Pre-implantation genetic diagnosis

Ian Findlay
Australian Genome Research Facility, Gehrmann Labs, University of Queensland, Queensland, Australia

Genetic disorders are a major cause of miscarriage and fetal death. Pre-implantation genetic diagnosis (PGD) can be used to diagnose genetic defects before pregnancy has occurred by creating embryos by IVF, then removing single cells which are genetically analysed using FISH or PCR. Although successful, the techniques have many difficulties because they are highly specialised and at the extreme limit of sensitivity. Newer techniques, however, can rapidly diagnose multiple defects including chromosomal aneuploidy, sex and single gene defects. Embryonic cells can also be DNA fingerprinted to ensure that contamination has not occurred. As embryo screening can increase IVF success rates and decrease miscarriage rates, it will be increasing offered in routine IVF rather than just those patients at high genetic risk. These new, low cost techniques may ultimately allow PGD to be offered to all IVF patients regardless of risk.

Over the last two decades, methods of ovarian stimulation, oocyte collection, IVF, embryo culture and transfer have been established for the treatment of infertility to the extent that the same techniques, albeit with minor modifications, are used world-wide. For example, the introduction of ovarian stimulation using FSH (follicle stimulating hormone) and LH (luteinising hormone), allows multiple oocytes to be produced per treatment cycle, therefore increasing the chance of pregnancy. The combination of these IVF techniques and the enormous advances in genomics in the last decade has allowed not only the study of the genetic aspects of embryogenesis but also prenatal diagnosis to be performed even before pregnancy by testing embryos created by IVF using pre-implantation genetic diagnosis (PGD). If genetic defects can be identified during early stages of embryonic development before implantation occurs, i.e. PGD, then embryos shown to be unaffected by disease could be selected for transfer, therefore greatly reducing the need for pregnancy termination.

The first real possibilities for PGD of human genetic disease were demonstrated in 1989 for X-linked diseases and in 1990 for autosomal recessive diseases. More than 200 children have now been born following these procedures demonstrating that embryo biopsy and PGD can be safely performed in humans.
Although PGD has been performed for more than 10 years, it is still fraught with difficulties. It is only by understanding what these difficulties are that new sampling or diagnostic techniques can be developed to improve PGD and perhaps ensure that PGD is as commonplace as ICSI is today.

I will firstly discuss difficulties with PGD and then the techniques that are currently used (both sampling and diagnostic) before discussing a new method that may revolutionise pre-implantation genetic diagnosis.

**Difficulties with PGD**

The clinical application of PGD has not been minor. There are a great many difficulties and the techniques require great expertise. These difficulties include:

**Difficulties in embryo research**

1. Difficulty in obtaining experimental material. The supply of human embryos for experimentation is extremely limited (perhaps only a few thousand over the last 10-20 years) unlike mouse embryos where there has been an almost unlimited supply over the last 60 years.

2. Standardisation. Not only are human embryos extremely variable, but only a few embryos can be obtained from each woman making comparison and experimental controls extremely difficult.

3. Ethical considerations which control the types of experiments performed on human embryos.

**Diagnostic difficulties**

1. PGD usually requires single cell analysis which is at the most extreme level of sensitivity. Very few techniques are sensitive enough to detect disorders from such minute amounts of material.

2. Single cell analysis must both be highly reliable (> 95%) as well as extremely accurate (> 99%) for clinical application. Very few techniques can achieve such high reliability and accuracy rates with the consistency required for clinical PGD.

3. Diagnosis must be performed in as short a time period as possible to allow embryo transfer the same day (preferably less than 8 h). Again, very few techniques allow diagnosis within 24 h.

4. Techniques must be wide ranging to allow a variety of disorders to be detected.
Clinical and practical difficulties

1 Embryo biopsy is a skilled technique as not only must single blastomeres be removed, but the embryo must remain intact and viable. It is useless to have excellent diagnostic techniques if embryos do not survive the biopsy procedure.

2 Very low efficiency as PGD is combined with IVF. The number of embryos available for biopsy can often be limited to less than 25% of oocytes collected. For example, only ~70% oocytes will fertilise, only ~70% of these will develop to the 6–8 cell stage and only ~80% of these will be suitable for biopsy. Of the biopsied embryos, ~5% may not be analysed either because the embryo or biopsied cell is destroyed during biopsy. Diagnosis will be obtained from 90% and approximately 50% of embryos will have the disorder tested for. Therefore, from 100 oocytes collected, only 17 will result in an embryo suitable for transfer. A pregnancy rate of 15% per embryo transferred decreases the chance of success even further. In total, perhaps only 2.5% of eggs collected will form a viable unaffected PGD pregnancy.

