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Preimplantation Genetic Testing

Good practice recommendations of the European Society of Human Reproduction and Embryology

2020

ESHRE Preimplantation Genetic Testing Guideline Development Group

The current brochure provides an overview of the good practice recommendation for PGT published in 4 separate papers.

The aim of the current brochure is to facilitate the use of the information, especially given the numerous cross references in the published papers. The current brochure includes 5 sections which can be used as stand-alone documents:

- SECTION A. ORGANISATION OF PREIMPLANTATION GENETIC TESTING
- SECTION B. POLAR BODY AND EMBRYO BIOPSY FOR PREIMPLANTATION GENETIC TESTING
- SECTION C. DETECTION OF MONOGENIC DISORDERS
- SECTION D: DETECTION OF STRUCTURAL CHROMOSOMAL ABERRATIONS
- SECTION E: DETECTION OF NUMERICAL CHROMOSOMAL ABERRATIONS

All information included in this brochure is based on a series of 4 papers, and these references should be used when referring to the information contained herein.

ESHRE PGT Consortium Steering committee, Carvalho F, Coonen E, Goossens V, Kokkali G, Rubio C, Meijer-Hoogeveen M, Moutou C, Vermeulen N, De Rycke M. ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing. *Hum Reprod Open 2020. doi: 10.1093/hropen/hoaa021*

ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group, Kokkali G, Coticchio G, Bronet F, Celebi C, Cimadomo D, Goossens V, Liss J, Sofia Nunes S, Sfontouris I *et al.* ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for preimplantation genetic testing. *Hum Reprod Open* 2020. doi: 10.1093/hropen/hoaa020

ESHRE PGT-M Working Group, Carvalho F, Moutou C, Dimitriadou E, Dreesen J, Giménez C, Goossens V, Kakourou G, Vermeulen N, Zuccarello D *et al.* ESHRE PGT Consortium good practice recommendations for the detection of monogenic disorders. *Hum Reprod Open* 2020. doi: 10.1093/hropen/hoaa018

ESHRE PGT-SR/PGT-A Working Group, Coonen E, Rubio C, Christopikou D, Dimitriadou E, Gontar J, Goossens V, Maurer M, Spinella F, Vermeulen N *et al.* ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations. *Hum Reprod Open* 2020. doi:10.1093/hropen/hoaa017

Disclaimer

This Good Practice Recommendations (GPR) document represents the views of ESHRE, which are the result of consensus between the relevant ESHRE stakeholders and are based on the scientific evidence available at the time of preparation.

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Furthermore, ESHREs GPRs do not constitute or imply the endorsement, or favouring of any of the included technologies by ESHRE.

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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 nineties 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 fertilization (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)***, ***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).

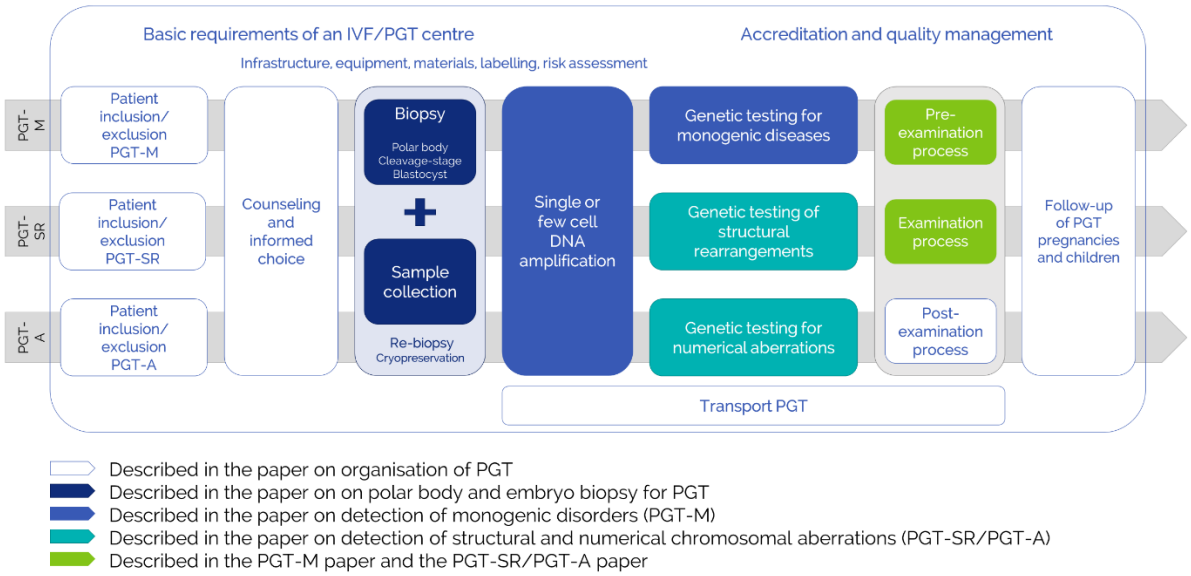
The goal of this series of papers is to bring forward best practices to be followed in all types of PGT services, offering PGT-A as well as PGT-M and PGT-SR.

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 drafted guidelines (2004, 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 European Society for Human Reproduction and Embryology (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 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 figure 1.

Figure 1: Overview of the IVF/PGT process, and how all aspects are covered by one of the 4 recommendations papers.



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 (editorial note, in the current brochures the cross references were removed and information was duplicated to facilitate use of the recommendations). Therefore, it is recommended to not consult the papers independently but always as a set when one is seeking guidance on a PGT issue.

SECTION A: ORGANISATION OF PREIMPLANTATION GENETIC TESTING

This section is entirely based on the paper:

ESHRE PGT Consortium Steering committee., Carvalho F, Coonen E, Goossens V, Kokkali G, Rubio C, Meijer-Hoogeveen M, Moutou C, Vermeulen N, De Rycke M. ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing. Human Reproduction Open 2020;doi: 10.1093/hropen/hoaa021.

1. Patient inclusion/exclusion criteria

The decision to accept or decline patients in PGT services should be undertaken by a team of dedicated healthcare professionals (including clinical geneticists or genetic counsellors, molecular biologists/cytogeneticists, mental health professionals, clinical IVF specialists and embryologists), based on well-defined inclusion/exclusion criteria. PGT requests should be compliant with national legislation, and, where needed, also be considered by local ethics boards or local/national regulatory agencies. Local regulations will vary from centre to centre as will criteria for inclusion and exclusion of patients. The following recommendations should be considered as a starting point for discussion.

1.1 General: inclusion/exclusion

It is recommended that PGT is only applied when genetic diagnosis is technically feasible, and the reliability of the diagnosis is high. Current procedures in most IVF/PGT centres allow for overall error rates (resulting in misdiagnosis) as low as 1 to 3% (De Rycke *et al.*, 2017). Each centre should be aware of their error rates and include this information in their ***informed consents*** and reports in an open communication with the patient.

When considering PGT, safety issues, female age, impossibility to retrieve male or female gametes, body mass index (BMI) and other contraindications for IVF, should be considered as possible exclusion criteria.

Furthermore, exclusion from PGT should be considered if the woman has serious signs and symptoms of an autosomal dominant or X-linked disorder (for which PGT is requested) which could introduce medical complications during ovarian stimulation, oocyte retrieval or pregnancy, or medical risks at birth. PGT should be carefully considered if one of the partners has serious physical or psychological problems, either linked to the tested disease, or due to other conditions.

1.2 PGT-M, mitochondrial disorders and HLA: inclusion/exclusion

PGT-M refers to testing for DNA pathogenic variant(s) causing (combinations of) monogenic disorders, X-linked, autosomal dominantly or recessively inherited, for which the disease-causing loci (nuclear or mitochondrial) has been unequivocally identified. In this respect, HLA typing of embryos is a different (no pathogenic variant detection) indication.

1.2.1 PGT-M

PGT-M testing can be carried out for (likely) pathogenic germline genetic variant(s) (Richards *et al.*, 2015), shown with high likelihood to be disease causing with serious health effects that may manifest at birth, in childhood or in adulthood. Further research (e.g. functional studies, family studies) may be indicated to prove the clinical significance of genetic variants. Cases of genetic variants of unknown significance that are not predictive of a phenotype should be excluded from PGT. PGT testing is inappropriate in case of uncertain genetic diagnosis (for example genetic/molecular heterogeneity), or in case of uncertain mode of inheritance.

For autosomal recessive disorders, where a single pathogenic variant has been diagnosed in the proband and only one parent, it is acceptable to offer PGT if the pathogenic genotype is attributed to a single gene and sufficient evidence from the family pedigree allows identification of the disease-associated haplotypes. Similarly, it is acceptable to offer PGT for known X-linked recessive single gene disorders with a clear unequivocal clinical diagnosis where no pathogenic variant was found in the proband, but low- and high-risk haplotypes can be identified

based on the family history.

Exclusion or **non-disclosure testing** can be indicated for late-onset disorders, such as Huntington's disease, to avoid pre-symptomatic testing of the partner with a family history of the disease. Exclusion testing is preferred over PGT with non-disclosure of the direct test results to the couple (Shenfield *et al.*, 2003).

1.2.2. PGT for mitochondrial disorders

PGT for mitochondrial disorders caused by mitochondrial DNA (mtDNA) pathogenic variant(s) allows to select for embryos with a mtDNA pathogenic variant load below the threshold of clinical expression, providing an effective risk reduction strategy for heteroplasmic mtDNA pathogenic variant(s). As this threshold is often not known for rare or private pathogenic variant(s), a meta-analysis was performed for all mtDNA pathogenic variant(s), showing that embryos with a pathogenic variant load of less than 18% have a likelihood of more than 95% of being unaffected, irrespective of the mtDNA pathogenic variant and can be considered for transfer. For all mtDNA pathogenic variant(s) tested so far, the pathogenic variant load in individual blastomeres is representative for the entire embryo, which was expected due to the absence of mtDNA replication in the cleavage stage. Whether the same is true for blastocysts remains to be established, as mtDNA replication has started in this stage, leading to increased variation. Therefore, it is warranted to assess the variation in pathogenic variant load within embryos.

PGT is not indicated in case of ***homoplasmy***.

In cases where the causative pathogenic variant of the mitochondrial disease is encoded by nuclear DNA, testing is the same as for other monogenic disorders.

1.2.3 HLA typing

When all other clinical options have been exhausted, selection of HLA-matched embryos via PGT is acceptable for couples who already have a child affected with a malignant, acquired disorder or a genetic disorder where the affected child is likely to be cured or life expectancy is substantially prolonged by transplantation with stem cells from an HLA-matched sibling. Testing can be performed for HLA typing alone, if the recurrence risk of the disease is low, or in combination with autosomal dominant/recessive or X-linked disorders.

Attention should be given to the time required for PGT workup, cycle(s) application and for an HLA-matched sibling to be born. Therefore, cases in which the affected child has an acute medical condition prohibiting safe stem cell transplantation or an extremely low life expectancy should be carefully considered for PGT. Any request for HLA typing in the absence of a specific disease to create a future donor for a sibling should be refused.

1.3 PGT-SR: inclusion/exclusion

PGT for chromosome structural rearrangements (PGT-SR) is an accepted and routine procedure in most IVF/PGT centres. It has been developed for patients, unable to achieve a pregnancy or at high risk of pregnancy loss and of abnormal live born births, resulting from inheritance of unbalanced products of the rearrangement.

Depending on the technology used (fluorescence in situ hybridisation (FISH), quantitative real-time PCR (qPCR), comprehensive testing methods [array-based comparative genomic hybridisation (aCGH), single nucleotide polymorphism (SNP) array or next generation sequencing (NGS)]), different inclusion/exclusion criteria may apply. In general, PGT-SR is only recommended if the technique

applied is able to detect all expected unbalanced forms of the chromosomal rearrangement. When comprehensive testing strategies are applied, it is acceptable to use information on copy number of non-indication chromosomes to refine embryo transfer strategies.

1.4 PGT-A: inclusion/exclusion

Although PGT-A remains heavily debated in clinical practice, the following indications for its use have been reported:

- Advanced female/maternal age (AMA)
- Recurrent implantation failure (RIF)
- Recurrent Miscarriage (RM). It should be noted that couples with a history of RM have a high chance of successfully conceiving naturally and that PGT-A for RM without a genetic cause is not recommended in a recent evidence-based guideline (The ESHRE Guideline Group on RPL *et al.*, 2018)
- Severe male factor (SMF)

The exact definition (e.g. age limit, number of losses) of these factors should be determined by each centre. International definitions are provided in the glossary (See Supplementary data 1. Glossary).

For all, but in particular for RIF, RM and SMF couples, a previous karyotype of both partners is recommended, since there is a higher chance of structural rearrangements for these indications. If an abnormal karyotype is identified, the technology for the detection of unbalanced abnormalities can differ from the regular PGT-A.

2. Counselling and informed choice

2.1. Relevant documents

The following documents should be available before starting PGT:

- original or copy of results of genetic testing, karyotypes, or other specific testing of the index patient, spouse or partner, children or other family members (when appropriate).
- female reproductive history, gynaecological and fertility status.
- male reproductive history, andrological history, fertility status, results of sperm analysis (especially in cases where the genetic disorders for which PGT is desired has effects on sperm parameters, e.g. monogenic diseases, such as myotonic dystrophy and cystic fibrosis/congenital bilateral absence of the vas deferens and some Robertsonian translocations).
- reports on health problems of female and male partners, that may affect genetic diagnosis, or the outcome of IVF and pregnancy (when appropriate). Health status may need to be re-evaluated over time.
- for PGT-M, PGT-SR: a genetic counselling report together with full pedigree and family data.
- for HLA testing: a medical report of the affected child, current situation, prognosis, options for treatment other than PGT, suitability for stem cell transplantation, results of previous HLA typing (serologic and/or DNA markers) in affected child, parents and siblings.

As laws and regulations on PGT vary internationally, the legality of undertaking PGT in a particular country for a specific indication should be verified. If required, licenses

or approval to carry out PGT should be obtained prior to the start of ovarian stimulation.

2.2. Counselling: General issues

- All information, oral and written, should be in language that can be understood by a layperson as technical terminology may lead to patient misunderstanding.
- Written information about treatment should be available prior to a consultation.
- When PGT involves the treatment of a couple, both partners should, when possible, attend consultations.
- An independent interpreter should be present when necessary, although a family member could act as translator in the absence of an alternative.
- Counselling should be offered both before and after each IVF/PGT cycle.
- Genetic counselling should be provided by a qualified clinical geneticist or genetic counsellor.
- A specialist in reproductive medicine should provide information regarding the IVF cycle.
- The counselling provided should be non-directive and include all reproductive options available to the couple, enabling them to reach their own conclusion about the suitability of treatment.
- Costs and timelines should also be discussed to ensure that patients are fully informed of all aspects of IVF and PGT before treatment starts. The social and psychological impact needs to be considered, especially in couples already responsible for the care of affected children.
- Additional counselling may be needed after completion of the laboratory work-up.
- Individualised post-consultation letters should contain a summary of the information discussed.
- The patients should sign a written consent for all procedures.

2.3. PGT-related counselling

PGT counselling includes counselling related to the IVF treatment on one side and genetic counselling on the other side.

2.3.1. Related to the IVF treatment

Counselling should include discussion of:

- the risk of medical complications for women during ovarian stimulation or oocyte retrieval;
- the risk of spontaneous pregnancy in the waiting time or during IVF treatment, and the need for contraception;
- the number of oocytes to be retrieved and the need to maximize this within the safe limits of medical practice. Different options for pooling oocytes or embryos before biopsy should be considered, when appropriate;
- the expected number of embryos for biopsy, the biopsy stage, the number of cells to be biopsied and the percentage of embryos expected to survive;
- the possibility that some embryos remain undiagnosed. In specific cases, re-biopsy is acceptable to achieve diagnosis. If no diagnosis is obtained, selection of these embryos for transfer is not acceptable. An exception can be made for PGT-A but requires patients' fully informed consent;
- the number of embryos to be transferred and the policy on elective single-embryo transfer in the centre. The risk of conceiving a multiple pregnancy should also be discussed;
- the possibility of having no embryos for transfer if all the embryos are morphologically and/or genetically unsuitable;

- the chance of pregnancy/live birth per cycle started and per embryo transfer, taking into account maternal age and indication;
- the risk of miscarriage and the importance of re-analysis of placental or fetal tissue, as a tool to assess false negative rates and to advise the couple for further treatment;
- cryopreservation following PGT and the predicted success of pregnancies from biopsied and cryopreserved embryos;
- follow-up of pregnancies and children born from PGT;
- options for embryos not transferred or frozen for future use, including donation to research, according to local regulations.

2.3.2. Related to the genetic analysis

Counselling should include discussion of:

- an updated review of the genetic risk and molecular or cytogenetic confirmation of the diagnosis when appropriate, the severity and variability of the condition, and presence or absence of genotype/phenotype correlation;
- the principle of the test; it should be explained that depending on the indication, biological samples and genetic reports from the couple and relevant family members may be required for the laboratory work-up;
- the condition(s) tested for, the testing method and the limitations of the test;
- the expected time-frame for the laboratory work-up and the treatment;
- the reporting of results and the centre's policy on incidental findings;
- decision-making about which embryos are acceptable for transfer/vitrification: this should be discussed with the patients before a treatment cycle begins and may need to be revisited. The fate of undiagnosed embryos and non-transferable embryos also needs to be addressed. It is acceptable to use non-transferable embryos for test optimisation;
- chromosomal mosaicism as an inherent biological phenomenon in human preimplantation embryos and when appropriate, how this may affect diagnosis and the centre's embryo transfer policy;
- the possibility of a misdiagnosis; error rates expressed as false negative or positive results should be based on 'in-house' work-up and follow-up analysis for specific diagnostic tests or strategies;
- the option and possible recommendation for prenatal diagnosis (in case of pregnancy) for confirmation of the PGT result.

Depending on the condition, and test to be used, the following issues should also be addressed in counselling:

- for structural chromosomal rearrangements, it is important to discuss that the applied technology may not allow to discriminate between normal and balanced results;
- for autosomal recessive, as well as for X-linked recessive disorders, the transfer of carrier embryos should be discussed, according to the local regulations;
- for X-linked diseases where specific pathogenic variant detection is not possible, the pros and cons of embryo ***sexing*** should be discussed: all male embryos, affected or unaffected, will be discarded and carrier females cannot be distinguished from unaffected female embryos;
- the option of revealing the sex of the embryo should be discussed within the local legal constraints;
- for monogenic disorders caused by dynamic pathogenic variants with repeat instability where testing involves repeat size determination, the couple should

be fully informed on the threshold of repeat expansions below which embryos can still be transferred;

- for HLA typing, the theoretical number of embryos suitable for transfer should be discussed. The fate of unaffected non-HLA-matched embryos should be discussed, taking local and national regulations into consideration. Due to the complexity of the procedure it is recommended to maintain close collaboration between specialists of the IVF, PGT and transplant units, and to minimise the time of the whole procedure;
    - All potential limitations should be communicated to the couple, including the chance of finding a transferable embryo and hematopoietic stem cell transplantation issues (potential stem cell source, timing, expected success rate).
    - It is recommended to counsel prospective parents on the genetic chance of identifying a transferable embryo:
        - 25% (1 out of 4) of biopsied embryos are genetically transferable when performing preimplantation HLA-typing only;
        - 18.8% (3 out of 16) when concurrently excluding an autosomal recessive or X-linked recessive disease;
        - 12.5% (1 out of 8) when concurrently excluding an autosomal dominant disease;
    - It is important to discuss the risk of a unique crossover in the proband, leading to very low likelihood of identifying a transferable embryo.
- for PGT-M or PGT-SR combined with PGT-A, the policy for embryo (ranking and) transfer should be discussed.

2.4. Psychological support and evaluation

When available in the centre, psychological support should be offered to every couple before, during and after PGT, including unsuccessful cycles.

Psychological evaluation should be considered for the following patients:

- couples for whom the geneticist, gynaecologist or other member of the IVF/PGT team has doubts regarding the welfare of existing or future children or the psychological and physical wellbeing or mental capacity of future parents;
- couples in whom one of the future parents is the carrier of an autosomal dominant disorder and may have signs and/or symptoms of this disorder as determined by the appropriate specialist physician (e.g. neurodegenerative/psychiatric diseases);
- couples who are undergoing PGT HLA-typing to evaluate their 'child wish' and the extent to which the new child is welcomed, not only as a donor but also as a full family member, appreciated for whom s/he is.

Psychological support and intervention are recommended for:

- couples with a history of reproductive failure;
- patients with past traumatic experiences;
- patients with current salient psychological distress;
- couples who actively request psychological intervention.

3. Basic requirements of an IVF/PGT centre

A close collaboration between the IVF centre and the PGT centre is essential, particularly in complex cases.

Oocyte retrieval, fertilisation, culture, biopsy and transfer of embryos and PGT diagnosis should be undertaken in a centre with suitable laboratory infrastructure, equipment and trained staff, in accordance with the European Union Tissue and Cells directive or other local laws. Adherence to published best practice guidance on PGT is recommended.

The following recommendations apply to the preclinical work-up and testing of clinical cases.

3.1. Laboratory infrastructure, equipment and materials

3.1.1. Laboratory infrastructure

Oocyte and/or embryo biopsy should be performed in a specifically designated laboratory setting. Collection of the biopsied samples and initial steps of genetic testing procedures should be carried out in laboratory settings dedicated for processing single and/or few cells. Appropriate precautions should be taken both to prevent contamination of samples by physical isolation, and to detect any such contamination. Licenses for offering embryo biopsy procedures and/or genetic testing by the centre may be obtained, according to local regulations.

3.1.2 Equipment

- All clinical equipment should meet the criteria set for the intended application, be appropriately calibrated, maintained and serviced, with all aspects supported by written ***standard operating procedures (SOPs).*** Equipment used for critical steps should have uninterrupted power supply (UPS).
- For areas within the IVF centre, whether it is a dedicated area or a room, all equipment should comply with "Revised Guidelines for good practice in IVF laboratories (2015)", section 3 "laboratory safety" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). Prior to the biopsy procedure, work surfaces, equipment and hoods should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory.
- For areas within the PGT centre, prior to each use, work surfaces and equipment should be cleaned and decontaminated with DNA ***decontamination*** solutions or 10% bleach, or by UV-C irradiation or autoclaving (when applicable, for example tube racks). It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.
- Multichannel pipettes or automated systems may be useful in the PGT laboratory to minimise the risks of mislabelling or misallocation of samples during the post-amplification steps, but they are not recommended in the pre-amplification steps.

3.1.3 Materials

- To prevent contamination, protective clothing for DNA amplification of a single and/or few cells 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). For areas within the IVF centre, protective clothing, preferably with low particle-shedding and non-powdered gloves and masks should be considered.
- The pre-amplification materials and reagents should be kept away from any DNA source and preferably stored in the pre-amplification area.

- 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 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 internal 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 all reagents.
- Reagents and solutions can be DNA decontaminated by UV-C irradiation. Alternatively, reagents and solutions made in-house can be autoclaved, preferably using a PGT-dedicated autoclave.
- Careful handling of all reagents employed must be ensured with regards to storage temperature and working conditions, following manufacturer's recommendations. Vortexing and quick temperature changes should be avoided for the most sensitive components.

Specific issues for handling of reaction tubes to reduce cross-contamination:

- It is recommended to avoid touching the inside or the lid of the tubes with your fingers.
- It is recommended to avoid touching the outside or the cap of the tubes with the tip of the pipette. If this happens, the pipette tip should be changed immediately.
- It is recommended to keep the reaction tubes open not longer than necessary.

3.1.4.Laboratory documentation

Well-structured (electronic and/or paper) laboratory forms should be available for recording wet-laboratory details of work-up and PGT cycle procedures.

Further specific requirements with respect to infrastructure, equipment, materials and documentation are discussed separately in the papers on embryo biopsy and PGT techniques (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).

