Mini Review

The Primed *In Situ* (PRINS) technique: an alternative approach for preimplantation chromosomal diagnosis

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Abstract
Preimplantation genetic diagnosis (PGD) is a novel approach for the prevention of genetic disorders in couples at risk of having offspring with genetic disease. Although the original idea dates back to early 1960s when sexing of rabbit blastocysts was attempted, the first clinical application of PGD was reported about three decades later, describing the use of PCR for sexing embryos from couples at risk of X-linked disease. The development of PCR-based tests led to PGD for screening well known monogenic diseases such as thalassaemia and cystic fibrosis. The introduction of fluorescence in situ hybridization (FISH) quickly replaced PCR-based methods which had led to misdiagnoses for sexing embryos. FISH can be used for aneuploidy screening of up to seven clinically significant chromosomes and translocation detection. The advent of molecular genetic techniques has brought forth new procedures for in situ chromosomal analysis. One of these techniques is the primed in situ labeling (PRINS) procedure which constitutes a fast and efficient alternative to conventional fluorescence in situ hybridization for nucleic acid detection. This technique has the potential to become a powerful tool for cytogenetic investigations. The recent achievements reported show that PRINS can constitute an efficient complement to PCR and FISH. Adaptation of this technique to preimplantation embryo screening both at chromosomal level and gene localization opens a promising perspective for being used in the field of PGD.
Keywords: *Preimplantation genetic diagnosis, PCR, FISH, PRINS, chromosomal abnormality.*

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INTRODUCTION

It has been estimated that 15% of all clinically recognized pregnancies spontaneously abort, and of these 50% are chromosomally abnormal (Andrews *et al*., 1984). The most common abnormality found is aneuploidy. Some couples may be at a higher risk of transmitting a chromosome abnormality to their offspring as they carry a balanced translocation. When the carrier tries to reproduce, chromosomes may be lost or gained in the abnormal gametes, causing chromosomal abnormalities in the embryo and fetus. If the chromosome abnormality is severe this may result in spontaneous miscarriage or the development of an abnormal fetus. Other groups of patients that transmit such abnormalities are gonadal mosaics. Their blood karyotype is normal but they repeatedly show the development of abnormal fetuses or offspring such as trisomy 21(Down syndrome) fetuses.

Such patients may have severe problems trying to produce a normal pregnancy. Their history often shows either failure to obtain a pregnancy repeated spontaneous miscarriage or an affected pregnancy after prenatal diagnosis, which may lead to repeated

induced abortions.

Until recently, couples known to be at a higher risk of giving birth to children suffering from autosomal recessive or X-linked disorders could only be offered the choice of prenatal diagnosis of those genetic diseases after the pregnancy has been established for some time.

Invasive tests in pregnancy are used to obtain fetal cells, fluids or tissues for prenatal diagnosis. This may be indicated when fetal karyotype or fetal DNA, is being investigated. The choice of invasive test is determined by the indication and fetal gestation balanced against the safety of the procedure. Amniotic fluid contains a variety of fetal cells that require 1-3 week's culture to provide sufficient dividing cells (metaphase nuclei) for karyotype analysis. Between 15 and 20 weeks gestation, the viable cell to amniotic fluid ratio optimizes culture yield. Chorionic villi from CVS consist of an inner mesenchymal core and an outer cytotrophoblast containing dividing cells. The cytotrophoblast will yield metaphases suitable for direct analysis and short-term culture. With any technique there is a risk of cell culture failure. Maternal cell contamination is a recognized complication, though rare, and particularly important in long-term culture of chorionic villi.

However, the duration until the diagnosis is established is 2-4 weeks depending on the technique, but once the diagnosis was established the parents would have to face the difficult decision of terminating the pregnancy if the fetus is affected. In some cases the termination of pregnancy is not a possible solution because of personal, moral or religious reasons. Therefore, for such couples preimplantation genetic diagnosis (PGD) is a very viable alternative.

