Preimplantation genetic diagnosis (PGD) has experienced a considerable technical evolution since its first application in the early 1990s. The technology for single-cell genetic analysis has reached an extremely high level of accuracy and enabled the possibility of performing multiple diagnoses from one cell. Diagnosis of a monogenic disease can now be combined with aneuploidy screening, human leukocyte antigen typing, and DNA fingerprinting. New technologies such as microarrays are opening the way for an increasing number of serious genetic defects to be detected in preimplantation embryos. The new PGD techniques will empower patients and clinicians to screen for almost any kind of genetic problem in embryos, with the potential to change completely the manner in which parents approach and manage genetic disease.

### Abstract

Preimplantation genetic diagnosis (PGD) has been introduced as an alternative to prenatal diagnosis to increase the options available for fertile couples who have a known genetically transmittable disease. Its intended goal is to reduce a couple’s risk of transmitting a genetic disorder significantly by diagnosing a specific genetic disease in oocytes or early human embryos that have been cultured in vitro before a clinical pregnancy has been established.1,2

Following its first application in 1990,3 PGD has become an important complement to the presently available approaches for the prevention of genetic disorders and an established clinical option in reproductive medicine. The range of genetic defects that can be diagnosed includes not only single-gene disorders4–7 but also structural chromosomal abnormalities such as reciprocal or Robertsonian translocations.8 The scope of PGD has also been extended to improve in vitro fertilization (IVF) success for infertile couples, by screening embryos for common or age-related chromosomal aneuploidies in patients who are deemed to be at increased risk, such as patients of advanced maternal age and those having a history of repeated miscarriage.9,10 More recently, PGD has been used not only to diagnose and avoid genetic disorders, but also to screen out embryos carrying a mutation predisposing to cancer or to a late-onset disease. Additionally, PGD permits selection for certain characteristics, such as human leukocyte antigen (HLA) matching for tissue type with the ultimate aim of recovering compatible stem cells from cord blood at birth for transplantation to an existing sick sibling.11–14

### Keywords

- PGD
- PGS
- minisequencing
- array-CGH
- WGA

### Stages for Single-Cell Testing

There are several stages during preimplantation development at which genetic testing can be performed. PGD is usually performed by testing single blastomeres removed from day 3 cleavage stage embryos (six to eight cells).

An alternative approach is represented by testing the first polar body (1PB) before oocyte fertilization (so-called preconception genetic diagnosis)15 or sequential analysis of both first and second (2PB) PBs,16 which are by-products of female meiosis as oocytes complete maturation upon fertilization. In women who are carriers for a genetic disease, genetic analysis of 1PB and 2PB allows the identification of oocytes that contain the maternal unaffected gene. Analysis of PBs might be considered an ethically preferable way to perform PGD for couples with moral objections to any micromanipulation and the potential discard of affected embryos.15,17 It may also be an acceptable alternative for countries in which genetic testing of embryos is prohibited.15,18 However, PB analysis is labor intensive because all oocytes must be tested despite the fact that a significant number will not fertilize or will fail to form normal embryos suitable for transfer. Furthermore, it cannot be used for conditions in which the male partner carries the genetic disorder because only the maternal genetic contribution can be studied.

Single cells for genetic analysis may also be obtained from the embryo at the blastocyst stage of development on day 5 or 6 after fertilization.19 Biopsy at this stage has the advantage of

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allowing more cells to be sampled (5 to 10 cells), making genetic tests more robust.

Diagnostic Methods

PGD tests have largely focused on two methodologies: fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR).

Methods Based on Polymerase Chain Reaction

PCR-based methodologies has been mainly used to diagnose single-gene disorders in single cells biopsied from embryos of couples at risk of transmitting a monogenic disease (autosomal recessive, autosomal dominant, or X-linked) to their offspring.

Almost all genetically inherited conditions diagnosed prenatally can also be detected with this approach. It can theoretically be performed for any genetic disease with an identifiable gene. Diagnostic protocols now exist for >200 monogenic disorders.4–7

Originally, X-linked diseases were avoided by selection of female embryos, by using the FISH procedure to identify embryos with two X chromosomes.20 A disadvantage of this approach is that half of the discarded male embryos will be healthy, a fact that gives rise to ethical criticism and reduces the chances of pregnancy by depleting the number of embryos suitable for transfer. In addition, half of the female embryos transferred are carriers of the condition. In several X-linked dominant disorders (e.g., fragile X syndrome), there is also the possibility that, to a varying degree, carrier females may manifest the disease. For many X-linked diseases, the specific genetic defect has now been identified allowing a specific DNA diagnosis. Therefore, there is now a consensus that it is preferable to use PCR-based tests for sex-linked disorders for which the causative gene is known, instead of performing sex selection.21

In PGD for single-gene defects, the disease-linked locus is amplified from blastomere DNA using targeted primers designed specifically for the mutation of interest.1,2,7,22–24 Amplified fragments of DNA can be then analyzed with different strategies to detect the disease-causing mutation(s).