3 Age of patient. PGD patients, particularly those for aneuploidy screening, are often in their mid-thirties or forties, which not only increases their risk of chromosomally abnormal embryos, but also decreases the chance of success after IVF.

4 Logistic considerations such as timing of egg collection, availability of diagnostic equipment, etc.

PGD techniques

Fortunately, our increased knowledge of human pre-implantation development gained through IVF and the enormous progress being made in genetic and molecular biology including mapping the entire human genome (97% completed at June 2000), means that diagnostic techniques are advancing at a tremendous rate.

The main techniques (PCR and FISH) currently used for PGD will now be discussed. A new technique, fluorescent multiplex PCR will then be discussed. Although other evolving techniques such as SNP (single nucleotide polymorphism) analysis to determine genetic predisposition, e.g. type II diabetes, spectral karyotyping and microarrays, may have huge potential in PGD, the impact of these highly sophisticated techniques on PGD is as yet unknown.

Sampling techniques

PGD of a disease can be achieved either indirectly by analysing biochemical markers or by-products of the embryo, such as enzymes/proteins, or directly by sampling part of the embryo itself.
**Indirect diagnosis**

Unfortunately measuring enzymes or protein is only possible when the biochemical defect is known at the protein level. This technique requires:

1. That the gene is actively transcribed and translated.
2. That the level of the gene product analysed in the embryo is measurably different from the gene product inherited in the maternal cytoplasm of the oocyte.
3. That a sensitive biochemical microassay is available which can detect the presence or absence of the gene product from single cells. Although microassays capable of measuring the activity of some enzymes involved in inherited syndromes at the single cell level have been available for some years, they have yet to be applied to clinical PGD due to the difficulties outlined above.

The obvious advantage of a non-invasive approach is that embryos are not biopsied and, therefore, the potential hazards of their future development caused by micromanipulation are minimal. However, only a few preliminary studies have investigated the release of factors synthesised by pre-implantation embryos *in vitro*. Also, since embryos may be able to adapt to their culture conditions, the uptake or release of factors could prove to be due to experimental *in vitro* artefact. Such assays, although potentially very promising for assessing the likelihood of an embryo reaching the blastocyst stage, would be unsuitable for PGD since products secreted into the media are unlikely to be linked to the genetic disorder.

Therefore, at least for the time being, PGD by measuring enzyme/protein activity is extremely limited and cannot yet be attempted in human embryos, not least because very little is known about human gene expression during pre-implantation development. The diagnosis of genetic defects, therefore, requires one or more cells to be biopsied from the embryo and several methods have been developed.

**Direct diagnosis**

To test for genetic diseases, a sample of genetic material must be obtained directly from the embryo. Genetic material can be obtained at three main stages (Fig. 1).

1. *Polar body biopsy*. Before fertilisation by sampling the gametes (oocytes only), particularly the polar bodies.

2. *Early cleavage stage*. Involving removal of 1 or 2 blastomeres at the early cleavage stage (6–8 cells) at about 3 days. Embryonic cells cannot be obtained earlier, as cell removal at this stage would be seriously detrimental to further embryonic development.
3 Blastocyst biopsy after about 5–7 days (~100 cell stage) by removing 5–12 trophectoderm cells. Cells cannot be removed at 3–5 days, as the embryo is at the morula stage of development where the cells are very closely compacted making biopsy extremely difficult.

Polar body biopsy
Although preconception genetic diagnosis could be achieved by genotyping either sperm or oocytes, the former approach is not realistic at the present time as it destroys the sperm. After extensive studies, claims to have reliably and successfully separated X and Y sperm to
prevent X-linked disorders have had variable success. The only approach for preconception diagnosis at present, therefore, seems to be genotyping oocytes by biopsing the polar bodies, with subsequent genetic analysis of biopsied material.

The biopsy of the polar body from oocytes provides the possibility for preconception diagnosis of inherited disease, therefore obviating some ethical concerns. However, this approach can be viewed as being wasteful in both time and resources as all available oocytes must be biopsied and tested even though only 50–60% will fertilise and form viable embryos.