Following its introduction, PGD has become an important complement to preventive measures for genetic disorders and an option for avoiding traditional prenatal diagnosis potentially leading to abortion (Handyside *et al.,* 1989). The advent of PGD has also presented the opportunity for preselection of aneuploidy free embryos in assisted reproduction practices, improving the effectiveness of *in vitro* fertilization (IVF) in low prognosis patients, such as those with advanced maternal age.

PGD offers the selection of female unaffected embryos in families with serious X-linked disorders for which the only option is termination of all male pregnancies (of which half would be unaffected). PGD with embryo selection may provide an acceptable solution for some couples who have normal or religious objections to abortion.

Embryo sexing for cases of X-linked disease were the first cycle of PGD to be performed by using PCR technology (Handyside *et al.,* 1989 and Kubo *et al.,* 2002), and now sexing accounts for a large number of cycles performed world wide and is probably the simplest diagnosis to perform. PCR-based PGD can also be used to detect single gene disorders, and diagnosis has been achieved for cystic fibrosis (Handyside *et al.,* 1992 and Goossens *et al.,* 2003), sickle cell disease (Xu *et al*. 1999), β-thalassaemia (Kuliev *et al.,* 1998 and Jiao *et al.,* 2003), Marfan's syndrome (Harton *et al.,* 1996) and many other genetic syndromes (e.g. Iacobelli *et al.,* 2003; Moutou *et al.,* 2003; Bermudez *et al.,* 2003 and Drusedau *et al.,* 2004).

One of the commonest reasons for requesting PGD is that one partner carries a structural rearrangement of chromosomes such as balanced reciprocal or Robertsonian translocations, so that there is a high risk that the fetus may have abnormal karyotype (Conn *et al.,* 1998 and 1999). This may have lead to repeated miscarriage or developmental abnormality. Infertile males are also at an increased risk of carrying a translocation and previously would not been able to reproduce. A real jump was observed in the number of PGD cycles after the introduction of the techniques of fluorescent *in situ* hybridization (FISH) for both sex and aneuploidy screening and translocation detection (Verlinsky and Kuliev, 1996; Velilla *et al.,* 2002; Staessen *et al.,* 2003; Pujol *et al.,* 2003 and Emiliani *et al.,* 2003). It is encouraging that many healthy children have been born, suggesting its safety and reliability.

Methods used in PGD: The best way to examine chromosomes is to perform a karyotype. However to perform a karyotype, the chromosomes have to be arrested in metaphase so that all the chromosomes can be clearly identified. With cultures for 1 week (e.g. blood or a prenatal diagnosis sample) about 10% of the nuclei give bandable metaphases (Harper and Wilton, 2001).

In PGD where only 1 or 2 cells are available, it is very difficult to obtain suitable metaphases from the preimplantation embryos. Therefore other approaches are used in studying chromosomal and genetic defects of embryos in PGD.

1. The use of PCR in PGD: The polymerase chain reaction (PCR) technique uses enzymatic amplification to increase the number of copies of a specific

DNA fragment at a level at which can be visualized and subjected to further genetic analysis.

The success of PCR in achieving this objective has enabled it to become one of the most important methods in genetic testing and mutation detection. Application of PCR protocols to single cell analysis has proven highly successful and remains the only means of detecting specific mutant alleles in human preimplantation embryos. Diagnosis of single gene defect is usually performed on one or two blastomers biopsied from 8-10 cell embryos on the third post-insemination day using nested PCR to amplify informative fragments (Wells and Sherlock, 2001).

PCR allows amplification from a limited number of target sequences (Verlinsky and Kuliev, 1996) and under carefully optimized conditions, amplification of as few as one or two target copies present in a single haploid or diploid cell is possible. In PGD, problems unique to single cell PCR must also be overcome.