Numerous variants of PCR-based protocols have been assayed at the single-cell level: procedures such as restriction enzyme digestion,25–27 single-stranded conformational polymorphism,28,29 denaturing gradient gel electrophoresis,30 and allele-specific amplification.31,32 Real-time PCR33,34 has been used to detect specific mutations.

The strategies just cited have been gradually replaced by minisequencing,23 a technique based on a single dideoxynucleotide primer extension that permits a quick and accurate detection of point mutations, as well as small deletions or duplications. The minisequencing primer is designed to anneal one base before the target site, and it will be elongated with only one dideoxynucleotide. The four different dideoxynucleotides are labeled with different fluorochromes, and the products can be distinguished on an automated DNA sequencing system. This technique is very versatile and has been broadly used in mutation and single nucleotide polymorphism (SNP) detection21,22,23 (Fig. 1).

Multiplex Fluorescent Polymerase Chain Reaction

The pioneering early PGD studies used nonfluorescent nested PCR and gel electrophoresis to look for the causative mutation(s). A major step forward came with the introduction of fluorescent PCR (F-PCR),24 which has become the gold standard in PGD for monogenic disorders, permitting the development of sophisticated and sensitive PGD assays. F-PCR is based on the 5’ labeling of one of the primers with a fluorochrome and the analysis of the PCR products on an automated sequencer. This system provides a very accurate molecular weight determination that is >1000 times more sensitive than the analysis on ethidium bromide-stained gels. In addition, F-PCR opened the way to semiautomated analysis using capillary electrophoresis systems and made possible the multiplexing of different primer pairs in a single PCR reaction (multiplex fluorescent PCR[MF-PCR]), enabling the simultaneous assessment of numerous loci.

The introduction of MF-PCR24 allowed testing of a panel of short tandem repeat (STR) polymorphic-linked markers in addition to direct mutation analysis. A MF-PCR protocol includes at least two polymorphic markers located very close to, or within (i.e., linked), the gene region surrounding the disease causing the mutation(s). The study of the DNA of the patients provides a priori knowledge of which alleles are to be expected in the embryos, as well as on which alleles the markers co-segregate with the mutation (Fig. 2).

This approach substantially improved the robustness of the PGD protocols and decreased the possibility of misdiagnosis enabling allele dropout (ADO)15 and contamination detection.

The ADO phenomenon consists of the random nonamplification of one of the alleles present in a heterozygous sample. ADO seriously compromises the reliability of PGD for single-gene disorders (SGDs) because a heterozygous embryo could potentially be diagnosed as either homozygous affected (in which case it would be lost from the cohort of available embryos) or homozygous normal (and therefore suitable for replacement) depending on which allele would fail to amplify. This is particularly concerning in PGD for autosomal dominant disorders, where ADO of the affected allele could lead to the transfer of an affected embryo.

MF-PCR obviates the ADO problem. In fact, determination of the specific STR haplotype associated with the mutation acts both as a diagnostic tool for indirect mutation analysis, providing an additional confirmation of the results obtained with the direct genotyping procedure, and as a control of misdiagnosis due to undetected ADO. Diagnosis is assigned only when haplotype profiles obtained from linked STR markers and mutation analysis profiles are concordant.

The multiplex STR markers system also provides an additional control for contamination with exogenous DNA because other alleles, differing in size from those of the parents, would be detected. The partial “fingerprint” of the embryo obtained with the MF-PCR strategy provides an added assurance, confirming that the amplified fragment is of embryonic origin. Furthermore, the polymorphic nature of STR markers also permits the detection of haploidy and uniparental disomy (UPD).
The experience of a large series of PGD cycles strongly suggests that PGD protocols for a SGD are not appropriate for clinical practice without including a set of linked STR markers. Consequently, this strategy is currently used by most PGD laboratories.

Indirect Testing by Linkage Analysis
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A further advantage of MF-PCR is the possibility of its use as a tool for indirect mutation analysis in linkage-based protocols. In fact, any informative polymorphism that lies in close proximity to the disease locus can be used as a tool to indicate the presence or absence of the mutation without its direct detection.

For disorders that are mostly caused by private mutations, the development of multiplex PCRs exclusively for linked markers has the advantage that they can be used for several couples because it omits the detection of the (private) mutation. However, the ability to use such indirect testing with linked markers depends on the availability of appropriate family samples to determine which chromosome carries the disease-causing mutation (s) (determination of phase). Nevertheless, the candidate couples must be informative for at least two of the markers included in the PCR. Moreover, the markers should be flanking each side of the mutation to be able to detect recombination.

For some couples, the disease-causing mutation may have arisen de novo in the individual. In this case, the couple should at least have had a child or a pregnancy from which DNA of the fetus is available. However, it is possible for the phase of paternal alleles to be determined by performing single sperm analysis; for maternal alleles, PB analysis can be performed, although this requires a cycle to be initiated prior to the determination of phase.

Nevertheless, the advantages of this approach largely compensate for the possible drawbacks, and the use of multiplex PCR for markers has become widespread in PGD for monogenic disorders, HLA typing, or aneuploidy screening.