If the polar body from a woman heterozygous for a mutant gene causing a disease is found to be homozygous for one allele, then it can be assumed that the oocyte is homozygous for the other allele, and that crossing-over did not occur. If crossing-over occurs, the polar body will be heterozygous with copies for both alleles and the genotype of the oocyte cannot be predicted. If crossing-over does not occur, the first polar will be homozygous for the allele not contained in the oocyte and second polar body. If crossing-over occurs, the oocyte will be heterozygous and the eventual genotype of the oocyte cannot be predicted. The frequency of crossing-over will vary with the distance between the locus and the centromere. For telomeric genes, the cross-over frequency approaches 50% whereas for genes close to the centromere the frequency may be very close to 0%.

However, in recessive disorders such as cystic fibrosis, 50% of oocytes will contain the abnormal gene and be fertilised with sperm carrying the normal gene and will thus be unaffected. Polar body biopsy is, therefore, not able to identify all unaffected embryos, which ultimately have to be identified by other biopsy methods.

Although polar body biopsy is undertaken clinically (and has become the most common form of PGD), it is not always possible to establish a genetic diagnosis for four reasons. Firstly, the paternal alleles cannot be analysed. Secondly, it is a very poor test for telomeric loci due to high frequency of crossing-over which can lead to heterozygous embryos and misdiagnosis. Thirdly, gender determination is not possible. Fourthly, polar body analysis cannot identify carriers of recessive disorders, such as cystic fibrosis because although 50% of affected oocytes will contain the mutation, they will be fertilised with unaffected spermatozoa and will, therefore, be unaffected. Polar body biopsy is, therefore, not able to identify all unaffected embryos, and so other biopsy methods are required in these cases.

Polar body analysis is, however, particularly useful in detecting aneuploidies since most (~90%) aneuploidies are maternally derived and thus already present in the oocyte. Most aneuploidy screening is now performed by polar body analysis. In most cases of PGD, however, polar biopsy has been either replaced by or followed by blastomere biopsy.
Cleavage stage biopsy

Rather than removing a cell before fertilisation, the embryo can be fertilised and allowed to develop in vitro for several days until the 6–8 cell stage (Fig. 1). At this stage, a blastomere can be removed and analysed allowing direct diagnosis of the embryo.

One advantage of cleavage stage biopsy is that pre-implantation development of human embryos does not appear to be adversely affected by biopsy at the 6–10 cell stage, since development to blastocyst appears unaffected and live-births have been obtained. A second advantage is that embryo transfer is performed routinely in IVF whilst the embryo is at the cleavage stage.

Cleavage stage biopsy does, however, have three main disadvantages:

1. The small amount of biopsy material obtained (often only a single cell), which may allow only a single analysis. It is advisable to perform replicate diagnoses in case of ambivalent results or to confirm the initial diagnosis.

2. The removal of cells that would have contributed to the fetus, which may have ethical implications.

3. The majority of cleavage stage embryos undergo developmental arrest in vitro, so the chance of implantation of a biopsied embryo might be limited. However, as most biopsied embryos are transferred to the uterus as quickly as possible after diagnosis, this does not appear to have a significant effect.

Blastomere biopsy, usually two cells, at the cleavage stage is the method of choice in the majority of PGD centres around the world.

Blastocyst biopsy

An alternative method of direct analysis is to remove cells at the blastocyst stage. Extensive studies on the development of blastocyst biopsy in human embryos have been undertaken.

There are several apparent advantages of blastocyst stage biopsy:

1. Trophectoderm cells can be removed easily with simple glass microneedles rather than having three different sized glass micropipettes attached to syringes (ease of biopsy is an important consideration since the techniques are used in IVF facilities).

2. The trophectoderm cells do not contribute to the embryo proper, but eventually form placenta and other extra-embryonic tissues thus reducing ethical considerations. This procedure is more akin to chorionic villus sampling prenatal diagnosis.

3. Multiple cells (as many as 15) can be removed allowing either several diagnoses or to provide confirmation of the original diagnosis.
Pre-implantation genetic diagnosis

4 Blastocysts transferred into the uterus may have higher pregnancy rates than to transfer rates at cleavage stage. In practice, however, there may not be a clinical advantage of transferring embryos at the blastocyst stage since pregnancy rate after blastocyst transfer appears to be similar to cleavage stage transfer. Recent work\(^9\) suggests that blastocyst transfer can have significant advantages.

5 The embryo will already have developed beyond the 8-cell block, where most human embryos fail to develop further or degenerate. Thus another parameter of embryo viability is achieved and may provide a better selection procedure as it is likely that those embryos that have survived to blastocyst are more likely to survive \textit{in vivo}.

