Comparison of FISH, PRINS, and Conventional and Fluorescent PCR for Single-Cell Sexing: Suitability for Preimplantation Genetic Diagnosis

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Purpose: Although conventional polymerase chain reaction (PCR) was the first method used for sexing in preimplantation genetic diagnosis, fluorescent in situ hybridization (FISH) has become the method of choice. Recently two new techniques, primed in situ synthesis (PRINS) and fluorescent PCR, have been developed. This study compares the reliability and accuracy of these four techniques in single cells.

Results: In buccal cells, fluorescent PCR and FISH had similar reliability (94 and 93%) and accuracy (97 and 96%) rates. The reliability and accuracy of PRINS (91 and 25%) and conventional PCR (79 and 89%) were lower. In human blastomeres, FISH and fluorescent PCR had similar reliability (100%, 7/7; 95%, 190/201) rates. Accuracy rates were 71% (5/7) and 99% (188/190) for FISH and fluorescent PCR, respectively, however, too few blastomeres were analyzed by FISH for meaningful comparison. However, when these data are compared with published data, the method of choice for blastomere sexing appears to be fluorescent PCR.

Conclusions: Fluorescent PCR has major implications for PGD.

KEY WORDS: fluorescent in situ hybridization; single-cell PCR; preimplantation genetic diagnosis; primed in situ synthesis; sexing.

INTRODUCTION

Although conventional polymerase chain reaction (PCR) was the first technique used for sexing in clinical

preimplantation genetic diagnosis (PGD), it was shown to be unreliable and, more importantly, inaccurate, due to amplification failure (1). However, PCR has been, and still is, used successfully to diagnose cystic fibrosis status (2–4) as well as other single-gene defects in PGD.

Fluorescent in situ hybridization (FISH) has now been adopted by most PGD centers worldwide as the method of choice for sexing (5–8).

The application of FISH in PGD has shown that chromosomal mosaicism is common at the cleavage stage of development (6). This finding has very important implications for diagnosis of dominant single-gene disorders, monosomies, and trisomies as well as for the understanding of early human development. These studies have now been confirmed (9,10), demonstrating that embryo aneuploidy and/or mosaicism, at least in humans, occurs at a rate of 40–80%, depending on the number of FISH probes and embryo quality. This has subsequently resulted in the increased use of polar body analysis for aneuploidy diagnosis. A further advantage of FISH is that the risk of contamination is reduced because the nucleus is observed throughout the process.

However, there are three main limitations to current FISH procedures for PGD.

- (1) FISH being generally limited to the diagnosis at the chromosomal rather than the single-gene level.
- (2) The loss of nuclei when spreading single blastomeres.
- (3) The time taken for the diagnosis.

Alternative methods for single-cell sexing are primed in situ synthesis (PRINS) and fluorescent PCR.

PRINS is a similar technique to FISH except that a previously labeled probe is not required (11), as the labeling reaction takes place in situ, usually with a biotin or digoxigenin dUTP (12). During the annealing

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and extension step, the oligonucleotide forms hybrids with its complementary sequences and then acts as a primer for the synthesis of labelled DNA. As the oligonucleotide is unlabeled, this results in a low background. The theoretical advantages of PRINS over FISH are twofold.

(1) As labeled nucleotides are incorporated only at the annealing site, nonhybridized or poorly hybridized probes result in a low background, unlike FISH, where such probes contribute to background staining and potential misdiagnosis.

(2) Unlike FISH, PRINS does not appear limited to chromosomal detection but can also label single-copy genes (13).

However, although the initial incubation steps are shorter in PRINS, the additional steps needed to label for more than one chromosome mean that it takes as much time as, if not longer than, FISH (12,14). PRINS has recently been applied to sexing human embryos with promising results (15,16).

PCR is an extremely powerful *in vitro* method for the amplification of specific DNA or RNA sequences. It can be used to amplify rapidly a minute amount of DNA, even single copies, more than a billionfold in a fully automated procedure. Genetic diagnoses can be obtained from a small number of cells (17) and even single cells (18,19). Apart from its great sensitivity, a very important advantage of PCR is that DNA fragments can be amplified within a few hours.