Exclusion Testing
Exclusion Testing

Linkage analysis has also been used for exclusion testing for late-onset Huntington’s disease (HD), where someone with a family history of HD does not wish to know their HD status but wishes to ensure that their children are not at risk.
Exclusion testing is based on a linkage analysis PGD protocol involving the use of polymorphic markers flanking the HD gene. This strategy allows tracking of the parental and grandparental origin of the chromosomes and can subsequently be used to identify the low-risk (grandparental) allele in derived offspring (Fig. 3). In this way, only embryos are replaced that do not contain the chromosome 4 derived from the affected grandparent because inheritance of this chromosome confers a 50% risk of HD. This method can be used to classify embryos as at low or high risk for developing the disease (29 Jasper), avoiding the need to detect the mutation itself.

PGD for HD is also possible by direct testing (i.e., evaluating the presence or absence of the expanded repeat) or by linkage-based indirect testing (i.e., ascertaining the presence or absence of the haplotype associated with triplet expansion). Another way of testing for HD is the so-called nondisclosure PGD that entails direct testing of the expanded repeat on the embryos without knowing the disease status. It is applied in those cases in which the prospective parent at risk does not wish to be informed about his or her own carrier status but wants to have offspring free of the disease (29,44). Embryos can be tested for the presence of the mutation without revealing any of the details of the cycle or diagnosis to the prospective parents. Nondisclosure testing is controversial and professional generally do not approve it (42,45) because it puts practitioners in an ethically difficult position, that is, when no embryos are available for transfer and a mock transfer has to be performed to avoid the patient suspecting that he or she is a carrier or having to undertake PGD cycles even when the results of previous cycles preclude the patient being a carrier. The European Society of Human Reproduction and Embryology (ESHRE) ethics task force (41) currently discourages nondisclosure testing, recommending the use of exclusion testing instead.

Preimplantation Human Leukocyte Antigen Matching
Linkage-based approach has also been used for preimplantation HLA matching. (11–14) This strategy has revealed a useful tool for couples at risk of transmitting a genetic disease, allowing selection of unaffected embryos that are HLA tissue type compatible with those of an existing affected child. In such cases, PGD is used not only to avoid the birth of affected children but also to conceive healthy children who may also be potential HLA-identical donors of hematopoietic stem cells for transplantation to siblings with a life-threatening disorder.

From a technical point of view, PGD for HLA typing is a difficult procedure due to the extreme polymorphism of the HLA region. Taking into account the complexity of the region (presence of a large number of loci and alleles), the use of a direct HLA typing approach would require standardization of a PCR protocol specific for each family, presenting different HLA allele combinations and making it time consuming and unfeasible. The use of a preimplantation HLA matching protocol irrespective of the specific genotypes involved makes the procedure more straightforward.

Currently, PGD laboratories use a strategy based on a flexible indirect HLA typing protocol applicable to a wide spectrum of possible HLA genotypes (11–14). The approach involves the testing of single blastomeres by fluorescent multiplex PCR analysis of polymorphic STR markers,
scattered throughout the HLA complex of chromosome 6, obtaining a “fingerprint” of the entire HLA region (►Fig. 4).

STR haplotyping for family members (father, mother, and affected child) is performed prior to preimplantation HLA typing to identify the most informative STR markers of the HLA complex to be used in the following clinical PGD cycles. A panel of STR markers is studied during the setup phase to ensure sufficient informativity in all families. For each family, only heterozygous markers presenting alleles not shared by the parents are selected, so that segregation of each allele and discrimination of the four parental HLA haplotypes can be clearly determined. Informativity is also evaluated for STR markers linked to the gene regions involved by mutation and is thus used to avoid possible misdiagnosis due to the well-known ADO phenomena.

By selecting a consistent number of STR markers evenly spaced throughout the HLA complex, an accurate mapping of the whole region can be achieved. Because genes in the HLA complex are tightly linked and usually inherited in a block, profiles obtained from such markers in father, mother, and affected child allow the determination of specific haplotypes. Thus the HLA region can be indirectly typed by segregation analysis of the STR alleles, and the HLA identity of the embryos matching the affected sibling can be ascertained by evaluating the inheritance of the matching haplotypes. Because segregation of the STR alleles fully corresponds to the direct HLA genotyping, STR haplotyping can be used as a reliable diagnostic tool for indirect HLA matching evaluation. The use of microsatellite markers for this purpose is very useful. They may provide information on identity over a greater distance within the HLA region compared with classical HLA genes alone, making haplotyping more accurate in predicting compatibility. Another important advantage of using STR markers in preimplantation HLA matching is that the whole HLA complex can be covered, which allows the detection of recombination events between HLA genes (►Fig. 4).

Preimplantation Genetic Diagnosis Using Whole Genome Amplification

One of the most exciting developments in single-cell analysis has been the introduction of protocols designed to amplify the entire genome from a single cell (whole genome amplification [WGA]). The idea was using WGA as a universal first step to enable secondary analysis of a range of sequences without the need to optimize primers and reaction conditions for multiplexing, also overcoming the difficulties of testing single cells.