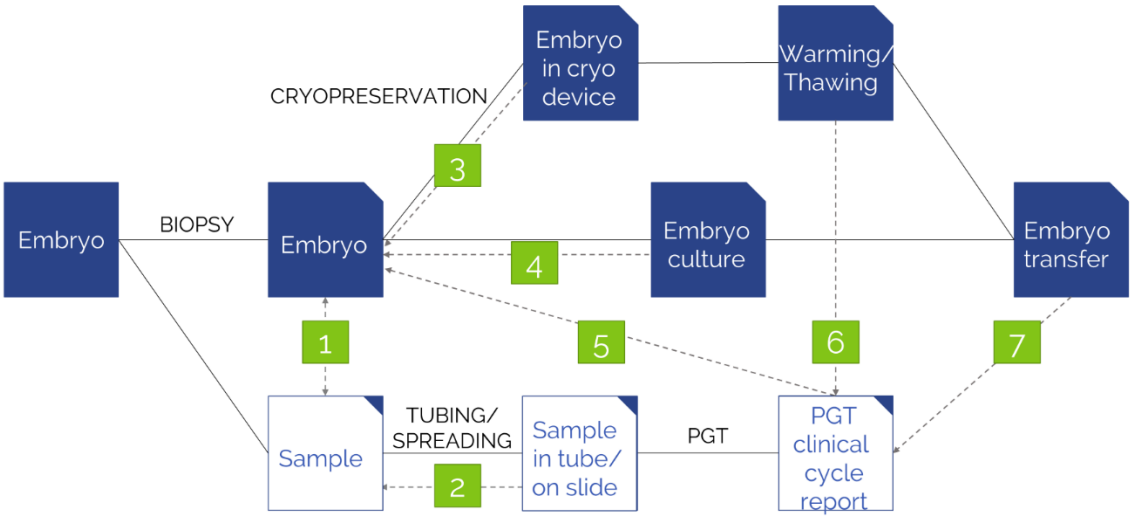
3.2. Training and personnel

- It is recommended that laboratory personnel performing clinical work should be supervised by an appropriately trained person.
- Staff training and competence: embryo biopsy procedures and genetic testing should be performed by competent and adequately trained laboratory staff, according to national legislation. Joining specific training programmes (workshops, hands-on training, one-to-one training) for embryology and PGT procedures is recommended. All staff should document their competence level and continuous professional development. The number of trained laboratory staff should reflect the number of PGT cycles performed per year and also consider other duties such as administration, quality management and communication with respect to the PGT work. For centres with a low number of PGT cycles, more than one individual should be trained, to avoid difficulties with absence.
- It is recommended for a member of the personnel with abstinence from a specific technique to demonstrate laboratory skills before working again with clinical cases.
- When the interpretation of results includes specific software, personnel may also be trained in management and interpretation of the software.
- Good laboratory practice and good scientific judgement are always required.

3.3. Labelling and witnessing

- It is recommended that an adequate labelling system, written or barcoded (electronic), with two unique patient identifiers plus the embryo/cell(s) number is used to match the sample's diagnostic result with the embryo from which that sample was taken. This should ensure traceability throughout the IVF and PGT process until reporting of the final results.
- The labelling system should be comprehensible and practical for both the IVF and the PGT centres. Printed sticker labelling may be superior to pens, as labelling should be legible and uneditable.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer and signed off (Figure 2). These critical steps are detailed in the technical papers for the various methods (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
- After biopsy, the sample may be analysed in house, or sent for genetic testing in another centre (see " Transport PGT").

Figure A.2: Outline of the biopsy and genetic testing procedure with indications of the 7 critical steps where labelling and sample identification should be confirmed. Further details on labelling and sample identification during the PGT testing are included in the specific sections of the PGT-A/SR paper.



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) In case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device; (4) For further embryo culture, at placing and labelling the embryo into the culture dish; (5) When diagnostic results are issued to ensure accuracy and correlation with the correct sample identification; (6) During the thawing/warming procedure to ensure accuracy and correlation with the correct embryo diagnostic result; and (7) At the time of selecting the embryo(s) for embryo transfer.

4. Preclinical work-up report, examination and post-examination process

4.1 Preclinical work-up

The PGT work-up report should contain at least the following information (Claustres *et al.*, 2014):

- administrative information:
  - title or name of the report;
  - number of the report (as used for document control, when available);
  - pagination including the actual and total number of pages (the patient identifier and report name/number must be present on each additional page);
  - full date of the report;
  - name and address of the physician referring the patient;
  - identification of the person(s) performing the diagnosis/authorizing the release of the report and their signature;
  - identity of the IVF/PGT centre with full contact details;
- patient (male and female)/sample identification:
  - full given name(s) and surname, or unique patient identification code;

    - unequivocal date of birth;
    - gender;
  - specific for the preclinical work-up report:
    - date of DNA sample collection;
    - date of DNA sample arrival in the laboratory;
    - samples and genetic status of relevant family members can be mentioned only with their informed consent and should be in accordance with general data protection regulations (GDPR) and/or local privacy regulations;
    - for PGT-SR, an overview of the most likely segregation products;
  - restatement of the clinical question, i.e. the indication(s) being requested for analysis, the type of required testing, the referral reason, parental karyotypes/genomes;
  - specification of genetic tests used:
    - brief information on the methods used in the analysis;
    - full details of the extent of the tests, including software, where appropriate;
    - where a commercially available kit is used, this should be clearly identified in the report, including the reference and version of the kit;
  - a clear description and interpretation of results;
  - a clear summary of the results;
  - error rates/limitations of the test/misdiagnosis (a general figure should be stated for the overall cycle/treatment).
- It is recommended that all reporting based on haplotyping clearly states that the accuracy of the results is based on the assumption that samples received were correctly identified, family relationships are true, and the clinical diagnosis of relatives is correct.
- It is recommended that any particularity of the protocol (e.g. specifying type of biopsy, number of cells) is clearly indicated and communicated to both the patient and the IVF centre, if needed.

4.2. Examination process

- The examination process and reporting of results, must comply with local guidelines or law, or with the ISO 15189 standard.
- Before starting a clinical PGT cycle, relevant documents should be available, labelling of samples should be checked, and genetic counselling provided to the couple.
- It is recommended that the PGT laboratory has clearly documented procedures for all steps of the examination process (explicit instructions and a summary of validation results) and release of results (diagnosis, reporting, embryos transfer policy). These procedures are preferably covered in a service-level agreement between the PGT and IVF centres.
- Many different methods have been published and all appropriately validated methods are acceptable for clinical cases. The method used should have been previously implemented, tested and validated in the PGT centre.

4.2.1. Scoring of clinical results

- It is recommended that results are reviewed and signed or electronically validated by a suitably qualified person (name, qualification, date).

4.2.2. PGT clinical cycle report

The PGT clinical cycle report contains interpretation of the clinical results and guidance on which embryos are genetically transferable. The same recommendations apply as specified for the preclinical work-up report (see section "Preclinical work-up"), together with the following items:

  - unique cycle/treatment code;
  - date of oocyte retrieval;
  - date of biopsy;
  - date of biopsy sample arrival in the laboratory;
  - information on the sample type (including number of samples and controls);
  - unique ID number for each cycle and/or biopsy sample tested;
  - indication for PGT.
- When scoring results from PB testing, it is recommended to report what was detected in each polar body and then infer the oocyte diagnosis. It is recommended to test both PBs.
- When scoring results from blastomere/TE testing, it is recommended to report what was detected in the sample and then infer the embryo diagnosis.
- When results are reported from 'pooling' of embryos, it is advisable to refer to each oocyte and sample collection date and clearly differentiate the embryo number between cycle/treatment.
- Reporting of clinical results to the IVF centre must follow local regulations or international accreditation guidelines, including GDPR.
- The embryo transfer policy should be agreed upon between stakeholders (IVF centre, genetic centre, genetic counsellors, clinicians and patients). In PGT-M and PGT-SR cases, embryos with no or inconclusive results are not recommended for transfer. Depending on local rules and following adequate counselling of the prospective parents, the carrier status of embryos (for autosomal recessive or X-linked recessive disorders) may be taken into consideration for embryo selection. In case of PGT-A in addition to PGT-M or PGT-SR, it is crucial that the centre has a clear policy on embryo (ranking and) transfer.
- A written or electronic report should be securely transmitted to the IVF centre to ensure transfer and/or cryopreservation of the correct embryos. Results should not be communicated orally.
- Reporting time should be kept as short as possible, and when fresh transfer is intended, reporting time should be adapted to allow the IVF centre to organise the embryo transfer.
- It is recommended that the report is clear, concise, accurate and easily understandable by non-geneticists.
- It is recommended that the overall result and interpretation (including transfer recommendation) are presented per embryo, preferably in tabulated form. Sufficient information for genetic counselling should be included, such as the chromosome(s) involved, chromosome band(s)/nucleotides, the size of the chromosomal aberration in Mb, and the correct identification of the genetic variant. Where applicable, the latest version of the international system for human cytogenetic nomenclature (ISCN)/Human genome variation society (HGVS) nomenclature can be used.
- In case of no diagnosis and re-biopsy to try and obtain a result, this should be included in the report.

- The final clinical cycle report must be signed by appropriately qualified (authorised) personnel (name, qualification, date).
- It is recommended that the clinical cycle results are discussed with the couple before embryo transfer.
- It is recommended that the report is stored in the patient file in the PGT centre, according to local regulations.
- It is recommended to include a disclaimer in the report to address limitations of the test and any other information that may be of significance to the addressee.
- It is acceptable to indicate in the report the need for prenatal testing to confirm the result in case of pregnancy.

Further details on the specific reporting of the results and interpretation of results are outlined in the technical recommendations papers (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020).

4.3 Post-examination process

4.3.1. PGT cycle follow-up

For quality purposes, it is recommended to confirm the PGT diagnosis on a subset of embryos not transferred or cryopreserved following diagnosis, in line with local regulations. Such confirmation aims to provide internal ***quality assurance (QA)*** as well as accurate and up-to-date misdiagnosis rates to prospective PGT patients. It is recommended that this is performed on as many embryos as is practicable. It is acceptable to perform this periodically.

When a pregnancy ensues following PGT testing, it is recommended that parents are (again) made aware of the chance and risks of a misdiagnosis and be informed on the possibilities for prenatal testing. PGT and IVF centres should make special efforts to follow-up with the parents following prenatal testing or birth, especially if confirmatory testing is not possible.

Follow-up data should be used for both internal ***quality control (QC)*** and QA purposes and documented in the ESHRE PGT Consortium online database for international data collection.

It is recommended that laboratories follow local regulations or accreditation schemes on storage of clinical samples and patient records. If no local regulations or guidelines exists on storage of clinical samples and patient records, it is recommended as follows.

- If embryos have been transferred and/or frozen, all relevant material (e.g. FISH slides, DNA amplification products) from the case should be retained and appropriately stored. Samples should be stored for at least 1 year. Prolonged sample storage could be considered, taking into account the availability of information on delivery and the duration of embryo cryopreservation.
- If there is no genetically suitable embryo for transfer or cryopreservation, it is not necessary to keep the samples.
- If there is no pregnancy after transfer of all genetically suitable embryos, samples can be discarded.

4.3.2. Misdiagnosis rate

- It is recommended that each PGT centre performs a prospective risk analysis in order to prevent and/or eliminate possible causes of ***misdiagnosis***.
- It is recommended that misdiagnosis rates should be calculated for each type of method and for all methods from a particular centre. Misdiagnosis rates include

those clinical cases in which affected pregnancies arose and cases for which re-analysis results were discordant with the biopsy result.
- It is recommended that confirmatory testing should be performed at least periodically as a QA.
- It is recommended that the published and in-house estimates of misdiagnosis rates should be available on request to prospective patients along with pregnancy rates and live birth rates, to allow informed consent for PGT.
- Following a misdiagnosis, the IVF/PGT centre should investigate the possible causes of the misdiagnosis and make changes to protocols to eliminate the risk in the future. Many of the causes of misdiagnosis are avoidable by taking preventive actions and following the principles of quality management.
- Misdiagnosis should be reported, for instance through the ESHRE PGT Consortium online database.

4.3.3. Baseline IVF live birth rates for PGT

- Setting appropriate baseline live birth rates should be left up to the individual centres. However, it is recommended that each IVF centre should compare PGT live birth rates and matched non-PGT [routine IVF or ***intracytoplasmic sperm injection (ICSI)***] live birth rates within that IVF centre.
- Comparison of live birth rates with those reported by the ESHRE PGT Consortium or comparable peers can also be carried out to set benchmarks for continual improvement of the PGT centre.

5. Transport PGT

- When in-house genetic analysis is not feasible, transport PGT is an option. In transport PGT, patients have the IVF treatment (oocyte retrieval, embryo culture, biopsy and transfer, pregnancy follow-up) at their local IVF centre, but genetic testing is performed at a collaborating PGT centre with significant experience in PGT.
- The IVF centre and PGT centre should have in place an official agreement (Service-Level Agreement) dealing with legal, insurance and accountability issues about the Transport PGT procedures.
- Transportation companies entitled to transport biopsied material should certify their suitability to transport the biopsied material, provide the likelihood of a sample loss or sample delivery delay and provide actions taken against these risks.
- The IVF centre and PGT centre should make arrangements to ensure that patients have had adequate PGT counselling.
- The IVF centre and PGT centre should have in place a set of clinical/laboratory validated protocols, including tubing/spreading protocols, and shipment protocols specifying approximate transportation time and ensuring cell and/or DNA integrity.
- In addition, practical and logistic arrangements on who will be responsible for the various stages of the PGT treatment should be clearly established.
- The IVF centre and PGT centre should delineate clear and sufficient lines of communication as documented in written procedures and compliant with the GDPR during all stages of a transport PGT treatment.
- Preclinical runs: before sending/receiving clinical samples from the treatment cycles, one or more 'preclinical runs' are recommended. This practice may detect issues related to the quality of biopsy, handling and labelling of biopsied samples, and the transport. Negative control specimens should be included in preclinical runs to assess contamination. The sensitivity and specificity of genetic testing

should be evaluated and compared with in-house samples and/or samples received from other IVF centres. The reporting of the results should be agreed upon.
- The IVF/PGT centres should agree on the feasibility, the number and the timing of transport PGT cycles, and define a schedule.
- It is recommended that all diagnostic results and reports are sent in written form (complying with the GDPR).
- The IVF centre and PGT centre should agree on who is responsible for the collection of PGT data and follow-up of PGT children (www.eshre.eu/data collection).

6. Follow-up of PGT pregnancies and children

6.1. Prenatal diagnosis

Prenatal diagnosis should be offered to all women who become pregnant following PGT. The discussion about the tests available should be undertaken by a suitably qualified professional to ensure that all available options are presented, including prenatal invasive diagnostic tests such as chorionic villus sampling and amniocentesis, and prenatal non-invasive diagnostic or screening tests such as ultrasound scanning or cell-free fetal DNA testing (NIPT screening for aneuploidies or NIPD diagnosis for monogenic disorders and sexing).

As an alternative to prenatal diagnosis, patients could choose to have postnatal confirmation by cord blood sampling. However, testing of minors for non-actionable conditions should be in line with local legislation.

6.2. Follow-up of PGT pregnancies and children

There have been concerns about the health of children after assisted reproductive technologies (ART), and in particular after embryo biopsy techniques, prolonged culture to blastocyst, and cryopreservation/vitrification.

So far there is no indication that embryo biopsy causes an increased risk for adverse neonatal outcome. However, PGT includes ART for which there is evidence that ART singleton children differ from spontaneously conceived children. It is unclear whether this difference is due to the infertility status of the couple and/or the ART procedure itself.

There is uncertainty about the long-term impact of ART and/or PGT and IVF/PGT centres should be encouraged to obtain follow-up data on babies born after treatment, preferably in collaborative prospective and retrospective studies. If this is not possible, the suggested minimum data set to collect should include:

- date of birth;
- singleton versus multiple pregnancy + chorionicity status;
- gestational age at birth;
- delivery mode;
- birthweight and length;
- sex;
- congenital abnormalities.

Neonatal complications and APGAR score can additionally be recorded.

7. Accreditation and Quality management

7.1. Accreditation

Accreditation, together with proficiency testing through internal (IQA) and external quality assessment (EQA), provides a means to achieve and maintain the highest quality standards. Accreditation is the formal recognition that an authoritative body gives to a laboratory/department/centre when it demonstrates competence to carry out defined tasks, and involves all aspects of management, along with technical requirements.

Where possible, IVF/PGT centres should be accredited/certified, even when it is not legally required.

Because PGT is of a multidisciplinary nature, the various units involved should each be accredited/certified for their defined tasks and according to the most appropriate quality standards. For each unit, responsibilities should be clearly outlined/described and transition of responsibility from one unit to the other during the PGT process should be well defined and guaranteed.

IVF/PGT laboratories should strive for accreditation conforming with the latest version of ISO15189 or equivalent international/local standards and work with international diagnostic laboratory accreditation schemes, if available.

IVF/PGT clinical units should strive for certification conforming with the latest version of ISO9001 or equivalent international/local standards and work with medical/clinical peer review, if available.

7.2. Quality management

It is recommended that a ***quality management system*** is integrated with the IVF/PGT centre. Quality management ensures that an IVF/PGT centre and the PGT service it provides, are of consistent quality. It has four main components: quality planning, QA, QC and quality improvement. To most if not all accreditation/certification schemes, QA and QC are prerequisites.

Aspects of quality management to be implemented include, among others, quality policy, quality manual, document control, compliance with SOPs, risk management, continual improvement, audits and management review. Technical requirements include personnel, laboratory conditions and environment, laboratory equipment, all stages of examination procedures, results reporting and QA.

- It is recommended that PGT centres participate on a regular basis in EQA schemes; GenQA offers schemes for PGT that cover all types of analysis performed (https://www.genqa.org/).
- Validation of all methods used is recommended.
- Written SOPs should be available for all steps of the PGT procedure. Laboratory staff should have profound knowledge of the SOPs as these are the fundamental backbone of the service. Deviations from protocols should be recorded.
- ***Risk assessment*** is part of the QC system and required for every stage of the PGT process. It should be integrated into the SOPs.
- Laboratories should perform a risk assessment analysis to estimate the probability of a putative hazard and the severity of their consequences, as well as the chances for detection of error. A collaborative and multidisciplinary approach between the different operators involved in the management of a PGT cycle would lead then to the prevention of any putative procedural risk and implementation of specific corrective measures.

SECTION B: POLAR BODY AND EMBRYO BIOPSY FOR PREIMPLANTATION GENETIC TESTING

This section is entirely based on the paper:

ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for preimplantation genetic testing" with some addition from the paper "ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing"

1. 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.

1.1. 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 power of the laser beam and exposure (pulse length/width) should be carefully addressed following the manufacturer's specifications to avoid damage to polar bodies or embryonic cells.

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

1.2. 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 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 ***trophectoderm (TE)*** cells (called flicking).

1.3. Time of biopsy

Biopsy can be performed by removal of one or two polar bodies 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 (Figure 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.

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 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.

1.4. 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 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.

1.5. 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.

2. 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).

2.1. 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.

2.2. 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.
- 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.

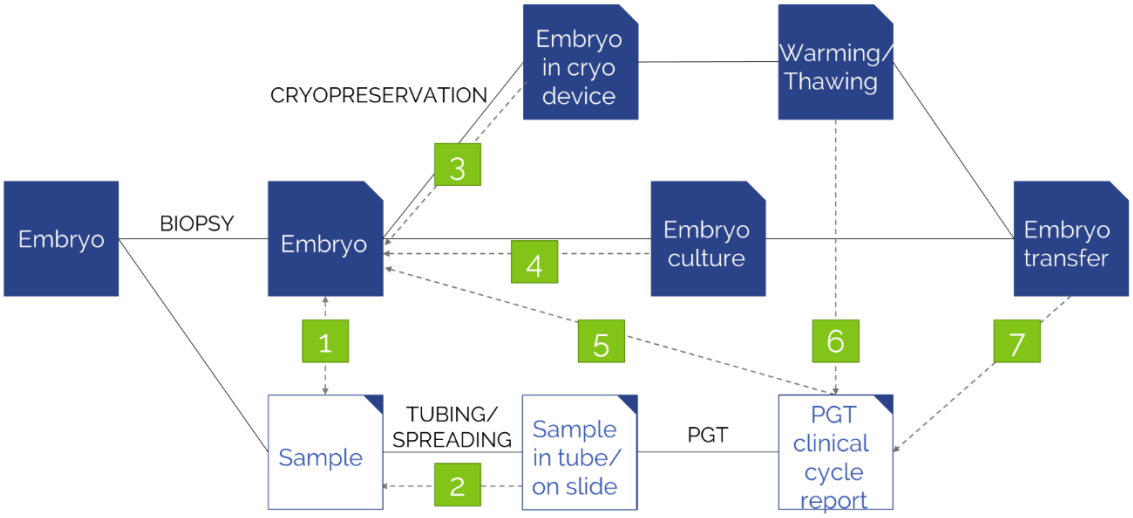
2.3. Labelling and witnessing

General recommendations on labelling and witnessing throughout the IVF-PGT :

- It is recommended that an adequate labelling system, written or barcoded (electronic), with two unique patient identifiers plus the embryo/cell(s) number is used to match the sample's diagnostic result with the embryo from which that sample was taken. This should ensure traceability throughout the IVF and PGT process until reporting of the final results.
- The labelling system should be comprehensible and practical for both the IVF and the PGT centres. Printed sticker labelling may be superior to pens, as labelling should be legible and uneditable.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer and signed off (Figure B1). These critical steps are detailed in the technical papers for the various methods (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
- After biopsy, the sample may be analysed in house, or sent for genetic testing in another centre (see " Transport PGT").

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; (vii) during the thawing/warming procedure and at the time of selecting the embryo(s) for transfer.

Figure B1: Outline of the biopsy and genetic testing procedure with indications of the 7 critical steps where labelling and sample identification should be confirmed.



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) In case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device; (4) For further embryo culture, at placing and labelling the embryo into the culture dish; (5) When diagnostic results are issued to ensure accuracy and correlation with the correct sample identification; (6) During the thawing/warming procedure to ensure accuracy and correlation with the correct embryo diagnostic result; and (7) At the time of selecting the embryo(s) for embryo 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.

2.4. 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, …) should be noted and shared with the genetic laboratory.

3. Biopsy laboratory infrastructure, equipment and materials

3.1. 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"*, 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 7. Sample collection).

3.2. Equipment

Biopsy equipment set up includes an inverted microscope with heated stage and three-dimensional micro-manipulators and micro-injectors (air or oil), placed on antivibration pads, equivalent to a setup 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 25x or 40x objective of an inverted microscope and piloted by a software and camera. The laser can be controlled either using mouse or foot switch.

3.3. 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. 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).