PGD was first achieved for X-linked diseases by determining the sex of embryo using a Y chromosome specific repetitive sequence. The first clinical applications of PGD utilized a simple PCR-based protocol for the avoidance of X-linked disorders such as Duchene muscular dystrophy (DMD) and retinitis pigmentosa. For this purpose the sex of embryos was determined by PCR using primers specific for DNA sequences found only on the Y chromosome. A blastomere which produced PCR amplification products was indicative of a male embryo and thus at a high (50%) risk of developing the disease (Handyside *et al.,* 1989 and 1990). Although several girls were born following this PCR sexing protocol, a misdiagnosis, presumably due to amplification failure did occur (Handyside and Delhanty, 1993). This experience demonstrates that it is best not to rely on a negative result to indicate a genotype of the tested cell.

Specific diagnosis has been achieved for cystic fibrosis transmembrane regulator gene (Kubo *et al*.,2002), and for Lesch-Nyhan syndrome by amplifying across a familial best substitution nullifying a natural restriction site in the HPRT gene (Ray *et al.,* 1994).

In both instances, nested PCR strategies were chosen to amplify the muted sequence allowing sufficient amplification for detection on ethidium bromide stained gels. Ray and Handyside (1996) reported that they performed PGD for 12 couples in which both partners carry the cystic fibrosis mutation in a total of 18 cycles, which resulted in 5 singleton births confirmed to be homozygoues normal. Single blastomeres from embryos that have not been transferred were analyzed to confirm the original and assess the reliability in clinical practice. Amplification efficiency and accuracy were high with blastomeres from embryos diagnosed as homozygous normal or affected. In a proportion of blastomeres from presumed carrier embryos, one of the parental alleles failed to amplify, apparently at random, allele dropout.

Amplification failure in PCR preparation is one of the major difficulties in PGD, because of the limitation in cell number. Therefore Gibbons *et al*. (1995) reported that the practical solution to such a problem would only be through using more than one blastomere, thereby taking more risk with the viability of the rest of the embryo but giving more chance to the success of the diagnosis.

Recently a new PCR procedure has been described, based on the use of fluorescently tagged primers and named fluorescent PCR (F-PCR). Findlay *et al.* (1998) described the first application of fluorescent PCR technique to the genetic analysis of single cells. With fluorescence PCR procedure, several primer set can be used in the same reaction, with sensitivity 1000 times greater than in the conventional PCR technique. This approach significantly decreases the possibility of misdiagnosis, such as allele drop-out and contamination. Also, because only a few PCR cycles are required, the overall time taken by the procedure is substantially reduced. Findlay *et al*., (1998) compared the reliability and accuracy of PCR techniques with FISH. In buccal cells fluorescent PCR and FISH had similar reliability (94 and 93%) and accuracy (97 and 96%) rates. In human blastomeres, FISH and fluorescent PCR had similar reliability (100 and 95%) rates. Accuracy rates were 71% and 99% for FISH and fluorescent PCR respectively; however the numbers were small for meaningful statistical analysis. Therefore fluorescent PCR is expected to be the method of choice in the future of PGD (Findlay *et al.,* 1998).

2. The use of FISH in PGD: Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique for enumerating chromosomes and rearrangement detection (Verlinsky and Kuliev, 1996; Velilla *et al*., 2002; Staessen *et al*., 2003; Pujol *et al.,* 2003 and Emiliani *et al.,* 2003). It is particularly useful in tissues that can not be karyotyped using routine cytogenetics because interpretable metaphase chromosomes are difficult to prepare, which includes blastomers from preimplantation embryos. Blastomeres can be exposed to mitotic inhibitors to induce metaphase arrest but this result in shortened chromosomes that

clump together when fixed; resulting in a very low efficiency of informative metaphase spreads (Witton 1993 and Jamieson *et al.,* 1994).

FISH uses DNA probes that are fluorescently labeled and *in situ* hybridized to complementary sequences on a specific chromosome. After hybridization, nuclei can be examined under a fluorescent microscope and the number of fluorescent signals will indicate the number of chromosomes present. Several probes can be hybridized simultaneously if each is labeled with a different colored flourochrome. This enables enumeration of more than one chromosome in a single cell and up to five chromosomes have been identified in a single round of FISH (Munne *et al*., 1998).

