

process. This may have been due to the unavailability of the DNA or, alternatively, to an impairment of normal cellular processes. If it is true that this unavailability/impairment interferes with normal embryonic development, causing embryo malformation or blockage at later stages of development, then this has very interesting possibilities. PCR testing could, at least in theory, provide an additional embryo selection procedure so that only those embryos that produced a signal could be selected for embryo transfer. This would also reduce the possibility of apparently morphologically normal, but genetically abnormal, embryos being transferred and may subsequently lead to an increase in implantation and pregnancy rates.

Preimplantation genetic diagnosis of sex using fluorescent PCR was also used clinically, resulting in a confirmed pregnancy after four cycles of in vitro fertilization (unpublished results). This is the first time that fluorescent PCR has been used for sexing in clinical PGD and demonstrated the wide potential of fluorescent PCR in PGD programs.

In the multiplex PCRs, the reliability, accuracy, and allele dropout were poorer for the multiple trisomy multiplex than for the DNA fingerprint multiplex. Reliability was 70–75% for the trisomy multiplex, compared with 80–85% with the DNA fingerprint multiplex. Accuracy in the trisomy multiplex was ~70%, compared with the ~80% observed in the DNA fingerprint multiplex, and allele dropout in the trisomy multiplex of ~30%, compared with allele dropout in the DNA fingerprint multiplex of ~20%. As previous work (15) has demonstrated that cell type appears to make little difference in reliability and allele dropout from the same single-cell multiplex PCR, these differences may be due to the fact that unlike the trisomy multiplex, the DNA fingerprint multiplex has undergone extensive optimizations. Optimization of the trisomy multiplex is ongoing and may reduce allele

dropout further, as well as increasing reliability and accuracy.

One possibility of allele dropout is that it may be linked to the size of the PCR fragment, with allelic dropout increasing as the fragment size increases. However, Figs. 1 and 2 show that allele dropout in both multiplex PCRs remains relatively constant as the fragment size increases.

One way of reducing allelic dropout, in sexing, for example, would be to use multiplex PCR for simultaneous amelogenin and X- and Y-chromosome microsatellites in a manner similar to that previously undertaken for chromosome 21 (15,16,20). If the allelic dropout rate of each allele should be similar, the combined dropout rate of two alleles, assuming independent reactions, should be 0.25% (5% of 5%). If this assumption is correct, a theoretical possible accuracy rate of 99.75% should be obtained using two primers, and 99.999875% using three primers.

These results suggest that fluorescent PCR is a very powerful technique for clinical PGD, not just because of its high reliability and accuracy, but also due to its potential in providing multiple diagnoses and contamination detection. Optimization of multiplex PCR may provide a very wide-ranging and versatile repertoire for genetic diagnosis.

Table VII. Fluorescent PCR Reliability and Accuracy in Diagnosing CF Carrier and Unaffected Blastomeres^a

Blastomere source	Reliability	Accuracy
All blastomeres	91% (40/44)	100% (40/40)
CF carrier blastomeres	100% (7/7)	100% (7/7)
CF homozygous, unaffected	89% (33/37)	100% (33/33)

^a Reliability and accuracy of CF diagnosis in CF carrier and unaffected embryos. Reliability and accuracy are high in all cases. Table reproduced with permission (13). It excludes two embryos which gave significantly aberrant results.

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