4. Training for biopsy

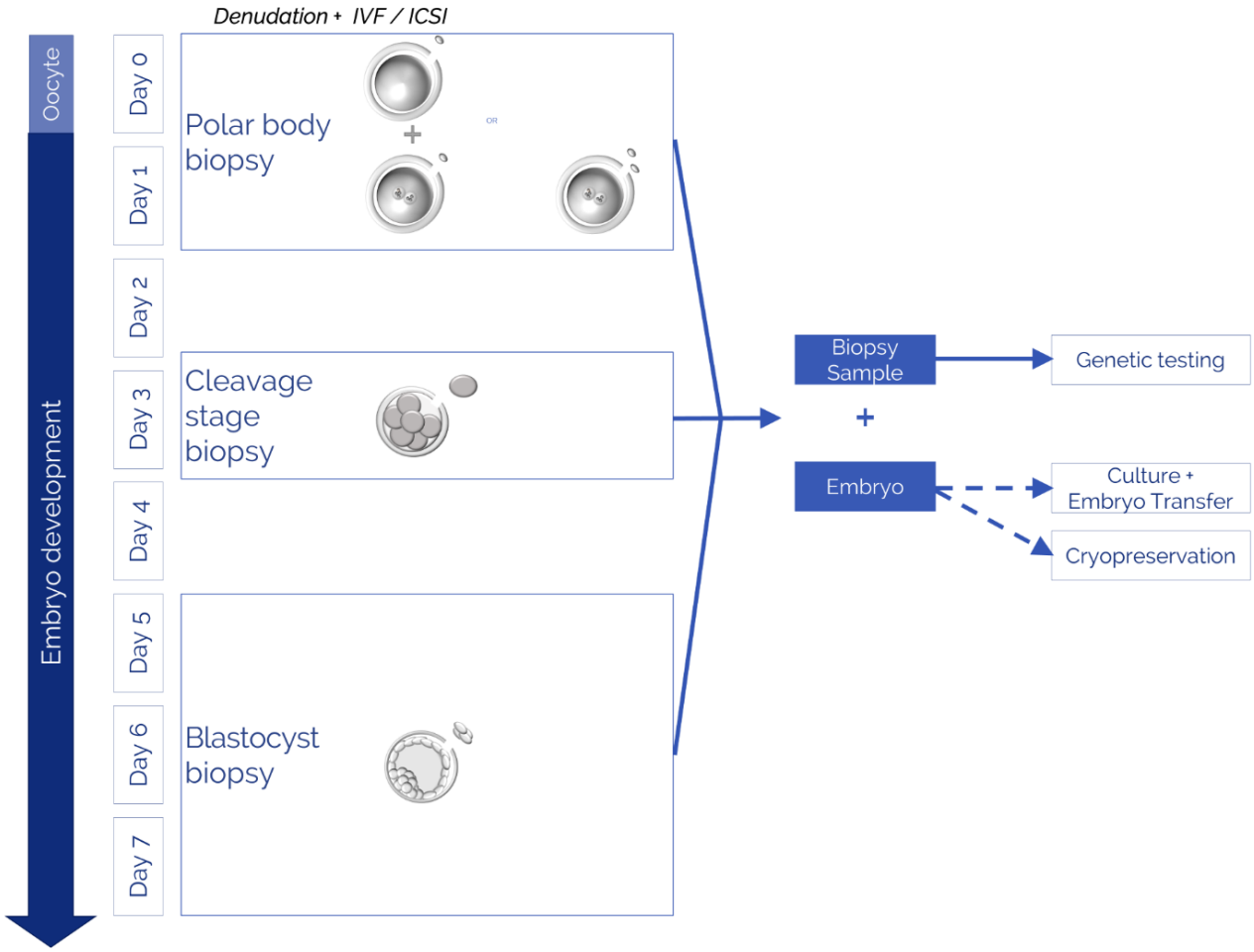
The embryo biopsy laboratory should be supervised by a person with recognised expertise in clinical embryology and preferably also basic knowledge in medical genetics.
The biopsy procedure should be performed by experienced practitioners with basic skills in general embryology and micromanipulation, 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.

5. Biopsy stage and procedure

Figure B2: Methods of oocyte and embryo biopsy



5.1. 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*.

5.1.1. Organisation of the biopsy

Polar bodies 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.

5.1.2. 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 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.

5.1.3. 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.

5.1.4. Rebiopsy of embryos

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

5.2. Cleavage-stage biopsy

5.2.1. 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.

5.2.2. Biopsy procedure

Biopsy is performed either directly in biopsy medium (Ca^{2+}/Mg^{2+}-free) or in HEPES-buffered medium after 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.

5.2.3. 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.

5.2.4. 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.

5.3 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.

5.3.1. 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 Day 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.

5.3.2. Biopsy procedure

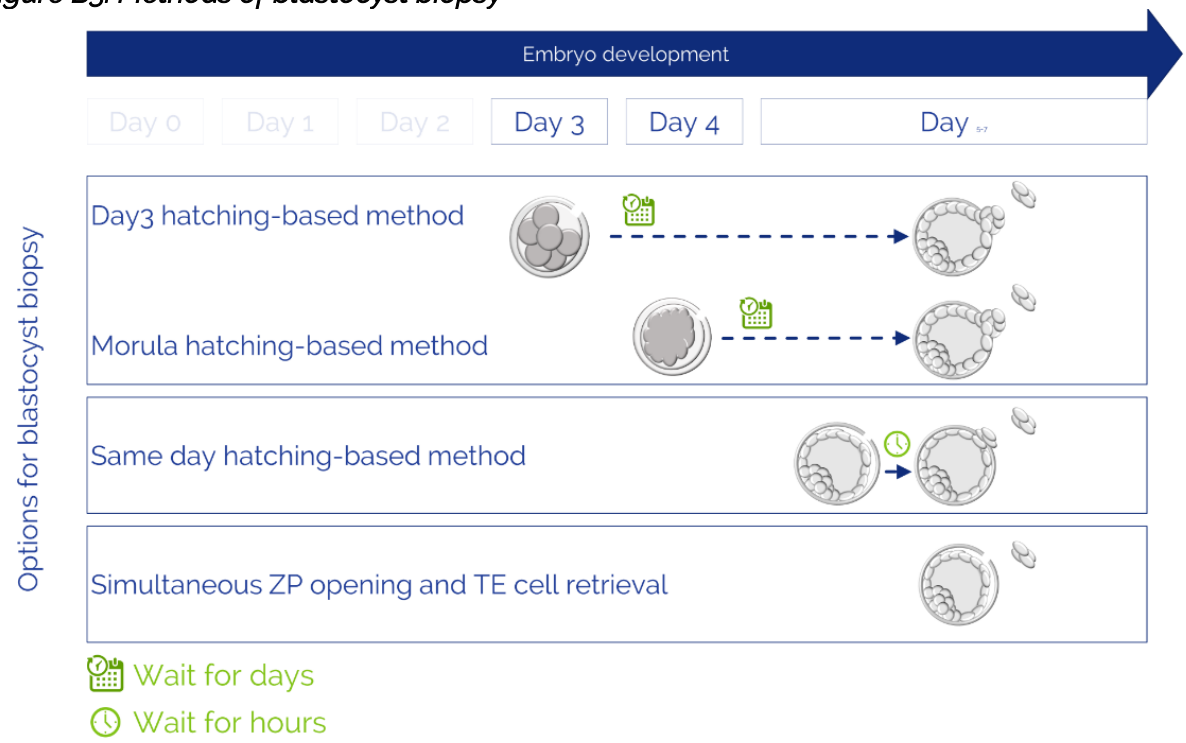
The biopsy procedure may vary depending on the morphology and quality of the blastocyst, expansion grade and the position of the ICM. Furthermore, there are some variations among operators and laboratories.

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 (Figure B3):

- 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 B3: Methods of blastocyst biopsy



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 by 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.
- 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, the blastocyst is immediately transferred in culture medium or cryopreserved.

5.3.3. 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.

5.3.4. 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.

6. General strengths and limitations

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

6.1. 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 on Day 1 or on both 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 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).

Table BI. 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 members 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 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 (Day 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%

6.2. 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 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.

6.3. Blastocyst biopsy

TE biopsy entails the collection of a multicellular section (5 to 10 cells) on Day 5-7 from a part of the blastocyst which gives rise 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 with alternative biopsy procedures, including higher reliability by the analysis of a higher number of cells.

Alternative blastocyst biopsy approaches (see figure 3) involve different learning curves and levels 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 a 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 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).

7. 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 require 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.

7.1. 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.

7.1.1. Labelling and witnessing

General recommendations on labelling and witnessing are presented in Section B.2.3

7.1.2. 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).

7.2. Laboratory infrastructure, equipment and materials

7.2.1. 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 a close proximity to the biopsy area.

7.2.2. 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.

7.3. 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 authorised 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.

7.4. 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 trophectoderm, 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 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 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 on two different timepoints 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 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 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. 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.

8. 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:

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 for the genetic laboratory to analyse the samples);
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 must be vitrified individually in 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.

9. Alternative Biopsy methods

Morula-stage biopsy is under validation as alternative biopsy method (Figure B4).

9.1. Morula-stage biopsy

The biopsy of morula-stage embryos on Day 4 is performed after artificial decompaction (requiring Ca^{2+}/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.

10. Alternative sampling methods

Cell free DNA analysis (blastocentesis and spent culture media) is under validation as alternative sampling method for genetic testing (Figure B4).

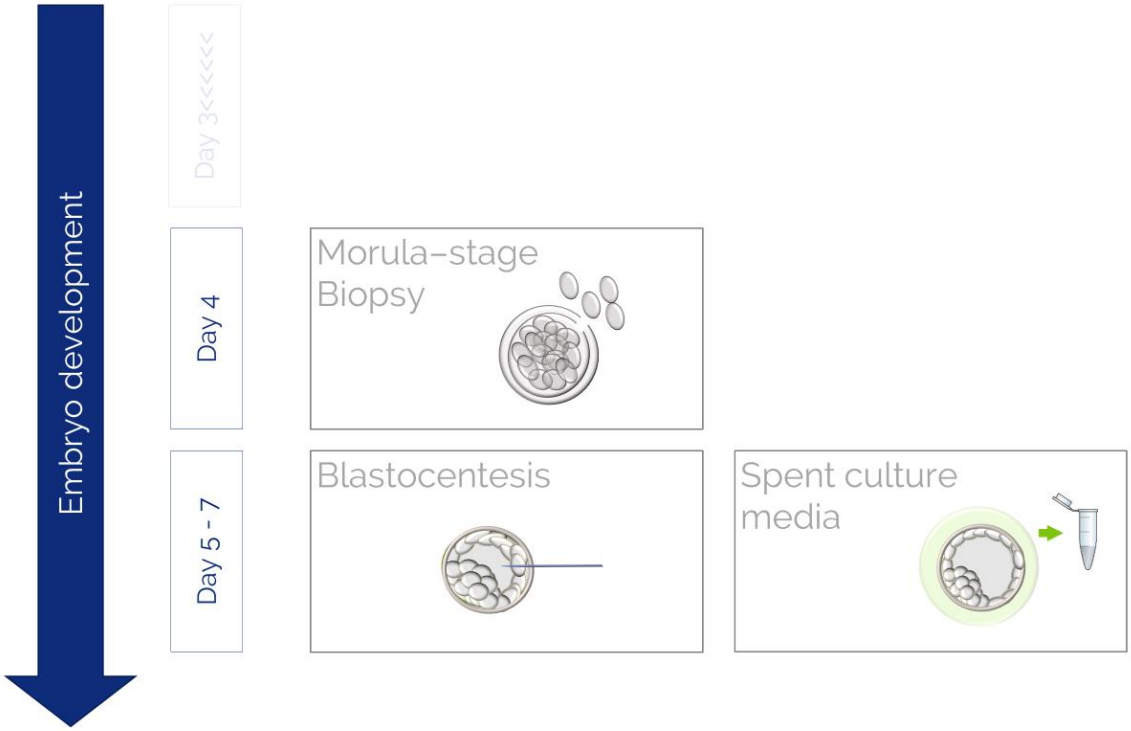
10.1. Blastocentesis

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.

10.2. 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.

Figure B4: Alternative biopsy and sampling methods



SECTION C: DETECTION OF MONOGENIC DISORDERS

This section is entirely based on the paper:

"ESHRE PGT Consortium good practice recommendations for the detection of monogenic disorders" with additions from the paper "ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing"

1. Introduction to PGT-M

This paper provides detailed technical recommendations for the most applied methods for PGT-M.

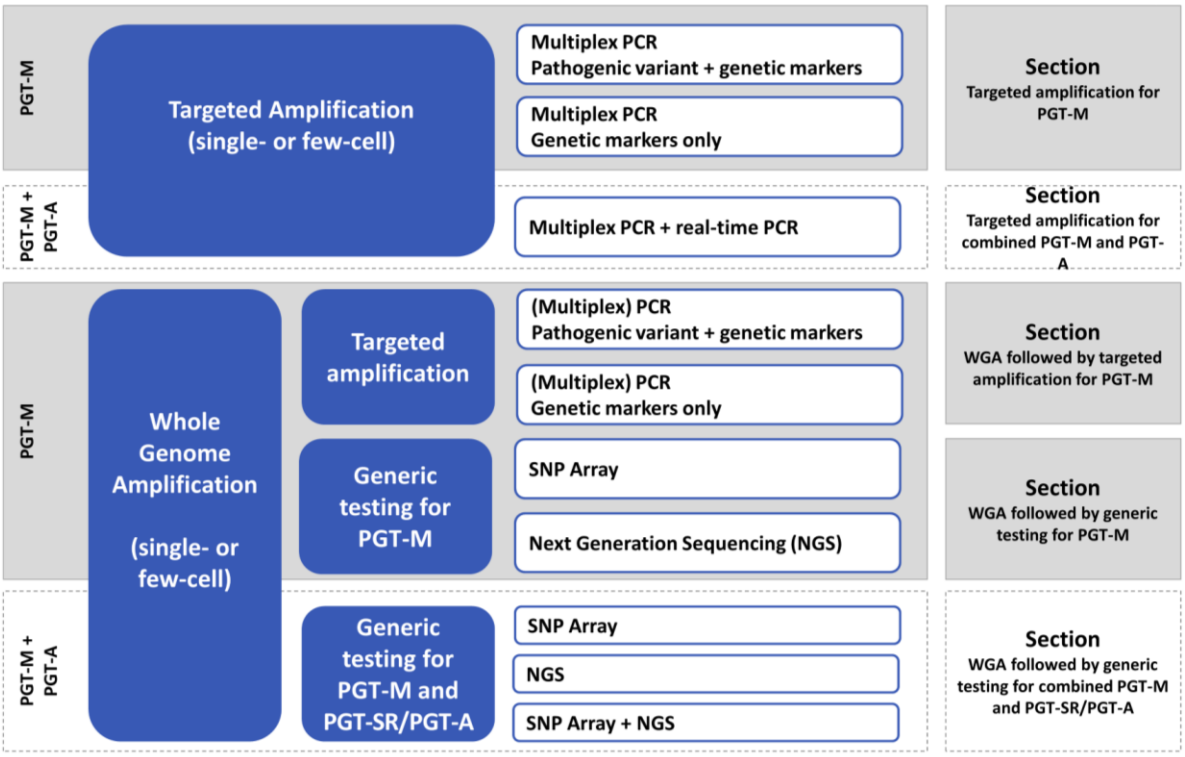
PGT-M refers to testing for nuclear DNA pathogenic variant(s) causing monogenic disorders, with an autosomal dominant, autosomal recessive or X-linked transmission pattern, but also mitochondrial DNA pathogenic variant(s). It also refers to exclusion testing and to HLA typing with or without concurrent testing for a monogenic disorder.

One of the greatest challenges for PGT-M is the low amount of input DNA for which sensitive DNA amplification techniques are needed. Biopsied single (after polar body or single blastomere biopsy) or few cells [i.e. 5-10 trophectoderm (TE) cells] undergo either a targeted amplification reaction via multiplex PCR or a whole-genome amplification (WGA) step followed by downstream applications (targeted or genome-wide) such as PCR, single nucleotide polymorphism (SNP) arrays or next-generation sequencing (NGS) (Figure C1). Each method has its advantages and its limitations. The principle of most of these methods is based on haplotyping (i.e. determination of the group of alleles within a genetic segment on a single chromosome being inherited together). Therefore, genetic markers located close to the gene of interest are genotyped in DNA samples of the couple and relevant family members with known genetic status during preclinical work-up. Genetic markers that are informative, flank the locus of interest and allow discrimination of the parental haplotypes, are selected for use in the clinical test. The haplotype which is common in the family members with the familial pathogenic variant is referred to as the high-risk haplotype (or mutant), whereas the haplotype without familial pathogenic variant is referred to as the wildtype or low-risk haplotype. The clinical test can be either direct, when pathogenic variant plus linked genetic markers are assessed, or indirect, when testing is based on haplotyping only.

The limitations of low DNA quantity are related to the increased risk of either DNA amplification failure (AF), DNA contamination or the phenomenon of allele drop-out (ADO), in which one of two alleles in a heterozygous sample is amplified while the other remains undetected. This is often more challenging for single-cell analysis compared with analysis of few cells. The occurrence of any of these events may have a severe impact on the reliability of the diagnostic result, and precautions must be taken to minimise their occurrence or improve their detection during the test set-up and its clinical implementation.

The recommendations formulated in this section are independent of the testing method applied.

Figure C1: Overview of the testing strategies that can be applied for PGT-M



1.1. Training and personnel

- Genetic testing procedures should be performed under the supervision of a specialised geneticist, competent and authorised to perform clinical diagnostics according to local or national regulations.
- All personnel undertaking genetic testing should be trained adequately as required in a genetic laboratory and should follow written standard operating procedures (SOPs).
- Training for each technique should be documented. Prior to working on clinical specimens, the following recommendations apply for each trainee.
    - For tubing, training is discussed in the paper on polar body and embryo biopsy for PGT (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
    - For targeted PCR, it is recommended that 30 to 50 single- or few-cell samples are subjected to multiplex PCR, in two or three separate testing rounds and successfully processed. Negative controls should be included to monitor contamination in each round.
    - For WGA, it is recommended that 30 to 50 single- or few-cell samples are subjected to WGA and that the WGA products are successfully processed in downstream application(s), in multiple separate testing rounds. Negative controls should be included to monitor contamination in each round.

1.2. Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

1.2.1. Laboratory infrastructure

Oocyte and/or embryo biopsy should be performed in a specifically designated laboratory setting. Collection of the biopsied samples and initial steps of genetic testing procedures should be carried out in laboratory settings dedicated for processing single and/or few cells. Appropriate precautions should be taken both to prevent contamination of samples by physical isolation, and to detect any such contamination. Licenses for offering embryo biopsy procedures and/or genetic testing by the centre may be obtained, according to local regulations.

1.2.2 Equipment

- All clinical equipment should meet the criteria set for the intended application, be appropriately calibrated, maintained and serviced, with all aspects supported by written ***standard operating procedures (SOPs).*** Equipment used for critical steps should have uninterrupted power supply (UPS).
- For areas within the IVF centre, whether it is a dedicated area or a room, all equipment should comply with "Revised Guidelines for good practice in IVF laboratories (2015)", section 3 "laboratory safety" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). Prior to the biopsy procedure, work surfaces, equipment and hoods should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory.
- For areas within the PGT centre, prior to each use, work surfaces and equipment should be cleaned and decontaminated with DNA ***decontamination*** solutions or 10% bleach, or by UV-C irradiation or autoclaving (when applicable, for example tube racks). It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.
- Multichannel pipettes or automated systems may be useful in the PGT laboratory to minimise the risks of mislabelling or misallocation of samples during the post-amplification steps, but they are not recommended in the pre-amplification steps.

1.2.3 Materials

- To prevent contamination, protective clothing for DNA amplification of a single and/or few cells 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). For areas within the IVF centre, protective clothing, preferably with low particle-shedding and non-powdered gloves and masks should be considered.
- The pre-amplification materials and reagents should be kept away from any DNA source and preferably stored in the pre-amplification area.
- 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 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 internal 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 all reagents.
- Reagents and solutions can be DNA decontaminated by UV-C irradiation. Alternatively, reagents and solutions made in-house can be autoclaved, preferably using a PGT-dedicated autoclave.
- Careful handling of all reagents employed must be ensured with regards to storage temperature and working conditions, following manufacturer's recommendations. Vortexing and quick temperature changes should be avoided for the most sensitive components.

Specific issues for handling of reaction tubes to reduce cross-contamination:

- It is recommended to avoid touching the inside or the lid of the tubes with your fingers.
- It is recommended to avoid touching the outside or the cap of the tubes with the tip of the pipette. If this happens, the pipette tip should be changed immediately.
- It is recommended to keep the reaction tubes open not longer than necessary.

1.2.4.Laboratory documentation

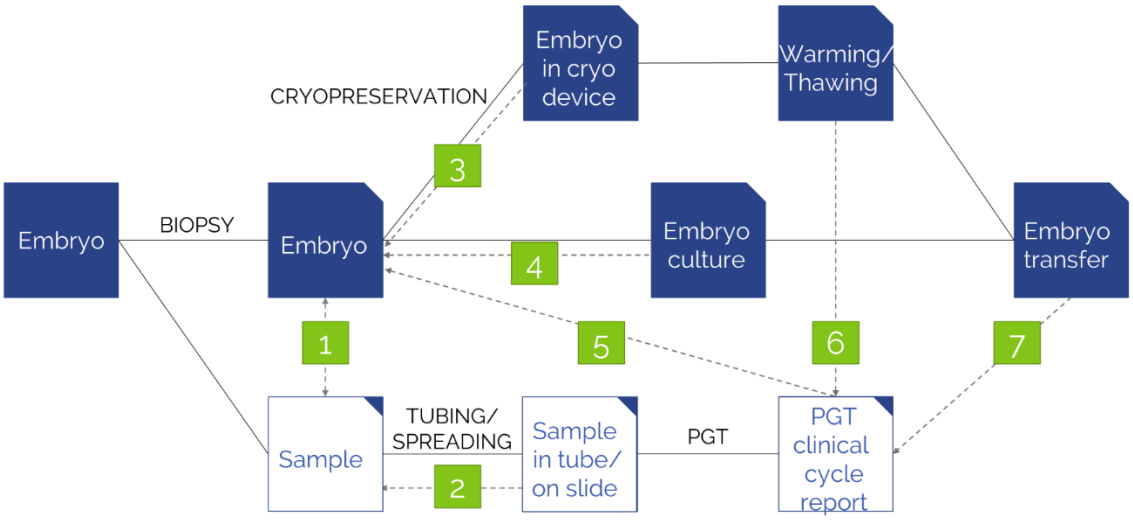
Well-structured (electronic and/or paper) laboratory forms should be available for recording wet-laboratory details of work-up and PGT cycle procedures.

1.3. Labelling and witnessing

General aspects on labelling and witnessing as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) :

- It is recommended that an adequate labelling system, written or barcoded (electronic), with two unique patient identifiers plus the embryo/cell(s) number is used to match the sample's diagnostic result with the embryo from which that sample was taken. This should ensure traceability throughout the IVF and PGT process until reporting of the final results.
- The labelling system should be comprehensible and practical for both the IVF and the PGT centres. Printed sticker labelling may be superior to pens, as labelling should be legible and uneditable.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer and signed off (Figure C.2). These critical steps are detailed in the technical papers for the various methods (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
- After biopsy, the sample may be analysed in house, or sent for genetic testing in another centre (see " Transport PGT").

Figure C.2: Outline of the biopsy and genetic testing procedure with indications of the 7 critical steps where labelling and sample identification should be confirmed.



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) In case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device; (4) For further embryo culture, at placing and labelling the embryo into the culture dish; (5) When diagnostic results are issued to ensure accuracy and correlation with the correct sample identification; (6) During the thawing/warming procedure to ensure accuracy and correlation with the correct embryo diagnostic result; and (7) At the time of selecting the embryo(s) for embryo transfer.

2. Single- or few-cell methods

PGT-M can be subdivided into the pre-examination process and the clinical cycle (examination process). The pre-examination process includes preclinical work-up with informativity/segregation analysis and eventually test development followed by validation. For informativity/segregation analysis, short tandem repeat markers (STR) or SNP marker genotyping is performed on DNA samples of the couple and related family member(s) to identify informative markers and to establish which combination of marker alleles (haplotype) segregates with the pathogenic variant. If the high-risk haplotype is determined during work-up, an indirect testing method can be applied. Alternatively, a direct method is chosen where the detection of the pathogenic variant is combined with the genetic markers for haplotype confirmation. For some cases, it will not be possible to determine the high-risk haplotype during work-up, for instance when a *de novo* pathogenic variant is present or when no relevant family DNA samples can be obtained (see also section De novo pathogenic variant(s)). In these cases, it may be determined during the clinical cycle based on the results from the biopsied embryo cells.

The following section describes pathogenic variant and genetic marker loci and the most applied methods for their detection.