The initial experiments using FISH on interphase blastomeres employed in directly labeled probes (Griffin *et al.,* 1991, 1992, 1993 and 1994). With the introduction of new fluorochromes and the improvement in the sensitivity of fluorescent detection system, now directly labeled probes where the fluorchrome is attached to the probe itself can be used (Harper *et al.,* 1994). There are three types of FISH probes: alpha-satellite or repeat sequence probes or unique sequence probes and chromosome paints.

FISH have been used for the PGD of sex since 1991 (Griffin *et al.,* 1991). Sexing for X-linked disease is one of the major indications for PGD (Verlinsky *et al.,* 1999; ESHRE PGD Consortium, 1994). More recently FISH has been used for the PGD of chromosome abnormalities for patients carrying translocations and other chromosome abnormalities and aneuploidy (Sermon, 2002).

The use of interphase FISH using probes for chromosomes X and Y was developed to sex embryos for patients at risk of transmitting X-linked disease. Most laboratories use three colors FISH for PGD of sex with probes for chromosomes X, Y and an autosome (usually 18) to provide more information concerning aneuploidy and polyploidy (Staessen *et al.* 1999). More recently five-color FISH with additional probes for chromosomes 13 and 21 has been used for blastomere analysis (Verlinsky *et al.,* 1999 and Gianaroli *et al.,* 1997).

3. The use of PRINS : The advent of molecular genetic techniques has brought forth-new procedures for *in situ* chromosomal analysis. One of these techniques is the primed *in situ* labeling (PRINS) procedure, which constitutes a fast and efficient alternative to conventional fluorescence *in situ* hybridization (FISH) for nucleic acid detection. Based on the use of chromosome - specific primers, the PRINS method combines the high sensitivity of the PCR reaction with the cytological localization of DNA sequences. Since, its introduction by Koch *et al.* in 1989, the PRINS protocol has been optimized and numerous applications have been developed. The technique has thus proved to be a useful tool for *in situ* chromosomal screening and can become a simple and efficient to conventional cytogenetic and FISH methods.

Principles and methodology of PRINS: The key to the PRINS technique is the use of unlabeled, short and specific oligonucleotides. Oligonucleotides are annealed *in situ* to complementary DNA targets on denatured chromosome spreads, nuclei or tissue sections and then act as primers for chain elongation catalyzed by a *Taq* DNA polymerase in the presence of free nucleotides. The visualization of generated fragments results from the incorporation of one labeled nucleotide. PRINS labeling of human chromosomes is obtained using oligonuclotide primers for repeated DNA sequences. Various repeat families are spread over the human genome and involve satellites, *Alu* or telomeric DNA sequences.

An advantage of primers is their ability to differentiate between closely related sequences. This feature has been utilized for generating chromosome – specific primers from the alpha-satellite DNA motifs (Pellestor *et al.,* 1995a) and variant telomeric repeats (Krejci and Koch, 1999). The complementation process between the primer and its centromeric target will be so specific that a simple mismatch between the 3´-end of the primer and the genomic sequence will prevent initiation of the elongation by the *Taq* DNA polymerase (Bottema and Sommer, 1993). Thus, it has been possible to define specific alpha-satellite primers for some chromosomes undistinguishable by FISH with centromeric probes, such as chromosomes 13 and 21 (Pellestor *et al.,* 1995b). The length of primers ranges from18 to 35 nucleotides; this small size greatly facilitates their accessibility to their genomic target sequences. This is particularly significant in cells with highly condensed nuclei, such as spermatozoa. To date, specific primers have been defined for 20 human chromosomes. As an alternative to oligonucleotides, cloned probes fragmented by restriction enzyme digestion can be used as primer (Koch *et al*., 1991 and 1995). Because they are unlabelled, high amounts of primers can also be used in PRINS reaction without inducing background signals. This makes the PRINS a very fast technique.