In the past few years, PCR analysis has been automated by modifications in PCR technology. One such modification is fluorescent PCR. This system uses fluorescent primers and an automated DNA sequencer to detect the PCR product (20), which has improved both the accuracy and the sensitivity (21,22). Fluorescent PCR is very similar to conventional PCR except that each primer is tagged with a fluorescent dye. Using a photo-multiplier and computer enhancement technology, the fluorescent dye is detected at a much lower threshold level than conventional agarose or acrylamide gel analysis (23). This system allows highly accurate and reliable detection even in heterozygote cells, where one allele is very weak or many times lower (to ~ 1%) compared to the other (24).

In recent years fluorescent PCR has been applied to sexing single buccal cells (24–26) as well as human blastomeres (27) for PGD. This method of single-cell sexing has been shown to have a high reliability (~ 97%) and accuracy (~97%) (24). Fluorescent PCR has also been used for the single-cell diagnosis of single-gene defects (24), trisomies (Matthews *et al.*,

submitted for publication, and DNA fingerprinting (25), even for forensic applications (27).

However, as yet, the four methods of single-cell sexing (FISH, PRINS, conventional and fluorescent PCR) have not been directly compared.

MATERIALS AND METHODS

FISH and PRINS were initially applied to cell smears to establish baseline reliability and accuracy rates.

Single-Cell Isolation

Buccal cells and human blastomeres were obtained and isolated as described previously (26). Oocytes were unfertilized oocytes from the *in vitro* fertilization (IVF) program at Leeds General Infirmary, Leeds, UK.

Ethical permission to perform these experiments was obtained in all cases from the Local Research Ethics committee and the UK Human Fertilisation and Embryology Authority.

FISH

Buccal Cells. FISH was performed on single cells in the manner described in Appendix 1.

Blastomeres. FISH was performed on blastomeres in a manner similar to that on buccal cells with the exception that blastomeres were digested with 100 µg/ml pepsin in 0.01 M HCl for 10 min at 37°C between fixing and FISH labeling.

Conventional PCR

Conventional PCR was performed on single buccal cells using DYZ1 primers as described previously (28).

Fluorescent PCR

Fluorescent PCR was performed on single buccal cells as described previously (26).

PRINS

Buccal Cells. PRINS was performed using a slightly modified system, as detailed in Appendix 2, based on the manufacturer's (Applied Biotechnology, Surrey, UK) protocols.

Blastomeres. FISH was performed on blastomeres in a manner similar to that on buccal cells with the excep-

tion that blastomeres were digested with 100 µg/ml pepsin in 0.01 M HCl for 10 min at 37°C between fixing and FISH labeling.

RESULTS

In all cases reliability is defined as the number of times a result is obtained from the number of samples analyzed. Accuracy is defined as the number of times the result is correct from the number of results obtained.

Smeared Buccal Cells

The results obtained for FISH and PRINS are shown in Table I. These results demonstrate that sufficiently reliable and accurate results could be obtained from these techniques to compare at the single-cell level.

Single Buccal Cells

Table II shows the result obtained for FISH, multi-PRINS, conventional PCR, and fluorescent PCR on single buccal cells. Results for single fluorescent PCR are shown in Figs. 1a and b.

Single-cell sexing using fluorescent PCR has a reliability rate of 94% and an accuracy rate of 97%, which are similar to the reliability (93%) and accuracy (94%) rates obtained by the gold standard, FISH.

This table also demonstrates that a reliability rate of 79% (19/24) and an accuracy rate of 89% (17/19) were obtained for conventional PCR. Two misdiagnoses (males diagnosed as females) were observed. The conventional PCR signals from single cells were often weak and difficult to resolve, resulting in difficulties in interpretation and decreased confidence in determining a diagnosis.