2.1. Pathogenic variant and genetic marker loci

Pathogenic variant loci can be nuclear or mitochondrial and involve germline genetic variant(s) proven to be disease causing (previously termed mutation). Whether the pathogenic variants themselves are incorporated in the clinical test depends on multiple factors, including the nature of the pathogenic variant (familial or *de novo*), the availability of relevant family DNA samples, the variant type and the preclinical work-up results. For mitochondrial diseases the variant is always included, because the test is based on the determination of the percentage of the genetic variant present in the embryo.

STR markers are short tandemly repeated DNA sequences (dinucleotides are the most common) which are highly polymorphic and quite abundant in the human genome (one STR per 2000-10 000 bp). Useful STR markers are taken from published papers or *in silico* selected from public databases and usually involve many alleles (high heterozygosity). STR loci are targeted with fluorescently labelled primers and coamplified in a multiplex PCR reaction.

A fully informative STR marker will have different amplicon sizes for each of the four parental alleles, allowing discrimination of all possible embryo genotypes and detection of problems of contamination, ADO, recombination and copy number aberrations. A partially informative (semi- or limited informative) STR marker indicates that not all embryo genotypes can be distinguished and is less powerful in detecting additional problems. A non-informative STR marker is a marker that cannot distinguish between an affected and an unaffected embryo. This is illustrated below in an example for an autosomal dominant disorder (Table C.I).

*Table C.I: Example of STR informativity results for an autosomal dominant disorder (the pathogenic allele is indicated with *after validation of segregation analysis with a suitable reference)*

Affected Male partner	Unaffected Female partner	Informa-tivity	ADO detection in the embryo	Detection of maternal contami-nation	Additional info on monosomy /trisomy	Comments	Recommendation for PGT-M (ranking1)
124 - 126*	120 - 122	Fully informative	Yes	Yes	Yes	4 distinctive parental alleles	Preferred marker (1)
124 - 126*	120 - 120	Informative	Yes	No	Partially	3 distinctive parental alleles, the affected partner is heterozygous, the unaffected partner is homozygous for a third allele. The wild-type allele is a unique allele.	Good marker (2)
124 - 126*	120 - 126	Partially informative	Partially	Partially	Partially	3 distinctive parental alleles, both partners are heterozygous, but the mutant allele of the affected partner is shared with an allele of the unaffected partner. The wild-type allele is a unique allele. Unaffected embryos (124-120 or 124-126) can be distinguished, as well as one genotype of affected embryos (126*-120). The second genotype of affected embryos is homozygous (126*-126), therefore it is uncertain if both paternal and maternal alleles are present.	Usable marker (3)
124 - 126*	126 - 126	Partially informative	Partially	No	Partially	2 distinctive parental alleles, the wildtype allele is a unique allele; the marker yields only information about the wildtype allele and is therefore limited in use	Usable marker (4)
124 - 126*	120 - 124	Partially informative	Partially	Partially	Partially	3 distinctive parental alleles, both partners are heterozygous, but the wildtype allele of the affected partner is shared by the unaffected partner. One genotype of unaffected embryos (124-120) can be distinguished from affected embryos (126*-120 or 126*-124); the second genotype of unaffected embryos is homozygous (124-124), therefore it is uncertain if both paternal and maternal alleles are present.	Usable marker (5)
124 - 126*	124 - 124	Partially informative	Partially	No	Partially	2 distinctive parental alleles, the mutant allele is a unique allele; the marker yields limited information about the mutant allele.	Usable marker (6)
124 - 126*	124 - 126	Partially informative	Partially	No	No	2 distinctive parental alleles, no unique alleles; the marker yields very limited information (to be used in combination with other markers)	Usable marker (7)
126 - 126*	Any genotype	Non-informative				No information about the monogenic disorder but may yield information on parental contribution.	Not recommended

[1]The utility of the markers is ranked from very good (1) to very low (7)

The ranking of the marker according to its informativity takes into consideration the presence of unique alleles on the low-risk haplotype, confirming the presence of this haplotype. The ranking can be used when developing a new test, but any (partially) informative marker included in an existing protocol can be helpful, independently of its ranking.

SNPs are mostly biallelic and have a lower information content per marker compared to STRs. Three informative SNPs provide equivalent information as a single informative STR, but SNPs are much more abundant (one SNP per 300-1000 bp), easier to interpret and amenable to high-throughput analysis.

A SNP combination in a couple is informative when a clear distinction between the high-risk and low-risk allele(s) can be made. An informative SNP marker in which the wildtype allele is unique, is the most powerful, as unaffected embryos are then distinguished by heterozygous SNPs, limiting the misdiagnosis risk due to ADO. This is illustrated below in an example for an autosomal dominant disorder (Table C.II).

Informativity results are first evaluated for each marker separately; afterwards, the overall effectiveness of the selected set of markers to be used in the clinical test is assessed for its ability to evaluate the status of the embryo relative to the monogenic disorder, as well as other parameters such as occurrence of ADO, monosomy, trisomy, and parental (mostly maternal) contamination.

2.2. Basic methods for allele discrimination

Pathogenic variant and marker loci are amplified with primer pairs in which one primer is fluorescently labelled, allowing sensitive detection of the amplification products afterwards. The method is designed so that wild-type and pathogenic or high-risk allele discrimination is part of the amplification itself [e.g. double amplification refractory mutation system (D-ARMS)], or allele discrimination is carried out in a post-amplification step (e.g. mini-sequencing). In some cases, a DNA purification step may be required to remove primers and buffer components of the amplification reaction, before starting the post-amplification reaction.

*Table C.II: Example of SNP informativity results for an autosomal dominant disorder (the pathogenic allele is indicated with *after validation of segregation with a suitable reference).*

Affected male partner	Unaffected female partner	Informativity	ADO detection in the embryo	Detection of maternal contamination	Additional info on Monosomy/ trisomy	Comments	Recommendation for PGT-M (ranking[1])
A*B	AA	Informative	Partially	No	Partially	The wildtype allele is a unique allele, therefore unaffected embryos are heterozygous and can be distinguished	Preferred marker (1)
AB*	BB						
AB*	AA	Informative	Partially	No	Partially	The mutant allele is a unique allele therefore unaffected embryos are homozygous; therefore, it is uncertain whether both paternal and maternal alleles are present	Usable marker (2)
A*B	BB						
A*B or AB*	AB	Non-informative	No	No	No	The marker yields very limited information (to be used in combination with other markers)	Usable marker (3)
AA or BB	Any genotype	Non-informative	-	-	-	No information	Not recommended

[1] The utility of the markers is ranked from very good (1) to very low (3)

2.2.1. Fragment length analysis

Principle of the test

This approach is based on different migration patterns of fluorescently labelled DNA molecules according to their molecular weight or size. Fragment length analysis is usually carried out via capillary electrophoresis on an automated sequencer. Allele discrimination for STR markers and insertion/deletion pathogenic variant loci is performed via fragment length analysis directly following PCR.

Limitations of the test

For pathogenic variant(s), direct allele discrimination following PCR via fragment length analysis is limited to variants that generate DNA fragments of different size. Although technically feasible to distinguish fragments differing by 1 bp, this may require another strategy for more accurate discrimination. For other loci such as SNPs and single nucleotide variant(s) which do not generate PCR products of different size, direct amplification methods exist (e.g. D-ARMS), but often amplification is followed by post-PCR reactions for allele discrimination. The direct detection of complex and/or larger gene rearrangements may not be feasible, as the exact break points are often unknown, or their amplification is not possible as single- or few-cell targeted PCR fragments usually remain below 500 bp.

For STRs, especially with dinucleotide repeats, stutter patterns (one repeat unit less in size) may complicate allele discrimination and make data interpretation more difficult.

2.2.2. Restriction enzyme digestion

Principle of the test

A common form of DNA sequence variation detection is based on the ability of restriction enzymes to recognise specific DNA sequences and cleave the strands very close to, or at the site of, the variant. As a variant can create or destroy a restriction site, fragment length analysis will reveal the presence or absence of the variant. This method is a post-PCR reaction which may require a prior DNA purification step. The restriction enzyme digestion is followed by fragment length analysis. It is recommended to always check for complete restriction digestion.

Limitations of the test

This approach can be used if the pathogenic variant creates or destroys a restriction site. If not, primer design may be adapted in order to generate an artificial restriction site.

It is preferable to apply this method in cases where the pathogenic variant destroys rather than when it creates a restriction site. In the first case, the normal allele will be digested whereas the mutant allele will remain undigested. When the pathogenic variant creates a restriction site, failed or incomplete digestion could lead to misdiagnosis.

2.2.3. Double amplification refractory mutation system (D-ARMS)

Principle of the test

Double amplification refractory mutation system (D-ARMS) allows the amplification of both the wild-type and the pathogenic or high-risk allele for single nucleotide pathogenic variant(s). The test relies on a set of three PCR primers: a common fluorescently labelled primer, and two primers located at the target site with the last 3' nucleotide overlapping the single nucleotide pathogenic variant, one primer being specific for the normal allele and one specific for the mutant allele. A tail is added at the 5′ end of one primer to enable sizing discrimination between wild-type and pathogenic or high-risk alleles following single-round PCR and fragment length analysis. For ARMS primers, it is recommended to introduce an additional mismatch between three and five nucleotides upstream of the 3′

end of each specific primer, to increase the discrimination potential between pathogenic or high-risk and wild-type alleles.

Limitations of the test

D-ARMS is not recommended when the pathogenic variant is part of a nucleotide stretch, since the difference in amplification specificity between pathogenic or high-risk and wild-type alleles may be insufficient.

2.2.4. Real-time PCR for pathogenic variant detection and genotyping

Principle of the test

Real-time PCR is a closed-tube system where amplification is monitored real-time and post-PCR processing steps are not required. A first-round multiplex PCR precedes the nested real-time PCR to enable multiplexing for concurrent amplification of the variant locus (or loci) and informative markers. Probe design is flexible and the most commonly used are hybridisation and hydrolysis probes. There are a number of real-time PCR platforms and chemistries suitable for PGT-M genotyping.

Limitations of the test

This approach requires dedicated instruments, and the possibility of multiplexing is limited depending on the real-time PCR platform (limited number of detector channels).

2.2.5. Mini-sequencing

Principle of the test

Mini-sequencing is based on Sanger sequencing but without sequencing the entire PCR product. The mini-sequencing reaction requires purified PCR products as template, together with a specific unlabelled mini-sequencing primer (forward and/or reverse), designed to anneal adjacent to the target site. The mini-sequencing primer is extended with a single dideoxy nucleotide, complementary to the target site. Each dideoxy nucleotide is labelled with a different fluorochrome, allowing alleles to be distinguished on an automated sequencer. This detection method is mainly used in cases of base substitutions, but it can also be applied for small insertions or deletions.

Limitations of the test

When applying this detection method in cases of small insertions or deletions, the nucleotide may be the same in the presence or absence of the pathogenic variant, and mini-sequencing primer design should be adapted.

2.3 Single- or few-cell targeted amplification

Following embryo biopsy, biopsied cell samples are washed, transferred to reaction tubes and lysed. Amplification reaction components are then added directly to the lysed cell(s) without prior DNA purification. Samples undergo either targeted amplification by means of multiplex PCR or WGA (see section "Single- or few-cell whole-genome amplification"). The prevention of external DNA contamination is mandatory, together with accurate and strict sample processing. This requires a specialised laboratory environment and working practice.

When performing targeted amplification on single or few cells, the following recommendations apply.

2.3.1. Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials are covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). For targeted amplification-based PGT specifically, the following recommendations are made.

Infrastructure

- There should be a physical separation between the genetic laboratories and the biopsy laboratory.
- There should be a physical separation of the pre-amplification (preferentially a positive pressure room with a dedicated laminar flow hood), and the post-amplification (preferentially a negative pressure room) areas. It is recommended to have the thermal cyclers in a dedicated room (amplification area). If this is not possible, it is acceptable to have them in the post-amplification area.
- When positive and negative pressure rooms are present, they are preferably enclosed by a lock chamber.
- Secondary amplification reactions can be performed in the post-amplification area in a simple cabinet like a PCR workstation or dedicated area in which one has a restricted area to process the samples.
- A dedicated set of equipment (including thermal cyclers), consumables and laboratory coats should be used for each designated area and not be exchanged between these areas.
- An appropriate unidirectional workflow should be in place, avoiding any backflow of amplified products to the pre-amplification area.
- Preferably, the pre- and post-amplification rooms/areas should be equipped with UV-C light for DNA decontamination.

Equipment

Equipment required for amplification-based analysis of samples includes:

- class II safety cabinets, preferably equipped with UV-C light, to prevent contamination of samples at the pre-amplification stage;
- simple cabinets;
- thermal cyclers with heated lids;
- micro-centrifuges, vortex and pipettes; and
- capillary gel electrophoresis equipment for fragment analysis following amplification.

Materials

Specific materials required for targeted amplification of samples include:

- lysis buffers, (pre-)amplification enzymes and primers/probes specific to each amplification method used; and
- capillary gel electrophoresis materials.

2.3.2. Tubing of samples

General recommendations about biopsy and transfer of samples to tubes (referred to as tubing) is provided in the paper on polar body and embryo biopsy for PGT (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).

2.3.3. Work practice controls

It is recommended to use positive and negative (no DNA) controls.

- As a positive control sample, diluted and/or undiluted genomic DNA from the couple is recommended. DNA samples from other family members may also be included. In addition, single- or few-cell samples can be used. Positive control cell samples can be lymphocytes, buccal cells or cultured cells. If the test includes the pathogenic variant detection, it is recommended to use:
    - For dominant diseases: DNA samples with high-risk and low-risk genotypes;
    - For X-linked diseases: DNA samples with high-risk, low-risk, male and female genotypes;
    - For recessive diseases: DNA samples with heterozygous pathogenic variant carrier, homozygous normal, and (if available) homozygous or compound heterozygous genotypes.
- Negative controls should be included to confirm that there was no contamination introduced from the procedure of sample collection or from the amplification reactions.
    - 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 step of cell sample collection (i.e. the IVF laboratory negative control); for example, collection on two different timepoints 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 few cells, the number of negative controls should preferably be increased, preferably one negative control per biopsied embryo.
    - A minimum of one negative control with amplification mixture only is recommended to control for contamination during setting up of amplification reactions (i.e. the genetic laboratory negative control).

2.4. Single- or few-cell whole-genome amplification

Following embryo biopsy, cell samples are washed and transferred to reaction tubes. After cell lysis, WGA reaction components are added without prior DNA purification. WGA allows provision of sufficient DNA template from minute DNA samples to carry out subsequent DNA amplifications or to be used with other downstream techniques like multiple standard PCR testing, array-based comparative genomic hybridisation (aCGH), SNP array or high-throughput assays like NGS. Moreover, WGA products should be stored, according to the local quality system or legislation, for years (at -20˚C) to facilitate the use later in time to confirm results/diagnosis or carry out new tests.

Several methods for WGA have been developed over time and are available as commercial kits. Any WGA technique should be evaluated with regards to genomic coverage, high fidelity of the sequence, reliable quantification of copy number variation and technical errors of ADO and allele drop-in (ADI). A WGA method should be selected in function of the downstream application, taking into account advantages and disadvantages. Currently, multiple displacement amplification (MDA) is recommended for PGT-M (e.g. SNP haplotyping), whereas displacement degenerate oligonucleotide-primed PCR (DOP-PCR) (marketed Picoplex/Sureplex) is the method of choice for the detection of chromosomal copy number variation.

When applying WGA on single or few cells, recommendations for laboratory infrastructure, equipment and materials, tubing and controls are described below.

2.4.1. Laboratory infrastructure, equipment and materials

In general, follow the recommendations as stated in section "Single- or few-cell targeted amplification".

The following additional recommendations are made for infrastructure, equipment and materials, specifically for WGA.

Infrastructure

As WGA is a first round (primary) amplification step, it should be performed in the pre-amplification room/area. Reactions starting from WGA products are considered secondary reactions and should be performed in a separated area. Successful amplification should be confirmed before proceeding to downstream applications.

Equipment

Additional equipment includes:

- gel electrophoresis equipment to check for successful amplification;
- fluorometer for DNA quantification; the use of a DNA quantification system (to determine the amount of amplified DNA after WGA) is optional;
- specific equipment, depending on the downstream application.

Materials

Specific materials required for WGA of samples include:

- reagents specific to each WGA method used;
- reagents for DNA quantification following WGA;
- specific reagents, depending on the downstream application.

2.4.2. Tubing of samples

General recommendations about biopsy and tubing are provided in the paper on polar body and embryo biopsy for PGT (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).

2.4.3. Work practice controls

Positive and negative controls should be included to monitor the WGA reaction, as described in section "Single- or few-cell targeted amplification".

It is acceptable to include these controls only at the level of the WGA reaction and omit them from downstream reactions.

3. Pre-examination process

The pre-examination process includes preclinical work-up with informativity/segregation analysis and test development followed by validation.

3.1. Informativity/segregation analysis

- It is recommended that the original molecular genetic reports including the description of identified variants together with the appropriate gene reference sequence are obtained from an accredited laboratory. It is advisable to confirm the pathogenic variant(s) whenever possible.
- It is recommended to perform a preclinical work-up to assess PGT-M feasibility, identify informative genetic markers, establish parental haplotypes (when possible) and work on a clinical testing strategy.

- It is recommended to perform the informativity/segregation analysis for STR markers as well as for SNP markers. The results allow evaluation of the expected genotypes in the embryos.
- A geneticist experienced in pedigree and linkage analysis should determine which familial DNA samples are needed for a reliable and accurate diagnosis.
- For all diseases, samples should be collected from the prospective parents and close relatives with known disease status (proven via genetic reports) to establish the high-risk and low-risk haplotypes.
    - For dominant diseases, it is recommended that these samples include DNA from at least one affected (ideally two) and/or one unaffected individual as a reference.
    - For recessive diseases, these would include at least a homozygous or compound heterozygous affected individual, one carrier and one non-carrier individual as a reference.
    - For X-linked diseases, an affected individual must be used as a reference and/or one unaffected individual. A proven carrier would also be recommended.
- When no suitable family members are available, the informativity analysis should be performed in the couple and the segregation established during the clinical cycle (or on single sperm).

3.2. Testing strategies and test development

A test strategy is determined based on informativity/segregation analysis results. Different strategies for amplification and allele discrimination have been clinically applied.

The three main testing strategies for PGT-M are:

i) targeted amplification of informative markers with or without the pathogenic variant(s) in a single-/few-cell multiplex PCR *(sections "Targeted amplification for PGT-M" and "Targeted amplification for combined PGT-M and PGT-A")*

ii) WGA followed by targeted amplification of informative markers with or without the pathogenic variant(s) *(section "WGA followed by targeted amplification for PGT-M")*

iii) WGA followed by a generic method such as SNP array or NGS-based sequencing *(sections "WGA followed by generic testing for PGT-M" and "WGA followed by generic testing for combined PGT-M and PGT-SR/PGT-A")*

The first strategy (i.e. targeted amplification of informative markers in a single-/few-cell multiplex PCR), including the development/validation of a new test, is more time consuming and labour intensive than the WGA-based strategies, and the turnaround time between referral and clinical cycle is significantly increased. The major disadvantage of this approach is that development and validation of the multiplex PCR to the single-/few-cell level has to be repeated with every new gene/locus/variant of interest. The second strategy (WGA followed by targeted amplification) is a step towards a more generic method, because the adaptation/validation of PCR reactions at the single cell level can be omitted from the preclinical work-up. Locus-specific information is available in both cases in the form of either genotypes (pathogenic variant detection, SNP) or allele length (STR). Nevertheless, due to their targeted nature, the majority of these tests do not provide a comprehensive view of the genome. The third approach, the development of genome-wide generic methods, tackled this issue. SNP arrays, as well as sequencing-based approaches, allow genome-wide haplotyping as well as copy number typing. The extent to which the whole genome is analysed depends on the platform and/or approach. SNP array-based methods are restricted by the fixed number of probes included on the platform of choice. Sequencing-based approaches can be more or less comprehensive, depending on the genome coverage, SNP density, and the depth of sequencing. Additionally, sequencing-based approaches are high-throughput and allow automation,

reducing hands-on time and minimising the possibility of human errors. The WGA-based strategies are mostly coupled with TE biopsy, which leaves often insufficient time for fresh embryo transfer. This is overcome by cryopreservation and embryo transfer in a deferred cycle.

Further recommendations for test development are given in the following sections.

3.2.1. Targeted amplification for PGT-M

For many years, the co-amplification of genetic markers alone or in combination with the pathogenic variant at the level of single/few cells has been the 'gold standard' procedure for PGT-M. The inclusion of genetic markers in the clinical test improves the accuracy, as it not only allows for indirect pathogenic variant analysis but also allows for detection of ADO, contamination and recombination.

Recommendations for single- or few-cell targeted amplification concerning infrastructure, equipment, materials, tubing and work practice controls are described in section "Single- or few-cell targeted amplification". At the preclinical work-up, informativity/segregation analysis is required, together with the development of a locus-specific test at the level of single or few cells. Based on the results of informativity/segregation analysis, suitable STR markers close to the locus of interest are selected for co-amplification in a multiplex PCR, alone or in combination with the pathogenic variant.

The adaptation of PCR reaction conditions is usually carried out in several steps. The selected -most suitable- amplicons are preferably first multiplexed on genomic DNA samples. Further finetuning is then carried out with single- or few-cell samples. For test development, processing of at least one negative control with amplification mixture only for each amplification reaction is recommended. When working with single or few cells, negative controls with sample collection buffer only should be added as well, to control for contamination during sample collection. The optimised single-/few-cell PCR protocol is then validated on a series of single or few cells along with positive and negative controls (see also section "Pre-examination validation").

Familial pathogenic variant + genetic markers (STRs and/or SNPs)

When developing pathogenic variant and STR and/or SNPs analysis for single or few cells, the following recommendations are made.

- Amplicons should be designed ideally to be sized between 100 and 500 bp, using combinations of fluorochromes allowing loci discrimination.
- Single-round multiplex PCR is preferred compared with nested or semi-nested PCR as it is less error prone. When available, the use of STRs with tri-, tetra- or penta-nucleotide repeats is preferable, to reduce any confounding ambivalence due to the phenomenon of stutter patterns and improve allele discrimination.
- It is recommended to avoid STRs with a very wide range of allele size since the ADO risk of the large alleles is increased even at the genomic DNA level, leading to false homozygous genotypes during pre-clinical work-up and PGT-M.
- Before moving on to single-cell validation, it is recommended to establish a correct discrimination of pathogenic variant/wild-type or marker alleles of the test at hand. It is recommended to test various genotypes concerning the pathogenic variant or marker of interest using the following DNA samples:
    - affected (autosomal dominant) DNA samples;
    - carrier (autosomal recessive, X-linked diseases) DNA samples;
    - unaffected DNA samples for the pathogenic variant to be tested; and
    - DNA samples with heterozygous markers for indirect tests.
- When a protocol is employed for PGT-M, it is recommended to apply the specific test to DNA or single cells from each particular couple, to discover any unexpected

test results which could render future blastomere results questionable (for example, a polymorphism which may exist under a primer used in the single-cell assay but not in the routine laboratory assay).

- Polymorphic markers should have a high degree of heterozygosity and produce a clearly interpretable peak pattern, and preferably be intragenic.
- When using extragenic markers, it is recommended to stay within 1-Mb (approximately 1 cM) distance from the pathogenic variant of interest, to reduce the misdiagnosis risk due to recombination events (on average, loci 1 cM apart are expected to show 1% recombination). If no suitable markers are available within 1Mb, markers within 2 Mb are acceptable but not advisable. This may be adapted in case of large genes or duplications.
- The risk of misdiagnosis due to recombination should be considered for every marker, and is especially important in case of large genes and genes with known recombination hot spots. Careful selection of markers flanking the pathogenic variant of interest will reduce the risk of misdiagnosis due to recombination.
- Defining the minimum number of informative markers required in the single-/few-cell test: assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended to include at least one STR or three SNPs proximal and one STR or three SNPs distal to the region of interest, together with the pathogenic variant locus (choose markers with rank 1 or 2 in Table 1 and rank 1 in Table 2). In case of AF and ADO rates between 5% and 10%, either the test should be further optimised, or a higher number of markers should be included. In case of insufficient markers of the highest rank, markers of lower rank can be selected for test development, but the number of markers should then be increased.
- More markers will make the test more robust; analysis of at least two loci closely linked to and flanking the gene will reduce the risk of unacceptable misdiagnosis owing to ADO. Also, the risk for no diagnosis due to AF of a single amplicon in the multiplex will decrease.

