ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for preimplantation genetic testing

AUTHORS

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GENERAL INTRODUCTION

The general introduction (including figure 1) is included in the Paper on Organisation of PGT, and will be copied to the final version of the 4 papers.

METHODS

A working group was composed of people with hands-on expertise on the described techniques, aiming at a representation of different settings and nationalities. The working group members assessed the previous guidelines (Harton *et al.*, 2011) and deducted an outline for the current paper. All group members according to their expertise, wrote a section that was later discussed in depth with the entire group until consensus. 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 XX and XX , 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.

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 laser.

Zona pellucida opening

Mechanical zona pellucida 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 have 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 exposure to the laser should be short and care should be taken to avoid damage of embryonic cells.

In case of polar body or cleavage stage biopsy, the size of the opening should not be too large as to avoid loss of ***blastomeres*** during embryo development, but neither too small, to allow embryo ***hatching*** at the blastocyst stage.

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 then pulled away from the embryo. Cell removal by extrusion or flow displacement has also been applied on 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 removal of the ***trophectoderm (TE)*** cells.

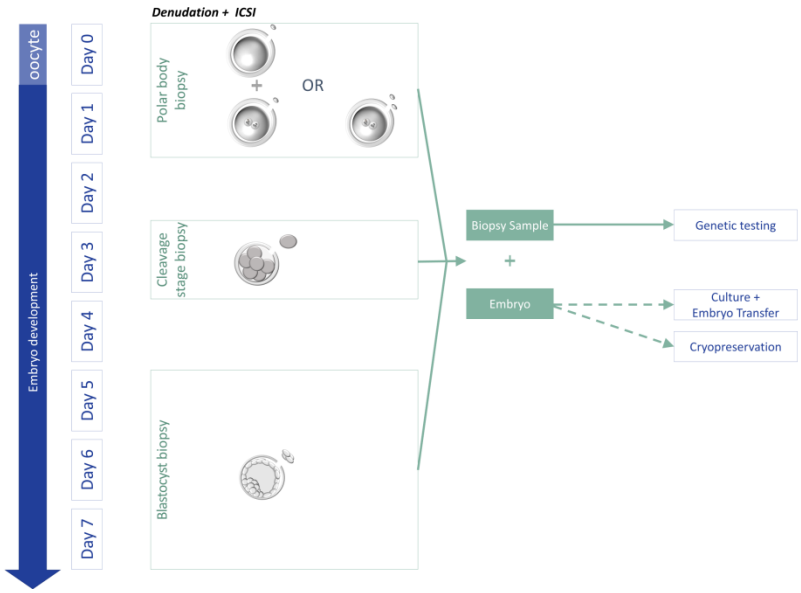
Stages of embryo biopsy

Biopsy can be performed by removal of one or two polar bodies from the unfertilized 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 (Figure 2). While cleavage-stage biopsy was the most widely practised form of embryo biopsy for over a decade (Harton *et al.*, 2011), its clinical use has now been reduced.

Polar body biopsy may be an alternative to embryo biopsy due to regulations that prohibit embryo biopsy in specific regions or countries, or if only maternal mutations 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.

Figure 2



Sample collection

After biopsy, cell(s) are washed and either fixed on a slide for fluorescence *in situ* hybridisation (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 last 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 in case of failed, incomplete, or inconclusive genetic diagnosis. The rebiopsy policy should be in accordance with the local legislation.

1. 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.

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).

1.1 Insemination and culture

- *Intracytoplasmic sperm injection (ICSI)* is mandatory for PGT to avoid the risk of both maternal contamination from residual cumulus cells and paternal contamination from extraneous sperm

attached to the ZP or non-decondensed sperm within blastomeres. Careful removal of cumulus cells (***denudation***) and rinsing of oocytes prior to ICSI 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. The exposure of the embryos to ***sub-optimal environmental conditions*** should be limited, whenever possible. If available, ***time-lapse imaging*** systems may be adopted to limit the exposure of the embryos to sub-optimal conditions.
- Following biopsy, oocytes and embryos should be thoroughly rinsed before culture or ***cryopreservation***.
- 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.
- 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.

1.2 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.
- It is recommended to minimize the duration of the biopsy procedure.
- 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.
- Ensure all micromanipulation equipment is installed correctly, calibrated and maintained per written procedures. Biopsies must be performed on a warmed stage.
- Ensure the appropriate reagents and micromanipulation tools are available, sterile and within their expiration date.
- Ensure that biopsy dishes are prepared, equilibrated and clearly labelled with at least the patient name and oocyte/embryo number. Dishes should contain rinsing drops and a drop of biopsy medium of sufficient size to maintain pH, osmolarity and temperature during the procedure, under oil.