6 The survival of the blastocyst, after biopsy, can be assessed within 4–5 h by the reformation of the blastocoel cavity, whereas it is more difficult to assess survival of an early cleavage stage embryo after biopsy.

However, the major problem with biopsy at this stage is the very low numbers of embryos reaching blastocyst stage (only 10–20%). This puts a severe limit on embryos available for biopsy and, therefore, available for transfer to the uterus. The use of blastocyst biopsy for PGD is thus extremely limited until greater numbers of embryos can develop to the blastocyst stage, perhaps by improved \textit{in vitro} culture conditions.

\textit{Embryo sampling techniques – the future}

The reliance of a diagnostic procedure which relies on a single analysis from a single cell has two main difficulties. Firstly, there is often only a single opportunity of obtaining both a result and, more importantly, the correct result. Secondly, the biopsied cell may not be representative of the entire embryo.

Three approaches can be used to alleviate these difficulties:

1 Increasing the number of cells by sampling at the later blastocyst stage\(^9\). However, either significant improvements in embryo culture media are required to maximise the number of embryos reaching the blastocyst stage \textit{in vitro}, or embryos can be obtained directly at the blastocyst stage by uterine lavage.

2 Blastomere replication. Single biopsied blastomere can be encouraged to divide several times \textit{in vitro} thus allowing either multiple analyses of single individual cells or, alternatively, adding to the original cell sample increasing the number of cells analysed with the single reaction\(^7\)\(^{–}\)\(^10\).

3 Improving diagnostic methods allowing multiple analyses from single cells. If the number of cells cannot be increased, then both the reliability and accuracy of testing and the number of diagnoses per cell can be increased. This is discussed further in the diagnostic section.
**Uterine lavage**
An alternative method of obtaining embryos, rather than by IVF, is by flushing embryos from the uterus by a non-surgical procedure known as uterine lavage\textsuperscript{11}. Uterine lavage has been used after artificial insemination to collect pre-implantation embryos from egg donors, which are then transferred to recipient mothers with synchronised cycles. The efficiency of obtaining pregnancy in recipient women, following transfer of morulae or blastocysts obtained by uterine lavage, appears to be high\textsuperscript{12}.

There are several advantages in collecting blastocysts in this way:
1. The procedure is non-surgical.
2. Only a single embryo is replaced.
3. At the blastocyst stage, the embryo has the maximum number of cells before implantation and the greater the number of biopsied cells for analysis, the more reliable the diagnosis is likely to be.
4. An ethical problem is avoided as the biopsied sample would be extra-embryonic, whereas cells biopsied from cleavage stage embryos have both embryonic and extra-embryonic potential.

Although pregnancy rates after uterine lavage are high, uterine lavage does have difficulties which must be resolved.
1. The rate of recovery of embryos is very poor, suggesting that uterine lavage following normal conception may not be the optimal source of embryos for biopsy, for example due to natural embryo wastage. Although ovarian hyperstimulation prior to lavage was suggested as an approach to increase the number of embryos recovered from a single attempt, results in hyperstimulated cycles have been disappointing\textsuperscript{12}.
2. Retained embryos which are not flushed out may result in pregnancies. As such pregnancies have not been tested for genetic defects, this is contradictory to the use of PGD.
3. Infections have been observed\textsuperscript{13}.
4. Fluid may be lost in the tubes during lavage; therefore, there is the theoretical risk of salpingitis and ectopic pregnancy.

If uterine lavage can be developed to be sufficiently safe and efficient, it could prove to be an alternative method for IVF for those couples requiring early testing of genetic disease. This would allow early genetic screening to become much more wide-spread, particularly for older mothers who are at higher risk from having a child with a chromosomal defect.

Although uterine lavage appears promising, it has not been widely adopted either for PGD or for the collection of embryos for research.
Pre-implantation genetic diagnosis

**Blastomere replication**
The principle behind blastomere replication is that a single blastomere can be biopsied from an embryo, the blastomere can be cultured *in vitro* allowing several cell divisions thus providing a much larger sample for either repeated testing or analysis of multiple disorders.

Biopsied mouse and human blastomeres have been cultured using a variety of substrates and culture conditions in an attempt to stimulate replication. Unfortunately, studies examining the replicative potential of single blastomeres from human embryos have had only limited success\(^7\)-\(^10\).