Genetic markers only (STRs and/or SNPs)

Targeted indirect haplotype-only analysis of single or few cell(s) is applied for (i) exclusion testing, (ii) HLA typing, (iii) in case of unknown pathogenic variant but the locus/genomic region of interest is proven causative, (iv) triplet repeat expansion (e.g. the FMR1 CGG repeat expansion reluctant to single cell amplification), (v) large deletions/insertions with unknown breakpoints, (vi) in case direct pathogenic variant testing is not feasible [presence of pseudogene(s), GC-rich sequences refractory to single-cell amplification], or (vii) linkage analysis in general (to avoid to develop a test including the pathogenic variant). An indirect testing strategy is only applicable when high-risk and low-risk haplotypes have been established during preclinical work-up (exception, see section "*de novo* pathogenic variant(s)").

In general, when developing an indirect test with STR and/or SNPs for single or few cells, follow the recommendations as stated in the previous section (Familial pathogenic variant + genetic markers), except for the minimum number of markers required.

- Assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended to include at least two STRs or six SNPs proximal and two STRs or six SNPs distal to the locus of interest (choose markers according to Tables I and II). Here too, more markers are required in case higher AF and ADO rates are obtained and more markers will make the test more robust.
- In cases where the region of interest is located close to a centromere or telomere, flanking markers may not be possible. It is then recommended to include the pathogenic variant in the test strategy and to combine the test with TE biopsy to limit the risk of allele dropout at the pathogenic variant locus. The risk of misdiagnosis due to recombination should be reconsidered. In exceptional cases

where flanking markers are not possible and the pathogenic variant locus cannot be included [e.g. Facioscapulohumeral Muscular Dystrophy (FSHD)], the test strategy will be linked with a higher risk of misdiagnosis. Such exceptional cases should be counselled in depth and the need for prenatal testing (of which the availability should be ascertained before the start of the PGT procedure) should be explained.

3.2.2. Targeted amplification for combined PGT-M and PGT-A

PGT-M and PGT-A can be analysed simultaneously on the same biopsy sample in a testing strategy based on real-time PCR (RT-PCR). The workflow involves four steps: cell sample lysis, multiplex pre-amplification, RT-PCR and analysis. After sample collection and cell lysis, samples are subjected to multiplex PCR pre-amplification for both PGT-A and PGT-M. For PGT-A, a pool of 96 loci are pre-amplified, representative of four independent regions for each chromosome. For PGT-M, a custom set of amplicons is added, based on preclinical work-up results. Aliquots of the pre-amplified samples are subsequently interrogated in triplicates or quadruplicates by RT-PCR and relative quantification. Only whole chromosome copy number changes can be detected for PGT-A by this strategy. Automation can be applied to streamline the procedure, which can be completed in 3-4 h and is compatible with fresh transfer, following biopsy and genetic analysis.

3.2.3. WGA followed by targeted amplification for PGT-M

The implementation of WGA for PGT-M has increased concomitantly with the development of trophectoderm (TE) biopsy and vitrification. The approach of prior single or few cell WGA followed by standard PCR reactions for a set of STRs flanking the region of interest with or without the pathogenic variant, is widely applied. The use of SNPs instead of STRs has been described but the clinical application has been very limited. It is being replaced by SNP array-based or NGS-based haplotyping, as these approaches allow assessment of a multitude of SNPs in a standardised way.

Recommendations for single- or few-cell WGA concerning infrastructure, equipment, materials, tubing and work practices are described in section "Single- or few-cell whole-genome amplification". The following recommendations are made.

- It is recommended to use an MDA-based WGA protocol for haplotyping applications because of better genome coverage and low genotyping error rates.
- At the preclinical work-up, informativity/segregation analysis is required, together with the development of a locus-specific test, using WGA products as template DNA.
- It is recommended to carry out a validation assay for the WGA protocol and the specific downstream test(s) with respect to the number of biopsied cells, to determine the rate of AF, ADO and preferential amplification.

Familial pathogenic variant + genetic markers (STRs and/or SNPs) after WGA

In general, when developing a test with WGA of single or few cells followed by familial pathogenic variant + STR and/or SNPs analysis, follow the recommendations as stated in section "Targeted amplification for PGT-M".

- ADO rates for WGA plus multiplex PCR at the single cell level are higher (20-30%) than for single-cell multiplex PCR. Biopsy of few cells is recommended for WGA application, as ADO rates will be lower than for single cells.
- Single-cell biopsy is acceptable, but the higher ADO risk should be taken into account when defining the number of markers required in the downstream test.
- Defining the minimum number of fully informative markers required in the few-cells test: assuming validation data of AF and ADO rates per locus remain below

5%, it is recommended to include at least one STRs or three SNPs proximal and one STRs or three SNPs distal to the locus of interest together with the pathogenic variant locus (choose markers according to Tables I and II). Again, more markers are required in case higher AF and ADO rates are obtained.

Genetic markers only (STRs and/or SNPs) after WGA

In general, when developing a test with WGA of single or few cells followed by indirect STR and/or SNPs analysis, follow the recommendations as stated in section "Targeted amplification for PGT-M" and in the previous paragraph [Familial pathogenic variant + genetic markers (STRs and/or SNPs) after WGA].

- ADO rates for WGA plus multiplex PCR at the single-cell level may be higher than for single cell multiplex PCR, and this should be taken into account when defining the number of markers required in the downstream test.
- Defining the minimum number of fully informative markers required in the few-cells test: assuming validation data of AF and ADO rates remain below 5%, it is recommended to include at least two STRs or six SNPs proximal and two STRs or six SNPs distal to the locus of interest (choose markers according to Tables I and II).
- Here too, more markers are required in case higher ADO rates are obtained.

3.2.4. WGA followed by generic testing for PGT-M

SNP arrays for PGT-M only

SNP arrays are high-density oligo-arrays containing up to several million probes, which allow genotyping of hundreds of thousands of selected SNPs across all chromosomes in a single reaction. The commercially available SNP arrays use various methods for SNP genotyping of sample DNA: hybridisation to SNP allele-specific probes or single-base extension reactions are often applied. A given platform has a preset number of SNPs and therefore the position and number of SNPs within the region of interest will be fixed.The arrays are scanned and SNP genotypes are called based on the total fluorescence and the ratio of hybridisation intensities for the two SNP alleles.

The following recommendations are made.

- A relatively high DNA input is necessary for SNP arrays, so that a prior WGA step is required.
- It is recommended to use an MDA-based WGA protocol for haplotyping applications because of better genome coverage and low genotyping error rates compared to other WGA methods.
- As SNP arrays are generic platforms, preclinical work-up only requires informativity/segregation analysis for the locus of interest; the locus-specific development can be omitted.
- It is recommended to carry out a validation assay for the WGA protocol and the SNP arrays in respect to the number of biopsied cells. No-call rates and ADO rates for WGA plus SNP arrays at the single-cell level will be higher than for few cells and this should be taken into account when defining the minimum number of informative SNPs required in the region of interest.
- When using commercially available SNP array protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet- and dry-laboratory workflow prior to clinical use. For specific recommendation regarding the implementation validation, see also section "Pre-examination validation".

- The turnaround time from sample processing to data analysis can vary from 24 h to several days, depending on the setting and the platform of choice. It is recommended that each laboratory validates in-house whether the implementation of shortened protocols has an effect on hybridisation efficiency and data quality.

Limitations of the test

SNP array haplotyping requires at least one first degree relative of the partner carrying the mutation for phase determination, as an indirect testing strategy is only applicable when high-risk and low-risk haplotypes have been established during preclinical work-up (exception, see section "*de novo* pathogenic variant(s)").

NGS for PGT-M only

In NGS, a DNA polymerase catalyses the incorporation of deoxyribonucleotide triphosphates (dNTPs) into a DNA template during sequential cycles of DNA synthesis. Depending on the sequencing platform, each cycle of nucleotide incorporation is followed by the release of fluorophores or hydrogen ions. This procedure can take place across millions of fragments/molecules in a massively parallel manner.

Several approaches have been developed in the context of PGT-M, including both targeted locus-specific and generic genome-wide haplotyping-based methods. Some of these are commercially available.

The following recommendations are made.

- A relatively high DNA input is necessary for NGS, so that a prior WGA step is required.
- If long-read sequencing is applied, it is recommended to use a suitable WGA to ensure amplification of high molecular weight DNA.
- Given that sequencing-based analysis is a generic approach, preclinical work-up only requires informativity/segregation testing; the locus-specific development can be omitted.
- It is recommended to carry out a validation assay for the WGA protocol and the NGS protocol in respect to the number of biopsied cells.
- When using commercially available NGS-based protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet and dry-laboratory workflow prior to clinical use. Specific recommendations regarding the implementation validation are provided in section "Pre-examination validation".
- Each step in the NGS protocol will contribute to the overall quality of the data set. QC metrics should be established throughout the procedure, among others including analysis of the fragment length before and after adapter incorporation as well as quantification of the prepared library before and after possible size selection, to ensure optimal sample quality and DNA fragment representation in the multiplexed library samples. QC metrics should be established regarding the quality of the final sequencing data.
- Optimal indexing of the samples should be used to ensure that different samples can be efficiently distinguished from each other during demultiplexing of the sequencing data.
- The turnaround time from sample processing to data analysis can vary from 24 h to several days, depending on the setting and the platform of choice. Consequently, an embryo transfer can be planned in the current or a subsequent cycle.

Further general recommendations on NGS are covered in the paper on detection of structural and numerical chromosomal aberrations (ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020).

Limitations of the test

Major limitation of NGS methods is the length of reads they produce, a challenge tackled by long read sequencing technologies that allow the sequencing of single DNA molecules.

Generic haplotyping-based approaches require at least one first degree relative for phase determination. As an indirect test, it is not applicable in case of *de novo* pathogenic variant(s) for couples without previous pregnancies (see also section " *de novo* pathogenic variant(s)")

Analysis software is only available for some of the developed approaches. In the absence of appropriate software, support of skilled bioinformaticians needs to be guaranteed and the software will require further validation.

3.2.5. WGA followed by generic testing for combined PGT-M and PGT-SR/PGT-A

Comprehensive PGT refers to the combination of PGT-M and PGT-A. Several methods have been developed towards that direction. These can be based on the parallel processing of the same WGA product with two different approaches, one aiming in PGT-M and the second in PGT-A. Alternatively, using genome-wide approaches enabling concurrent haplotyping and detection of copy number changes, allow PGT-M and PGT-A to be simultaneously performed in the same test. These generic approaches can be SNP array-based, sequencing-based or a combination of the two.

The following recommendations are made.

- When combined PGT-M and PGT-A are offered, it is recommended that the couple receives comprehensive counselling regarding the possible findings and the consequences on the transfer policy, according to the method used.
- Regardless of the centre-specific transfer policy, it is recommended that if following analysis unaffected embryos free of aneuploidies are available, they are given priority for transfer.
- The preclinical work-up for PGT-M should be performed, as described in section "WGA followed by generic testing for PGT-M".
- These approaches can also be used for inherited chromosomal structural variants in PGT-SR. Depending on the size of the involved segments, aberrant intensity ratios may or may not be detectable for the region(s) of interest. If detectable, it is recommended that the diagnosis is supported by Log R ratio and B allele frequency values.
- Additionally, even if a commercially available platform is used, an implementation validation to determine or confirm the lower size limit for the detection of segmental aneuploidies is recommended. These values may differ between platforms. It is recommended to perform the validation assay with WGA products from single or few cell samples of known karyotype and/or WGA products from embryonic cell(s) diagnosed with a formerly validated method.

Further recommendations specific to PGT-A are covered in the paper on detection of structural and numerical chromosomal aberrations (ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020).

Limitations of the test (for combined PGT-M and PGT-A)

- As these tests (for combined PGT-M and PGT-A) require the presence of phasing reference(s), they are not applicable to all PGT-A indications.
- Ploidy changes cannot be detected by all approaches; methods based on aCGH or NGS cannot reliably detect all types of polyploidy and haploidy (see also Table I in the paper on detection of structural and numerical chromosomal aberrations (ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020); SNP arrays and NGS-based haplotyping can identify polyploidy and haploidy.
- Meiotic errors cannot be distinguished from mitotic in all cases and by all approaches.
- Defining the threshold of mosaicism detection is recommended.

3.3. Pre-examination validation

3.3.1. For PGT-M

- Validation criteria are dependent on the number of cells biopsied (single cell at cleavage stage, or few cells at blastocyst stage) and on the type of strategy used for PGT-M. It is acceptable to perform the validation on cell(s) from embryos donated to research or on other cell types such as peripheral blood lymphocytes.
- Misdiagnosis risk needs to be established.
- The following criteria apply for targeted STR-based testing and variant analysis, with or without prior WGA.
    - Validation assays will determine amplification efficiency, accuracy and ADO rate. Accuracy should be >99% for single- or few-cell samples of known genotype.
    - The amplification efficiency per locus should be >95%. An amplification efficiency of >90% is acceptable, but more markers need to be included.
    - The ADO rate per locus should be <5%. An ADO rate less than 10% is acceptable, but more markers need to be included.
- Every new test based on targeted amplification should be validated.
- For targeted amplification, validation assays should be performed in 50 single- or few-cell samples, in two or three separate runs prior to clinical use. It is acceptable to validate updated protocols (i.e. adaptations of existing protocols) with fewer samples.
- No validation is needed on few-cell samples when the protocol has been previously validated on single cells.
- The following criteria apply for generic strategies, such as SNP arrays with prior WGA.
    - When using commercially available SNP arrays or NGS-based protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet- and dry-laboratory workflow prior to clinical use.
    - It is recommended to perform the validation assay with WGA products from single- or few-cell samples of known genotype and/or WGA products from embryonic cell(s) diagnosed with a formerly validated method.
    - The validation assay should be performed with a minimum of 50 WGA samples, ideally covering various indications.
    - Validation assays will determine amplification efficiency, accuracy and minimum of genetic markers in the region of interest required for diagnosis.
    - The amplification efficiency should be >95% for good quality samples (this may not be achievable for biopsy samples from embryos donated for research/training). Accuracy should be >99% for WGA samples from single

or few cells of known genotype. Similarly, for WGA products from embryonic cell(s) formerly diagnosed, concordance with another validated method should be >99%.
    - If both single- and few-cell analyses are to be performed clinically, it is necessary to validate each separately.

3.3.2. For combined PGT-M and PGT-A

- It is necessary to validate both indications. Again, validation criteria are dependent on the number of cells biopsied (single cell at cleavage stage, or few cells at blastocyst stage) and on the type of strategy used. For PGT-M, the above-mentioned recommendations apply. For PGT-A, recommendations for validation are described in the paper on detection of structural and numerical chromosomal aberrations (ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020).
- Once validated, preclinical work-up and testing of PGT-M conditions on five WGA products is sufficient.

3.4. Risk assessment

Assessment of the risk of misdiagnosis with PGT-M depends on the analysis strategy followed. The residual risk of a protocol with targeted amplification of genetic markers and pathogenic variant(s) has to take into account the genetic distance of the flanking markers towards the variant or gene of interest and the ADO rate of the pathogenic variant(s). Undetected recombination or double recombination and ADO of the pathogenic variant(s) may result in a misdiagnosis. Recombination may go unnoticed when using partially informative markers and imply an elevated residual risk. If a marker-only protocol is used, an undetected recombination or double recombination may also result in a misdiagnosis.

For the genome-wide SNP array- or NGS-based haplotyping strategies, the residual risk may be lower compared with the conventional targeted amplification strategies. This is due to the presence of multiple SNPs flanking a gene or locus of interest, thereby eliminating the effect of ADO of an individual marker. Also, by using multiple SNP markers the effect of a recombination event may less frequently result in an inconclusive result. Still, the distance of the used informative SNP markers to the gene are crucial for the residual recombination risk.

Risk assessment should also cover:

- risks caused by errors in sample tracking;
- risks caused by handling biopsy samples prior to DNA analysis which, if not performed with care, may compromise DNA integrity;
- risk of inconclusive or false results due to sub-optimal experimental conditions (contamination, ADO, ADI) or due to biological reasons (recombination, double recombination, meiotic or mitotic chromosomal aberrations);
- risk of incidental findings;
- risk of test failure (i.e. insufficient markers and/or sequencing to produce a diagnosis).

3.5. Preclinical work-up report

General guidance and recommendations on administration and patient information for the preclinical work-up report are provided in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). For PGT-M, the preclinical work-up report should also include a summary of the work-up and specify the test strategy for the clinical cycle.

It is recommended that the following are clearly stated in the report:

- Indication and gene (with OMIM number when possible), pathogenic variant(s) nomenclature using Human Genome Variation Society (HGVS) recommendations;
- Gene reference sequence, genome build, inheritance mode, polymorphic marker selection when using STRs, number of informative SNPs, distance from marker to gene or pathogenic variant(s), results from informativity testing on all available family members, pathogenic variant(s) detection and linkage analysis (depending on the strategy chosen for the PGT cycle); and
- Test limitations and residual risk of PGT misdiagnosis, including a figure

4. Special cases

4.1. De novo pathogenic variant(s)

In case of a *de novo* pathogenic variant(s) in one partner or in a child, it is mandatory to include mutation detection in the test strategy. Determination of high-risk and low-risk haplotypes or phasing may be completed only during PGT cycle(s).

4.1.1. de novo pathogenic variant(s) in a prospective parent

If DNA samples from affected offspring are available, the case can be dealt with as a usual PGT-M request. If no DNA samples from affected offspring are available, the following recommendations apply.

- It is mandatory to include the mutation detection in the test strategy and diagnosis will depend on the presence or absence of the mutation. Amplification failure at the mutation locus will yield no diagnosis.
- It is recommended to try to, when possible, establish the high-risk and low-risk haplotypes prior to clinical application. In case of a *de novo* pathogenic variant(s) in the male partner, it is recommended to establish phasing from single sperm analysis. Establishing phase from polar bodies for a *de novo* pathogenic variant(s) in the female partner is also an option, but it may be much more complex (requires an extra biopsy procedure and haplotypes in the oocytes are deduced from haplotypes in the polar bodies where recombinations may be present). Phasing can also be deduced by long-read sequencing by NGS of disease-specific amplicons from the affected partner and his/her parents. This will indicate the grandparental haplotype on which the *de novo* pathogenic variant(s) arose in the prospective parent. High-risk and low-risk haplotypes should be confirmed in the clinical cycles.
- Alternatively, it is acceptable to establish genetic marker haplotypes using DNA from the affected partner and his/her parents prior to the clinical cycle, and then complete phasing during the PGT cycle(s). In the scenario when only DNA samples of the prospective parents are available, establishing the haplotypes and phasing needs to be based on the genotypes of the embryos.

When phasing is unknown at the start of the clinical cycle, the following recommendations apply.

- It is mandatory to include pathogenic variant(s) detection in the test strategy.
- TE biopsy is recommended to limit the risk for ADO at the pathogenic variant(s) locus. If cleavage-stage biopsy is performed, two independent cells should be tested.
- When too few embryos are available for biopsy, it is recommended to biopsy and analyse unfertilised oocytes (if pathogenic variant in the female partner) and/or embryos which are non-suitable for biopsy, to support phasing.

- Ideally, at least one affected embryo and one unaffected embryo are needed to establish the correct phase and detect recombination events. The pathogenic variant(s) should be consistently detected in the presence of the same parental haplotype. If this is not possible, it is recommended to cryopreserve the embryos and to wait for the analysis of embryos from next cycle(s). Couples should be counselled upfront about this possibility. Alternatively, it is acceptable to transfer embryos after extended counselling and strongly recommend confirmation by prenatal diagnosis.
- As germline mosaicism due to post-zygotic *de novo* pathogenic variant(s) in the prospective parent cannot be excluded, it is not recommended to use an unaffected child/prenatal/embryo sample as phasing reference. If mosaicism has been detected, the transmission risk has to be evaluated.
- Germline mosaicism detected in the prospective parents can be an indicator of somatic mosaicism and vice versa. In the single cell validation of the PGT protocol for the ADO rate of the pathogenic variant(s) tested, using single cells of such an individual can lead to increased ADO rates of the mutant allele, depending of the degree of mosaicism.
- Following single-cell analysis, it is not recommended to transfer embryos who carry the wild-type allele for the pathogenic variant(s) locus and the high-risk haplotype because of the ADO risk. It is acceptable to transfer such embryos following analysis of TE samples. Prenatal diagnosis is then strongly recommended.

4.1.2. de novo pathogenic variant in an affected child

- When a *de novo* pathogenic variant(s) is detected in a child, it is important to thoroughly counsel the couple with regards to the possibility of recurrence and to assist them in making a well-informed reproductive choice. Achieving a pregnancy and performing prenatal diagnosis should be considered in all cases prior to initiating a PGT-M procedure.
- The decision on whether PGT-M is permitted for cases of a *de novo* pathogenic variant(s) in a child may vary depending on local regulation.
- It is recommended to exclude a post-zygotic origin of the *de novo* pathogenic variant(s) in the previously affected child of the couple. In this case, the recurrence risk is minimum and the option of IVF treatment with PGT should be carefully evaluated. An initial evaluation of the couple's reproductive history may provide evidence of potential germline mosaicism in the parents, for example through evidence of recurrent transmission in previous pregnancies. If DNA from previous terminated cases is available, the case can be dealt with as a usual PGT-M protocol. Further evidence of potential germline mosaicism in the parents may come from evaluation of the pathogenic variant(s) in various parental tissues. If germline mosaicism is detected, the recommendations from the above section on a *de novo* pathogenic variant(s) in a prospective parent apply.

4.2. Consanguineous families

It may be necessary to adapt the testing strategy when consanguineous relationships are present in the pedigree, especially in case of targeted amplification.

4.2.1. Consanguineous grandparents

A prospective parent may have two identical haplotypes in the region of interest because of a consanguinity between his/her parents, and it may be difficult to find informative genetic markers within the 1-2 Mb flanking region. In case of autosomal dominant disease, the pathogenic variant(s) analysis should be included in the test strategy and ADO rates

for the pathogenic variant(s) locus after validation should be low (TE sample analysis is preferable to a two-cell analysis at Day 3). In case of autosomal recessive disease, diagnosis should be based on the low-risk haplotype of both partners.

4.2.2. Consanguineous couple

In case the prospective parents share the high-risk haplotype for an autosomal recessive disorder, parental contamination (most often maternal) in a homozygous affected embryo cannot be distinguished from a carrier embryo, and this may lead to adverse misdiagnosis with transfer of an affected embryo. It is recommended to adapt the testing strategy by either including analysis of unlinked informative polymorphic marker(s) or by performing the analysis on two independent biopsy samples. If this is not done, it is acceptable to prioritise the transfer of healthy embryos compared with carrier embryos.
When SNP markers are used after WGA, parental contamination can be detected.

4.3. HLA typing

The aim of HLA testing of preimplantation embryos is to establish a pregnancy with an embryo that is HLA-compatible with an affected child in need of haematopoietic stem cell transplantation. Haematopoietic stem cells are collected from the umbilical cord blood or the bone marrow of the HLA-matched donor sibling born (or a combination of both sources) and are used for transplantation to and cure of the affected sibling. Recommendations on counselling and important considerations prior to embarking on the PGT-HLA procedure are discussed in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). The following recommendations are relevant as well.