A recent important evolution of these WGA protocols is multiple displacement amplification (MDA), a technique that may offer greater accuracy and more rapid throughput in the future. Amplification of DNA for a single biopsied blastomere gives ∼10^6-fold amplification. Aliquots of MDA products can be taken and used as a source of templates for subsequent locus-specific PCRs, allowing many individual DNA sequences or genes to be analyzed in the same cell. The principal advantage of MDA for PGD is that sufficient amplified DNA is produced to allow extensive parallel genetic testing and accurate mutation detection by conventional relatively low sensitivity methods. A further advantage is that WGA provides a sufficient supply of sample DNA available for other applications, simplifying the combination of different indications in one PGD cycle, such as combining mutation with aneuploidy screening by array CGH technique.
or HLA haplotyping. It could therefore be envisaged that in the future every embryo tested for a monogenic disorder would also be routinely screened for aneuploidy, investigating all chromosomes.

The main limitations of using this method on single cells are the high ADO and preferential amplification rates compared with direct PCR on DNA from a single cell. In fact, PCR on MDA products gives ADO rates of between 5% and 31%, which is unacceptable for direct mutation testing, due to the likelihood of exclusion of embryos from transfer following inconclusive results. Nevertheless, MDA has been used clinically in PGD of cystic fibrosis, β-thalassemia, and fragile X syndrome. However, this approach has not been taken further owing to the effect of high ADO on the reliability of results.

Limitations of the PCR-Based Preimplantation Genetic Diagnosis Protocols
Genetic diagnosis of single cells is technically demanding, and protocols have to be stringently standardized before clinical application.

To establish a diagnostic PGD protocol, extensive preclinical experiments are performed on single cells (lymphocytes, fibroblasts, cheek cells, or spare blastomeres from research embryos) to evaluate the efficiency and reliability of the procedure.

Many genetic disorders can now be diagnosed using DNA from single cells. However, when using PCR in PGD, one is faced with a problem that is nonexistent in routine genetic analysis, namely the minute amounts of available genomic DNA. In fact, because PGD is performed on single cells, PCR has to be adapted and pushed to its physical limits. This entails a long process of fine-tuning the PCR conditions to optimize and validate the PGD protocol before clinical application.

Three main difficulties are inherently associated with single-cell DNA amplification. The limited amount of template makes single-cell PCR very sensitive to contamination. The presence of extraneous DNA can easily lead to a misdiagnosis in clinical PGD. Cellular DNA from excess sperm or maternal cumulus cells that surround the oocyte is a potential source of contamination. These cells can be sampled accidentally during the biopsy procedure. For these reasons oocytes used for PGD of single-gene defects should always be stripped of their cumulus cells and fertilized by the use of intra-cytoplasmic sperm injection (ICSI) in which only a single sperm is inserted into the oocyte. Furthermore, any biopsied cells should be washed through a series of droplets of medium before transfer to the PCR tube, and the wash drop should be tested for contamination.

Other sources of contamination include skin cells from the operators performing the IVF/PGD procedure or so-called carryover contamination of previous PCR products, but this type of contamination can be minimized or eliminated by following the strict guidelines prescribed by the ESHRE PGD Consortium.

Another problem specific to single-cell PCR is represented by the previously mentioned ADO phenomenon, which is
particularly concerning in PGD for autosomal dominant disorders, where ADO of the affected allele could lead to the transfer of an affected embryo.

**Fluorescent in Situ Hybridization Methods**

FISH is a cytogenetic technique that uses a set of DNA probes labeled with distinctly colored fluorochromes that bind to specific DNA sequences unique to each chromosome. Imaging systems enable the fluorescent probe signals to be identified and counted to detect missing or excess chromosomal material (Fig. 5).

**Fish-Based Preimplantation Genetic Diagnosis for Structural Chromosomal Abnormalities**

FISH, to date, is the most widely used method for detecting unbalanced chromosome rearrangements in embryos produced from carriers of structural chromosomal abnormalities, such as reciprocal and Robertsonian translocations, chromosomal deletions, or duplications. This method requires the PBs or biopsied cells to be spread on a microscope slide and then hybridized to chromosome-specific DNA probes.

A commonly used FISH strategy for detection of the abnormal segregation in reciprocal and Robertsonian translocations involves the use of commercially available centromeric, locus-specific, and subtelomere probes, allowing for a simplified approach that laboratories can apply routinely.

FISH strategies for the assessment of reciprocal translocations use three differentially labeled probes: two probes specific for the subtelomeric regions of the translocated segments combined with a centromeric probe. Analysis of Robertsonian translocations is simpler, involving the use of specific probes chosen to bind at any point on the long arm of each chromosome involved in the translocation (Fig. 5).
This combination of probes allows embryos that carry an unbalanced chromosome complement to be distinguished from healthy ones. However, both strategies do not discriminate between noncarrier embryos and those that carry the balanced form of the translocation.