Single-cell sexing using PRINS has a reliability rate of 79% (79/100) and an accuracy rate of 82% (65/79) when single PRINS (X probe only) is used or a

Table I. Comparison of Sexing from Smeared Cells

Technique	Reliability (%)	Accuracy (%)	Time taken (hr)
FISH (X + Y probes)	96 (2713/2839)	90 (2454/2713)	7
PRINS (X + Y probes)	84 (837/995)	90 (757/837)	5

Table II. Comparison Among Fluorescent PCR, Conventional PCR, FISH and PRINS in Sexing Single Buccal Cells

Technique	Reliability (%)	Accuracy (%)	Time taken (hr)
Fluorescent PCR (female)	93 (93/100)	100 (93/93)	5
Fluorescent PCR (male)	92 (92/100)	95 (87/92)	5
Fluorescent PCR (male) [from previous studies (26)]	97 (144/149)	97 (140/144)	5
Overall fluorescent PCR	94 (329/349)	97 (320/329)	5
FISH (male)	93 (93/100)	96 (89/93)	7
Conventional PCR (male)	79 (19/24)*	89 (17/19)	8
Single PRINS (male)	79 (79/100)*	82 (65/79)*	5
MultiPRINS (male)	91 (91/100)	25 (23/91)*	6

* Statistically significant difference ($P < 0.05$, χ^2 test) between the result and that obtained by fluorescent PCR.

reliability rate of 91% (91/100) and an accuracy rate of 25% (23/91) when multiPRINS was used.

Blastomeres and Oocytes

When blastomeres and oocytes were analyzed, the results shown in Table III were obtained.

DISCUSSION

These results show that single-cell sexing using fluorescent PCR or FISH is more reliable and/or more accurate than either conventional PCR or single PRINS. Although fluorescent PCR and FISH have reliability rates similar to that of multiPRINS, their accuracy, at 96 and 97%, respectively, far exceeds that of multiPRINS (25%).

As a method for looking at single chromosomes, PRINS is quick and efficient. However, when used as a technique for sexing single cells, the method is laborious, lengthy, and inaccurate and has many difficulties that must be overcome before it can be seriously considered as a diagnostic procedure for sex determination. Although single-chromosome detection (single PRINS) is relatively rapid and the method is relatively simple to perform, reliability and accuracy rates of approximately 80% are not sufficiently high to con-

Fig. 1. (a) Sexing of male cell using fluorescent PCR. Using the SS standard, the X signal is sized at 103 bp and the Y signal at 110 bp. (b) Sexing of female cell using fluorescent PCR. Using the ROX standard, the X signal is sized at 107 bp.

Table III. Sexing of Blastomeres and Oocytes^a

Technique	Reliability (%)	Accuracy (%)	Time taken (hr)
Fluorescent PCR (female) blastomeres	96% (86/90)	98% (84/86)	5
Fluorescent PCR (male) blastomeres	94% (104/111)	100% (104/104)	5
Total fluorescent PCR (blastomeres)	95% (190/201)	99% (188/190)	5
FISH (blastomeres)	100% (7/7)	71% (5/7)	7
FISH (oocytes)	96% (96/100)	—	7
PRINS (oocytes)	75% (88/118)	—	6

^a Accuracy could not be obtained for oocytes since confirmation of the diagnosis could not be undertaken.

sider using for PGD. MultiPRINS, using both X and Y probes, although more reliable (91%), is more time intensive and technically difficult. As with single PRINS, the accuracy of this technique, at 25%, is insufficient for PGD. Applying the different chromosome labels is time-consuming, and the risk of signal carryover from one reaction application to another is high, especially if a sufficient blocking step is not included. As PRINS offers little more than traditional FISH, it seems unlikely at this time that PRINS will become the preferred method over traditional FISH. Diagrams showing differences among the PRINS, FISH, and fluorescent PCR techniques are shown in Fig. 2.