However, the major difficulty of using blastomere replication for PGD is the 2–3 days' delay waiting for the blastomeres to replicate, since embryo transfer is normally performed the same day as embryo biopsy. Although this difficulty could be resolved, at least in part, by cryopreserving the biopsied embryos for several days, this approach has the disadvantage that only \(\sim60–70\%\) of embryos survive the freeze-thawing process – a further reduction in the very limited number of embryos available for transfer after IVF and PGD.

**Current diagnostic methods**

The number of diagnostic techniques that can be used for PGD is restricted by three main factors:

1. The small number of cells in embryos. This not only making embryo biopsy difficult, but also eliminates the possibility of repeat sampling.

2. The small numbers of cells that can be biopsied without seriously affecting the viability and development of the embryos. Often only single cells can be removed.

3. Significant difficulties of analysing genetic material at the very extreme limits of sensitivity.

Despite these difficulties, a number of methods have been attempted.

**Karyotype analysis**

Karyotype analysis for chromosomal abnormalities is a well-established diagnostic method, usually performed on chorionic villus and amniocentesis samples. The advantages of a cytogenetic approach for PGD would have enormous benefits as karyotype information would allow selection of only those embryos without chromosomal abnormalities, therefore, increasing pregnancy rates and decreasing miscarriage rates.

Although karyotyping generally requires large numbers of cells, metaphase spreads from human 8-cell embryos have been examined\(^14\),\(^15\).
However, karyotyping of human pre-implantation embryos and oocytes has proved to be very difficult for the following reasons:

1. Obtaining chromosomal preparations from single embryonic cells is still difficult and involves high-quality equipment and reagents as well as considerable technical skill.

2. The embryo's chromosomes are short and often overlapping making complete visualisation, and thus diagnosis, extremely difficult.

3. Interpretation of the results can also be difficult because of the occurrence of chromosomal mosaicism. To detect chromosomal mosaicism, more than one metaphase per embryo must be available. However, individual blastomere division may not be synchronised and removal of two or more blastomeres may not always help in diagnosing mosaicism.

It is, therefore, unlikely that pre-implantation diagnosis by karyotype analysis would be reliable, mainly because of poor quality chromosome preparations or loss of chromosomes during spreading. However, many problems still remain to be solved and much effort is needed before cytogenetic diagnosis can be used for routine PGD.

**Polymerase chain reaction (PCR)**

In 1985, a procedure known as polymerase chain reaction (PCR) was described\(^\text{17}\) that allowed the amplification of specific DNA sequences \textit{in vitro} using DNA polymerase and short oligonucleotide primers flanking the DNA region of interest. Repeated cycles of enzymatic amplification were increase the quantity of the target DNA fragment many millions of times. PCR has two major advantages for PGD:

1. **Sensitivity.** It is possible to detect a single copy sequence in a single cell or a few cells. A variety of sequences have been amplified from single cells, including human sperm, fibroblasts, human unfertilised oocytes and polar bodies\(^\text{2,3,18,19}\).

2. **Speed.** Specific DNA fragments to be amplified within a few hours so that analysis can be completed within the time available for transfer later the same day.

**Conventional PCR**

The first human PGD used PCR for both embryo sexing\(^\text{2}\) and autosomal recessive diseases\(^\text{3}\). Sexing was performed by removing a blastomere from the embryo, this cell then underwent PCR amplification with Y-chromosome specific primers. Although this approach resulted in the birth of several normal girls, a misdiagnosis was reported when a lack of signal was misinterpreted as being from a female cell rather than from amplification failure\(^\text{20}\).
Pre-implantation genetic diagnosis

This misdiagnosis emphasised the disadvantage of reliance on a single probe PCR system. Several methods were then used to minimise the possibility of misdiagnosis, including both modifications of the PCR technique itself such as fluorescent PCR, primer extension pre-amplification (PEP), as well as alternative techniques such as fluorescent \textit{in situ} hybridisation (FISH).

**Fluorescent PCR**

In the last 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\textsuperscript{21} which improves both the accuracy and sensitivity of PCR\textsuperscript{22,23}.

Fluorescent PCR is very similar to conventional PCR except that each primer is tagged with a fluorescent dye. The resulting fluorescent product is then electrophoresed on a gel passing a scanning laser beam, which makes the product fluoresce. Using a photomultiplier and computer enhancement technology, the fluorescent dye is detected at a much lower threshold level than conventional agarose or acrylamide gel analysis. Fluorescent PCR is highly sensitive being approximately 1000 times more sensitive than conventional gel analysis\textsuperscript{24}, which allows the detection of a signal far below the threshold that can be obtained from conventional methods. This results in highly accurate and reliable detection even when the signal is very weak or much lower (< 1\%) than that of the other allele.

