

ESHRE PGD Consortium/Embryology Special Interest Group—best practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS)[†]

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ABSTRACT: In 2005, the European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis (PGD) Consortium published a set of Guidelines for Best Practice to give information, support and guidance to potential, existing and fledgling PGD programmes (Thornhill AR, De Die-Smulders CE, Geraedts JP, Harper JC, Harton GL, Lavery SA, Moutou C, Robinson MD, Schmutzler AG, Scriven PN *et al.* ESHRE PGD Consortium best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS). *Hum Reprod* 2005;**20**:35–48.). The subsequent years have seen the introduction of a number of new technologies as well as the evolution of current techniques. Additionally, in light of ESHRE's recent advice on how practice guidelines should be written and formulated, the Consortium believed it was timely to revise and update the PGD guidelines. Rather than one document that covers all of PGD as in the original publication, these guidelines are separated into four new documents that apply to different aspects of a PGD programme; Organization of a PGD centre, fluorescence *in situ* hybridization-based testing, amplification-based testing and polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). Here we have updated the sections that pertain to embryology (including cryopreservation) and biopsy of embryos prior to PGD or PGS. Topics covered in this guideline include uses of embryo biopsy, laboratory issues relating to biopsy, timing of biopsy, biopsy procedure and cryopreserving biopsied embryos.

Key words: embryology / PGD / assisted reproduction

Introduction

The rapidly changing nature of preimplantation genetic diagnosis (PGD), specifically the technologies associated with its use and increasing patient access, has necessitated review and revision of the original the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium guidelines (Thornhill *et al.*, 2005). As a result, the ESHRE PGD Consortium has prepared four guidelines: one relating to the Organization of the PGD Centre (Harton *et al.*, 2010a) and three relating to the methods used: DNA amplification (Harton

et al., 2010b), fluorescence *in situ* hybridization (FISH) (Harton *et al.*, 2010c) and biopsy/embryology. The method guidelines (i.e. FISH-based testing) should be read in conjunction with the Organization of the PGD Centre guidelines, which contains information on personnel, inclusion/exclusion criteria, genetic counseling and informed consent, setting up an IVF centre, transport PGD, quality assurance/quality control (QA/QC) and accreditation (which is also further discussed in the paper by Harper *et al.*, 2010a).

PGD has been developed for patients at high risk of transmitting a genetic abnormality to their children, which includes all monogenic

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defects (autosomal recessive, autosomal dominant and X-linked disorders) and carriers of balanced translocations, which are at high risk of implantation failure and recurrent abortions. Preimplantation genetic screening (PGS), called 'low-risk PGD' in the original guidelines, has been carried out for infertile patients undergoing IVF with the aim of increasing the IVF pregnancy and delivery rates. Cited indications for PGS include advanced maternal age, repeated implantation failure, severe male factor and couples with normal karyotypes who have experienced repeated miscarriages. The clinical use of PGS remains in question (Harper et al., 2008, 2010b; Mastenbroek et al., 2008; Simpson, 2008; Hernandez, 2009) and recently there have been calls for laboratories interested in PGS to move to new technology (array-based testing for 24 chromosomes) and explore different biopsy timing (polar body or trophectoderm) to show whether PGS may increase delivery rates in a randomized controlled trial (Geraedts et al., 2010; Harper and Harton, 2010).

PGD is still relatively unregulated and lacks standardization compared with other forms of diagnostic testing, however, more federal, state and local governments are beginning to take an interest in PGD and some have begun accrediting laboratories that offer PGD (Harper et al., 2010a). This is a logical step considering the comparative difficulty in achieving the highest levels of accuracy and reliability with single cells as part of PGD versus more routine genetic testing. Many regulations, laws and voluntary networks exist in the mainstream diagnostic community to maintain the highest quality in diagnostic testing. For example, the European Quality Molecular Network has attempted to improve and standardize molecular diagnostic testing across Europe (Dequeker et al., 2001). One step towards higher quality overall and standardization for PGD is to build consensus opinion on best practices within the PGD community; a component of the mission of the ESHRE PGD Consortium (hereafter referred to as the Consortium—ESHRE PGD Consortium Steering Committee, 1999, 2000, 2002).