The reliability of fluorescent PCR, although slightly higher, was statistically similar to that of FISH, the main technique currently used for single-cell sexing. The accuracy of these two techniques is also similar,

Fig. 2. (a) Simplified diagram of fluorescent in situ hybridization. Note that the sample DNA and previously labeled probe are denatured separately. (b) Simplified diagram of primed in situ synthesis. Note that the sample DNA, the unlabeled probe, and the labeling mixture are all added together and denatured simultaneously. (c) Simplified diagram of a FPCR reaction.

at 96 and 97% for FISH and fluorescent PCR, respectively. In summary, therefore, at least from these data, both FISH and fluorescent PCR are the methods of choice for single-cell sexing.

Although the results in this study are consistent with those in other studies (Table IV), the FISH reliability

Table IV. Comparison of FISH and Fluorescent PCR for Sexing Human Blastomeres

Technique	Reliability (%)	Accuracy (%)
Fluorescent PCR (this study)	95 (190/201)	99 (188/190)
FISH (this study)	93 (93/100)	96 (89/93)
FISH ^a	~70	>90
FISH ^b	84	96
FISH ^c	86 (424/492)*	Not known
Conventional PCR (this study)	79 (19/24)*	89 (17/19)
Conventional PCR ³	78 (324/417)*	Not known

^a Data from ESHRE Campus Workshop on Preimplantation Diagnosis of Genetic Disease, London, Sept. 1993.

^b Delhanty J: Blastomere data presented at Special Interest Group in Reproductive Genetics, ESHRE, Edinburgh, 1997.

^c Harper J: Blastomere data presented at Special Interest Group in Reproductive Genetics, ESHRE, Edinburgh, 1997.

* Statistically significant difference ($P < 0.05$, χ^2 test) between the result and that obtained by fluorescent PCR.

obtained (93%) appears to be higher than that in other studies (70–86%). However, this is not statistically significant.

Of the five methods tested, only fluorescent PCR and FISH have rates of reliability high enough to make them viable diagnostic options. Of these two methods, FISH is easier to perform when dealing with low sample results.

A further advantage of FISH is that of detecting sex chromosome aneuploidies. Difficulties of quantification using fluorescent PCR mean that this approach, as yet, cannot distinguish sex-chromosome aneuploidies such as XX, XXX, XO or XY, XYY, XXY. Although there is an identifiable risk of misdiagnosis of these aneuploidies, this risk is relatively minor.

Although FISH reliability and accuracy rates are high in this and previous studies, the technique does have some difficulties. First, the hybridization efficiency may vary with the probe type. Alpha satellite probes (used in this study) have efficiency rates of approximately 95%, whereas locus specific probes have only an 80–85% efficiency. Second, the pH of the washes must be monitored closely because an increase in the pH leads to increased stringency and poorer reliability. Although an increase in the NaOH content is beneficial because it increases the stability of the formamide, the NaOH concentration must be relatively low after the sample has been hybridized to the probe so that the hybridized DNA is not washed

away. Third, the formamide concentration must be optimized, because the higher the concentration in the solution, the lower the temperature needs to be for efficient denaturation. Finally, although the rate of false positives is similar to that of fluorescent PCR, at approximately 1–4%, FISH, unlike fluorescent PCR, cannot determine a DNA fingerprint of the cell and thus determine the risk of contamination. Although contamination by erroneous cells such as cumulus cells in PGD is unlikely, because the cell is visualized throughout the procedure, it cannot be ruled out.

Fluorescent PCR has the advantages of a high reliability and accuracy and that many samples (up to 36) can be run without greatly increasing the run time. This allows for a significantly higher number of analyses (36) than can be achieved with FISH (5–10) in the same amount of time.

One potential disadvantage of fluorescent PCR, like all PCR techniques, is the possibility of contamination. Although contamination can occur with FISH, the risk is higher with PCR. Fluorescent PCR, unlike conventional PCR, can provide a DNA fingerprint of a single cell (25,27), which virtually eliminates the risk of cellular contamination. The only significant disadvantage of fluorescent PCR is that of the capital cost, which can be significantly higher than the costs of FISH workstations.

