



ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for PGT[†]

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ABSTRACT: The field of preimplantation genetic testing (PGT) is evolving fast, and best practice advice is essential for regulation and standardisation of diagnostic testing. The previous ESHRE guidelines on best practice for PGD, published in 2005 and 2011, are considered outdated, and the development of new papers outlining recommendations for good practice in PGT was necessary.

The current paper provides recommendations on the technical aspects of embryo biopsy and covers recommendations on the biopsy procedure, cryopreservation and laboratory issues and training, in addition to technical aspects and strengths and limitations specific for currently used techniques at different stages (polar body, cleavage stage and blastocyst biopsy). Furthermore, alternative sampling methods are briefly described. This paper is one of a series of four papers on good practice recommendations on PGT. The other papers cover the organisation of PGT, and the different technical aspects of PGT for monogenic/single-gene defects (PGT-M) and PGT for chromosomal structural rearrangements/aneuploidies (PGT-SR/PGT-A).

Together, these papers should assist everyone interested in PGT in developing the best laboratory and clinical practice possible.

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Key words: ESHRE / preimplantation genetic testing / biopsy / tubing / good practice

WHAT DOES THIS MEAN FOR PATIENTS?

The paper describes good practice recommendations for preimplantation genetic testing (or PGT). Similar documents have been published in 2011, but these needed updating to the new techniques used in IVF and genetics labs.

The recommendations should help laboratory personnel and geneticist to perform PGT according to the best laboratory and clinical practice possible. The current paper provides recommendations on the technical aspects of embryo biopsy, which is the removal of a sample from an egg or embryo. This sample can then be tested in the genetics laboratory.

These technical recommendations are not directly relevant for patients, but they should ensure that PGT patients receive the best care possible.

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Introduction

The previous terms of preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) have been replaced by the term preimplantation genetic testing (PGT), following a revision of terminology used in infertility care (Zegers-Hochschild et al., 2017). PGT is defined as a test performed to analyse the DNA from oocytes (polar bodies) or embryos (cleavage stage or blastocyst) for HLA typing or for determining genetic abnormalities. This includes PGT for aneuploidy (PGT-A), PGT for monogenic/single gene defects (PGT-M) and PGT for chromosomal structural rearrangements (PGT-SR) (Zegers-Hochschild et al., 2017). PGT for chromosomal numerical aberrations of high genetic risk are included within PGT-SR in the data collections of the ESHRE PGT consortium.

PGT began as an experimental procedure in the 1990s with polymerase chain reaction (PCR)-based methods used for sex selection and the detection of monogenic diseases. Interphase fluorescence *in situ* hybridisation (FISH) was introduced a few years later and became the standard method for sexing embryos and for detecting numerical and structural chromosomal aberrations. Genome-wide technologies began to replace the gold standard methods of FISH and PCR over the last decade and this trend was most apparent for PGT-A. PGT-A has been carried out mainly for *in vitro* fertilisation (IVF) patients with original aims of increasing pregnancy rates per embryo transfer and decreasing miscarriage rates. Other outcome measures such as increasing elective single embryo transfer and reduced time to pregnancy have been added more recently. Cited indications for PGT-A include advanced maternal age (AMA), recurrent implantation failure (RIF) and severe male factor (SMF) and couples with normal karyotypes who have experienced recurrent miscarriage (RM). The value of the procedure for all IVF patients and/or appropriate patient selection remains an ongoing discussion, but this is outside the scope of this manuscript (Harper et al., 2018).

The goal of this series of papers is to bring forward best practices to be followed in all types of PGT services, offering PGT-A as well as PGT-M and PGT-SR.

In order to take PGT to the same high-quality level as routine genetic testing, guidelines for best practice have been designed by several societies. The PGD International Society has drafted guidelines (The Preimplantation Genetic Diagnosis International Society (PGDIS)

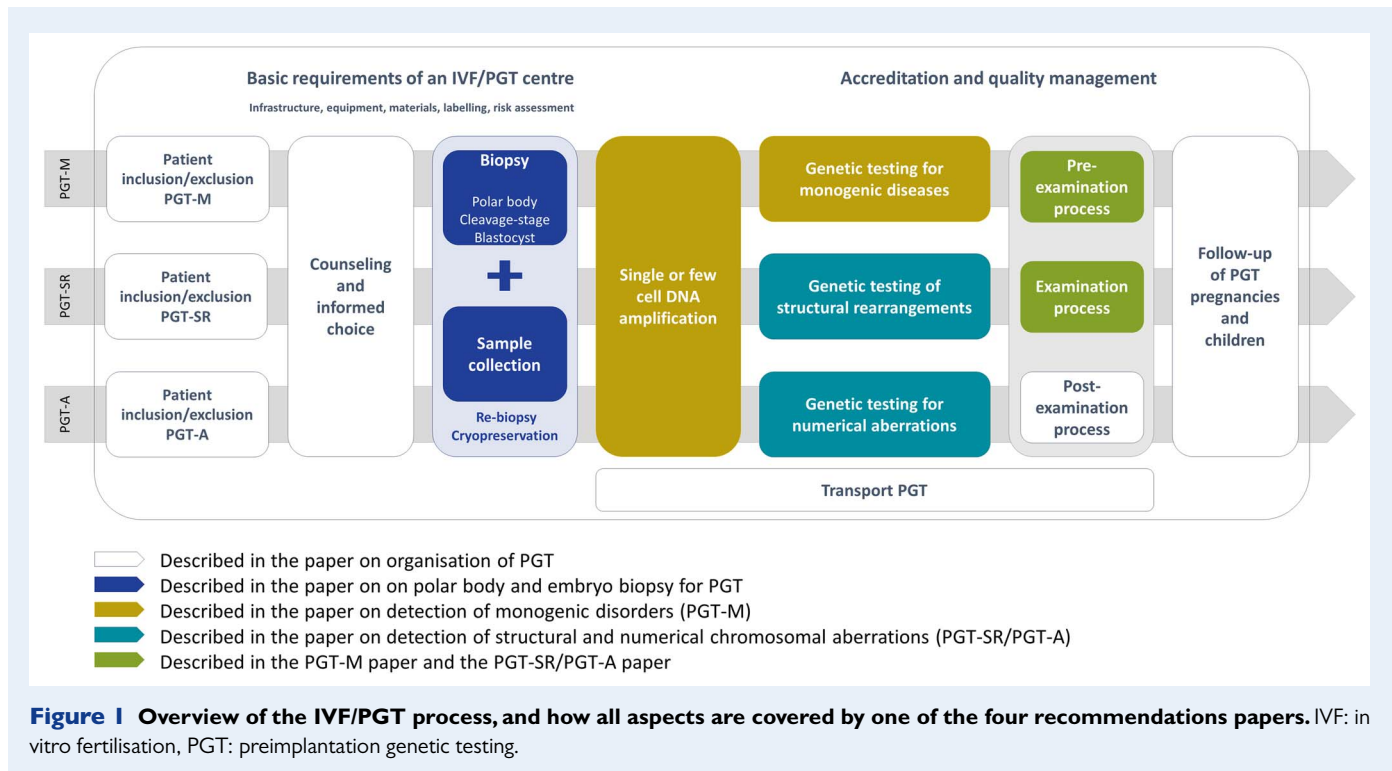
2004, Preimplantation Genetic Diagnosis International Society 2008) while the American Society for Reproductive Medicine reviewed PGT practice in the USA Practice Committee of the Society for Assisted Reproductive Technology and Practice Committee of the American Society for Reproductive Medicine (2008) and published several opinion papers (on blastocyst culture, embryo transfer and on PGT-A). The first guidelines of the ESHRE PGT Consortium were published in 2005, as one of the missions of the Consortium was to bring overall standardisation and improve quality standards (Thornhill et al., 2005). In collaboration with the Cytogenetics European Quality Assessment (CEQA) and the UK National External Quality Assessment Service (UKNEQAS), now together in Genomics Quality Assessment (GenQA), the ESHRE PGT Consortium also initiated External Quality Assessment (EQA) schemes to provide an independent evaluation of laboratories and help them improving their techniques and reports. A review of the original guidelines yielded four sets of recommendations on different aspects of PGT: one on the organisation of PGT and three relating to the methods used: embryo biopsy, amplification-based testing and FISH-based testing (Harton et al., 2011a; Harton et al., 2011b; Harton et al., 2011c; Harton et al., 2011d). These four guidelines are now being updated and extended, taking into account the fast changes in the provision of PGT services. In these updated guidelines, the laboratory performing the diagnosis will be referred to as the PGT centre and the centre performing the IVF as the IVF centre.