The biopsy procedure

Biopsy can be performed by different methods: removal of one or two polar body(ies), from the unfertilized oocyte or the zygote, removal of one or two blastomeres at the cleavage stage or removal of several cells at the blastocyst stage. Cleavage-stage biopsy remains the most widely practised form of embryo biopsy worldwide, accounting for approximately 90% of all reported PGD cycles (ESHRE PGD Consortium: Harper et al., 2010c). Polar body biopsy may be chosen as an alternative to embryo biopsy if only maternal mutations or aneuploidies are investigated (Montag et al., 2009). Some centres perform polar body biopsy as a means to avoid removing embryonic cells (Verlinsky et al., 1990, 1997), others use the technique exclusively due to regulations that prohibit embryo biopsy in their region or country (for example in Germany: K pker et al., 2001). In other countries, first polar body testing before fertilization (so-called preconception genetic diagnosis) was the only procedure permitted (for example in Italy: Fiorentino et al., 2008; Gianaroli et al., 2010). Blastocyst biopsy is usually performed on the morning of Day 5 or 6 post-insemination. Experience from two centres routinely applying blastocyst biopsy indicate that the limitations to the procedure (not all preimplantation embryos develop to this stage *in vitro*) may be outweighed by the advantages (more cells may be removed for

analysis, the biopsy procedure is reported to be easier to perform than the procedure for polar bodies or individual blastomeres, and the sampled cells (trophectoderm) do not contribute to the embryo proper). Accumulating evidence highlights that blastocyst biopsy has no adverse affect on either embryo implantation or development to term (Kokkali et al., 2005; McArthur et al., 2005; Kokkali et al., 2007; Schoolcraft et al., *in press*).

Recently, many groups have been moving away from cleavage stage biopsy for some types of preimplantation testing, specifically aneuploidy screening, due to the issue of mosaicism. It is well known that 40–60% of embryos are mosaic (i.e. contain more than one cell line), which can lead to an increase in false-positive/false-negative results in PGS (Colls et al., 2007; DeUgarte et al., 2008; Hanson et al., 2009). Removal of polar bodies prior to cleavage, or trophectoderm biopsy and testing of multiple cells from an embryo containing approximately 100 cells, should reduce or eliminate the issue of false-positive/false-negative results in PGS. Each of these biopsy techniques also brings unique challenges to preimplantation testing. Polar body testing only allows for testing of the maternal complement of chromosomes, leaving the male's contribution untested, while trophectoderm biopsy could entail cryopreservation of biopsied embryos while genetic testing is performed. These cryopreserved embryos are then thawed or warmed and transferred in a separate transfer cycle, and this extra step may lower implantation and/or pregnancy rates.

The Consortium recognizes that owing to variations in local or national regulations and specific laboratory practices, there will remain differences in the ways in which PGD is practised (from initial referral through IVF treatment, single cell analysis to follow-up of pregnancies, births and children). However, this does not preclude a series of consensus opinions on best practice based upon experience and available evidence. Indeed, the American Society for Reproductive Medicine (ASRM) published a practice committee report for PGD in 2001 (ASRM and Society for Assisted Reproductive Technology, Practice Committee Report, 2008) reviewing PGD practice in the USA. The PGD International Society, has also drafted guidelines that were recently updated and although more in-depth than the ASRM report, these guidelines are concise and remain so in their recent revised edition (Preimplantation Genetic Diagnosis International Society, 2008). The consensus opinions provided in this document and the accompanying guidelines, not only reflect current use of PGD but also offer consensus-based specific guidance regarding how best to practice clinical PGD based on clinical experience and data, both published and unpublished.