Initially, PRINS reaction was performed either in a thermo-block or a water bath. The weakness of these methods was in the lack of stringency of primer annealing. In fact, these procedures did not allow precise and durable temperature control. Indeed, although the range of *Taq* DNA polymerase activity is large enough, the optimization of the annealing and its stringency in order to increase specificity need accurate temperature control. The procedure has been considerably improved by using programmable thermal-cyclers, equipped with flat plate block. With this equipment, the precision of temperature control may reach 0.2 ¦C and the required temperature changes are both easy to program and rapidly carried out. Thus, semi automatic PRINS protocols have been developed offering a high reproducibility of the labeling reaction. The PRINS method was also extended to allow the detection of low copy number repeats by repeating the initial extension step for a variable number of times (10, 20 or 30 cycles). This method, called cycling PRINS, takes a little longer but has considerably increased sensitivity (Gosden and Lawson 1994). An additional improvement was the use of various fluorochromes in sequential PRINS reactions (Multi-PRINS). In this procedure, multiple PRINS reactions are performed to label different, specific target chromosomal DNA sequences with different colors. The multi -PRINS procedure is capable of identifying at least 2 pairs of chromosomes, but with the appropriate selection of primers, reporters and counterstains, may be capable of identifying three or more chromosomes (Gosden and Lawson 1994, Hindkjaer *et al*., 1994, Speel *et al*., 1995). Examples of pictures showing chromosomes identified using the PRINS technique is shown in figure 1 (A-C).