In conclusion, this study demonstrates that only FISH or fluorescent PCR should be used for single-cell sexing. Although each method has its disadvantages, fluorescent PCR appears to be the method of choice for sexing blastomeres. Fluorescent PCR also has the advantage that is a multipurpose technique that can also be used to diagnose single-gene defects such as cystic fibrosis and trisomies as well as providing a DNA fingerprint.

APPENDIX 1: FISH PROTOCOLS

1. Single buccal cells are isolated with a mouth pipette and transferred to a four-well Nunc dish (Nunc, Denmark), where they are washed in PBS for 2 min.

2. The cell is then transferred to a slide in spreading solution (0.01 M HCl, 0.01% Tween 20).

3. The cell is then observed under an inverted microscope until it lyses, and the spreading solution and the excess cytoplasm are removed and replaced with PBS and allowed to air-dry.

4. The slide is then fixed in methanol acetic acid 3:1 for 30 min and then treated with 100 µg/ml pepsin in 0.01 M HCl for 10 min at 37°C.

5. The slide is then rinsed in PBS and distilled water for 2 min before being dehydrated through an ethanol series (70–90–100%).

6. Slides are then transferred to 2× SSC wash at 37°C and dehydrated again.

7. Slides are then air-dried and denatured by immersing in 70% formamide and 2× SSC at 70°C for 2 min.

8. Slides are then plunged into ice-cold 100% ethanol for 2 min and air-dried again.

9. Twelve microliters of the hybridization mix (1 µl X, µl Y, 10 µl hydriol) (Oncor, UK) is then added, and the coverslip, and incubated at 37°C for 2 hr.

10. Slides are then immersed in 2× SSC at room temperature to remove coverslips.

11. Slides are placed in SSC at 70°C for 5 min, then drain.

12. Twenty microliters of the detection stain is added, then the coverslips, and incubated at 37°C for 20 min.

13. Slides are placed in SSC for 2 min remove coverslips, then washed and allowed to drain.

14. Ten microliters of DAPI in antifade is then added, and a coverslip is added and sealed with nail varnish.

15. The slide is then viewed using a fluorescent microscope with the appropriate filters.

APPENDIX 2: PRINS PROTOCOLS

PRINS protocols are based on those supplied by the manufacturer (Applied Biotechnology, Surrey, UK).

1. Single buccal cells are isolated with a mouth pipette and transferred to a four-well Nunc dish (Nunc, Denmark), where they are washed in PBS for 2 min.

2. The cell is then transferred to a slide in spreading solution (0.01 M HCl, 0.01% Tween 20).

3. The cell is then observed under an inverted microscope until it lyses, and the spreading solution and the excess cytoplasm is removed and replaced with PBS and allowed to air-dry.

4. The slide is then fixed in methanol acetic acid, 3:1, for 30 min and treated with 100 µg/ml pepsin in 0.01 M HCl for 10 min at 37°C.

5. The slide is then rinsed in PBS and distilled water for 2 min before being dehydrated through an ethanol series (70–90–100%).

6. Fifty microliters of the reaction mix (1 slide = 43 µl labeling mix, 5 µl primer, 1 µl Taq polymerase, 1 µl of 1 mM labeled dNTP) is then applied to each slide.

7. The slides are then heated for 3.5 min at 93°C, 10 min at 60°C (e.g., for biotin labeled Y probes), and then 15 min at 72°C.

8. The slides are then washed in 0.1x SSC at 65°C and 50 µl of second reaction mix (using a different label, i.e., digoxigenin for the X label) is applied to each slide.

9. The slides are then heated for 10 min at 50°C (X) and 15 min at 72°C.

10. Slides are then washed in 0.1x SSC at 65°C (X2) and transferred into the stop solution at 65°C for 5 min and then to the wash solution for at least 2 min.

11. Forty microliters of the blocking reagent is then applied and the slides are incubated at room temperature for 5 min.

12. Forty microliters of FITC/rhodamine is then added and the slides are incubated at 37°C for 30 min. The slides are washed in wash solution at 45°C, mounted, and viewed using a fluorescent microscope with the appropriate filters.

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