General aspects of PGT, including patient selection, counselling, pregnancy and children follow-up and transport PGT, will be covered in the paper on organisation of PGT. Technical recommendations for embryo biopsy and tubing will be covered in the paper on embryo biopsy. Recommendations for genetic testing will be covered in the papers on detection of numerical and structural chromosomal aberrations, and on detection of monogenic disorders. The content of the different papers is aligned with the IVF/PGT clinical procedure in Fig. 1.

The ESHRE PGT Consortium recognises that owing to variations in local or national regulations and specific laboratory practices, there will remain differences in the ways in which PGT is practiced (from initial referral through IVF treatment, genetic testing to follow-up of pregnancies, births and children). This does not preclude a series of consensus recommendations for best practice based on experience and available evidence. These recommendations are not intended as the only approved standard of practice, nor are they legally binding. The unique needs of individual patients may justify deviation, and the recommendations must be applied according to individual patient's needs using professional judgement. However, recommendations and opinions may be used to frame laws and regulations, and practitioners should ensure that they comply with statutory requirements or clinical practice guidelines in their own countries. To keep the papers concise, repetitions have been excluded as much as possible and many cross-references were included. Therefore, it is recommended to not consult the papers independently but always as a set when one is seeking guidance on a PGT issue.

Materials and Methods

The current paper was developed according to the published methodology for ESHRE Recommendations for good practice papers (Vermeulen et al., 2019). A working group was composed of people



with hands-on expertise in the described techniques, aiming at a representation of different settings and nationalities. The working group members assessed the previous guidelines (Harton et al., 2011d) and deduced an outline for the current paper. As the aim was to provide technical guidance and support, it was not considered relevant to perform a formal literature search and as a result, no references were added, except for references to other guidance documents. All group members, according to their expertise, wrote a section that was later discussed in depth with the entire group until consensus was reached. Eleven online meetings were organised for discussion. The final draft of the paper was checked for consistency with the other papers of the series. The draft was then submitted for stakeholder review; it was published on the ESHRE website between 10 June and 10 July 2019, and ESHRE members were invited to send in comments. All comments were checked by the working group and incorporated in the final version where relevant. A review report is published on the ESHRE website.

For easier use of the recommendations, specific terms are explained in a glossary (Supplementary Table S1) and abbreviations are listed (Supplementary Table SII).

Results/Recommendations

Introduction to biopsy and sample collection

This paper provides detailed technical recommendations for the most applied biopsy methods and collection of biopsied samples for genetic testing.

The biopsy procedure of preimplantation embryos consists of two main steps: creating an opening in the zona pellucida (ZP) and removing polar bodies (PBs) or embryonic cells.

ZP opening may be performed either mechanically, chemically or using a laser.

ZP opening

Mechanical ZP opening (also termed partial zona dissection) was the first method used for opening the ZP and is still applied clinically, although to a lesser extent. The method involves creating a slit in the ZP using a sharp micropipette.

Chemical zona drilling involves the use of an acidic solution (acid Tyrode's) to locally dissolve the ZP. The method was widely used during the early era of cleavage-stage embryo biopsies. However, the subsequent implementation of laser technology, and concerns about potential toxicity of acid Tyrode's on embryo viability, has led the majority of laboratories to move away from chemical ZP drilling.

Laser is at present the most popular method of ZP opening for PB, cleavage stage and blastocyst biopsy. The method involves the use of a guided non-contact laser beam, which can be adjusted to create a ZP opening of the desired size in an accurate and rapid manner. The power of the laser beam and exposure (pulse length/width) should be carefully addressed following the manufacturer's specifications to avoid damage to PBs or embryonic cells.

In case of PB or cleavage stage biopsy, the size of the opening should not be too large, so as to avoid loss of blastomeres during embryo development.

Sample (PB or embryonic cell) removal

Several methods have been described for cell removal, depending on the stage and morphology of the embryo to be biopsied. Cell removal by aspiration inside the biopsy micropipette is the most widely used method and is applicable for all stages of biopsy (PB, cleavage stage and blastocyst biopsy). Alternatively, cells may be partially aspirated and