4.3.1. Test strategy

- The preferred PGT methodology is indirect HLA haplotyping (using STR markers or genome-wide SNP haplotyping), which involves linkage analysis of genetic markers flanking the HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ regions, to identify matching haplotypes between the tested embryos and the affected child.
- The PGT protocol must include a minimum of one fully informative marker located at each of the following regions: telomeric to the HLA-A, between HLA-A and HLA-B, between HLA-B and HLA-DRA, between HLA-DRA and HLA-DQB1 and downstream to HLA-DQB1. It must be noted that some difficulty in finding markers between HLA-DRA and DQB1 has been encountered. In this case, two fully informative markers flanking the HLA-DQB1 must be included.
- If a fully informative marker is not available for each of the regions above, a combination of partially informative markers must be included to provide adequate information on the parental HLA haplotypes.
- A highly multiplexed protocol for the amplification of selected STRs or genome-wide SNP must be preferred where possible (i.e. more than one marker per region) to make the test more robust and to assist in detecting potential recombination that can occur throughout the whole major histocompatibility complex (MHC) region.
- As HLA haplotyping may be compromised by genetic recombination, it is recommended that during preclinical PGT work-up, protocol testing includes testing of any additional available first-degree family members, aside from the parents and the affected child, to be able to detect recombination having occurred in the affected child.
- In case recombination is detected in the affected child or in case recombination is detected in embryos during the PGT cycle, any decision on PGT and selection of embryos for transfer must be carefully discussed with the haematologist and transplantation experts, as it may be that a certain mismatch is permissive of haematopoietic stem cell transplantation. The location of the recombination event

is of major significance for this purpose. This further highlights the importance of a highly multiplexed protocol.

- HLA typing of preimplantation embryos can be performed as a sole indication when the affected child requires transplantation to treat an acquired disease, or in combination with PGT-M when there is a need to concurrently avoid transmission of an inherited disease in the family. This requires combining in one protocol the HLA typing approach with the recommended PGT-M strategies. For this purpose, the most comprehensive approaches, NGS and SNP haplotyping, are advantageous by allowing whole genome haplotyping from a single data set.

4.4. Exclusion testing

In families with a history of late-onset diseases, individuals at risk who want to avoid pre-symptomatic testing but wish for their own biological children to be free of the disease, may opt for PGT. Exclusion testing is preferred over PGT with non-disclosure of the direct test results to the couple.

- It is recommended to apply indirect testing with selection of embryos carrying the haplotype of the unaffected prospective grandparent for transfer. Haplotyping can be performed with STR or with SNP markers, relying on targeted amplification at the single or few-cell level or on targeted amplification following WGA.
- Preclinical informativity/segregation testing is applied to DNA samples of the couple and the grandparents (parents of the partner at risk) only; other relatives of the partner at risk should not be tested.

4.5. Mitochondrial DNA Disorders

Maternally inherited mitochondrial DNA (mtDNA) mutations are a frequent cause of mitochondrial disorders. The great majority of pathogenic mtDNA mutations show ***heteroplasmy***, a coexistence of wild-type and mutated mtDNA. PGT based on quantifying mutation load is an acceptable reproductive option for female carriers of heteroplasmic mtDNA point mutations which requires case-by-case counselling, considering the uncertainties linked to this risk-reduction strategy. The key factor is selecting embryos with a mutation load below the threshold of phenotypic expression. For common mutations (e.g. m.3243A>G and m.8993T>G), a mutation-specific heteroplasmy threshold can be established based on available data. For rare or private mutations, the correlation between mutation load and phenotype should be investigated on a case-by-case basis, and literature should be reviewed in order to establish an acceptable expression threshold (see also in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

Next to the recommendations for targeted amplification of nuclear monogenic disorders, the following recommendations are relevant for the quantitative analysis of monogenic mtDNA disorders by restriction enzyme digestion (see also section "Basic methods for allele discrimination").

- PCR master mix should be decontaminated by restriction enzyme treatment prior to PCR amplification to eliminate external mtDNA contamination.
- Complete restriction enzyme digestion should be checked by spiking all the amplification products with an amplicon containing the restriction site of interest if no control site is present in the amplification product.
- Reproducibility of the mtDNA mutation protocol based on restriction enzyme digestion should be validated preclinically, using replicates of mixes of wild-type and mutant mtDNA molecules in a broad range from 0% to 100% mutant and especially for the mutation load around the expression threshold.

- In addition to the controls described in section "Single- or few-cell targeted amplification" (diluted and/or undiluted genomic DNA, IVF and genetic laboratory negative controls and single cell control samples), control samples with a known mutation load (preferably around the expression threshold) should be used.
- Biopsy at the cleavage stage is recommended. It is acceptable to biopsy one blastomere. Biopsy at the preconception stage (first and second PB) is not recommended. For biopsy at the blastocyst stage, sufficient data on the representability of the TE biopsy for the embryo as a whole is currently lacking to make recommendations at this point (ESHRE PGT Consortium Steering committee. *et al.*, 2020).
- It should be taken into account that the cytoplasm of lysed blastomeres may no longer fully represent the embryo mutation load.

5. Examination process

- The examination process and reporting of results, must comply with local guidelines or law, or with the ISO 15189 standard.
- Before starting a clinical PGT cycle, relevant documents should be available, labelling of samples should be checked, and genetic counselling provided to the couple.
- It is recommended that the PGT laboratory has clearly documented procedures for all steps of the examination process (explicit instructions and a summary of validation results) and release of results (diagnosis, reporting, embryos transfer policy). These procedures are preferably covered in a service-level agreement between the PGT and IVF centres.
- Many different methods have been published and all appropriately validated methods are acceptable for clinical cases. The method used should have been previously implemented, tested and validated in the PGT centre.

5.1. Scoring of clinical results

- It is recommended that results are reviewed and signed or electronically validated by a suitably qualified person (name, qualification, date).
- When suboptimal samples or samples not meeting the preferred requirements (e.g. mislabelled samples, lysed cells or when a nucleus is not observed) are received for testing, this should be documented by the PGT lab and a procedure how to further process and interpret these samples should be in place.
- It is recommended that results are analysed by two independent observers and discrepancies adjudicated by a third observer (where possible). If no consensus is reached the embryo should not be recommended for transfer and should therefore be given the diagnosis of uninterpretable or inconclusive.
- Haplotyping scoring criteria should be established in a written protocol and adhered to for the interpretation of the results.
- It is acceptable to attempt to reduce the number of embryos with no result or no clear diagnosis following PGT, by adopting a "no result rescue" approach. This could involve either a second biopsy step or repetition of the analysis after WGA. For practical recommendations regarding the re-biopsy procedure please refer to the PGT biopsy paper. In case of targeted amplification following cleavage stage biopsy, a second biopsy (at the blastocyst stage) may be performed, followed by a second analysis. In case of genome-wide testing, a second analysis of the existing WGA and/or a second biopsy, followed by WGA and a second analysis, may be performed.

5.2. Clinical cycle report

General items required on PGT work-up and clinical cycle reports are included in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

The PGT clinical cycle report contains interpretation of the clinical results and guidance on which embryos are genetically transferable. The same recommendations apply as specified for the preclinical work-up report (see section "Preclinical work-up"), together with the following items:

  - unique cycle/treatment code;
  - date of oocyte retrieval;
  - date of biopsy;
  - date of biopsy sample arrival in the laboratory;
  - information on the sample type (including number of samples and controls);
  - unique ID number for each cycle and/or biopsy sample tested;
  - indication for PGT.

- When scoring results from PB testing, it is recommended to report what was detected in each polar body and then infer the oocyte diagnosis. It is recommended to test both PBs.
- When scoring results from blastomere/TE testing, it is recommended to report what was detected in the sample and then infer the embryo diagnosis.
- When results are reported from 'pooling' of embryos, it is advisable to refer to each oocyte and sample collection date and clearly differentiate the embryo number between cycle/treatment.
- Reporting of clinical results to the IVF centre must follow local regulations or international accreditation guidelines, including GDPR.
- The embryo transfer policy should be agreed upon between stakeholders (IVF centre, genetic centre, genetic counsellors, clinicians and patients). In PGT-M and PGT-SR cases, embryos with no or inconclusive results are not recommended for transfer. Depending on local rules and following adequate counselling of the prospective parents, the carrier status of embryos (for autosomal recessive or X-linked recessive disorders) may be taken into consideration for embryo selection. In case of PGT-A in addition to PGT-M or PGT-SR, it is crucial that the centre has a clear policy on embryo (ranking and) transfer.
- A written or electronic report should be securely transmitted to the IVF centre to ensure transfer and/or cryopreservation of the correct embryos. Results should not be communicated orally.
- Reporting time should be kept as short as possible, and when fresh transfer is intended, reporting time should be adapted to allow the IVF centre to organise the embryo transfer.
- It is recommended that the report is clear, concise, accurate and easily understandable by non-geneticists.
- It is recommended that the overall result and interpretation (including transfer recommendation) are presented per embryo, preferably in tabulated form. Sufficient information for genetic counselling should be included, such as the chromosome(s) involved, chromosome band(s)/nucleotides, the size of the chromosomal aberration in Mb, and the correct identification of the genetic variant. Where applicable, the latest version of the international system for human cytogenetic nomenclature (ISCN)/Human genome variation society (HGVS) nomenclature can be used.
- In case of no diagnosis and re-biopsy to try and obtain a result, this should be included in the report.
- The final clinical cycle report must be signed by appropriately qualified (authorised) personnel (name, qualification, date).

- It is recommended that the clinical cycle results are discussed with the couple before embryo transfer.
- It is recommended that the report is stored in the patient file in the PGT centre, according to local regulations.
- It is recommended to include a disclaimer in the report to address limitations of the test and any other information that may be of significance to the addressee.
- It is acceptable to indicate in the report the need for prenatal testing to confirm the result in case of pregnancy.

It is recommended that the following nomenclature is used in the clinical cycle report:

- unaffected carrier or affected when reporting for monogenic disease;
- low-risk or high-risk when reporting for mitochondrial disorder;
- at risk or not at risk (for exclusion testing);
- HLA compatible or HLA not compatible (for HLA typing);
- no amplification (no result);
- inconclusive (results but no diagnosis due to AF, ADO or recombination events); or
- abnormal (or aberrant): when a numerical or structural abnormality involves the chromosome(s) carrying the disease locus.

6. Post-examination process

Recommendations on PGT follow-up, baseline IVF live birth rates for PGT and misdiagnosis as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) included:

6.1. PGT cycle follow-up

For quality purposes, it is recommended to confirm the PGT diagnosis on a subset of embryos not transferred or cryopreserved following diagnosis, in line with local regulations. Such confirmation aims to provide internal ***quality assurance (QA)*** as well as accurate and up-to-date misdiagnosis rates to prospective PGT patients. It is recommended that this is performed on as many embryos as is practicable. It is acceptable to perform this periodically.

When a pregnancy ensues following PGT testing, it is recommended that parents are (again) made aware of the chance and risks of a misdiagnosis and be informed on the possibilities for prenatal testing. PGT and IVF centres should make special efforts to follow-up with the parents following prenatal testing or birth, especially if confirmatory testing is not possible.

Follow-up data should be used for both internal ***quality control (QC)*** and QA purposes and documented in the ESHRE PGT Consortium online database for international data collection.

It is recommended that laboratories follow local regulations or accreditation schemes on storage of clinical samples and patient records. If no local regulations or guidelines exists on storage of clinical samples and patient records, it is recommended as follows.

- If embryos have been transferred and/or frozen, all relevant material (e.g. FISH slides, DNA amplification products) from the case should be retained and appropriately stored. Samples should be stored for at least 1 year. Prolonged sample storage could be considered, taking into account the availability of information on delivery and the duration of embryo cryopreservation.

- If there is no genetically suitable embryo for transfer or cryopreservation, it is not necessary to keep the samples.
- If there is no pregnancy after transfer of all genetically suitable embryos, samples can be discarded.

6.2. Misdiagnosis rate

- It is recommended that each PGT centre performs a prospective risk analysis in order to prevent and/or eliminate possible causes of ***misdiagnosis***.
- It is recommended that misdiagnosis rates should be calculated for each type of method and for all methods from a particular centre. Misdiagnosis rates include those clinical cases in which affected pregnancies arose and cases for which re-analysis results were discordant with the biopsy result.
- It is recommended that confirmatory testing should be performed at least periodically as a QA.
- It is recommended that the published and in-house estimates of misdiagnosis rates should be available on request to prospective patients along with pregnancy rates and live birth rates, to allow informed consent for PGT.
- Following a misdiagnosis, the IVF/PGT centre should investigate the possible causes of the misdiagnosis and make changes to protocols to eliminate the risk in the future. Many of the causes of misdiagnosis are avoidable by taking preventive actions and following the principles of quality management.
- Misdiagnosis should be reported, for instance through the ESHRE PGT Consortium online database.

6.3. Baseline IVF live birth rates for PGT

- Setting appropriate baseline live birth rates should be left up to the individual centres. However, it is recommended that each IVF centre should compare PGT live birth rates and matched non-PGT [routine IVF or ***intracytoplasmic sperm injection (ICSI)***] live birth rates within that IVF centre.
- Comparison of live birth rates with those reported by the ESHRE PGT Consortium or comparable peers can also be carried out to set benchmarks for continual improvement of the PGT centre.

SECTION D: DETECTION OF STRUCTURAL CHROMOSOMAL ABERRATIONS

This section is entirely based on the paper:

ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations" with additions from the paper "ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing

1. Introduction to PGT-SR techniques

This paper provides detailed technical recommendations for the most applied methods for PGT-SR, including fluorescence *in situ* hybridisation (FISH), array-based comparative genomic hybridisation (aCGH), next-generation sequencing (NGS) and single nucleotide polymorphism (SNP) array. Detailed technical recommendations for SNP array are covered in the paper on detection of monogenic disorders (ESHRE PGT-M Working Group *et al.*, 2020).

General recommendations for PGT-SR and PGT-A are formulated, independent of the testing method applied.

1.1. Training and personnel

- Genetic testing procedures should be performed under the supervision of a (cyto)geneticist, competent or authorized to perform clinical diagnostics.
- All personnel undertaking genetic testing should be trained adequately as required in a clinical molecular cytogenetic laboratory and should follow written ***standard operating procedures (SOPs)***.
- Training for each technique should be documented.
    - Training for tubing is discussed in the paper on polar body and embryo biopsy for PGT (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
    - For FISH, training should be at least to the standard required for routine testing in a clinical cytogenetic laboratory. It is recommended that at least 30 samples are successfully spread or fixed and subjected to FISH by each trainee during preclinical training. Supervised clinical training should include at least an additional 20 samples.
    - For aCGH and NGS, it is recommended that at least 30 samples are subjected to WGA, followed by aCGH or NGS by each trainee during preclinical training. Supervised clinical training should include at least an additional 20 samples.

1.2. Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

1.2.1. Laboratory infrastructure

Oocyte and/or embryo biopsy should be performed in a specifically designated laboratory setting. Collection of the biopsied samples and initial steps of genetic testing procedures should be carried out in laboratory settings dedicated for processing single and/or few cells. Appropriate precautions should be taken both to prevent contamination of samples by physical isolation, and to detect any such contamination. Licenses for offering embryo biopsy procedures and/or genetic testing by the centre may be obtained, according to local regulations.

1.2.2 Equipment

- All clinical equipment should meet the criteria set for the intended application, be appropriately calibrated, maintained and serviced, with all aspects supported by written ***standard operating procedures (SOPs).*** Equipment used for critical steps should have uninterrupted power supply (UPS).

- For areas within the IVF centre, whether it is a dedicated area or a room, all equipment should comply with "Revised Guidelines for good practice in IVF laboratories (2015)", section 3 "laboratory safety" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). Prior to the biopsy procedure, work surfaces, equipment and hoods should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory.
- For areas within the PGT centre, prior to each use, work surfaces and equipment should be cleaned and decontaminated with DNA ***decontamination*** solutions or 10% bleach, or by UV-C irradiation or autoclaving (when applicable, for example tube racks). It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.
- Multichannel pipettes or automated systems may be useful in the PGT laboratory to minimise the risks of mislabelling or misallocation of samples during the post-amplification steps, but they are not recommended in the pre-amplification steps.

1.2.3 Materials

- To prevent contamination, protective clothing for DNA amplification of a single and/or few cells 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). For areas within the IVF centre, protective clothing, preferably with low particle-shedding and non-powdered gloves and masks should be considered.
- The pre-amplification materials and reagents should be kept away from any DNA source and preferably stored in the pre-amplification area.
- 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 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 internal 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 all reagents.
- Reagents and solutions can be DNA decontaminated by UV-C irradiation. Alternatively, reagents and solutions made in-house can be autoclaved, preferably using a PGT-dedicated autoclave.
- Careful handling of all reagents employed must be ensured with regards to storage temperature and working conditions, following manufacturer's recommendations. Vortexing and quick temperature changes should be avoided for the most sensitive components.

Specific issues for handling of reaction tubes to reduce cross-contamination:

- It is recommended to avoid touching the inside or the lid of the tubes with your fingers.
- It is recommended to avoid touching the outside or the cap of the tubes with the tip of the pipette. If this happens, the pipette tip should be changed immediately.
- It is recommended to keep the reaction tubes open not longer than necessary.

1.2.4. Laboratory documentation

Well-structured (electronic and/or paper) laboratory forms should be available for recording wet-laboratory details of work-up and PGT cycle procedures.

General aspects on infrastructure, equipment and materials are covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020).

1.3. Labelling and witnessing

General aspects on labelling and witnessing as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) :

- It is recommended that an adequate labelling system, written or barcoded (electronic), with two unique patient identifiers plus the embryo/cell(s) number is used to match the sample's diagnostic result with the embryo from which that sample was taken. This should ensure traceability throughout the IVF and PGT process until reporting of the final results.
- The labelling system should be comprehensible and practical for both the IVF and the PGT centres. Printed sticker labelling may be superior to pens, as labelling should be legible and uneditable.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer and signed off (Figure D1). These critical steps are detailed in the technical papers for the various methods (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
- After biopsy, the sample may be analysed in house, or sent for genetic testing in another centre (see " Transport PGT").

1.4. Risk assessment

- When sub-optimal samples or samples not meeting the internal requirements (f.ex. lysed cells, nucleus not seen) are received for testing, this should be documented and a procedure on how to further process these samples should be in place.

Specifically, for PGT-SR:

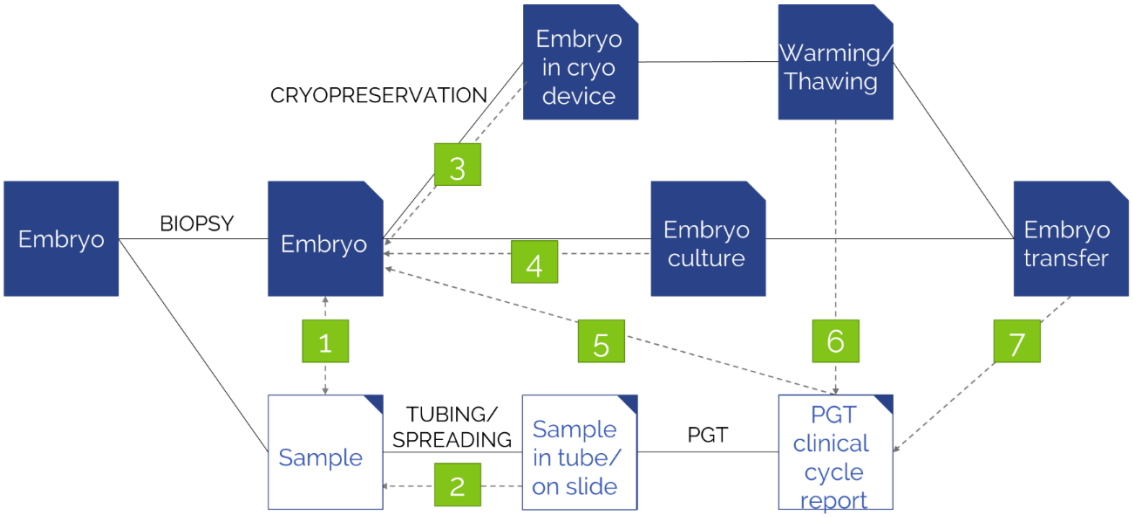
- ***Risk assessment*** for the patient should include figures on the potential risk of a viable unbalanced offspring due to failure to detect any of the unbalanced segregation products.
- In case only one of the two translocation segments can be detected, not all possible unbalanced segregation products can be identified. A test which cannot detect all the segments, and possibly some unbalanced products, may be less effective in decreasing the risk of a viable unbalanced offspring, first-trimester miscarriage and stillbirth. This should be mentioned in the preclinical work-up report.

1.5. Appropriate indications for specific tests

It is recommended that specific indications for PGT should remain within the scope of individual clinics.

- FISH is not recommended for PGT-A as only a subset of chromosomes can be tested, and better comprehensive molecular approaches to detect aneuploidy for all 24 chromosomes are available.
- Selection of embryos based on sex for social reasons is not acceptable.

Figure D1: Outline of the biopsy and genetic testing procedure with indications of the 7 critical steps where labelling and sample identification should be confirmed.



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) In case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device; (4) For further embryo culture, at placing and labelling the embryo into the culture dish; (5) When diagnostic results are issued to ensure accuracy and correlation with the correct sample identification; (6) During the thawing/warming procedure to ensure accuracy and correlation with the correct embryo diagnostic result; and (7) At the time of selecting the embryo(s) for embryo transfer.

2. Preimplantation testing for structural chromosomal rearrangements (PGT-SR)

Structural chromosomal rearrangements form a major indication category for preimplantation genetic testing. There are different types of structural chromosomal rearrangements: reciprocal and Robertsonian translocations, insertional translocations, deletions, duplications and inversions, all of which may be inheritable or occur *de novo*. Familial reciprocal and Robertsonian translocations constitute the most common indications for PGT-SR.

In case of familial rearrangements, preimplantation testing for structural rearrangements (PGT-SR) provides an opportunity to identify chromosomally unbalanced progeny at the

earliest stages of embryo development. Preconception testing of polar bodies provides a means to indirectly identify chromosomally unbalanced oocytes.

Several methods are applied to perform PGT-SR, among which are FISH, aCGH and NGS. PGT-SR is mostly performed on embryonic biopsies taken at the cleavage stage (Day 3 post insemination) or the blastocyst stage (Day 5-7 post insemination). PGT-SR on polar bodies is less applied and involves a different kind of analysis as the genomic content of the oocyte (and corresponding embryo) is inferred from that of the first and second polar body (indirect test). Detailed information on polar body-based PGT is available in the paper by Magli et. al (2011) and Geraedts et. al (2011) (Geraedts *et al.*, 2011, Magli *et al.*, 2011).

2.1. FISH-based PGT-SR

FISH-based PGT is mainly applied for inherited chromosomal rearrangements but can also be used for embryo sexing in X-linked diseases (if direct ***mutation*** testing is not applicable) (PGT-M).

FISH enables enumeration of chromosomal loci that are involved in structural rearrangements or are indicative of sex chromosomes. Based on signal scoring chromosomal imbalance or embryo sex can be established, and subsequently balanced embryos or embryos of the non-affected sex can be selected for transfer.

Disadvantages of the FISH technique constitute its technical nature: diagnosis is based on visual inspection of fluorescent signals, making loss of DNA integrity and overlapping signals two of the major problems. Furthermore, genomic information is limited to the loci targeted by the probes used.

Therefore, FISH-based PGT is acceptable for rearrangements involving small fragments or subtelomeric regions of chromosomes that are difficult or impossible to detect using other methods (e.g.<10Mb).

2.1.1. Laboratory issues

The principle of the FISH technology is based on the use of specific DNA probes that are labelled with distinctive fluorochromes (either direct or indirect via a hapten). The DNA probes and the target DNA, typically embryonic interphase nuclei, are (simultaneously) denatured and left to anneal. Following hybridisation, results are visualised via fluorescence microscopy.

Many variations in FISH methods have been published and all appropriately validated methods are acceptable. The method used should have been previously implemented, tested and validated in the PGT centre.