Although relatively successful, the FISH procedure is technically demanding and harbors several technical limitations that are well documented and include hybridization failure (lack of FISH signals), signal overlap, signal splitting, and poor probe hybridization, as well as problems related with the fixation process, such as cell loss and variable cell fixation. Since FISH was first introduced in clinical diagnosis, improvements have been established to diminish the error rate of the technique. Technical skill and sound laboratory practices can minimize most of the limitations of FISH-based methods, but certain shortcomings remain. Interpretation errors due to the technical issues described previously can affect the accuracy of the interpretation of the results, leading to a misdiagnosis of embryos both in eliminating suitable (normal/balanced) embryos for transfer, or worse, including abnormal embryos in the transfer cohort errantly. Error rates of FISH protocols for translocation have been reported in some studies to range from 0% to 10%, with an average error rate of 6%. Overlapping signals may be a source of misdiagnosis resulting in the false diagnosis of monosomies. Signal splitting has also been described, resulting in the detection of false trisomies. Finally, evidence suggests that up to half of all embryos identified as aneuploid at the cleavage stage and survive to the blastocyst stage will “self-correct.” Therefore an abnormal result may not necessarily indicate that the embryo is abnormal and ill-fated.

As a consequence of the limitations just described, it has been questioned whether some normal embryos might be excluded from the cohort that is considered suitable for embryo transfer, which, especially in older women who might have small embryo cohorts, could result in the failure to reach embryo transfer.

Since the publication of the first articles on PGS using cleavage-stage embryos and PEs, there have been several nonrandomized comparative studies of IVF/ICSI with or without PGS for advanced maternal age or repeated implantation failure. Most of these studies reported that PGS increases the implantation rate, decreases the abortion rate, and reduces trisomic conceptions. There are now 11 randomized controlled trials (RCTs) published on PGS, 10 using cleavage-stage biopsy and one using blastocyst biopsy. All have used FISH testing of a limited number of chromosomes, and none have shown an improvement in delivery rates, with some showing a significant decrease in delivery rates after PGS. Most of the RCTs have been for patients of advanced maternal age. These RCTs have shown that PGS, as it was practiced, has not provided the expected benefits. There are many possible reasons why these clinical studies failed to deliver the expected improvements in IVF outcome. Putative explanations for this poor clinical performance could be attributed to an incomplete understanding of important aspects of embryo biology, such as embryonic chromosomal mosaicism, which is present on day 3 of development (i.e., the tested blastomere is not representative of the whole embryo) or self-correction of aneuploidy within the embryo, which may decrease the chances of a live birth by prematurely labeling an embryo.

Detection of Chromosomal Aneuploidy by Fish

FISH is also able to detect aneuploidy in single blastomeres, and it was the most common method for aneuploidy screening of embryos derived from subfertile patients undergoing IVF (preimplantation genetic screening [PGS]). PGS enables the assessment of the numerical chromosomal constitution of embryos. It aims to identify and select for transfer only chromosomally normal (euploid) embryos to increase the implantation and pregnancy rate for IVF patients, lower the risk for miscarriage, and reduce the risk of having an infant with an aneuploidy condition.

Essentially, FISH probes to detect those aneuploidies most commonly observed after birth or in miscarriages (involving detection of chromosomes X, Y, 13, 16, 18, 21, and 22) were used for the purpose of PGS. This panel of probes has the potential of detecting >70% of the aneuploidies found in spontaneous abortions. Aneuploidy conditions (involving chromosomes 8, 9, 15, and 17) that cause lack of implantation or can result in a miscarriage early in pregnancy have also been tested.

However, FISH-based PGS allows identification of aneuploidies only for a limited number of chromosomes (5, 9, or 12 chromosomes). Commercial FISH probe panels are available that will identify five to six chromosomes at a time by different colors (fluorochromes). The number of chromosomes that can be tested at any one time is limited by the range of fluorochromes available and the eye’s ability to distinguish between different colors. The numbers of chromosomes investigated may be increased by washing off the first panel of probes and rehybridizing with an additional panel of probes, but this increases the time taken to arrive at a diagnosis. Rehybridization can only be performed with any clinical accuracy once, which therefore limits the number of chromosomes that can be analyzed.

Several studies have demonstrated that aneuploidies may involve all 24 chromosomes. Therefore FISH analysis may result in the transfer of reproductively incompetent embryos with aneuploidy present in the chromosomes not analyzed.

PGS currently has several further disadvantages that limit its clinical value. The main concern is the elevated mosaicism rate observed in the human cleavage-stage embryo. Mosaicism is defined as the embryo having cells with different chromosome makeup, and it has been found in up to 57% of day 3 biopsied embryos. Mosaicism may represent a major source of misdiagnosis (60%) because of both false-positive and false-negative results.

Besides mosaicism, several technical limitations inherent to the FISH technique have been described. FISH is considered to have an error rate between 5% and 10% Overlapping signals may be a source of misdiagnosis resulting in the false diagnosis of monosomies. Signal splitting has also been described, resulting in the detection of false trisomies. Finally, evidence suggests that up to half of all embryos identified as aneuploid at the cleavage stage and survive to the blastocyst stage will “self-correct.” Therefore an abnormal result may not necessarily indicate that the embryo is abnormal and ill-fated.