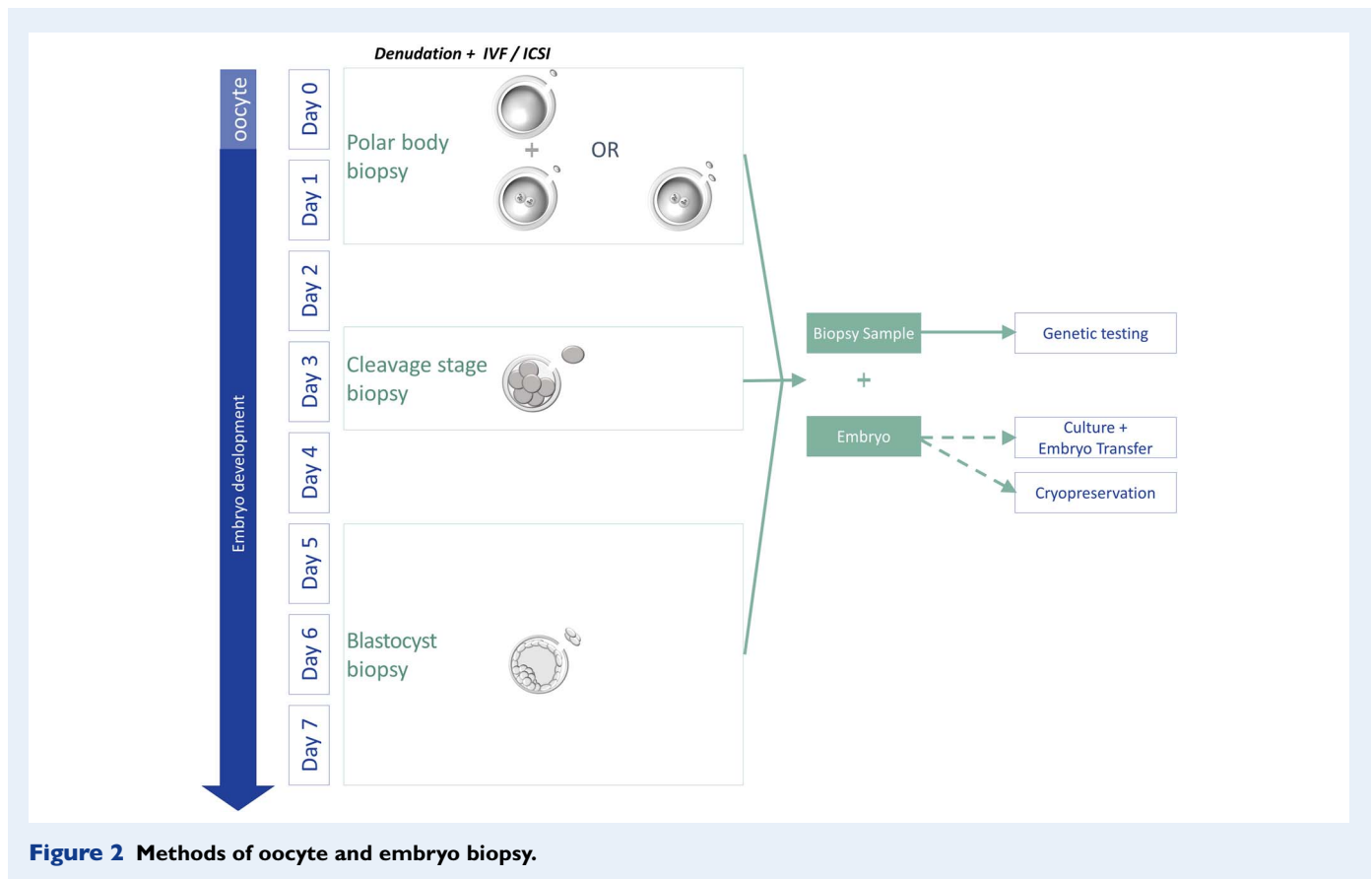


Figure 2 Methods of oocyte and embryo biopsy.

then pulled away from the embryo. Cell removal by extrusion or flow displacement has also been applied to cleavage-stage embryos, but the clinical application of these techniques has remained rather limited.

For blastocyst biopsy, aspiration and excision with a laser can be used, or aspiration in combination with mechanical detachment of the trophoctoderm (TE) cells (called flicking).

Time of biopsy

Biopsy can be performed by removal of one or two PBs from the unfertilised oocyte or the zygote, respectively, removal of one or two blastomeres at the cleavage stage or removal of several (5–10) TE cells at the blastocyst stage (Fig. 2). Although cleavage-stage biopsy was the most widely practised form of embryo biopsy for over a decade (Harton et al., 2011d), its clinical use has now been reduced.

PB biopsy may be an alternative to embryo biopsy, due to regulations that prohibit embryo biopsy in specific regions or countries, or if only maternal pathogenic variants, structural rearrangements or aneuploidies are investigated.

Blastocyst biopsy or TE biopsy is at present the most widely used technique (De Rycke et al., 2017). It provides more cells and is at an embryonic stage, more amenable for genetic analysis and less sensitive to possible damage.

Sample collection

After biopsy, cell(s) are washed and either fixed on a slide for FISH analysis or collected in small reaction tubes for amplification-based testing (called 'tubing'). As genome-wide technologies began to replace

the FISH method over the past decade and these technologies require whole-genome amplification (WGA) as a first step, tubing has become the most widely applied method for collection of biopsied samples. General recommendations on tubing have been formulated in this guidance paper.

Rebiopsy of embryos

Rebiopsy of embryos could be considered only in case of failed, incomplete or inconclusive genetic diagnosis, as the impact on further embryo development remains an area of investigation. The rebiopsy policy should be in accordance with local legislation.

Laboratory issues related to biopsy

Prior to the biopsy procedure, work surfaces, equipment and materials should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory.

During PGT-related procedures, protective clothing should be worn, including full surgical gown (clean, not sterile and changed regularly), hair cover/hat, face mask (covering nose and mouth) and preferably shoe covers or dedicated shoes. Gloves should be worn at all times and changed frequently. Gloves should be powder-free and well-fitting (e.g. nitrile, but not vinyl).

Insemination and culture

- Intracytoplasmic sperm injection (ICSI) is preferable for PGT, to minimise the risk of both maternal contamination from residual

cumulus cells and paternal contamination from surplus sperm attached to the ZP. Careful removal of cumulus cells (denudation) and rinsing of oocytes prior to ICSI and of zygotes in case of IVF after fertilisation check, are critical to avoid residual maternal contamination in the biopsy samples.

- Until time of biopsy, routine IVF culture conditions apply. The most adequate culture conditions, strategies and media should be used. If available, time-lapse imaging systems with a 'closed' culture system may be adopted to limit the exposure of the embryos to sub-optimal conditions and more easily decide on the optimal time for biopsy.
- Following biopsy, oocytes and embryos should be thoroughly rinsed to remove the biopsy medium before culture or cryopreservation.
- To culture embryos individually, the use of multiple-well dishes or droplets in separate dishes is advisable, to prevent mixing of embryos due to accidental movement during handling.