A further advantage of fluorescent PCR is that several primers can be multiplexed together since different fluorescent dyes can be simultaneously determined even if the product ranges overlap each other\textsuperscript{23}. These different coloured dyes allow identification of one product from the others even if the product sizes are within 1–2 bp of each other. This method has been applied to multiplexing as many as 15 sets of primers although high amounts of DNA are required.

Fluorescent PCR has already been successfully applied to genetic screening for cystic fibrosis\textsuperscript{25}, Down syndrome\textsuperscript{26}, muscular dystrophies\textsuperscript{27} and Lesch-Nyhan disease\textsuperscript{28} even at the single cell level\textsuperscript{29–31}.

As fluorescent PCR provides accurate quantitative measurements, it is possible to determine the product ratio of one allele relative to the other. These quantitative measurements allow difficulties of single cell PCR such as allelic drop-out and preferential amplification\textsuperscript{32} to be investigated.

**Primer extension pre-amplification (PEP)**

An alternative method that can be used for amplification of low copy numbers is whole genome amplification\textsuperscript{33,34} or more correctly termed...
primer extension pre-amplification (PEP). In this technique, the entire genome is theoretically amplified using PCR, thereby increasing the amount of template available from 1 copy to 50–100 copies for subsequent PCR reactions. PEP can be viewed as essentially a pre-diagnostic PCR treatment because the PEP product is then used in a further PCR to diagnose the specific defect. Many aliquots can be removed from the reaction mix and multiple diagnoses can be subsequently run in parallel. Unfortunately PEP has two major disadvantages:

1. The time taken for the diagnosis, usually 8–12 h for the PEP and the subsequent ~5–10 h for the diagnostic PCR and analysis, makes it very difficult to perform embryo transfer the same day.

2. Although the whole genome can be amplified, the amplification appears to be random and, in practice, only ~80% of the genome is effectively amplified. The remaining 20% of the genome, which may contain the gene of interest, may not be amplified at all. This is a particular problem in heterozygotes and may result in misdiagnosis when one allele is amongst the 80% that amplifies and the other is amongst the 20% not amplified – resulting in a carrier being misdiagnosed as affected or, arguably, even worse as unaffected.

Although very promising as a technique, these disadvantages make PEP unsuitable for clinical PGD.

**Fluorescent in situ hybridisation (FISH)**

By 1988, FISH had become established for prenatal diagnosis and was soon applied to single human blastomeres\(^3^5\). To ensure reliability, a dual technique with simultaneous hybridisation of a biotinylated X probe and two digoxigenin labelled Y probes, to guard against Y hybridisation failure, was developed\(^3^6\). Since then, FISH has been adopted by most PGD centres world-wide as the method of choice for the diagnosis of sex and for the detection of aneuploidies. The application of FISH for PGD has also identified that chromosomal mosaicism is common at the cleavage stage of development\(^3^7\). A finding that has very important implications for diagnosis of dominant single gene disorders, monosomies and trisomies as well as for our understanding of early human development.

However, there are several limitations to the use of FISH at the single cell level:

1. Cost. FISH probes are expensive resulting in cost of ~$300–400 per sample.

2. FISH is generally limited to diagnosis at the chromosomal rather than the single-gene level. Additional tests, e.g. PCR, are required for single gene defects (such as cystic fibrosis).
3 The loss of nuclei when spreading single blastomeres. This may result in diagnosis of disomic blastomeres as monosomy significantly reducing the number of embryos available for transfer.

4 Misdiagnosis. Recent reports using multiple FISH probes have demonstrated that as many as 21% of single cell diagnoses made by FISH are incorrect. This would lead to significant decrease in embryos available for transfer and thus a decrease in pregnancy rates.

5 Very specific testing. Analysis is often limited to only 5 chromosomes (13, 18, 21, X and Y) due to a limited number of fluorochromes. Additional probes (with significant additional costs) are required for further testing.

6 Results can be difficult to interpret due to signal overlap. Accurate interpretation requires the availability of many cells for analysis.