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There are now 11 randomized controlled trials (RCTs) published on PGS, 10 using cleavage-stage biopsy and one using blastocyst biopsy. All have used FISH testing of a limited number of chromosomes, and none have shown an improvement in delivery rates, with some showing a significant decrease in delivery rates after PGS. Most of the RCTs have been for patients of advanced maternal age. These RCTs have shown that PGS, as it was practiced, has not provided the expected benefits. There are many possible reasons why these clinical studies failed to deliver the expected improvements in IVF outcome. Putative explanations for this poor clinical performance could be attributed to an incomplete understanding of important aspects of embryo biology, such as embryonic chromosomal mosaicism, which is present on day 3 of development (i.e., the tested blastomere is not representative of the whole embryo).
as abnormal. Indeed, high levels of chromosomal mosaicism have been observed in blastomeres from cleavage-stage embryos.\textsuperscript{74,81,82,99}

Alternatively, it has been argued that inadequate cytogenetic methods may have led to reduced diagnostic accuracy and elimination of any potential benefit of screening.\textsuperscript{100} In fact, all prior clinical trials using PGS for IVF have used FISH, which screens for a minority of chromosomes (up to 12 chromosomes).\textsuperscript{87} Most FISH-based methods focus on the chromosomes most often found to be aneuploid in prenatal samples or material from miscarriages. However, these chromosomes are not necessarily the most relevant for early embryos. Thus the limitations of the FISH technology could have compromised the results of these studies.\textsuperscript{101,102}

**Emerging Technologies**

Increasingly, new techniques for chromosome analysis in embryos are being sought in an attempt to improve on current FISH test method performance and now are suitable for PGD/PGS use.

**MF-PCR in Preimplantation Genetic Diagnosis for Chromosomal Translocation**

Recently, a MF-PCR–based PGD approach for detection of chromosomal imbalances on embryos derived from both reciprocal and Robertsonian translocation carriers was proposed as a valuable alternative to the FISH-based PGD protocols.\textsuperscript{103} For reciprocal translocations, the strategy consisted of a MF-PCR using STR markers flanking the translocation breakpoints on both chromosomes involved, which tracks all meiotic segregations. For Robertsonian translocations, STR markers located at any point along the chromosomes involved allowing for differentiation between aneuploid embryos and normal/balanced embryos by simply enumerating peak signals (\textsuperscript{\textbullet} Fig. 5). This approach aimed to overcome several of the previously listed limitations related to the FISH technique while providing significant improvements in terms of test performance, automation, turnaround time, cost effectiveness, sensitivity, and reliability of the information obtained.

STR genotyping for both partners of each couple is performed to identify the most informative STR markers to be used in the clinical PGD cycles. For each couple, only fully informative heterozygous markers presenting nonshared alleles (i.e., four different alleles, male partner a/b and female partner c/d; or three different alleles, translocation carrier a/b, other partner c/c) are selected so that segregation of each allele could be clearly determined.

Embryos are diagnosed as “normal/balanced” if PCR results clearly indicated two signals (peaks) for each chromosome tested. Embryos are, instead, diagnosed as “unbalanced” if the PCR results showed a clear and consistent deviation from the “normal/balanced” signal pattern, such as (partial or full) trisomies (three peaks), (partial or full) monosomies (one peak), and nullisomies (no PCR signals) (\textsuperscript{\textbullet} Fig. 5).

Using STR markers simultaneously for each arm involved in the translocation, one can detect all abnormal segregations for any translocation. The co-amplification of at least three fully informative STR markers lying on either side of the translocation breakpoints increases the accuracy of the test, avoiding misdiagnosis due to possible ADO occurrences. In fact, ADO in any one marker is not likely to be repeated in the others; it would lead to misdiagnosis only if all markers tested are affected simultaneously. The multiplex STR marker system also provides an additional control for contamination with exogenous DNA because other alleles, differing in size from those of the parents, would be detected.\textsuperscript{7} However, the approach does not discriminate between noncarrier embryos and those that carry the balanced form of the translocation.

The MF-PCR-based approach not only diagnoses unbalanced inheritance of chromosomes in translocation carriers, it also allows for tracking of inheritance of each chromosome. This tracking of parental origin allows for the diagnosis of UPD where both chromosomes are inherited from one parent and no chromosomes are inherited from the other.\textsuperscript{104,105} FISH-based techniques cannot discern the origin of the chromosome, which can lead to chromosomally balanced embryos for transfer with from UPD.

Finally, the molecular approach is also amenable to automation and allows for easy data interpretation. It may also make transport PGD easier because placing a cell in a tube is far easier to train and monitor than teaching any of the current spreading methods.