1.3 Labelling and witnessing

General recommendations on labelling and witnessing throughout the IVF-PGT procedure are outlined in the paper on organisation of PGT (refer ORG paper). Specifically for the biopsy/tubing procedures, witnessing is recommended during the following stages: (1) immediately after biopsy to confirm the embryo and sample number match; (2) during spreading or tubing, to confirm that the sample identification matches the labelling on the relevant slide or tube, respectively; (3) for further embryo culture, at placing and labelling the embryo into the culture dish, and (4) in case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device.

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 embryo number should be written on the cryo-support, preferably in both numbers and letters.
- To ensure an embryo-based traceability, a witness is mandatory, even when an ***electronic witnessing system*** is in place.

1.4 Quality control

General recommendations on quality management and risk assessment are presented in the paper on organisation of PGT (refer ORG paper).

- Since biopsy is invasive, it could damage cells and DNA. Therefore, the impact of the laser on the integrity of biopsy samples should be validated before clinical application

2. BIOPSY LABORATORY INFRASTRUCTURE, EQUIPMENT AND MATERIALS

2.1 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 (2015)", 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 a close proximity of the biopsy area (see section 6).

2.2 Equipment

Biopsy equipment set up includes an inverted microscope with heated stage and three-dimensional micro-manipulators and micro-injectors, placed on antivibration pads. This equipment can be used either for ICSI or for any stage of oocyte/embryo biopsy. Air and oil micro-injectors are acceptable for biopsy. In addition, a stereoscope (for transferring oocytes/embryos in biopsy dishes and for tubing) and incubators should be available adjacent to the working area.

Special equipment is requested:

- Laser for ***assisted hatching*** and blastocyst biopsy: laser included in a 25x or 40x objective of the inverted microscope, piloted by a software and camera. The lowest amount of heat is recommended to avoid any embryo damage risk. A CE mark would be an advantage. Laser use can be performed either using mouse or foot switch.

2.3 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 differ according to biopsy stages. Holding pipette can be the same as for ICSI or use one with an adapted diameter. The biopsy pipette has a special diameter according to the biopsy stage (10-15µm for PB biopsy, 30-35µm for cleavage stage biopsy, 25-30µm for blastocyst biopsy).

3. TRAINING FOR BIOPSY

The embryo biopsy laboratory should be supervised by a person with recognized expertise in clinical embryology and preferable also qualifications in medical genetics.

The biopsy procedure should be performed by experienced practitioners with basic skills of 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 to 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 2 steps: preclinical training and supervised clinical training.

- For preclinical training, it is recommended that at least 50 oocytes or embryos are used to practice 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 to the standards of the laboratory and the PGT consortium data.
- Biopsy should be supervised by a clinical embryologist, who holds the relevant certification for their own country, and/or, where none exist, the ESHRE certification for clinical embryologists.

4. BIOPSY STAGE AND PROCEDURE

4.1 Polar body biopsy

Polar bodies 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***.

Organization of the biopsy

Polar bodies biopsy can be performed simultaneously or sequentially.

- In simultaneous biopsy PB1 and PB2 are removed between 6 and 9 hours after insemination.

- In sequential biopsy, PB1 is removed within 4 hours following oocyte retrieval and PB2 is removed following fertilization assessment (16 to 18 hours after insemination). Earlier removal of PB2 (6 to 9 hours 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 fertilized 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 impact blastocyst hatching.
- In simultaneous biopsy, the PBs should be positioned in the same focal plane to allow 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.

4.2 Cleavage stage biopsy

Organization of the biopsy

Cleavage stage biopsy is done on Day 3 post-insemination, between the 6-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 only embryos at 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, taken 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.

Embryos that did not reach the 6-cell stage on the time of biopsy may be included to help establish haplotypes, for instance in ***de novo mutation*** cases (see also the paper on detection of monogenic disorders (refer PGT-M paper), but they should not be transferred.

Biopsy procedure

Biopsy is performed either directly in biopsy medium (Ca^{2+}/Mg^{2+}-free medium) or in HEPES-buffered medium after incubation in biopsy medium according to manufacturer's recommendations.

Zona 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 a nucleated cell is removed, and binucleated cells are avoided. 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 rinsed in culture medium at least once 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. Supernumerary embryos can be cryopreserved at any stage up to the blastocyst stage according to cryopreservation procedures.

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.

4.3 Blastocyst biopsy

Trophectoderm 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.

Organization 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 Day 5-7 post-insemination, according to their rate of development, once the inner cell mass 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.