7 FISH cannot detect maternal contamination. There is, therefore, a potential for misdiagnosis.

8 Low through-put/highly labour intensive. FISH is generally limited to processing < 20 samples/person/day.

Although single-cell FISH can have a relatively low reliability rate ranging from 70–95% and be difficult to interpret, reported accuracy can be relatively high (> 90%)\(^{20,39}\). Recently though, even the accuracy of FISH has been brought into some doubt as recent reports\(^{38}\) from large centres have demonstrated that FISH can have misdiagnosis rates as high as 21% from single cells in a clinical PGD setting.

**Developing techniques**

**Multiplex PCR**

Multiplex PCR involves a PCR containing several different primer sets in the hope that each primer set will amplify sufficiently so that simultaneous detection can be performed. This can facilitate the diagnosis of either specific genetic disease or multiple diseases as it can provide information for many loci at the same time. However, multiplex PCR is a very sophisticated technique that requires very exacting reaction conditions that permit equal amplification of all fragments; careful design of the primers so that artefacts formed between the primers and non-specific amplification are avoided; and generation of products of different size to facilitate analysis.

An other alternative method, which is both rapid and inexpensive, is quantitative PCR using polymorphic STRs\(^{40}\). Quantitative PCR accurately determines the amount of PCR product from each allele permitting the ratio of product quantity between alleles to be calculated thus detecting aneuploidy status. Even though quantitative PCR was first
described almost 7 years ago, there were few reports in prenatal diagnosis\textsuperscript{41}. More recently, a modification of quantitative PCR known as multiplex fluorescent PCR (MF-PCR) has demonstrated the feasibility of using quantitative PCR in clinical prenatal diagnosis\textsuperscript{29,32,42-45} even though several thousand cells are still required. Until recently, it was not possible to use these techniques to diagnose trisomies at or near the single cell level, although very promising results have now been published\textsuperscript{42,44}.

More recently multiple fluorescent PCR techniques, used to detect aneuploidies in amniotic samples, have been adapted to provide diagnosis of chromosomal aneuploidies in single cells. This provides simultaneous amplification of multiple markers for each chromosome, therefore, minimising the risk of misdiagnosis from allele dropout as well as confirming diagnosis\textsuperscript{42}. A further advantage of using multiple markers for each chromosome is that the effects of allele drop-out will be minimised, as it is extremely unlikely that each marker would suffer from allelic dropout simultaneously.

Multiplex fluorescent PCR testing has the following advantages:

1. Rapid. Same day (within 5 h) detection of major abnormalities. Rapid patient re-assurance and rapid assessment.
2. Safer. A single blastomere can be used to diagnose multiple genetic defects as well as chromosome screening.
3. Inexpensive. Low cost could allow resources to be used more efficiently.
4. A single technique to detects most major defects (sex, cystic fibrosis, major trisomies).
5. Low sample size (< 1 ml) could make prenatal diagnosis safer.
6. DNA fingerprinting would exclude problems related to maternal contamination.
7. Simultaneous diagnosis and confirmation of multiple chromosomes. For example, 21, 18, 13, X and Y (Fig. 2) unlike other techniques.
8. DNA fingerprinting can confirm embryonic origin (rather than contamination), unlike other techniques.
9. Reliability and accuracy of fluorescent PCR equal or exceed other techniques.
10. Can provide additional information, for example the parental origin of the additional chromosome in trisomies when compared with maternal and paternal DNA.
11. Much less labour intensive than FISH/PRINS allowing high through-put using DNA sequencer. Can process more than 550 samples per 8 h day compared to 20–30 with FISH/PRINS.

Although several groups, have demonstrated that MF-PCR is an ideal marker system for providing rapid, wide-ranging diagnoses as well as the feasibility of using MF-PCR for prenatal diagnosis, large scale prospective
Table 1 Comparison of techniques at the single cell level

<table>
<thead>
<tr>
<th>Method</th>
<th>Reliability</th>
<th>Accuracy</th>
<th>Method of choice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sexing</td>
<td>94%</td>
<td>97%</td>
<td>F-PCR &amp; FISH</td>
</tr>
<tr>
<td></td>
<td>(329/349)</td>
<td>(320/329)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>93%</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(93/100)</td>
<td>(89/93)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91%</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(91/100)</td>
<td>(23/91)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>79%</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(19/23)</td>
<td>(17/19)</td>
<td></td>
</tr>
<tr>
<td>CF diagnosis from heterozygous cells</td>
<td>96%</td>
<td>97%</td>
<td>F-PCR</td>
</tr>
<tr>
<td></td>
<td>(157/163)</td>
<td>(150/157)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44%</td>
<td>74%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(35/80)</td>
<td>(26/35)</td>
<td></td>
</tr>
<tr>
<td>Chromosome 21 status (F-PCR (singleplex))</td>
<td>87%</td>
<td>90%</td>
<td>F-PCR</td>
</tr>
<tr>
<td></td>
<td>(206/238)</td>
<td>(185/206)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(53/59)</td>
<td>(16/53)</td>
<td></td>
</tr>
<tr>
<td>DNA fingerprinting (F-PCR)</td>
<td>96%</td>
<td>100%</td>
<td>F-PCR</td>
</tr>
<tr>
<td></td>
<td>(144/149)</td>
<td>(144/149)</td>
<td></td>
</tr>
</tbody>
</table>