**Detection of Chromosomal Abnormalities by Microarrays**

Comprehensive chromosome screening techniques\textsuperscript{75,106–108} have also been recently introduced into current routine PGD laboratory practices. Some of the most promising progress toward developing a comprehensive 24-chromosome analysis method has been made possible through the combination of WGA, a protocol able to amplify the entire genome from a single cell, and array comparative genomic hybridization (array-CGH).\textsuperscript{106,108–110}

Array-CGH involves a competitive hybridization of two differentially labeled DNA samples, one derived from the embryo (test DNA) and the other from a euploid DNA sample (reference DNA). The two samples are competitively hybridized to genomic probes of known sequence, immobilized on a glass microarray, each corresponding to a specific part of a chromosome. Software analysis of the scanned microarray is used to estimate the amount of test DNA and control DNA bound to each probe location, the copy number of the genomic sequence(s) represented by that probe, and hence the ploidy of the chromosomal region. This method has a high resolution, the analysis is fully automated, and the whole procedure can be performed within 24 hours. For day 3 or day 5 biopsy, it is possible to perform the embryo transfer within day 5 or day 6 of embryo development, in a fresh cycle.

A major advantage of the array-CGH approach over FISH is that it does not depend on cell fixation onto a microscope slide, a critical step that requires skill and experience. Because
the reliability of FISH analysis is linked to the quality of cell fixation, poorly fixed single cells will affect probe hybridization. The removal of a requirement for the more technically demanding cell fixation step notably simplifies the sample preparation procedure, with the potential to increase the percentage of embryos with a positive result. It may also make transport PGD easier, enabling broader use of PGD in IVF programs, because placing single cells into PCR tubes is far easier to train and monitor than teaching any of the current spreading methods.72

In addition, data analysis is performed by computational analysis of signal intensities and not by subjective signal scoring, as occurs with FISH analysis, allowing for easier and more reliable data interpretation. Finally, array-CGH is also amenable to automation for high throughput processing. The use of automated workstations for all manipulation greatly increases the number of samples a laboratory can process and also reduces the risk of mishandling the samples.

Beyond the technical advantages just described lies another important advantage. The WGA plus array-CGH approach not only diagnoses chromosomal abnormalities, it also enables the simultaneous screening of the embryos for single-gene disorders by PCR-based methods (such as for cases with combined indications for the couple, e.g., translocation plus carrier status for a SGD), using the same DNA produced after the WGA reaction. It could therefore be envisaged that in the future every embryo tested for a monogenic disorder would also be routinely screened for aneuploidy.

Preimplantation Genetic Diagnosis for Chromosomal Translocation Using Array-CGH

Recently, the array-CGH technique was clinically applied for the detection of chromosomal imbalances in embryos derived from translocation carriers.114 This approach was proposed as a valuable alternative to the FISH-based and PCR-based PGD protocols.

Array-CGH has several advantages compared with the other methods. In contrast to FISH and PCR, array-CGH allows screening for all aneuploidies in addition to the unbalanced derivatives associated with the specific translocation (►Fig. 6). It is a relatively common for PGD of chromosomal translocations to be combined with aneuploidy screening to assess aneuploidies for patients of advanced maternal age.67,111 With FISH, aneuploidy screening is typically done by sequential rounds of hybridization followed by chemical stripping of bound FISH probes. However, it is well recognized that the accuracy of the FISH analysis decreases with each additional round of hybridization because the DNA degenerates and hybridization efficiency is reduced.112 Furthermore, the persistence of signals from the first round of FISH could result in an incorrect interpretation of signals in the second round, thereby leading to a higher risk of misdiagnosis or a loss of normal embryos to false-positive errors. However, FISH allows the identification of aneuploidies only for a limited number of chromosomes (5, 9, or 12 chromosomes), most often found to be aneuploid in prenatal samples or material from miscarriages. Several studies have demonstrated that aneuploidies may involve all 24 chromosomes.71–75,94,114 This may result in the transfer of reproductively incompetent embryos with aneuploidy for chromosomes not analyzed.

Another advantage on the use of array-CGH technology is that it does not require preclinical validation before each IVF cycle, which is required for FISH or PCR-based methods of translocation screening. This spares couples the cost of setup testing, also allowing a rapid starting of the IVF treatments. In fact, a PGD cycle can be scheduled directly on the day of biopsy, based on the number of embryos available for biopsy.

Finally, array-CGH can also be used for more complex karyotypes, with multiple rearrangements, whereas FISH testing is generally very complicated, although the chances of getting euploid normal/balanced embryos from these cases is rare.

Although these results indicate that the array-CGH procedure is reliable and suitable for routine clinical application, the limitations must be considered. Array-CGH cannot detect haploidy and some polyplodieties, such as 69,XXX, 92,XXXX, or 92,XXYY, as well as balanced translocations, because there is no imbalance in the total DNA content. Additionally, the occurrence of a contamination event with exogenous DNA may reduce the number of embryos with a conclusive diagnosis using this technique.

Another potential limitation for routine application of this approach is that DNA amplification protocols require a minimum level of molecular biology experience as well as a laboratory environment where DNA contamination is avoided.

Unlike PCR and SNP microarrays, array-CGH does not allow for tracking of inheritance of each chromosome, so it cannot detect UPD.104,105

Finally, microarrays represent at this time an expensive option for embryo testing compared with the other conventional methods, although the cost of array-based testing will probably continue to drop as most new technologies do. The initial setup costs as well as the expertise levels required may mean that FISH- or PCR-based protocols are still appropriate for some PGD laboratories.