Setting up for biopsy

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

- Ensure that biopsy is performed according to written procedures by a suitably qualified practitioner.
- Minimise the duration of the biopsy procedure.
- Set biopsy criteria prior to performing clinical cases and adhere to them for all clinical cases. Routine updating of criteria should be done as necessary.
- Ensure all micromanipulation equipment is installed correctly, calibrated and maintained as per written procedures. Biopsies must be performed on a warmed stage.
- Ensure the appropriate reagents and micromanipulation tools are available, sterile and within their expiry date.
- Ensure that biopsy dishes are prepared, equilibrated and clearly labelled with at least the patient name and surname (female partner only or both female and male partners, according to each laboratory's policy), and oocyte/embryo number. Dishes should contain rinsing drops and a drop of biopsy medium of sufficient size to maintain pH, osmolality, osmolarity and temperature during the procedure, under oil.

Labelling and witnessing

General recommendations on labelling and witnessing throughout the IVF-PGT procedure are outlined in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). Specifically for the biopsy/tubing procedures, witnessing is recommended during the following stages: (i) immediately after biopsy to confirm the oocyte/embryo and sample number match; (ii) during spreading or tubing, to confirm that the sample identification matches the labelling on the relevant slide or tube, respectively; (iii) for further oocyte/embryo culture, at placing and labelling the oocyte/embryo into the culture dish, and (iv) in case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the oocyte/embryo into the cryopreservation device; (v)

for further embryo culture, at placing and labelling the embryo into the culture dish; (vi) after the diagnostic results are issued to ensure accuracy and correlation with the correct sample and/or embryo identification; and (vii) during the thawing/warming procedure and at the time of selecting the embryo(s) for transfer.

Other specific issues related to labelling and witnessing for biopsy:

- Biopsied oocytes and embryos must be cultured or cryopreserved individually with a clear identification system to ensure tracking of the biopsy sample (PB, blastomere or TE cells) and unambiguous post-diagnosis identification.
- When printed labels or barcodes are not feasible, the oocyte/embryo number should be written on the cryo-support, preferably in both numbers and letters.
- To ensure an oocyte/embryo-based traceability, a witness is mandatory, even when an electronic witnessing system is in place.

Quality control

General recommendations on quality management and risk assessment are presented in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

- Since biopsy is invasive, it could damage cells and DNA. Therefore, information about the integrity of biopsy samples (cell lysis, degeneration, degradation, etc.) should be noted and shared with the genetic laboratory.

Biopsy laboratory infrastructure, equipment and materials

Infrastructure

The embryology laboratory design should include a dedicated area for biopsy. A separate biopsy laboratory room may be advisable to provide adequate functionalities in IVF centres with high workload. The biopsy laboratory, whether it is a dedicated area or a room, should be designed taking into account all safety and environmental standards (air quality, positive pressure, laboratory access etc) as recommended in the 'Revised Guidelines for good practice in IVF laboratories', section 3 called 'Laboratory safety' to ensure good laboratory practice and to minimise any damaging effects on biological material (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016).

It is advised that tubing is performed in a dedicated area or room, in close proximity to the biopsy area (see section 'Sample collection').

Equipment

The biopsy equipment set-up includes an inverted microscope with heated stage and three-dimensional micromanipulators and microinjectors (air or oil), placed on antivibration pads, equivalent to a set-up for ICSI procedures. In addition, a stereoscope (for transferring oocytes/embryos in biopsy dishes and for tubing) and incubators should be available adjacent to the working area. A CE mark is recommended for all equipment, taking into consideration local legislation.

Special equipment such as a laser might be required for assisted hatching and blastocyst biopsy. The laser is usually included in a $\times 25$ or $\times 40$ objective of an inverted microscope and piloted by a software and camera. The laser can be controlled either using mouse or foot switch.

Materials

The following materials should be available before starting the biopsy procedure:

- capillaries;
- IVF certified dishes;
- IVF certified mineral oil;
- buffered media (HEPES, MOPS or other);
- micropipettes, which differ according to biopsy stages. The holding pipette can be the same as for ICSI or one with an adapted diameter can be used. The biopsy pipette has a special diameter according to the biopsy stage (10–15 µm for PB biopsy, 30–35 µm for cleavage stage biopsy, 25–30 µm for blastocyst biopsy).

Training for biopsy

The embryo biopsy laboratory should be supervised by a person with recognised expertise in clinical embryology and preferably also basic knowledge in medical genetics.

The biopsy procedure should be performed by experienced practitioners with basic skills in general embryology and micromanipulation, after appropriate training and following standard operating procedures (SOPs). The number of experienced practitioners is dependent on the number of procedures. At least one back-up practitioner is recommended. Deviations from SOPs and protocols should be properly documented and justified.

Training for biopsy should be to the standards required for certification in routine embryology and should be documented. Training for each biopsy stage (PB, cleavage stage, blastocyst stage) should consist of two steps: preclinical training and supervised clinical training.

- For preclinical training, it is recommended that at least 50 oocytes or 50 embryos are used to practise all steps (i.e. opening of the ZP, removal of cells) of the biopsy procedure. The source of the material will depend on local regulations. Trainees can proceed to the clinical training once they meet the procedure requirements.
- The supervised clinical training should include at least an additional 20 oocytes or embryos if the practitioner has extensive experience with micromanipulation and 40 oocytes or embryos for practitioners without experience. To evaluate clinical training, post-biopsy damage and survival after continued culture or after thawing/warming need to be monitored. In addition, damage/lysis of the biopsy sample and amplification outcomes should be evaluated. All parameters should be comparable with the standards of the laboratory and the PGT consortium data (De Rycke et al., 2017).
- Biopsy should be supervised by a clinical embryologist, preferably holding the relevant certification for their own country, and/or the ESHRE certification for clinical embryology.

Biopsy stage and procedure

PB biopsy

PBs are the by-products of female meiosis, which allows predicting the resulting genotype of the maternal contribution to the embryo. In most

cases, polar body 1 (PB1) can be distinguished from polar body 2 (PB2), based on size, shape and position within the perivitelline space.

Organisation of the biopsy. PB biopsy can be performed simultaneously or sequentially.

- In simultaneous biopsy, PB1 and PB2 are removed between 6 and 9 h after insemination.
- In sequential biopsy, PB1 is removed within 4 h following oocyte retrieval and PB2 is removed following fertilisation assessment (16 to 18 h after insemination). Earlier removal of PB2 (6 to 9 h after insemination) is also acceptable.

Cryopreserved/warmed oocytes can be biopsied similarly to fresh oocytes.

Biopsy procedure.