FISH protocol: structural rearrangements

For structural rearrangements, it is recommended that the probe set contains at least sufficient probes to detect all expected unbalanced variants of the chromosomal rearrangement. The analysis of the predicting segregation outcomes for carriers of a structural rearrangement should include an assessment of the plausible mechanism for chromosome pairing and the products of disjunction following the first and second meiotic divisions.

It is recommended that a combination of three informative probes (two distal and one proximal, or two proximal and one distal probe relative to the translocation break points) be used to detect all unbalanced segregation products as for more common two-way reciprocal translocation. For Robertsonian translocations and inversions, two probes are acceptable. For deletions, duplications and insertions, locus-specific probes for the target region should be used and a control probe should be included in the diagnostic cycle.

Where suitable probes are not available, it is acceptable to use probe combinations that cannot detect some unbalanced forms of a rearrangement, provided that they have been

assessed to be non-viable in a recognisable pregnancy or to have a very low prevalence. It has to be mentioned in the (pre-validation) report that there are unbalanced forms that cannot be detected, and patients should be counselled to this effect. A cytogeneticist or suitably qualified person should determine which probe combination to use.

For cleavage-stage embryos, PGT diagnosis on a single mononucleate blastomere is acceptable for chromosomal rearrangements provided that there are informative probes for at least two unbalanced segments for those products considered likely to be prevalent or viable in a recognisable pregnancy. PGT diagnosis based on concordant results from two mononucleate blastomeres is recommended where there is only one informative probe available for the chromosome imbalance involved that is considered likely to be prevalent or viable in a recognisable pregnancy.

For preconception PGT diagnosis, both polar bodies are required for analysis and all unbalanced products of meiotic segregation should be detectable so that it is possible to know the contents of the oocyte. However, it is important to point out that PGT-SR performed on polar bodies carries a risk of ***misdiagnosis*** for the carriers of structural rearrangements due to an uneven number of crossovers that may occur in meiosis I which may be undetectable through FISH. The presence of cumulus cells attached to the ***zona pellucida (ZP)*** could heavily affect the result of the PGT-SR analysis.

Blastocyst biopsy for a FISH-based PGT diagnosis is acceptable, provided that special care is taken to avoid overlapping cells. On average a trophectoderm (TE) sample contains 5-10 cells, which in theory allows for a more reliable diagnosis. However, the multi-cell nature bears the possibility of discordant results in the different cells because of a technical failure (sub-optimal FISH conditions) or true ***chromosomal mosaicism***. Reporting of discordant results should be regulated and genetic counselling should be provided to the couple to explain the possible impact on the reliability of the PGT diagnosis.

The use of additional probes to screen for aneuploidies of chromosomes not involved in the rearrangement is acceptable. If multiple rounds of FISH are being applied, the probes indicative of the rearrangement should be included in the first round.

FISH protocol: sexing in case of X-linked diseases (PGT-M)

For embryo sexing, it is recommended that the probe set contains at least probes specific for the centromere region of the X and Y chromosomes and one autosome.

The use of additional probes to screen for aneuploidies of autosomes is acceptable. If multiple rounds of FISH are being applied, the probes indicative of embryo sex (X and Y) should be included in the first round.

PGT diagnosis on a single mononucleate cell is acceptable for sexing.

It should be noted that FISH-based PGT for sexing to exclude transmission of X-linked diseases could be less advantageous when compared with amplification-based diagnosis of the disease-associated mutation alongside gender determination. A haplotyping-based diagnosis allows for identification of unaffected males as well as carrier females.

Turnaround time

The turnaround time for FISH-based PGT-SR depends on the number of embryos analysed and the number of hybridisation rounds applied. According to recommendations from commercial probe manufacturers the hybridisation time for each round should be at least 4 h, but laboratories may develop and validate their own protocol that will shorten the time for hybridisation while maintaining the intensity and brightness of the fluorescent signals. Thus, a clinical cycle report can be obtained within 4-48 h from sample fixation to signal scoring.

Documentation

The patient's file should include relevant laboratory documentation:

- high resolution (550-800 bands) parental karyotype, preferable with FISH verification of chromosome regions involved in structural rearrangements; also, it may include a karyotype of the affected child or other family member;
- results of cytogenetic analysis of any previous unbalanced pregnancies or preimplantation embryos;
- genetic counselling report with recommendations for PGT-SR, an indication of the testing method and the benefits and the limitations of the test;
- the ***informed consent*** of the couple with risk assessment and indication of test limitations.

2.1.2. Laboratory infrastructure, equipment and materials

Infrastructure

The following recommendations are for the laboratory space.

- The laboratory should be well-ventilated to minimise the effect of any noxious fumes. This is particularly important if cells are fixed using methanol and acetic acid. In this case the use of a fume cabinet for the fixation steps is recommended.
- FISH outcomes, including cell spreading and fixation, are dependent on humidity. The humidity in the FISH laboratory should be controlled and stable. FISH protocols should be optimised in these conditions.
- FISH signals may be bleached or weakened in bright light. It is recommended that the FISH laboratory be fitted with variable intensity incandescent lighting. Fluorescent lighting is acceptable. The slides should be stored cool and in light-tight storage boxes or folders.

Equipment

- A FISH-based PGT diagnosis requires the following equipment: a fluorescence microscope equipped with appropriate filters for the fluorescent dyes used, a water bath and a hybridisation device. A fluorescent image capture system is preferred for documenting FISH images.

Materials and reagents

- Required materials are glass slides and coverslips, and a probe set specific for the chromosomal structural rearrangement of interest.
- Daylight should be avoided during hybridisation and post-hybridisation steps.
- The use of commercial probes is recommended since they generally come with ***quality control (QC)*** and validation reports.
- The use of homemade probes is acceptable with appropriate preclinical ***quality assurance (QA)***/QC and validation.
- It is recommended that all probe vials be tested before clinical application, to confirm that they contain the correct chromosome-specific probe and are labelled

with the correct fluorochrome or hapten. Furthermore, it is recommended that they be informative for the intended PGT-SR couple, and meet documented acceptance levels for signal specificity, brightness and discreteness. Batch numbers should be recorded to ensure continuous traceability.

- It is recommended that only appropriately qualified personnel (as documented in written competency lists) authorise selection of probes for clinical use.
- In case of a Robertsonian translocation, fluorescent probes for any locus on the long arm of the two acrocentric chromosomes involved in the rearrangement can be used. For reciprocal translocations, alpha-satellite probes, locus-specific probes, or sub-telomere probes indicative of the translocated regions may be used. For inversions, mostly locus-specific probes for the short and the long arm of the intended chromosome are used, possibly combined with alpha-satellite repeat probes. For the detection of deletions, duplications, or insertions, it is preferable to use locus-specific probes indicative of the target chromosomal region, combined with a control probe (alpha-satellite or subtelomere probe) to discriminate between a true deletion/duplication and a whole chromosome copy number change.
- It is recommended that for each round of FISH, all probes be labelled with a different fluorochrome or combination of fluorochromes so that the colour of different probe signals can be distinguished from one another. The signals should be one domain apart.
- When using prehybridisation steps, such as pepsin and paraformaldehyde, it is recommended that measures should be taken to ensure appropriate QC for these solutions. The temperature ranges and pH values of solution should be verified before using in every round of FISH. Creation dates of solutions for all steps should be recorded and the solutions should be checked for possible cellular contamination prior to use.
- Mounting medium containing antifade (with or without DAPI, depending on the probe combination) is recommended to allow maintenance of fluorescent signals. It is recommended that prior to each FISH procedure, denaturation, hybridisation, the pH values of solution and wash temperatures be verified.

2.1.3. Work practice controls

Identification and witnessing

- The use of an adequate labelling system, written or barcoded (electronic), using two unique patient and embryo/cell(s) identifiers, is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in FISH. It is recommended that the unique patient identifier and embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing [see also in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). Witnessing is also indicated at the following steps of the FISH procedure:
  - at probe preparation, to check that the correct FISH probes (patient-specific pre-validated probe mixes should be correctly labelled in advance) are used for the case,
  - when diagnostic FISH results are recorded to ensure that FISH images correspond to the correct cell and/or embryo.
- The location of the fixed/spread cell on the slide may be recorded to facilitate tracing.

Intra-assay controls

The use of positive and ***negative controls*** in a FISH-based PGT diagnosis may be considered.

- Suitable positive controls are not readily available (i.e. unbalanced single human blastomeres, TE cells or other cell types to represent unbalanced human blastomeres or TE cells).
- Normal or carrier human metaphase lymphocytes may serve as control to ascertain that the probes in the hybridisation mixture identify the expected chromosomes/chromosomal regions.

2.1.4. Pre-examination process

Pre-examination process includes preclinical work-up, test development and validation.

Preclinical work-up and test development

- It is recommended to perform a preclinical work-up to assess PGT-SR feasibility, identify informative probes and work on a clinical testing strategy. It is recommended to perform segregation analysis for the intended structural rearrangement to ensure that the testing strategy allows for the detection of all expected genotypes in the embryos.
- It is acceptable to carry out FISH tests on sperm cells from male translocation carriers in an attempt to predict the efficacy of PGT-SR for these cases.
- When using a probe set previously shown to have a very low polymorphism rate, it is acceptable to forego any preclinical work-up. Other probes may be more prone to polymorphism and preclinical testing of peripheral blood lymphocytes is then recommended. Sequences in the heterochromatin regions of chromosomes 1, 9, 16 and Y are closely related and therefore cross-hybridisation among those chromosomes is frequently observed. In addition, the D15Z1 region on the short arm of chromosome 15 cross-hybridizes with the short arm regions of other acrocentric chromosomes, especially chromosome 14. Moreover, the centromeric probes D1Z7 (chromosome 1), D5Z2 (chromosome 5) and D19Z3 (chromosome 19) occasionally show cross-hybridisation. Finally, an overlap of signals generated by probes specific for the centromeres of chromosome 18 and chromosome 16 is frequently observed.
- Following the fixation procedure and following each round of FISH the location and integrity of the cells should be checked.

Pre-examination validation

- It is recommended to perform the validation on both the carrier of the rearrangement and the partner, but it is acceptable to perform the validation on the carrier only.
- It is acceptable to perform the validation on blastomeres and TE cells from embryos donated to research prior to clinical PGT-SR testing. It is also acceptable to perform the validation on other cell types such as peripheral blood lymphocytes and fibroblasts.
- It is recommended that at least 10 metaphase spreads are examined: (i) to ensure that the probes are specific for the correct chromosomes; (ii) to assess chromosome ***polymorphism*** and signal cross-hybridisation; and (iii) with respect to carriers of a chromosome rearrangement, to ensure that the probes hybridise to the expected segments of the rearrangement.
- In addition, it is recommended that at least 100 interphase nuclei are scored using appropriate scoring criteria (signal specificity, brightness and discreteness)

- Acceptable ranges of FISH hybridisation efficiency should be determined in each laboratory for each FISH probe and combined probe set. Validation tests should at least confirm that the probes hybridize as expected, that they are informative for the rearrangement and that >95% of the cells shows the expected number of signals for each of the probes used.
- It is recommended that scoring criteria are determined ahead of time (published or 'in-house') and should be adhered to as per written procedure.

Preclinical work-up report

General guidance and recommendations on administration and patient information for the preclinical work-up report is given in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). A preclinical work-up report should also include a summary of the PGT-SR work-up with details on the protocol and validation steps. It should further describe the FISH probes used and the hybridisation efficiency, the false-positive rate and the false-negative rate of the probe set. Where applicable, the latest version of the international system for human cytogenetic nomenclature (ISCN)/ Human genome variation society (HGVS) nomenclature can be used. Finally, the report should include potential limitations of the test.

2.1.5. Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking
- risks caused by handling biopsy samples prior to FISH analysis that, if not performed with care, may compromise DNA integrity;
- risk of inconclusive or false results due to sub-optimal experimental conditions; the reliability of the FISH diagnosis may be negatively influenced by the inability to accurately interpret signals, inconsistent fixation or suboptimal hybridisation; signal overlap may lead to an underestimation of the actual chromosome (region) copy number; and in addition, locus-specific and sub-telomere probes produce less bright signals when compared with alpha-satellite probes and show a higher rate of split signals, which compromises correct signal scoring;
- risk of inconclusive or false results due to biological reasons: (i) unbalanced segregations may arise from crossing over during meiosis I in the gametes of the carrier of the rearrangement; (ii) chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype;
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is unrelated to the test, whether biological or caused by a technical error.

2.1.6. Limitations of the test

The limitations of the FISH technique should be clearly mentioned in the preclinical work-up report and/or be discussed with the patients during genetic counselling.

- FISH-based PGT-SR analysis does not allow for a distinction between embryos with a normal or a balanced karyotype.
- FISH-based PGT-SR analysis does not allow for the detection of ***uniparental disomy (UPD)***.
- FISH-based PGT analysis can only assess the copy number of the chromosomes targeted by the DNA probes used.
- Due to the limited number of available fluorochromes, the number of chromosomes that can be simultaneously detected is also restricted. Sequential

rounds of FISH may therefore be required, which negatively affect DNA integrity and signal quality.
- Commercial probes are available for only a limited number of loci, which may complicate the selection of probes for the analysis of rare chromosomal rearrangements.
- Impossibility to detect mosaicism if FISH is performed in a single cell biopsy.

2.2. Array-based PGT-SR

Array-based comparative genomic hybridisation (aCGH) involves the competitive hybridisation of differentially labelled sample and reference DNA on a microscope slide with fixed DNA probes. DNA probes correspond to specific chromosomal regions and occupy discrete spots on the slide. Each spot has a colour that results from the fluorescence ratio of the two colours after hybridisation. The evaluation of fluorescence ratios is automated and indicative of chromosomal loss or gain.

Arrays are considered a more reliable approach for PGT-SR when compared with FISH since they provide multiple points of measure for each translocation segment. Furthermore, they allow for simultaneous copy number assessment of the chromosomes not involved in the rearrangement.

Currently, two types of commercial array platforms are being used. The first is an aCGH platform based on oligonucleotides-providing a resolution of 5 to 10 Mb. The second is a single nucleotide polymorphism (SNP) array platform based on oligonucleotides providing a resolution of 2.4 to 5 Mb (see also the paper on detection of monogenic disorders (ESHRE PGT-M Working Group *et al.*, 2020).

2.2.1. Laboratory issues

The aCGH workflow involves: (i) sample cell lysis and whole genome amplification (WGA); (ii) labelling of sample and reference DNA with different fluorochromes (e.g. green and red); (iii) purification of labelled DNA; (iv) microarray processing (hybridisation of biopsied and reference DNA samples followed by washing of microarray slides); (v) scanning; and (vi) analysis of scanned microarray tiff images where data is extracted to fluorescence ratio. The resulting ***log2 of fluorescence ratios*** is computed by specific software to identify structural and numerical chromosome copy number aberrations.

aCGH protocol

- It is recommended that wet-laboratory experimental conditions be established for all steps in the aCGH workflow followed by a preclinical assessment of the accuracy of the test to detect a chromosome aberration.
- It is acceptable to perform aCGH-based PGT-SR on polar body biopsies, provided that both polar bodies can be analysed, and all unbalanced products of meiotic segregation can be detected so that it is possible to know the contents of the oocyte. However, it is important to point out that PGT-SR performed on polar bodies carries a higher risk of ***misdiagnosis*** for the carriers of structural rearrangements due to an uneven number of crossovers that may occur in meiosis I which may be undetectable through aCGH. The presence of cumulus cells attached to the ***zona pellucida (ZP)*** could heavily affect the result of the PGT-SR analysis.
- It is acceptable to perform aCGH-based PGT-SR on single cell biopsies, although they present with an overall increased noise and step change chromosome artefacts in the aCGH profile. Acceptance criteria for noise level should be part of the QA/QC parameters.
- Blastocyst biopsy for an aCGH-based PGT diagnosis allows for a more reliable diagnosis as on average a TE sample contains 5-10 cells.

- It is recommended to use a WGA protocol which is compatible with the specific aCGH platform that has been used for validation.

Turnaround time

The net aCGH turnaround time from sample processing to comprehensive chromosome analysis is 24 h, although results can be obtained within 8-12 h. However, each laboratory needs to validate whether shorter hybridisation times affect hybridisation efficiency.

Documentation

Relevant laboratory documentation should include:

- a patients' karyotype, preferably at high resolution (550-800 bands), if available with FISH verification of the breakpoints;
- a report on any previous unbalanced products of conception;
- genetic counselling report with possibly a recommendation for PGT-SR, an indication of the testing method and the benefits and the limitations of the test;
- the informed consent of the couple with risk assessment and indication of test limitation.

2.2.2. Laboratory infrastructure, equipment and materials

Infrastructure

- To prevent carry-over of amplified DNA, the laboratory space should be divided into pre- and post- amplification rooms that are physically separated.
- Preferably the pre- and post-amplification rooms/areas should be equipped with UV-C light for DNA ***decontamination***.
- Positive air-pressure is recommended for the pre-amplification room. When positive and negative pressure rooms are present, they are preferably enclosed by a lock chamber.
- A dedicated set of equipment, consumables and laboratory coats should be used for each designated area and not be exchanged between the pre- and post-amplification rooms.
- Pre-amplification steps should be carried out in a laminar downflow cabinet. The workflow between the pre- and post-amplification areas should be unidirectional, from the pre-amplification room (clean room) to the post-amplification room.
- Constant regulation of environmental conditions (ozone, temperature and humidity) is recommended during all steps to ensure efficient hybridisation results.

Equipment

- Equipment required for WGA and aCGH analysis of biopsied samples includes:
  - a class II safety cabinet, preferably equipped with UV-C light, to prevent contamination of samples at the pre-amplification stage;
  - thermal cyclers with heated lids (one for the pre- and one for the post-amplification room);
  - microcentrifuges (one for pre-amplification, one for all the following stages) and a benchtop swingout centrifuge;
  - a magnetic stirrer, fume cabinet, hybridisation oven/incubator, water bath, gel electrophoresis equipment to check successful amplification and a vortex mixer;
  - a scanner, equipped with the corresponding lasers, to excite the hybridised fluorophores to read and store the resulting images of the hybridisations, placed in the post-amplification room in an atmosphere with low ozone parameters, regulated temperature and protected from daylight and

validated and adjusted to the required resolution for the specific PGT protocols.

- The use of a DNA quantification system (to determine the amount of amplified DNA after WGA) and a vacuum concentrator (to reduce the time required to process high numbers of samples) is optional.
- Associated servers should be also allocated in proper conditions and instruments used in critical steps should be UPS-connected.
- It is recommended that prior to each step of the protocol, the temperature ranges and/or pH values of equipment and solution are verified. Specific temperature and thermocycler programmes should be validated in individual PGT centres for all equipment, and instruments should be serviced and calibrated regularly to ensure accuracy.
- Software for automatic calling of structural aberrations is not always available and therefore segmental aneuploidies need to be manually called by the operator.

Materials

Materials required for WGA and aCGH analysis of biopsied samples include:

- cell lysis, pre-amplification and amplification enzymes and buffers specific to each amplification method used;
- DNA labelling reaction buffers, enzymes, dNTPs and fluorophore-marked dUTP that should be used under minimal light exposure since they are light sensitive;
- hybridisation and washing buffers, human Cot-1 DNA, and DNase/RNase-free distilled water;
- microarray slides.

2.2.3. Work practice controls

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell (s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifiers plus the embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing (see also the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). Witnessing is also indicated at the following steps of the aCGH procedure:
  - at the start of the WGA procedure to ensure that the correct volume of PCR master mixture is loaded into each tube;
  - at the start of the labelling procedure to ensure that the correct volume of labelling mixture is loaded into each tube;
  - at loading of the labelled DNA samples on array slides to ensure that each sample matches the sample identifier on the slide;
  - and when recording aCGH results to ensure that aCGH files correspond to the correct cell and/or embryo.

Intra-assay controls

- Suitable positive controls are not readily available (i.e. unbalanced single human blastomeres, TE cells or other cell types to represent unbalanced human blastomeres or TE cells).

- Negative controls serve to confirm that no contamination is present in the 'no-template' tube, which does not confirm the absence of contamination for the rest of reaction tubes carrying the biopsied samples.
- Diluted genomic DNA is recommended for positive intra-assay controls to check successful amplification of single or few cells and a successful reaction, respectively.
- Negative controls with sample collection buffer, biopsy media, or washing media (based on the protocols of the PGT centre) are recommended to control for contamination for each biopsy sample cohort (i.e. the IVF laboratory negative control).
- A minimum of one negative control with amplification mixture only is recommended to control for contamination during setting up of amplification reactions. (i.e. the genetic laboratory negative control).

2.2.4. Pre-examination process

Internal quality control

When using aCGH for PGT-SR, the challenge is to reliably call an unbalanced chromosomal rearrangement while avoiding false positives or false negatives.

The probability of detecting (small) unbalanced chromosomal segments depends on the performance parameters of the platform used.

- It is recommended to determine the effective resolution threshold as well as the percentage false-negative and false-positive results, the specificity and the sensitivity of the platform in a series of experiments using DNA from:
  - isolated single cells from cell lines with established structural copy number changes;
  - previous unbalanced pregnancies, when available;
  - cells isolated from donated embryos from previously performed PGT-SR cases. Initial PGT results obtained with a validated technique should be used as a reference to determine the false positive/negative detection rate for the particular chromosome regions involved in the rearrangement.
- It is recommended to test replicates of the same DNA sample in order to affirm that deviating ratios most likely represent a true copy number change.
- Following DNA amplification, a clear agarose gel band should be visible and/or quantitative measurement of DNA concentration should at least be 20-50 ng/µl.
- It is recommended to test the quality of each batch of arrays.
- It is recommended to use hybridisation template forms to record sample tracking.
- Barcoding of aCGH slides is mandatory to maintain the correlation between the sample and the array slide used for hybridisation.
- It is acceptable to re-analyse unbalanced embryos for QA/QC purposes.

Test efficiency

- To check for amplification efficiency, it is recommended that samples and intra-assay controls (if used) be put on an agarose gel and/or quantified by Qubit Fluorometer.
- The use of male and female reference DNA is recommended to assess hybridisation efficiency and interpret the results. Marked X/Y chromosome separation is indicative of a successful experiment in gender-mismatched samples, and the corresponding levels of gain for the X chromosome and loss for the Y chromosome are used as a reference to evaluate aneuploidy events for the autosomes.

- Gender-matched samples must show consistently no change on chromosome X or Y and none of the probes in the array should report a change.
- Negative amplification, negative intra-assay control or failed hybridisation should show a consistent noisy profile where no significant pattern is observed.
- Storage time and temperature have an impact on the integrity of cells, DNA and/or solutions and laboratories should validate that the conditions used in their protocols are fit for purpose. Furthermore, it is not recommended to use repeatedly frozen-thawed solutions containing DNA or enzymes.
- Hybridisation bias due to drying out of the microarray surface could lead to signal loss, degradation of fluorophore-marked dUTP and suboptimal scanned images.
- It is recommended to stringently wash the aCGH slides with minimum light exposure and under controlled ozone concentration, temperature and humidity. The use of laboratory carbon-loaded non-woven filters is recommended in case of high ozone levels.
- It is recommended to avoid the use of detergents to clean the wash equipment, as this may interfere with signal intensity.
- Washing and scanning of slides in small batches (2-3 slides) is recommended to minimise the exposure of slides and of labelling dyes to air.
- It is critical that slides are dried by centrifugation shortly after the final washing step, to avoid drying through evaporation.
- Scan images should have defined features with red and green images well registered and the colours evenly balanced.
- The assay signal to background noise ratio (SBR) should be sufficiently high for the log2 ratio change to be observed. In case of low SBR, additional washing of the slides and rescanning are acceptable.
- It is recommended to calculate the acceptable and optimum ranges of QCs for every array experiment. The QC measures of array data for every experiment are extrapolated by specific software and are indicative for the successful calling of all target probes. The QC measures will vary between array types and different scanners.