The Consortium hopes that a minimum standard might be achieved across all centres actively providing clinical PGD. Achieving this goal should ultimately have the net effect that patients receive the best care possible regardless of the centre at which they are treated. Rather than a drift towards the lowest common denominator, established and fledgling centres alike can learn from global experiences and be guided by consensus opinion.

These opinions are not intended as rules or fixed protocols that must be followed, nor are they legally binding. The unique needs of individual patients may justify deviation from these opinions, and they must be applied according to individual patient needs using professional judgement. However, guidelines and opinions may be incorporated into laws and regulations and practitioners should check the

status of clinical practice guidelines in their own countries to determine the status of this document.

1. Laboratory issues relating to biopsy

General

1.1. Until the time of biopsy, routine IVF culture conditions apply (Magli *et al.*, 2008). It is usual to transfer embryos at the morula/blastocyst stage following biopsy so the most adequate culture conditions in each embryology laboratory should be used.

1.2. It is 'recommended' that an experienced embryologist (i.e. general embryology and micromanipulation of embryos) performs the biopsy procedure after appropriate training (Harton *et al.*, 2010a) and follow standard operating procedures. Deviations to SOPs and protocols should be documented.

1.3. The embryologist may also be trained in spreading cells for FISH (Harton *et al.*, 2010c) and/or tubing cells for amplification-based PGD (Harton *et al.*, 2010b).

1.4. Training for biopsy personnel should be documented. It is 'recommended' that at least 100 oocytes/embryos are successfully biopsied prior to clinical work resulting in the removal of >90% intact cells. Training for biopsy should be at least to the standard required for certification in routine embryology.

1.5. It is 'essential' to ensure that an adequate labelling system is used to identify the cell number and the oocyte/embryo from which it was biopsied and it is critical that all stages have appropriate and recorded witnessing. This must include documented matching of the cell and oocyte/embryo after biopsy, of the cell and slide/tube during preparation and finally of the embryos recommended for transfer on the PGD report prior to embryo transfer.

1.6. It is strongly 'recommended' that all cumulus cells are removed before biopsy as these cells can contaminate FISH and PCR diagnoses and lead to misdiagnosis (Wilton *et al.*, 2009).

Insemination

1.7. ICSI is 'recommended' for all PCR cases to reduce the chance of paternal contamination from extraneous sperm attached to the zona pellucida or non-decondensed sperm within blastomeres (Harton *et al.*, 2010b).

1.8. ICSI and conventional insemination are both 'acceptable' for FISH cases.

Embryo culture

1.9. Standard IVF culture conditions are 'acceptable' until the day of biopsy but following biopsy, the following 'recommendations' are made:

1.9.1. Appropriate culture medium and biopsy medium for oocytes/embryos should be used.

1.9.2. Biopsied oocytes and embryos must be cultured singly in individual drops or dishes with a clear identification system to ensure tracking of polar bodies or blastomeres removed and easy identification of oocytes and embryos post-diagnosis.

1.9.3. The use of culture wells instead of droplets would decrease the possible mixing of embryo in culture dishes due to possible movement of droplets during handling.

1.9.4. Oocytes and embryos are rinsed post-biopsy to remove traces of acid or biopsy medium as applicable.

2. Timing of biopsy

Polar body biopsy

2.1. The first polar body can be removed from the oocyte on the day of the oocyte collection between 36 and 42 h post-hCG injection (Verlinsky *et al.*, 1990).

2.2. The first and second polar body can be removed simultaneously from the zygote between 9 and 22 h post-insemination, but at 22 h the first polar body may have degenerated (Verlinsky *et al.*, 1997).

2.3. Sequential removal of the polar bodies where the first polar body is removed on Day 0 and the second polar body on Day 1 is also acceptable (Strom *et al.*, 1998)

2.4. Simultaneous biopsy of the two polar bodies is 'acceptable' for FISH analysis since they can provide distinguishable results.