- The ZP opening should be performed with laser or mechanically and the diameter of the hole should be adapted to the diameter of the biopsy pipette.
- In sequential biopsy, after aspiration of PB1 the oocyte is fertilised and examined for the presence of pronuclei and extrusion of PB2, which is removed in the same manner as PB1. Although a second slit may be necessary to reach the second PB, it should be avoided as it may affect blastocyst hatching.
- In simultaneous biopsy, the PBs should be positioned in the same focal plane to low removal through a single slit in the ZP.
- PB1 and PB2 should be clearly distinguished and identified before they are transferred to separate tubes or fixed, according to the method of PGT analysis. When biopsy is performed simultaneously, discrimination of PB1 and PB2 should be reported.
- The biopsied oocytes/zygotes are then cryopreserved or returned to culture.

Embryo transfer and cryopreservation. Embryo transfer is possible at cleavage stage or blastocyst stage, according to the policy of the centre. Cryopreservation of zygotes or supernumerary embryos can be performed according to IVF laboratory policy and patient's preference.

Rebiopsy of embryos. If allowed by local regulations, rebiopsy could be considered at the cleavage or blastocyst stage.

Cleavage-stage biopsy

Organisation of the biopsy. Cleavage-stage biopsy is performed on Day 3 post-insemination, between the six-cell stage and the pre-compaction stage of embryo development. The exact timing varies according to timings of laboratory procedures. Cryopreserved/warmed embryos can be biopsied on Day 3 similarly to fresh embryos. It is recommended to biopsy embryos at the six or more cell stage on Day 3 with an acceptable grade (fragmentation limited to 25%) and according to the laboratory policy. Embryos with a degree of fragmentation between 25 and 50% can be biopsied, taking into account lower chances of implantation and possible issues with genetic diagnosis (misdiagnosis, failed diagnosis). Alternatively, these embryos may be cultured to the blastocyst stage for biopsy.

Biopsy procedure. Biopsy is performed either directly in biopsy medium (Ca²⁺/Mg²⁺-free) or in HEPES-buffered medium after

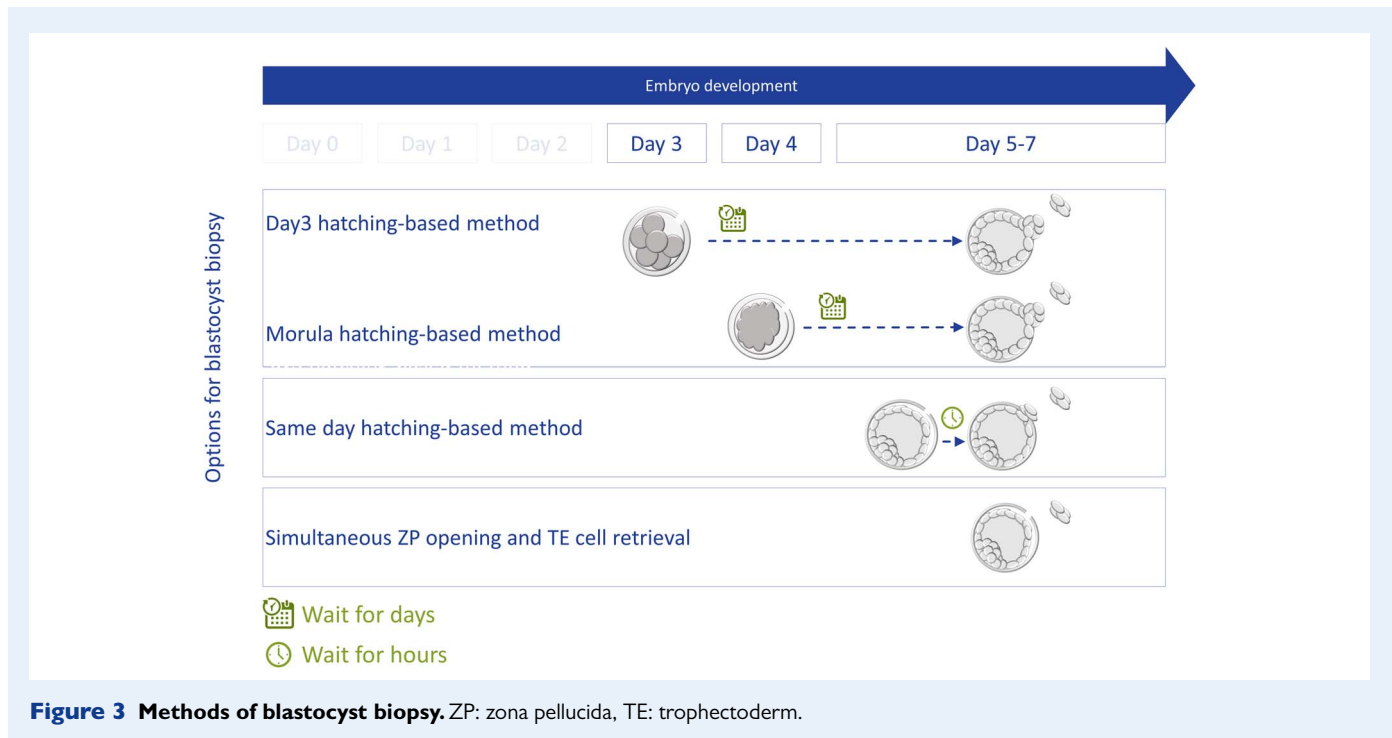


Figure 3 Methods of blastocyst biopsy. ZP: zona pellucida, TE: trophectoderm.

incubation in biopsy medium, according to manufacturers' recommendations.

ZP hatching/opening/breaching is performed with the laser or mechanically. The ZP opening should be up to the diameter of the biopsy pipette. It is recommended to visualise the nucleus to ensure that a nucleated cell is removed and to avoid binucleated cells for FISH. If the blastomere lyses, it is recommended to change the biopsy pipette. Biopsied blastomeres are then fixed or tubed for further PGT analysis. The biopsied embryo should be gently, but thoroughly, rinsed in culture medium before continuing culture.

It is recommended to biopsy only one cell. Nevertheless, two-cell biopsy may be required to bring the genetic testing accuracy to an acceptable level, or in case of cell lysis or absence of a nucleus.

Embryo transfer and cryopreservation. After biopsy, embryos are cultured according to standard IVF culture conditions. Transfer is possible on Day 4 post insemination or at the blastocyst stage. It is recommended to cryopreserve supernumerary embryos at the blastocyst stage.

Rebiopsy of embryos. Rebiopsy could be considered at a later stage, according to embryo morphology and development and embryo transfer policies. It is recommended to use the original ZP opening site.

Blastocyst biopsy

TE biopsy at the blastocyst stage enables the removal of several cells for genetic testing while being non-invasive to the inner cell mass (ICM), which is destined for foetal development.

Organisation of the biopsy. Blastocyst biopsy may be performed on fresh or previously cryopreserved embryos that have been assessed

for blastocyst formation. Blastocyst biopsy is performed on Days 5–7 post insemination, according to their rate of development, once the ICM is clearly visible. Alternatively, these embryos can be further cultured up to expansion. The exact timing varies according to timings of laboratory procedures. Cryopreserved/warmed blastocysts may be biopsied once they have reached re-expansion, similarly to fresh blastocysts.