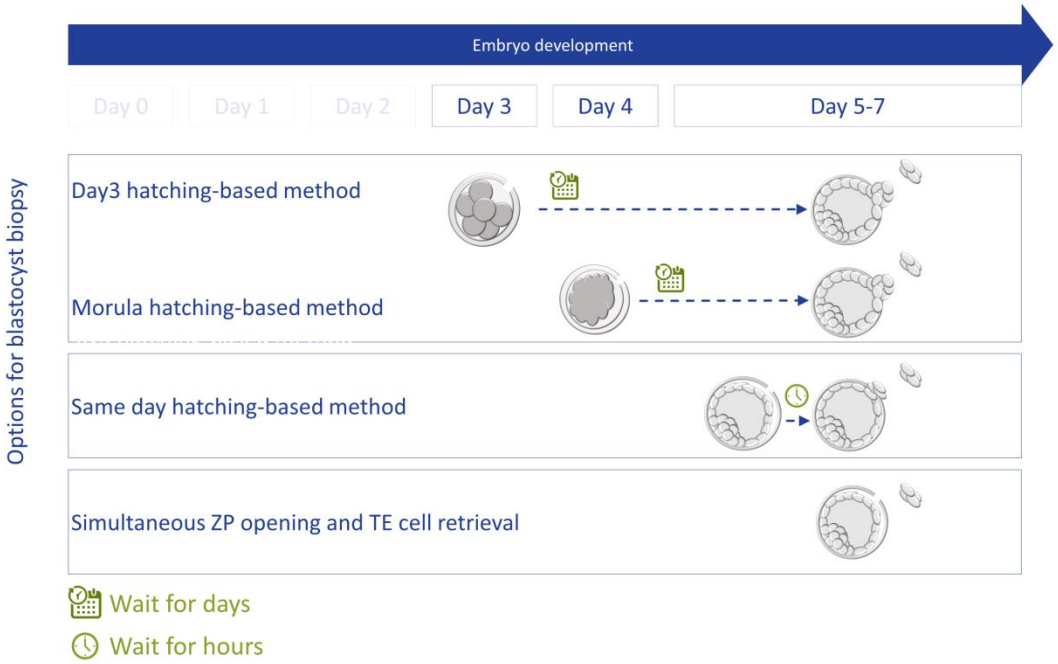
Biopsy is performed in HEPES-buffered medium.

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

- The ZP opening may be performed on Day 3-4 post-insemination, with removal of the TE cells on Day 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.

Figure 3: Methods of blastocyst biopsy



For biopsy, the ICM of the blastocysts should be positioned between 7 to 11 o'clock so that it is clearly visible and distant from the ZP opening. 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 minimize cell damage while detaching TE cells mechanically via a quick flicking movement of the biopsy pipette against the holding pipette.

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.
- Ca^{2+}/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, blastocyst is immediately transferred in culture medium or cryopreserved.

Embryo transfer and cryopreservation

Embryo transfer can be performed 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.

5. GENERAL STRENGTHS AND LIMITATIONS

The main characteristics of the three biopsy approaches are summarized in Table 1.

5.1 Polar body 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 in Day 1 or both in Day 0 and Day 1. 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 the latter sequential, as discrimination of PB1 and PB2 may be problematic.

Mitotic errors and paternally-derived meiotic errors and mutations cannot be detected from PBs. Nonetheless, in case of maternally-derived meiotic aneuploidies or maternal mutations, 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 ***developmental competence***, which is unpredictable at this stage. Moreover, there is a moderate risk for technical complications, such as fragmentation or degeneration.

Following biopsy, extended embryo culture might be performed while waiting for genetic results, but this is not mandatory. If required, 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 regulation) and still within the timing to allow fresh embryo transfer (if required).

To date, the impact of PB biopsy on embryo reproductive competence has not been studied.

5.2 Cleavage stage biopsy

Cleavage stage biopsy results in the collection of a single blastomere (the removal of 2 cells is discouraged). At this stage of development, blastomeres have the potential to contribute to the embryo proper since their commitment to either the inner cell mass 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 moderate to high workload as it is not frequent that zygotes arrest before Day 3 and all must be biopsied regardless of their developmental competence, which is unpredictable at this stage. Following biopsy, embryos may be either subjected to extended embryo culture while waiting for genetic result and used in fresh embryo transfers or cryopreserved.

This approach is characterized by the highest worldwide experience and its complexity is moderate low.

To date, an impact of cleavage stage biopsy upon embryo competence has been reported, but more data are required to investigate this issue.

5.3 Blastocyst biopsy

TE biopsy entails the collection of a multicellular fragment (5 to 10 cells) on Day 5-7 from a part of the blastocyst which gives origin to the placenta and the extra-embryonic membranes (the foetus originates from the inner cells mass, which is kept intact).

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

Alternative biopsy approaches involve different learning curves and level of skills, specifically, 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 cells retrieval strategy is the least time-consuming, but also difficult to acquire as skill for the laboratory personnel. 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 should have in place an efficient cryopreservation program.

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 platform used to conduct PGT, on the ***validation*** parameters defined within each genetic laboratory, as well as 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%. Blastocyst biopsy allows running multiple analyses for different indications from the same sample (for instance chromosomal abnormalities and mutations). Rebiopsy cannot be performed within the timing to allow fresh embryo transfer.