2.5. Sequential biopsy of polar bodies is 'recommended' for PCR analysis to determine recombination events between the first and second polar body.

2.6. Cleavage stage biopsy may be required to confirm the polar body diagnosis (Magli *et al.*, 2004; Cieslak-Janzen *et al.*, 2006). The limitations of polar body biopsy have been documented previously (De Vos and Van Steirteghem, 2001; Harper and Doshi, 2003; Soini *et al.*, 2006).

Cleavage stage biopsy

2.7. Biopsy on the morning of Day 3 post-insemination is 'recommended' but the exact timing varies according to timings of procedures in different laboratories.

2.8. It is 'acceptable' to exclude very poor quality embryos from the embryo biopsy procedure (Magli *et al.*, 2007).

2.9. It is 'recommended' to set biopsy criteria prior to performing clinical cases and to adhere to them for all clinical cases. Routine updating of criteria should be done as necessary.

Blastocyst biopsy

2.10. Several methods of blastocyst biopsy have been reported (Veiga *et al.*, 1997; Kokkali *et al.*, 2005; McArthur *et al.*, 2005; Kokkali *et al.*, 2007; Jones *et al.*, 2008). This may include breaching of the zona on Day 3 and removal of the trophoctoderm on Day 5 or zona breaching and trophoctoderm removal on Day 5.

3. Biopsy procedure

3.1. The following 'recommendations' are made for preparations prior to any biopsy procedure on human oocytes or embryos

3.1.1. Ensure all micromanipulation equipment is installed correctly, calibrated and maintained per written procedures. Biopsies must be performed on a warmed stage.

3.1.2. Ensure the appropriate reagents and micromanipulation tools are available, sterile and within their expiration date

3.1.3. Ensure that biopsy is performed by a suitably qualified person who is trained to a written procedure and adheres to that procedure

3.1.4. Biopsy dishes should be made up before the procedure, and clearly labelled with the patient name and oocyte or embryo numbers.

3.1.5. Biopsy dishes should contain a drop of biopsy medium of sufficient size to maintain pH, osmolarity and temperature during the procedure

3.1.6. Sufficient rinse drops comprising culture medium should be available to rinse oocytes and embryos after the biopsy procedure.

3.1.7. Acidified Tyrodes solution (if applicable) should also be readily available to allow pipette priming between biopsies.

Zona breaching

3.2. Historically, zona opening for polar body biopsy has been performed mechanically, while for preimplantation embryos most clinics have used zona drilling with acid Tyrodes. However, in the most recent data collection, zona drilling with a laser was the predominant method used to open the zona for preimplantation embryos (Harper et al., 2010c).

3.3. Mechanical zona drilling is 'acceptable' for first polar body biopsy when performed before fertilization, but acidic Tyrodes or laser are 'not recommended' as they may adversely affect the spindle (Malter and Cohen, 1989; Montag et al., 2004).

3.4. Mechanical, chemical or laser zona breaching are 'acceptable' for polar body biopsy when performed after fertilization (Magli et al., 2004; Montag et al., 2004).

3.5. Acceptable methods for zona breaching during cleavage stage or blastocyst biopsy include acidified Tyrodes solution, laser or mechanical methods (Gianaroli et al., 2002).

3.6. Zona drilling for trophectoderm biopsy can be performed on Day 3 or on the morning of Day 5 (McArthur et al., 2005; Kokkali et al., 2007).

Cell removal

3.7. Incubation and biopsy in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free medium and aspiration of the chosen blastomeres remain the most common practices for cleavage-stage embryo biopsy (Harper et al., 2010c).

3.8. The following methods of removal are 'recommended':

3.8.1. Removal of polar bodies by aspiration (Verlinsky et al., 1990).

3.8.2. Removal of cleavage stage blastomeres by aspiration (Harper et al., 2010c).

3.8.3. Removal of trophectoderm cells during blastocyst biopsy by herniation following drilling with laser (McArthur et al., 2005) or mechanical excision (Dokras et al., 1990).