Biopsy procedure. The biopsy procedure may vary depending on the morphology and quality of the blastocyst, expansion grade and the position of the ICM. Furthermore, there are some variations among operators and laboratories.

Biopsy is performed in buffered medium.

For blastocyst biopsy, the use of non-contact lasers is highly recommended, first to make a hole in the ZP and second to excise TE cells. There are several methods described for biopsy of blastocysts that are not fully hatched (Fig. 3):

- The ZP opening may be performed on Days 3–4 post insemination, with removal of the TE cells on Days 5–7 post insemination.
- The ZP opening may be performed early on the day of blastocyst formation, followed by a period of culture to allow herniation of TE cells from the ZP and TE cell removal.
- Simultaneous ZP opening and TE cell excision on the day of full blastocyst expansion.

For biopsy, the ICM of the blastocysts should be positioned between 7 and 11 o'clock so that it is clearly visible and distant from the ZP opening and avoiding the ICM by the suction from the holding pipette. TE cells are then aspirated into the biopsy pipette with gentle suction. Laser pulses are directed at the junctions between cells to either excise the aspirated cells from the blastocyst or to minimise cell damage while detaching TE cells mechanically

via a quick flicking movement of the biopsy pipette against the holding pipette. It is recommended to fire as few laser shots as possible.

If blastocysts are fully hatched, biopsy is still feasible and excision of TE cells is advisable using a combination of laser pulses and a flicking movement.

- It is recommended to biopsy 5 to 10 TE cells for genetic testing (according to the stage of development and number of cells constituting the blastocyst). The impact of removal of more than 10 TE cells on embryo development remains an area of further investigation.
- $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium should not be used for blastocyst biopsy.
- To avoid cross-contamination during biopsy, it is recommended to change the biopsy pipette for each blastocyst. Alternatively, it is acceptable to thoroughly rinse the biopsy pipette, but it should be verified in the laboratory that this suffices to avoid cross-contamination.
- It is also recommended that following biopsy, the blastocyst is immediately transferred in culture medium or cryopreserved.

Embryo transfer and cryopreservation. It is acceptable to perform embryo transfer in a fresh cycle if genetic testing results are available in a short time and embryos are not in an advanced stage (totally hatched at biopsy time). If the results are only available after several days, embryos have to be cryopreserved. Vitrification is the established technique for blastocyst cryopreservation. Blastocysts should be cryopreserved immediately after the biopsy according to cryopreservation procedures.

Time between blastocyst biopsy and cryopreservation is very important; it is recommended to cryopreserve them as soon as possible before re-expansion, particularly in those cases where blastocysts are totally hatched.

Rebiopsy of embryos. Rebiopsy at the blastocyst stage could be considered, according to blastocyst morphology, before or after cryopreservation. Before rebiopsy, adequate time is needed for blastocyst cavity re-expansion to occur. It is recommended to use the original ZP opening site. Following rebiopsy, it is recommended to proceed immediately to cryopreservation.

General strengths and limitations

The main characteristics of the three biopsy approaches are summarised in Table I.

PB biopsy

PB biopsy is the only option for PGT when, according to the local regulation, biopsy can be done only before syngamy. PBs are waste products of maternal meiosis. The biopsy might be performed only on Day I or on both Day 0 and Day I. In any case, both PBs are required for a successful/accurate diagnosis and must be reliably distinguished and identified. Simultaneous biopsy is less time-consuming but more complex than sequential biopsies, as discrimination of PB1 and PB2 may be problematic, especially in the case of PB fragmentation.

Mitotic errors and paternally derived meiotic errors and pathogenic variants cannot be detected from PBs. Nonetheless, in case of maternally derived meiotic aneuploidies or maternal pathogenic variants, this biopsy strategy is sufficient for testing.

PB biopsy entails a high workload since all oocytes and/or zygotes must be biopsied regardless of their further development, which is unpredictable at this stage. Moreover, there is a moderate risk for technical complications, such as fragmentation or degeneration of the PBs.

Following biopsy, extended embryo culture might be performed while waiting for genetic results, but this is not mandatory. If required, the PB biopsy approach is compatible with fresh embryo transfer.

The amount of DNA is limited, since single cells are analysed, and the estimated rate of inconclusive diagnosis is expected to be lower than 10%. Nonetheless, rebiopsy can be performed at a later developmental stage (if allowed by local regulations) and still within the timing to allow fresh embryo transfer (if required).

Cleavage-stage biopsy

Cleavage-stage biopsy results in the collection of a single blastomere (the removal of two cells is discouraged). At this stage of development, blastomeres have the potential to contribute to the embryo proper since their commitment to either the ICM or TE is not firmly established.

Meiotic errors from both parents can be detected, but mitotic errors leading to chromosomal mosaicism cannot be estimated from a single blastomere.

The amount of DNA is limited since a single cell is analysed and the estimated rate of inconclusive diagnosis is expected to be lower than 10%. Nonetheless, rebiopsy can be performed at the blastocyst stage and still within the timing to allow fresh embryo transfer (if required).

Cleavage-stage biopsy is performed on Day 3 only. Cleavage-stage biopsy entails a moderate to high workload, as it is not frequent that zygotes arrest before Day 3 and all must be biopsied regardless of their further development, which is unpredictable at this stage. Following biopsy, embryos may be either subjected to extended embryo culture while waiting for the genetic result and used in fresh embryo transfers or cryopreserved.

This approach is characterised by the highest worldwide experience until now and its complexity is moderately low.

Blastocyst biopsy

TE biopsy entails the collection of a multicellular section (5 to 10 cells) on Days 5–7 from a part of the blastocyst, which gives rise to the placenta and the extra-embryonic membranes (the foetus originates from the ICM, which is kept intact).

Blastocyst biopsy offers several advantages compared with alternative biopsy procedures, including higher reliability by the analysis of a higher number of cells.

Alternative blastocyst biopsy approaches (Fig. 3) involve different learning curves and levels of skills, specifically the following: (i) the Day 3 and Day 4 hatching-based strategies are more time-consuming but easier unless hatching starts from the ICM; (ii) the same-day hatching-based strategy is also more time-consuming since it requires a constant check of the blastocyst (ideally to be conducted via a time-lapse incubator), but it is also the easiest approach; (iii) the simultaneous ZP opening and TE cell retrieval strategy is the least time-consuming but also difficult to acquire as a skill for the laboratory personnel.

Table 1 The main oocyte and embryo biopsy approaches to conduct preimplantation genetic testing.