Overall reliability and accuracy for a variety of techniques used in single cell genetic diagnosis. In almost all cases, the reliability and accuracy rates of fluorescent PCR are higher than that of other techniques tested. Fluorescent PCR is, therefore, a method of choice in each case. PRINS data previously published.33

Studies have not yet been performed. Detection rates, reliability and accuracy (false negative/false positive rates) must be formally measured in large scale prospective studies so that prospective parents can make informed reproductive choices based on adequate information.

These results (Table 1) demonstrate that fluorescent PCR is a multipurpose technique that can diagnose each of the three main disorder types (sexing, single gene defects and trisomies). In addition, fluorescent PCR has many additional advantages over other techniques.

![Fig. 2](image_url)

**Fig. 2** Multiplex PCR (8-plex) of a single cell. Each peak indicates a fluorescent marker. Two markers for sex (sex), two for chromosome 21 (Ch 21), two for chromosome 18 (Ch 18) and two for chromosome 13 (Ch 13).
Even though recent evidence suggests that screening for the major chromosomal aneuploidies can increase pregnancy rates and decrease miscarriage rates, the limited number of available fluorochromes severely limits the number of chromosomes that can be tested. The other restrictions imposed by FISH such as high cost and low-volume currently restricts PGD to an extremely small group of patients, those who are at high risk of genetic disorder due to previous history or maternal age.

However, if a low cost, high through-put system, such as multiplex fluorescent PCR, were used, the implications for PGD and IVF are enormous since PGD could potentially be offered to all patients regardless of risk. This is currently not feasible using FISH. For example, an IVF unit performing 1000 cycles per year (25 patients per 40 working-week year) with 10 eggs/embryos per patient each with 2 cells (polar bodies or blastomeres) to be analysed would require $25 \times 10 \times 2 = 500$ tests per week – well in excess of the 10–20 sample daily (50–100 weekly) capability of FISH. These weekly 500 samples could be easily accommodated using a fluorescent PCR system capable of performing $> 2750$ (550 x 5) PCR samples per week. Such a system, resulting in transfer of single embryos, could result in significantly increased pregnancy rates, decreased miscarriage rates and decreased multiple birth rates.

Other new and potentially very exciting techniques such as SNP (single nucleotide polymorphisms) analysis, microarrays and SKY (spectral karyotyping) are on the horizon and are currently being developed but as yet have yet to be applied to human PGD.

References

1 Trounson AO, Calabrese R. Changes in plasma progesterone concentrations around the time of the luteinizing hormone surge in women superovulated for in vitro fertilization. / Clin Endocrinol Metab 1984; 59: 1073–80
6 Muggleton-Harris AL, Glazier AM, Pickering S, Wall M. Genetic diagnosis using polymerase chain-reaction and fluorescent in-situ hybridization analysis of biopsied cells from both the cleavage and blastocyst stages of individual cultured human preimplantation embryos. Hum Reprod 1995; 10. 183–92
8 Pickering SJ, Muggleton-Harris AL. Reliability and accuracy of polymerase chain-reaction amplification of 2 unique target sequences from biopsies of cleavage-stage and blastocyst-stage human embryos. Hum Reprod 1995; 10. 1021–9
Pre-implantation genetic diagnosis

15 Tracy TE, Mulcahy LS. A simple method for direct automated sequencing of PCR fragments. Biotechniques 1991; 1: 68-75
22 Findlay I, Matthews P, Toth T, Quirke P, Papp Z. Same day diagnosis of Downs syndrome and sex in single cells using multiplex fluorescent PCR. Mol Pathol 1998; 51: 164-8
41 Adinolfi M, Sherlock J, Pertl B. Rapid detection of selected aneuploidies by quantitative fluorescent PCR. *Bioessays* 1995; 17: 661-4