3.9. Removal of cleavage stage blastomeres by extrusion (Fallon et al., 1999) or displacement techniques (Pierce et al., 1997) have also been reported, but they are not very common methods and data are very scarce to evaluate the corresponding performance.

Number of cells to remove safely

3.10. The removal of more than one cell from Day 3 embryos has a negative impact on the clinical outcome (Goossens et al., 2008; De Vos et al., 2009). Nevertheless, some tests may require the use of two cells from each embryo to bring the diagnostic accuracy to an acceptable level. If removal of two cells is considered, it is recommended to be undertaken only on embryos with six or more cells (Van de Velde et al., 2000; Goossens et al., 2008).

Rebiopsy of embryos

3.11. This practice is 'acceptable' in the case of lost or anucleate blastomeres and failed diagnosis, but the embryo cell number and timing of rebiopsy should be considered.

3.12. Use of the original zona breach site is 'recommended'

Selection of cell for removal

3.13. When possible, the removal of mononucleate cells is recommended (Munné and Cohen, 1993).

Biopsy medium

3.14. The use of standard IVF culture medium during biopsy is 'acceptable' but its effectiveness may be highly dependent upon the developmental stage of the embryo biopsied.

3.15. The use of commercial $\text{Ca}^{2+}/\text{Mg}^{2+}$ free biopsy medium (Dumoulin et al., 1998) is common practice for biopsy at the cleavage stage, but some reports discourage its use (McArthur et al., 2005).

Time out of incubator

3.16. No specific recommendations for maximum time out of the incubator can be given but ideally biopsy should be performed as quickly as possible to ensure pH, temperature and osmolality are maintained.

3.17. A documented record for biopsy timings and operators is 'recommended' for QA/QC purposes.

4. Cryopreservation of biopsied embryos

There are several situations when embryos may be frozen in cases of PGD: prior to the biopsy (for example in cases of ovarian hyperstimulation syndrome), after the biopsy to give more time to perform the diagnosis, or after the biopsy and diagnosis where fresh embryos have been transferred but 'unaffected' surplus embryos remain. In any of these cases, cryopreservation is 'recommended'. Little data are available regarding the success rates of cryopreserved biopsied embryos. The same cryopreservation protocols are used for biopsied and intact embryos at present.

Cleavage stage embryos

4.1. Cryopreserved biopsied cleavage stage embryos show a lower survival rate than cryopreserved intact embryos (Magli et al., 1999; Stachecki et al., 2005). Vitrification of biopsied cleavage stage embryos has been shown to result in higher survival rates than slow-freezing methods (Zheng et al., 2005). Embryos biopsied at Day 3 and cryopreserved at blastocyst stage showed a higher survival rate than if cryopreserved on Day 3 (Zhang et al., 2009). In two other studies, where slow-freezing was performed on blastocysts having been biopsied on Day 3, similar survival and implantation rates were found compared with intact blastocyst (Magli et al., 2006; El-Thoukhy et al., 2009).

Blastocysts

4.2. Results published regarding the survival rate of biopsied blastocysts are conflicting. In a recent study a lower survival rate was found for cryopreserved biopsied blastocysts compared with intact blastocysts, while vitrification showed a higher survival rate than slow freezing (Keskinetepe et al., 2009).

4.3. If cryopreservation is performed because the transfer procedure is cancelled/delayed (due to OHSS for example), since several studies show a lower survival rate for biopsied embryos/blastocysts, it may be of advantage to perform the biopsy after thawing. On the other hand, doing the biopsy before cryopreservation ensures that only embryos with a positive diagnosis are cryopreserved, and will speed up the

process of the thawing cycle. For the time being, it is recommended that each centre decide its own policy regarding the cryopreservation of PGD embryos based on its experience and performance on embryo cryopreservation.

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