	PB biopsy	Blastomere/cleavage stage biopsy	Blastocyst/TE biopsy
Fragment origin	Waste products of maternal meiosis	Totipotent cells	TE gives origin to the placenta and the extra-embryonic membranes
Number of cells retrieved	2 (both required)	1 Two might be retrieved, but it is discouraged	5–10 TE cells
Complexity in the acquisition of the skill	Day 0 + Day 1 approach: Moderate Day 1-only approach: Moderate to high (PB1 and PB2 should be reliably recognised)	Moderate	Day 3 hatching-based strategy: Low to moderate Morula hatching-based strategy: Low to moderate Same day hatching-based strategy: Low to moderate Simultaneous ZP opening and TE cells retrieval strategy: Moderate to high
Complexity in the performance of tubing	Moderate to high	Moderate	Moderate to high
Embryo development	Unpredictable at this stage	Only cleaved embryos of a certain morphological quality are biopsied	Only embryos developing to the blastocyst stage are biopsied
Laboratory workload	Very high to high (all oocytes/zygotes should be biopsied regardless of their further development)	High to moderate (all embryos should be biopsied regardless of their further development)	Multiple time slots required (Days 5–7) and cryopreservation mostly mandatory Day 3 hatching-based strategy: High to moderate (all embryos should undergo ZP opening at the cleavage stage regardless of their further development) Morula hatching-based strategy: Moderate (all morulas should undergo ZP opening regardless of their further development) Same day hatching-based strategy: Moderate (all blastocysts should undergo ZP opening and monitoring of TE cells hatching) Simultaneous ZP opening and TE cells retrieval strategy: Moderate to low
Extended embryo culture	Suggested, but not mandatory	Suggested, but not mandatory	Mandatory
Cryopreservation following biopsy	According to laboratory/country policy	According to laboratory/country policy	Mostly mandatory
Meiotic errors assessed	Only maternal	Yes	Yes
Mitotic errors assessed	No	No	Possible within given technical, methodological and biological limitations (e.g. molecular platform- and bioinformatic parameters-dependent, inevitable sampling bias)
Inconclusive diagnoses	~10%	~10%	<5%

The parameters 'low', 'moderate' and 'high' were agreed unanimously after a thorough discussion among all the members of the working group. TE, trophectoderm; PB, polar body; ZP, zona pellucida

The choice among these three protocols depends on the laboratory policy.

More time slots should be planned from Day 5 to Day 7 to conduct TE biopsy in a busy IVF clinic; yet, less embryos per patient are biopsied, namely only the ones reaching this stage of development. Following TE biopsy, cryopreservation is mostly mandatory due to the turn-around time of the testing strategies required for PGT. Therefore, laboratories must have in place an efficient cryopreservation programme.

Meiotic errors are reliably assessed from the TE. Mitotic errors leading to chromosomal mosaicism might be detected within given technical, methodological and biological limitations, mainly depending on the technique used to conduct PGT, on the validation parameters defined within each genetic laboratory, and on the inevitable sampling bias underlying the retrieval of a biopsy from a mosaic blastocyst.

The amount of DNA is higher since a multiple cell fragment is analysed and the estimated rate of inconclusive diagnosis is expected

to be lower than 5%. Furthermore, blastocyst biopsy allows for an efficient way to run multiple analyses for different indications from the same sample after WGA (for instance chromosomal abnormalities and pathogenic variants).

Sample collection

After biopsy, cell(s) are washed and either fixed on a slide for FISH analysis (called 'spreading/fixation') or collected in small reaction tubes for amplification-based testing (called 'tubing'). Efficient transfer of biopsied cells to slides or reaction tubes is a critical step towards the success of a PGT cycle. Spreading/fixation or tubing requires careful and accurate handling of the sample to prevent exogenous DNA contamination.

With regards to spreading and fixation of samples for FISH, several methods have been described and these are still acceptable (Harton et al., 2011c). As genome-wide technologies have largely replaced the methods of FISH, the remainder of the current section is dedicated to tubing.

Laboratory issues related to tubing

Tubing should be carried out under stringent precautions to minimise contamination and maximise chances for amplification.

Personnel should wear protective clothing including full surgical gown (clean, not sterile and changed regularly), hair cover/hat, face mask (covering nose and mouth) and preferably shoe covers or dedicated shoes. Gloves should be worn at all times and changed frequently. These should be well-fitting (e.g. nitrile, but not vinyl, examination gloves).

The materials and reagents for tubing should be prepared in advance by the staff of the PGT centre, or by the staff of the IVF centre according to the instructions of the reference genetic laboratory.

Labelling and witnessing. General recommendations on labelling and witnessing are presented in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. et al., 2020).

Quality control. General recommendations on quality management and risk assessment are presented in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. et al., 2020).

Laboratory infrastructure, equipment and materials

Infrastructure. The tubing area should be in a DNA-free environment. DNA decontamination measures required for the tubing area are mostly incompatible with IVF good laboratory practices.

It is therefore advised that tubing is performed in a dedicated area or room in close proximity to the biopsy area.

Equipment and materials. Work surfaces, equipment, etc., should be cleaned with DNA decontamination solutions or 10% bleach prior to each use, although the use of the latter is not recommended within the embryology laboratory. It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.

To minimise contamination, the preparation of materials and reagents, and the tubing of biopsied cells, should be performed in a dedicated laminar flow hood or dedicated clean area, which is irradiated with UV-C light for DNA decontamination prior to each

use. Tubing equipment set-up further includes a microcentrifuge and a stereoscope or an inverted microscope.

- Whenever possible, all solutions or reagents should be purchased 'ready to use' and should be of 'molecular biology' grade or equivalent. All reagents (purchased and in-house) should be tested (for efficiency and contamination) and validated. All plasticware used, including filter tips, should be certified DNA-free and DNase-free.
- Batch or lot numbers should be recorded for traceability, according to the quality standards in the laboratory.
- Whenever possible, solutions or reagents should be split into small aliquots and no aliquot should be re-used for a clinical case.
- It is recommended to avoid repeated freeze-thaw cycles of reagents.
- Materials and reagents may be UV-C irradiated or autoclaved (when applicable, for example tube racks). Alternatively, reagents and solutions made in-house can be autoclaved, preferably using a PGT-dedicated autoclave or filter-sterilised followed by UV-C irradiation.

The tubing materials and reagents should be kept away from any DNA source and preferably stored in the pre-amplification area.

The following materials should be available before starting the tubing procedure:

- IVF certified dishes;
- IVF certified mineral oil;
- transfer pipettes.