To date, no impact of blastocyst biopsy upon embryo competence has been reported when less than 15 cells are retrieved especially from excellent/good quality embryos. However, more data are still required.

Table 1. The main oocyte and embryo biopsy approaches to conduct PGT. *The parameters "low", "moderate" and "high" were agreed unanimously after a thorough discussion among all the components of the working group. TE, trophectoderm; PB, polar body; ZP, zona pellucida.*

	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 recognized)	*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 developmental competence	Unpredictable at this stage	Unpredictable at this stage	Only embryos developing to the blastocyst stage are biopsied
Laboratory workload	*High* (all oocytes/zygotes should be biopsied regardless of their developmental competence)	*Moderate* (all embryos should be biopsied regardless of their developmental competence)	Multiple time slots required (Day 5-7) and cryopreservation mostly mandatory **Day 3 hatching-based strategy:** *Moderate to high* (all embryos should undergo ZP opening at the cleavage stage regardless of their developmental competence) **Morula hatching-based strategy:** *Moderate to high* (all morulas should undergo ZP opening regardless of their developmental competence) **Same day hatching-based strategy:** *Moderate to high* (all blastocysts should undergo ZP opening and monitoring of TE cells hatching) **Simultaneous ZP opening and TE cells retrieval strategy:** *Moderate*
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	Not	Not	Possible within given technical, methodological and biological limitations (e.g. molecular platform- and bioinformatic parameters-dependent, inevitable sampling bias)
Inconclusive diagnoses	~10%	~10%	<5%
Impact on embryo reproductive competence	Not reported, but more data are still required	Reported, but more data are still required	Not reported, but more data are still required

6. TUBING OF CELLS

An efficient transfer of biopsied cells to reaction tubes, i.e. tubing, is a critical step and a deciding factor for a successful PGT cycle. In principle, tubing requires careful and accurate handling of embryonic cells to prevent exogenous DNA contamination.

Laboratory issues related to tubing

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

To prevent contamination, 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. These should be well-fitting (e.g. nitrile, but not vinyl examination gloves).

Work surfaces, equipment, etc. should be cleaned with DNA ***decontamination*** solutions or 10% bleach prior to each use. It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.

The materials and reagents for tubing should be prepared in advance by the staff of the PGT centre.

Labelling and witnessing

General recommendations on labelling and witnessing are presented in the paper on organisation of PGT (refer to ORG paper).

Quality control

General recommendations on quality management and risk assessment are presented in the paper on organisation of PGT (refer to ORG paper).

Laboratory infrastructure, equipment and materials

Infrastructure

The tubing area should be in a DNA-free environment (pre-amplification area). 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 a close proximity of the biopsy area.

Equipment and materials

To minimize contamination, the preparation of materials and reagents, and the tubing of biopsied cells should be performed in a dedicated class-II laminar flow hood, 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.

- 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 plastic-ware 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.

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 or/and a specialised geneticist, competent and authorized to perform clinical diagnostics according to local or national regulations (see also 3. 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. Similarly, 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 trophectoderm, as they are usually sticky.
- it is recommended that a new pipette is used for each embryo to prevent DNA carry over.
- 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 (<1µl). 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 visualization.
- Tubing can be performed in PBS or directly in lysis buffer, depending on the protocol requirements of the PGT centre. Both alkaline lysis and proteinase K/sodium dodecyl sulphate are acceptable for cell lysis.
- A minimum of one negative control per buffer (sample collection buffer, biopsy media, 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); e.g. collection on two different timepoints for a specific cohort of embryos should

yield minimum 2 negative controls of this type. As the contamination risk is substantially higher when working with single cells in comparison to few cells, the number of negative controls should preferably be increased.

For transport of biopsied cells, the shipment can be done 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. If shipment of the cells is done using dry-ice (solid carbondioxide) 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 carbondioxide to get in contact with the sample.

7. CRYOPRESERVATION OF BIOPSIED EMBRYOS

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

i) prior to the biopsy (e.g. accumulation of oocytes/embryos; surplus oocytes/embryos from previous non-PGT cycles),

ii) after the biopsy (i.e. testing platforms often require cryopreservation as a mandatory step to give time to the genetic laboratory to analyse the samples; in general, cryopreservation is required also if implementing a ***freeze-all strategy***);

iii) 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 should be vitrified individually on a cryo-support properly labelled and witnessing is mandatory.

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.

8. ALTERNATIVE SAMPLING METHODS

Cell free DNA analysis (blastocentesis, spent media) and morula stage biopsy are under validation as alternative sampling methods for genetic testing (Figure 4).

Morula-stage biopsy

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

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 still controversial and need further investigation.

Spent media analysis

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 optimization of the methodology is required.

Figure 4: Alternative sampling methods

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