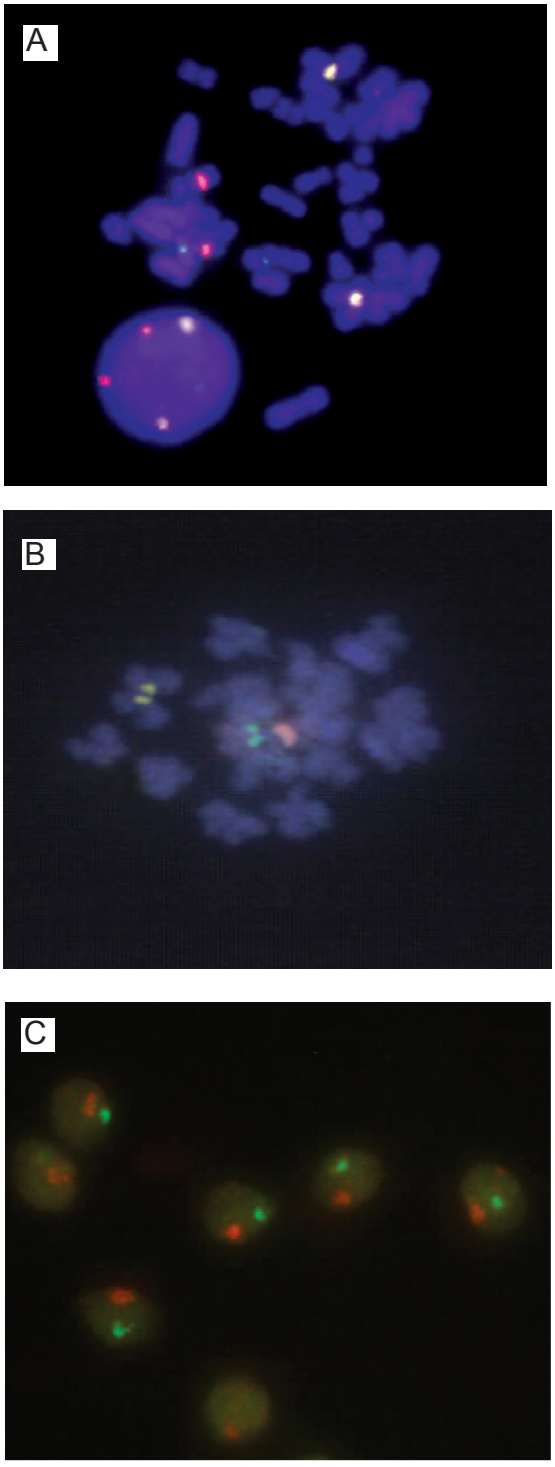

Figure 1: (A), a diploid set of chromosomes with an interphase nuclei showing three different signals; for chromosome 1 in Yellow, chromosome 7 in green and chromosome 9 in red, using the fast 3 color new PRINS reaction in which the 2 red and green colors are mixed in order to generate the 3rd color yellow. (B), specific labeling of an human oocyte with the chromosome 1 in yellow, the chromosome 9 in green and the chromosome 16 in red. (C), A dual color PRINS reaction on human spermatozoa with chromosome 9 in red and chromosome 18 in green.

Applications of PRINS

The PRINS procedure combines several features that make it very attractive for a number of cytogenetic purposes. Various applications have already been developed in humans, mammalians (Kipling *et al.,* 1994 and Coignet *et al*., 1996) and plants (Abbo *et al.,* 1993 and Pich *et al.,* 1995), demonstrating that PRINS could be easily adapted to various types of cells. In human, the PRINS method has been successfully tested for the assessment of aneuploidy in lymphocytes, fibroblastes (Gosden *et al.,* 1991 and Velagaleti *et al*., 1999) and amniocytes (Pellestor *et al*., 1995c). Since the PRINS reaction with *Alu* primers give high quality R-like banding on human chromosomes, this procedure has been adapted for the

cytogenetic screening of somatic hybrid cell lines (Coullin *et al.,* 1997) and identification of euchromatin in aberrant short arms of acrocentric chromosomes and small ring chromosomes (Callen *et al*., 1997). The use of PRINS has also been reported for analysis of structural aberrations, such as translocations, marker chromosomes and ring chromosomes (Hindkjaer *et al.,* 1995 and Velagaleti *et al*., 1997), and recently, the PRINS adapted to detect fetal cells among maternal nucleated cells from peripheral venous blood of pregnant women (Orsetti *et al.,* 1998). All these applications point out the potential efficiency of the PRINS method for diagnostic use. Some research studies have also reported the use of PRINS for the direct estimation of disomy rates in human sperm (Pellestor *et al*., 1996a and 2002 a, b). The PRINS methodology was combined with an efficient 3M NaOH treatment allowing the simultaneous decondensation and denaturaton of sperm nuclei. In PRINS, the decondensation of the sperm head is a less limiting factor than in FISH because of the small size of the oligonucleotide primers. This facilitates their penetration into sperm nuclei and their access to the genomic target sequences, resulting in a more efficient and more rapid labeling of sperm nuclei.

The PRINS technique was also adapted to preimplantation embryo screening (Pellestor *et al.,* 1996b). Various combinations of primer were used in double and triple PRINS reactions. In these preliminary results, a labeling efficiency of 100% was obtained. This indicated that PRINS is a well adapted tool for chromosome screening on isolated cells and in a limited time period such as in preimplantation genetic diagnosis.

CONCLUSION

PRINS has emerged as a research technique. With the development of rapid and simplified protocols producing reliable and reproducible results (Yan *et al*., 2001 and Pellestor *et al*., 2002c), this technique as the potential to become a powerful tool for cytogenetic investigations. The recent achievements reported show that PRINS can constitute an efficient complement to PCR and FISH. The adaptation of PRINS to the *in situ* detection of unique sequences constitutes an important challenge. This approach would allow doing away with tedious protocols for preparing probes and so, to rapidly map newly discovered genes on chromosomes by using synthetic primers derived from sequenced DNA. Preliminary results have been obtained on porcine chromosomes (Troyer *et al.,* 1994) and recently efficient PRINS localizations of several genes have been reported on human metaphases (Kadandale *et al.,* 2002 and Tharapel *et al.,* 2002). This opens news and promising perspectives for PRINS in the field of genetic diagnosis.

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