24-Chromosome Preimplantation Genetic Screening by Array-CGH

Recently, microarray-based approaches for 24-chromosome PGS have been proposed to overcome the technical difficulties that beset earlier PGS studies, allowing screening of the entire chromosome complement, rather than the limited chromosome assessment (►Fig. 7).72,73,75 Data from comprehensive aneuploidy screening showed that aneuploidies may occur in preimplantation embryos in any of the 24 chromosomes, indicating that aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal.72,75,114

However, screening for all 24 chromosomes in a single blastomere biopsied from a cleavage stage embryo may still be compromised by the degree of mosaicism that has been observed in such embryos or the possibility of self-correction of aneuploidy within the embryo. Although a follow-up study
of nontransferred chromosomically unbalanced embryos derived from translocation carriers revealed that all embryos that were classified as aneuploid after day 3 diagnosis were diagnosed again as abnormal after reanalysis, confirming at the end the previous results regardless of the actual abnormal genotype.

Because mosaicism is not present at the zygote stage, aneuploidy screening in PB1 and PB2 has been proposed to detect most of the aneuploidies that arise from the maternal genome, which is particularly relevant for patients of advanced maternal age. However, this technique ignores the possible contribution from the paternal genome and any aneuploidies that may arise during aberrant mitotic division.

Trophectoderm biopsy at the blastocyst stage of development is another alternative, but too few studies have been undertaken to evaluate if this is a more accurate reflection of the chromosomal status of the inner cell mass and ultimately the developing fetus.

Several RCTs performed by array-CGH technique, at different cell biopsy stage and categories of patients, are currently ongoing with the aim to establish whether PGS results in an enhanced live-birth rate and, if this is the case, to identify which patients may benefit from the procedure.

24-Chromosome Preimplantation Genetic Screening Combined with Preimplantation Genetic Diagnosis for Single-Gene Disorders

In the last few years, the use of SNP microarrays for PGD has been introduced. SNPs are common polymorphic DNA sequences found throughout the genome.

The microarray platforms typically involve oligonucleotide probes that are attached directly to the surface of solid supports, for example on slides or on beads, so each probe has a specific known location.

SNP arrays rely on a WGA step to amplify the single cell or small number of cells removed from a developing embryo.
After amplification, the DNA is fluorescently labeled and hybridized to the probes attached to the surface of the microarrays. This DNA is then assessed with automated fluorescent readers that analyze the probes to determine hundreds of thousands to millions of genotypes, and then they are compared with a control population using powerful computer software to allow for diagnosis of inheritance.

SNP-based arrays offer other options for testing that are not available on CGH-based systems. SNP-based arrays allow for simultaneous testing of specific genetic diseases and aneuploidy in each embryo. This will enable the selective transfer of genetically and chromosomally normal embryos for patients undergoing IVF with PGD for monogenic diseases. Simple haplotyping of SNPs surrounding and embedded in disease-causing genes allows for selection of embryos that have not inherited the affected chromosome. In addition, SNPs genotyping produces a unique DNA fingerprint for each embryo tested, enabling determination of which embryo was implanted in any given assisted reproductive technique cycle.

SNP arrays are just beginning to be used clinically in PGD and await clinical validation studies before they become more commonplace at IVF clinics. However, encouraging data from preclinical studies have begun to emerge.115

Karyomapping
Karyomapping is a universal method for the combined detection of chromosome aneuploidy and linkage-based PGD of single-gene defects that uses genome-wide, high-density SNP

Figure 7 Examples of array-CGH–based preimplantation genetic screening (PGS) results from day 3 biopsy. (A, B) Embryo with a complex aneuploidy involving chromosomes 15, 21 (loss) and 18 (gain).
genotyping. By genotyping the parents and appropriate family member(s) of known disease status, the parental origin of chromosomes and chromosome segments is identified in each embryo by Mendelian analysis of informative SNP loci. Informative loci are mainly those in which one parent is homozygous and the other parent heterozygous for the two SNP alleles. Using the genotype of an affected child, for example, as a reference, the linkage of the two alleles at each of these loci to one of the chromosomes in the heterozygous parent can then be established and compared with the alleles inherited in an embryo. Thus four sets of SNP markers for the four parental chromosomes are identified and by linkage can be used to diagnose embryos inheriting the same parental chromosome haplotypes in the region of the affected gene. At the same time, the inheritance of two chromosome haplotypes from one parent, with different patterns of recombination, trisomy, are identified by overlapping regions in which both haplotypes are present. Conversely, the absence of either parental chromosome haplotype enables the detection of monosomy or partial deletions.

Karyomapping is broadly applicable to any single-gene defect within the region covered by the SNP genotyping without the need for family- or disease-specific test development. Although diagnosis of SGDs is limited to those cases in which linkage can be established within the family, even with de novo mutations, it can still provide linkage information to which linkage can be established within the family, even with pregnancy

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