Training for tubing

The process of tubing requires adequate training, which is separate from the embryo biopsy training. Similar to embryo biopsy, training for tubing should be supervised by an experienced certified clinical embryologist/biopsy practitioner and/or a specialised geneticist, competent and authorised to perform clinical diagnostics according to local or national regulations (see also [Training for biopsy](#)). Training for tubing should evaluate amplification outcomes and ensure absence of exogenous DNA contamination.

Tubing procedure

- Prior to the biopsy procedure, dishes with numbered drops of washing buffer under mineral oil should be prepared. Alternatively, dishes with numbered drops of washing buffers should be prepared immediately before the tubing procedure without using mineral oil.
- Tubes should be clearly numbered and be readily available.
- Biopsied cells should be washed at least twice using a sterile transfer pipette before transfer into reaction tubes. Special care must be taken while washing cells from the TE, as they are usually sticky. However, care should be taken to avoid losing genetic material between consecutive washing steps.
- It is recommended that a new pipette is used for each embryo to prevent DNA carryover.
- If the single cell is lysed or part of the cell sample is lysed during washing or transfer, the pipette is possibly contaminated and has

to be discarded. For cleavage-stage biopsy, another blastomere should be sampled, whenever possible.

- The amount of medium co-transferred with the biopsied cell(s) into the tube should be minimal ($< 1 \mu\text{l}$). Tubes may be centrifuged in a microfuge before being stored or processed.
- It is acceptable to transfer biopsied cells to tubes with or without microscopic visualisation.
- Tubing can be performed in PBS, or directly in lysis buffer, depending on the protocol requirements of the PGT centre. Both alkaline and proteinase K/sodium dodecyl sulphate treatment are acceptable for cell lysis.
- A minimum of one negative control per buffer (sample collection buffer or washing media, depending on the protocols of the PGT centre) is recommended to control for contamination during each procedure of cell sample collection (i.e. the IVF laboratory negative control); for example, collection at two different time points for a specific cohort of embryos should yield a minimum of two negative controls of this type. As the contamination risk is substantially higher when working with single cells in comparison with a few cells, the number of negative controls should preferably be increased.

After tubing, the samples can be kept at room temperature, cooled or frozen, depending on the duration of storage, the laboratory conditions and recommendations of the genetic laboratory.

For transport of biopsied cells, the shipment can be at room temperature, cooled or frozen, in accordance with the logistic arrangements of the service-level agreement between the IVF centre and the PGT centre. The buffer containing the biopsied material within the reaction tube may be covered with mineral oil during transport. If shipment of the cells is done using dry ice (solid carbon dioxide), it is recommended that the tubes are well closed and packaged thoroughly, preferably in a suitable rack with lid, packaged in a plastic sealable bag to prevent carbon dioxide getting in contact with the sample.

Cryopreservation of biopsied oocytes/embryos

There are several situations when oocytes/embryos may be frozen in cases of PGT, depending on laboratory strategy and local regulations:

- prior to the biopsy (e.g. accumulation of oocytes/embryos; surplus oocytes/embryos from previous non-PGT cycles);
- after the biopsy (i.e. testing platforms often require cryopreservation as a mandatory step to give time for the genetic laboratory to analyse the samples);
- or after the biopsy and diagnosis (e.g. fresh embryos have been transferred but supernumerary tested embryos need to be stored).

At any stage along preimplantation development, cryopreservation via vitrification is recommended and the same protocol applies to biopsied and non-biopsied embryos. Biopsied embryos must be vitrified individually in a cryo-support properly labelled, and witnessing is mandatory.

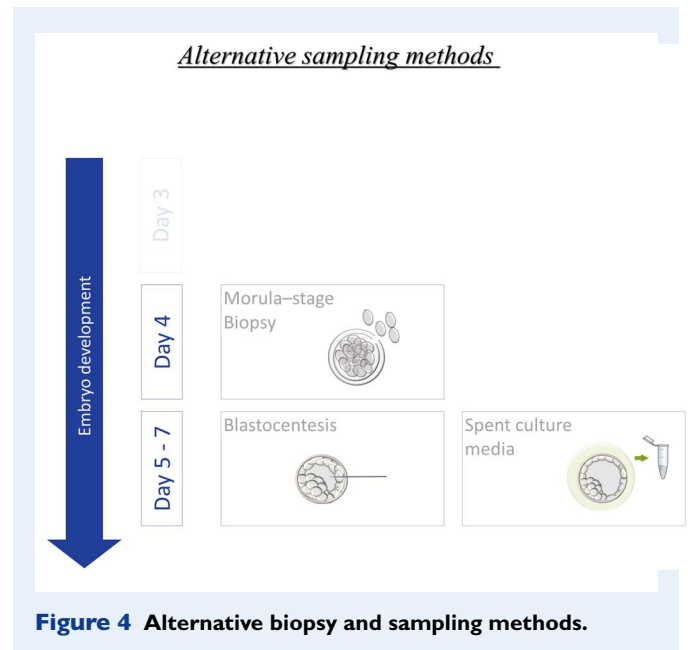


Figure 4 Alternative biopsy and sampling methods.

Multiple vitrification-warming cycles may be necessary in a minority of PGT cases; however, the influence of this approach on embryo viability/implantation and clinical outcomes still needs further investigation.

It is recommended that each centre decides its own policy regarding the cryopreservation/vitrification of PGT embryos, based on its experience and performance.

Alternative biopsy methods

Morula-stage biopsy is under validation as an alternative biopsy method (Fig. 4).

Morula-stage biopsy

The biopsy of morula-stage embryos on Day 4 is performed after artificial decompaction (requiring $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium), characterised by the loss of intercellular contacts and re-establishment of a spherical cellular shape. It is technically similar to cleavage-stage biopsy but allows procurement of the same number of cells as blastocyst biopsy. This technique requires more evidence before broad clinical implementation.

Alternative sampling methods

Cell-free DNA analysis (blastocentesis and spent culture media) is under validation as an alternative sampling method for genetic testing (Fig. 4).

Blastocentesis

Blastocoel fluid contains cell-free genomic DNA, which can be collected using a minimally invasive approach. The DNA can be purified and amplified for downstream genetic testing. According to the results to date, the efficacy and accuracy of this technique are insufficient and need further elaboration before being clinically applicable.

Spent culture media

Cell-free genomic DNA obtained (in a non-invasive way) from the embryo culture medium may be potentially used for genetic testing. One of the limitations of the technique is the current inability to discriminate embryonic DNA from sources of DNA contamination. Further optimisation of the methodology is required.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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G.K. and G.C. chaired the working group. All authors contributed to conception and design, drafting the content and discussing it. All authors approved the final version.

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Dr Cimadomo reports personal fees from Irvine Scientific, outside the submitted work; Dr Coticchio reports personal fees from IBSA and Excemed, outside and not related to the submitted work. The other authors had nothing to disclose.

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