2.2.5. Preclinical work-up and report

Preclinical work-up

- Karyotype reports should be obtained for both partners from an accredited/certified cytogenetics laboratory.
- A case-specific work-up is not required when performing aCGH for structural rearrangements, unless the carrier has an unbalanced karyotype.
- It is recommended to upfront ensure that all unbalanced products of the specific rearrangement can be identified with the platform used. The ability to detect an unbalanced product depends on the effective resolution and the coverage of the array used. This needs to be established prior to clinical application by using DNA from cell lines with well-established segmental aneuploidy to validate the presence and the number of all (consecutive) clones/probes representing the respective chromosome regions.
- It is acceptable that three out of four segments for two-way reciprocal translocations are detected to reliably identify unbalanced segregation products
- It is not acceptable to perform a clinical PGT-SR test if the size of the translocation segments, inferred from the karyotype, is below the threshold of resolution of the platform used.
- It is acceptable to forego any additional work-up when performing aCGH for structural rearrangements.

Preclinical work-up report

A case-specific preclinical wet-laboratory work-up report is not required, provided that no particularities have come to light during the work-up. However, a report on the theoretical evaluation of the preclinical work-up should be available.

2.2.6. Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking,
- risks caused by handling biopsy samples prior to aCGH analysis (tubing, washing) that, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA;
- risks that the size of the structural rearrangement is different from the one expected based on non-uniform reporting of parental karyotypes and therefore may remain undetected by the aCGH protocol (if they are below the resolution of the platform used);
- risk of inconclusive or false results due to suboptimal experimental conditions,
- risk of inconclusive or false results due to biological reasons: (i) unbalanced segregations may arise from crossing-over during meiosis I in the gametes of the carrier of the rearrangement; (ii) chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype; (iii) embryos of poor morphology are at risk of containing cells with degraded DNA;
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is below the resolution of the test, whether biological or caused by a technical error.

2.2.7. Limitations of the test

- Detection of translocation segments is limited by the resolution of the platform. If the size of more than one out of the four translocated segments is below this resolution limit, aCGH-based PGT is not possible.
- Detection of unbalanced segregations that have breakpoints near the telomere or in the sub-telomere region is not always possible, since the probe coverage in these regions is low. For each aCGH-based PGT-SR case, limitations should be investigated during preclinical work-up.
- aCGH-based PGT-SR analysis does not allow for a distinction between embryos with a normal or a balanced karyotype.
- aCGH-based PGT-SR analysis does not allow for the detection of UPD. There is an increased risk of UPD in carriers of chromosomal rearrangements when clinically relevant chromosomes (i.e. 6,7,11,14,15,20) are involved in the imbalance or a Robertsonian translocation, which involves chromosomes 14 or 15 (Kotzot, 2008). Prenatal diagnosis for UPD is acceptable but should be assessed critically on an individual basis.
- Array-based PGT-SR analysis is less sensitive to detect mosaicism than NGS.

2.3. SNP array

SNP array-based PGT-SR is not based on the detection of the actual chromosomes. The embryo karyotype is merely inferred from the haplotypes detected in DNA from the embryo biopsy.

SNP array-based PGT-SR requires a preclinical work-up to phase the imbalance. Phasing is performed using DNA from the couple and one reference (a balanced reference is

recommended, but an unbalanced is acceptable). If no reference is available, diagnosis can be performed during the clinical cycle and requires at least one unbalanced embryo or well-defined breakpoints to distinguish unbalanced embryos.

All samples need to be subjected to WGA prior to SNP array analysis.

- In case of PGT-SR for carriers of inherited balanced rearrangements, an added value of the approach is that, based on haplotype information, embryos carrying the balanced form of the rearrangement can be distinguished from normal diploid non-carrier embryos.
- Depending on the size of the involved segments, aberrant intensity ratios may or may not be detectable for the region(s) of interest. If detectable, it is recommended that the diagnosis is supported by Log ratio and B allele frequency values.

Further recommendations on SNP array are covered in the paper on detection of monogenic disorders (ESHRE PGT-M Working Group *et al.*, 2020).

2.3.1. Laboratory issues

Protocol

The protocol can vary significantly depending on the platform used. Independent of the platform, it includes: (i) sample pre-processing, (ii) hybridisation on the slides, (iii) SNP staining and detection, and (iv) data analysis.

Sample pre-processing and hybridisation generally includes any or all of the following processes: handling of biopsy samples (PB, single blastomere or TE cells); cell lysis and whole genome amplification; loading of the sample on the slides. Generation of reliable SNP calls is crucial and the process for generating them can vary depending on the platform.

WGA material of insufficient quality and/or quantity as well as contamination of starting material can lead to poor genotyping data.

Raw data produced after reading the SNP calls from the array are further processed by computational analyses and bioinformatics using a variety of algorithms to optimise genotyping and enable haplotyping.

As these processes may vary depending on the platform, it is recommended to optimise and validate each step individually (including the entire wet-bench process as well as the bioinformatic analyses) to empirically determine optimal assay conditions and analysis settings. For each platform, the SNP calling threshold and minimum SNP call rate should be defined with validation experiments (see pre-examination process section).

Turnaround time

The turnaround time from sample processing to data analysis can vary from 24 h to several days, depending on the setting and the platform of choice. It is recommended that each laboratory validates in-house whether the implementation of shortened protocols has an effect on hybridisation efficiency and data quality.

With the aim of accumulating samples for a SNP array run, biopsy samples can be stored short-term (weeks), and WGA samples can be stored long-term (years) at -20˚C or -80˚C.

Documentation

Relevant laboratory documentation should include:

- a karyotype, preferably at high resolution (550-800 bands), if available with FISH verification of the breakpoints from the patient and the phasing reference;
- a report on any previous unbalanced products of conception;

- genetic counselling report with possibly a recommendation for PGT-SR, an indication of the testing method and the benefits and the limitations of the test;
- the ***informed consent*** of the couple with risk assessment and indication of test limitation.

2.3.2. Laboratory infrastructure, equipment and materials

Infrastructure

General aspects on infrastructure are covered in the paper on organisation of PGT (refer ORG paper), and in section "Array-based PGT-SR".

Equipment

SNP array platforms differ, among others, in price, resolution (number of SNPs on the arrays) and chemistry. Initial set-up should follow manufacturer's instructions and it is recommended to collaborate with the manufacturer to ensure that the laboratory space has been optimised to meet the requirements. In addition, it is recommended to involve informaticians with relevant expertise to make sure all required elements (hardware, servers, data storage, internet) are in place.

- Equipment required for WGA and SNP array analysis of biopsied samples includes:
  - a class II safety cabinet, preferably equipped with UV-C light, to prevent contamination of samples during WGA;
  - thermal cycler with heated lid;
  - fume cabinet, hybridisation oven/incubator, water bath, gel electrophoresis equipment to check successful amplification and vortex mixers for plates and tubes;
  - a scanner, equipped with the corresponding lasers and suitable for the specific slide type, to excite the hybridised fluorophores to read and store the resulting images of the hybridisations, placed in the post-amplification room in an atmosphere with low ozone parameters, regulated temperature and protected from daylight.
- The use of a DNA quantification system (to determine the amount of amplified DNA after WGA) is optional
- Associated servers should be also allocated in proper conditions and instruments used in critical steps should be UPS-connected.
- It is recommended that prior to each step of the protocol, the temperature ranges and/or pH values of equipment and solution are verified. Specific temperature and thermocycler programmes should be validated in individual PGT centres for all equipment, and instruments should be serviced and calibrated regularly to ensure accuracy.
- Haplotyping analysis software is not always commercially available, therefore close collaboration with bioinformaticians needs to be guaranteed.

Materials

For all reagents employed in the different steps of the protocol, the lot numbers and expiration dates should be recorded.

Depending on the platform used and the manufacturer, materials required for WGA and SNP array analysis can vary substantially and may include one or more of the following constituents:

- cell lysis, amplification enzymes and buffers;

- DNA fragmentation buffers and enzymes, fluorophores and modified dNTPs that should be used under minimal light exposure since they are light sensitive; hybridisation and washing buffers;
- microarray slides.

2.3.3. Work practice controls

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell (s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifiers plus the embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing (see also the paper on organisation of PGT (refer org paper). Witnessing is also indicated at the following steps of the WGA/SNP array procedure:
  - at the start of the WGA procedure to ensure that the correct volume of reaction master mixture is loaded into each tube;
  - at the start of the SNP array protocol to ensure that that the correct volume of sample is transferred to the correct reaction tube / plate;
  - at loading of the DNA samples on SNP array slides to ensure that each sample matches the sample identifier on the slide (slide number and position per sample should be monitored and registered);
  - and when recording SNP array results to ensure that scanned raw files correspond to the correct cell and/or embryo.

Intra-assay controls

It is recommended to use negative and positive controls alongside the test samples to check if contamination or amplification failure has occurred.

- Suitable positive controls are not readily available (i.e. unbalanced single human blastomeres, TE cells or other cell types to represent unbalanced human blastomeres or TE cells).
- Diluted genomic DNA inputs are recommended as positive intra-assay controls to check successful amplification of single/few cells and a successful reaction, respectively.
- Negative controls serve to confirm that no contamination is present in the 'no-template' tube, which does not confirm the absence of contamination for the rest of reaction tubes carrying the biopsied samples.
- At least one 'no-template' reaction tube with washing buffer only (i.e. the IVF laboratory negative control) and one negative control with amplification mixture only (i.e. the genetic laboratory negative control) are recommended to exclude DNA contamination of these media.

2.3.4. Pre-examination process

Internal quality control

QC parameters define the overall quality profile of the samples. Depending on the platform, QCs should be defined by the user lab regarding acceptable call rate and level of noise of the samples. When using SNP arrays for PGT-SR, depending on the quality parameters, the chromosomal localization of the aberration(s) and the size of the involved segments, aberrant intensity ratios may or may not be detectable for the region(s) of

interest. If detectable, it is recommended that the diagnosis is supported by Log ratio and B allele frequency values.

- It is recommended to validate the protocol using single cells from cell lines with a known karyotype, or the same WGA products from embryos containing known deletions or duplications diagnosed with a previously validated technique.
- It is recommended to perform accuracy assessment, including both normal and abnormal samples. As different chromosome regions may have different SNP coverage, the series of abnormal samples should represent the range of structural rearrangements that the test is required to detect. It is recommended to use a minimum of three positive samples for each rearrangement type.
- Following DNA amplification, a clear agarose gel pattern should be visible and/or quantitative measurement of DNA concentration should be sufficient for further testing.
- Following accuracy assessment tests, it is recommended to calculate the performance (sensitivity, specificity, positive predictive value and negative predictive value) of the protocol.
- It is recommended to test the quality of each batch of SNP arrays.
- It is recommended to use hybridisation template forms to record sample tracking.
- Barcoding of SNP array slides is mandatory to maintain the correlation between the sample and the SNP array slide used for hybridisation.

Test efficiency

- It is recommended that the WGA procedure be performed in the same tube that the sample was collected in.
- To check for amplification efficiency, it is recommended that samples and intra-assay controls (if used) be put on an agarose gel and/or quantified by Qubit Fluorometer.
- Negative amplification, negative intra-assay control or failed hybridisation should show a consistent noisy profile where no significant pattern is observed.
- Storage time and temperature have an impact on the integrity of cells, DNA and/or solutions and laboratories should validate that the conditions used in their protocols are fit for purpose. Furthermore, it is not recommended to use repeatedly frozen-thawed solutions containing DNA or enzymes.
- It is recommended to calculate the acceptable and optimum ranges of QCs for every SNP array experiment. The QC measures will vary between array types and different scanners. It is recommended to perform an internal validation to establish a test-specific threshold for the overall noise value.

2.3.5. Preclinical work-up and report

Preclinical work-up

It is recommended that the following steps are taken during preclinical work-up:

- It is recommended to check whether the chromosomal segments involved in the rearrangement are adequately covered on the SNP array of interest.
- Parental and phasing reference karyotypes may facilitate testing and genetic counselling.

Preclinical work-up report

General guidance and recommendations on administration and patient information for the preclinical work-up report are provided in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). For PGT-SR using SNP array, the preclinical work-up report should also include a summary of the work-up.

It is recommended that the following are clearly stated in the report:

- indication and karyotype of the patient (ISCN nomenclature can be used);
- test limitations and residual risk of PGT misdiagnosis, including a figure.

2.3.6. Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking,
- risks caused by handling biopsy samples prior to SNP array analysis (tubing, washing) which, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA;
- risk of inconclusive or false results due to suboptimal experimental conditions at WGA or due to high background noise;
- risk of inconclusive results due to homologous recombination events in the vicinity of the fragments of interest;
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is below the resolution of the test, whether biological or caused by a technical error;
- risk of incidental findings.

2.3.7. Limitations of the test

SNP array haplotyping requires at least one first degree relative of the partner carrying the rearrangement of interest for phase determination.

2.4. Next generation sequencing

Next generation sequencing (NGS) allows for direct reading of sequenced DNA fragments and their quantification based on sequence read numbers. Depending on the sequencing read depth, NGS can be applied in different assays from whole chromosome aneuploidy to medium size deletions or insertions in chromosomes and detection of single gene disorders. Compared with aCGH, chromosomal copy number assessment based on NGS may offer several advantages including: (i) reduced DNA sequencing cost made possible by high throughput sequencing technologies and the larger number of samples that can be simultaneously sequenced during a single experiment (the latter requires adding a unique tag); (ii) enhanced detection of deletions and duplications because of the potential increase in resolution (as assessed in the pre-examination validation); (iii) increased dynamic range enabling enhanced detection of chromosomal mosaicism in TE samples; (iv) the potential automation of the sequencing library preparation to minimise human errors, reduce hands-on time and enable higher throughput and consistency.

2.4.1. Laboratory issues

NGS protocol

The sequencing by NGS protocol comprises five steps: (i) sample processing; (ii) initial quality analysis; (iii) library preparation; (iv) sequencing; and (v) data analysis.

The sample processing and sequencing generally includes any or all of the following processes: handling of biopsy samples (PB, single blastomere or TE cells); cell lysis; barcoding (molecular indexing) of samples; adapter ligation; amplification; library preparation; flow cell loading; and generation of sequence reads. It is recommended to perform initial quality analysis of DNA; contamination of starting material can lead to poor sequencing data quality.

DNA sequence generation by NGS platforms is almost entirely automated and the output consists of millions to billions of short sequence-reads. Raw data produced after

sequencing are further processed by computational analyses and bioinformatics using a variety of algorithms to map and align the short sequence reads to a linear reference human genome sequence.
As these processes may vary depending on the platform, it is recommended to optimise and validate each step individually (including the entire wet-bench process as well as the bioinformatic analyses) to empirically determine optimal assay conditions and analysis settings.

For each platform, the ***genome coverage, average read depth*** and minimum number of reads should be defined with validation experiments (see pre-examination process section).

Turnaround time

The turnaround time of NGS (from DNA amplification to reporting) can vary according to the platform, but currently it is at least 12 h. Turnaround time is expected to significantly decrease in the future.

With the aim of accumulating samples for an NGS run, biopsy samples can be stored short-term (weeks), and WGA samples can be stored long-term (years) at -20˚C or -80˚C.

Documentation

Relevant laboratory documentation should include:

- a patients' karyotype, preferably at high resolution (550-800 bands), if available with verified breakpoints from an accredited/certified cytogenetics laboratory; often, the rearrangement breakpoints are defined based on GTG-banded chromosomes and as the resolution of this technique is quite low, there is a potential risk that the actual translocation segments are (much) smaller than expected and hence the probability of detection of all the unbalanced segregation products of the structural rearrangement (much) lower;
- a report on any previous unbalanced products of conception;
- genetic counselling report with possibly recommendations for PGT-SR, an indication of the testing method and the benefits and the limitations of the test;
- the informed consent of the couple with risk assessment and indication of test limitation.

2.4.2. Laboratory infrastructure, equipment and materials

Infrastructure

General aspects on infrastructure as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020), and in section "Array-based PGT-SR:.

Equipment

NGS platforms differ, among others, in price, capacity, chemistry and read length. Initial set-up of an NGS system should follow manufacturer's instructions and it is recommended to collaborate with the manufacturer to ensure that the laboratory space has been optimised to meet the requirements. In addition, it is recommended to involve informaticians with relevant expertise to make sure all required elements (hardware, servers, data storage, internet) are in place.

NGS-based PGT requires the following equipment:

- A DNA quantitation instrument; it is crucial to accurately determine the amount of starting DNA for library preparation. There are several options that give highly accurate quantitation of low amounts of DNA. Amongst those is the Qubit high-

sensitivity double-stranded DNA (HS dsDNA) fluorometer, which measures dsDNA. HS dsDNA has been found to give a much more accurate estimation of the amount of DNA present in the sample, compared with standard spectrophotometry. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. It is recommended to use DNA with absorbance ratio values ranging from 1.8 to 2.0.

- Thermocyclers–DNA amplification and labelling are necessary steps during the library preparation, therefore requiring the use of a thermocycler.
- Pipettors or pipetting robots–dedicated multi-channel and single-channel pipettes are a necessity for NGS.
- Multichannel pipette or automated systems are recommended to minimise the risks of mislabelling or misallocation of samples during the different steps of the protocol.
- Sequencers should be allocated in a specifically designed room, with modulated light exposure and regulated temperature according to manufacturers´ instructions. Associated servers should also be kept under proper conditions and instruments used in critical steps should be UPS-connected.
- Sequencers should be validated for the specific PGT protocols and incorporate the latest version of the specified software, allowing proper performance of the PGT protocol.
- It is recommended that prior to each step of the protocol, the temperature ranges and or pH values of equipment and solution are verified. Specific temperature and thermocycler programmes should be validated in individual PGT centres for all equipment, and instruments serviced and calibrated regularly to ensure accuracy.
- Software for automatic calling of structural aberrations is not always available and therefore segmental aneuploidies need to be manually called by the operator.

Materials

For all reagents employed in the different steps of the protocol, the lot numbers and expiration dates should be recorded.

Depending on the manufacturer, NGS kits may include one or more of the following constituents:

- cell lysis and DNA extraction media; lysis buffer and specific enzymes for DNA extraction;
- DNA amplification media: some WGA protocols are PCR-based whereas others are not, and it is recommended to use a WGA protocol which is compatible with the specific NGS platform has been validated;
- library preparation media: although many methods are available, some preparation procedures are specific for a particular NGS platform, and therefore, it is recommended to pay attention to the compatibility of the libraries with the sequencing platforms.

2.4.3. Work practice controls

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell(s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifier and embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing (see also the paper on organisation of PGT (refer org

paper). Witnessing is also indicated at the following steps of the WGA/NGS procedure:

- at the start of the WGA procedure to ensure that the correct volume of PCR master mixture is loaded into each reaction tube;
- at the start of the library preparation to ensure that embryo identification corresponds with a dedicated barcode or index primers;
- at pooling, to make sure that all barcoded libraries are included in the pool before the start of the NGS run;
- during NGS run preparation; data input for each sample should be checked to ensure that samples match their identifier on the plate.

Intra-assay controls

It is recommended to use negative and positive controls alongside the test samples to check if contamination or amplification failure has occurred.

- As suitable positive controls are not readily available, it is recommended to use validated samples containing deletions or duplications (from very small size 5Mb to 20Mb), and a ***diploid*** control sample.
- Diluted genomic DNA inputs are recommended as positive intra-assay controls to check successful amplification of single/few cells and a successful reaction, respectively.
- One 'no-template' reaction tube with washing buffer only (i.e. the IVF laboratory negative control) and one negative control with amplification mixture only (i.e. the genetic laboratory negative control) are recommended to exclude DNA contamination of these media.

2.4.4. Pre-examination process

Internal quality control

QC parameters define the overall quality profile of the samples. Platforms have proper QCs defined as the minimum reading value and the lowest noise value needed to detect a copy number variation. Because the genomic resolution of NGS for PGT-SR can be an issue for small segmental abnormalities, NGS platforms may have already been validated for sensitivity, specificity and, negative and positive predictive values. Despite the information provided by the manufacturer, an implementation validation with respect to the resolution is necessary. These values may vary between NGS platforms depending on coverage, insert size, WGA methodology, and single versus paired-end sequencing.

Before testing patient samples, the analytical validity of the intended tests needs to be established with appropriate QC/QA.

- It is recommended to validate the protocol using single cells from cell lines with a known karyotype, or the same WGA products from embryos containing known deletions or duplications diagnosed with a previously validated technique.
- It is recommended to perform accuracy assessment, including both normal and abnormal samples. As different chromosome regions may have different coverage, the series of abnormal samples should represent the range of structural rearrangements that the test is required to detect. It is recommended to use a minimum of three positive samples for each rearrangement type.
- Following amplification, it is recommended to quantify DNA. DNA concentration should at least be 20-50 ng/µl.
- In general, poor-quality or failed WGA products should be excluded from further analysis as these samples may affect the sequencing read distribution per sample after library pooling and sequencing.

- Following accuracy assessment tests, it is recommended to calculate the performance (sensitivity, specificity, positive predictive value and negative predictive value) of the protocol.
- As the presence of chromosomal mosaicism is an issue when analysing TE biopsy samples, it is recommended to include mosaic samples (i.e. a mixture of cells with known segmental aneuploidies and euploid cells) in the validation study (see also section "Array-based and NGS-based PGT-A").

Test efficiency

For amplification efficiency checking, gel electrophoresis is recommended for samples and intra-assay controls using proper standards.

- It is recommended that the WGA procedure be performed in the same tube that the sample was collected in.
- After preparation the library should be quantified and normalised for each sample before creating the library pool.
- It is recommended to have high coverage for the region of interest and ascertain that the expected translocation is covered by a sufficient number of sequenced fragments.
- Sequencing by NGS comprises a series of steps that uniquely contribute to the overall quality of the data set. Thus, each individual step needs to be controlled to ensure high-quality results.
- NGS run parameters (coverage, number of reads, noise) should be monitored before the analysis of raw sequencing data to ascertain that the overall and individual run parameters for each sample correspond to the platform-specific required criteria. These sequencing quality metrics can provide important information about the accuracy of each step in this process, including library preparation, base calling, and read alignment.
- From the total number of reads, 70-80% should align to the genome. Lower percentages indicate contamination in the DNA sample, degraded DNA or suboptimal WGA.
- Each run should have an acceptable, previously established level of noise. It is recommended to perform an internal validation to establish a test-specific threshold for the overall noise value.
- Various amplification protocols are in use, which may be affected by single cell artefacts, such as allele drop out (ADO), ***amplification bias*** or allele drop in (ADI), that might affect the accuracy of the diagnostic test, and therefore extensive validation of WGA is required.
- It is recommended to calculate the acceptable and optimum ranges of QCs for every NGS experiment. The QC measures of NGS data for every experiment are extrapolated by specific software and are indicative for the successful calling of all target DNA sequencing. The QC measures will vary between NGS platforms and different software versions.

2.4.5. Preclinical work-up and report

Preclinical work-up

- It is recommended to check whether the chromosomal segments involved in the rearrangement are adequately covered, in terms of the number of sequence reads.
- Parental karyotypes may facilitate testing and genetic counselling.
- It is acceptable that at least three out of four segments for two-way reciprocal translocations can be detected to reliably identify unbalanced segregation products.
- It is not acceptable to perform a clinical PGT-SR test if the size of the translocation segments, inferred from the karyotype, is below the threshold of resolution of the platform used.
- It is acceptable to adjust the lower detection limit provided by the platform's manufacturer, based on a feasibility study using DNA from previous unbalanced products of conception.
- It is acceptable to forego any additional work-up when performing NGS for structural rearrangements.

Preclinical work-up report

A case-specific preclinical work-up report is not required, provided that no particularities have come to light during the work-up. However, a report on the theoretical evaluation of the work-up should be available.

2.4.6. Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking,
- risks caused by handling biopsy samples prior to NGS analysis (tubing, washing) which, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA;
- risk of inconclusive or false results due to suboptimal experimental conditions at WGA or due to high background noise or low coverage;
- risk that the size of the deletion or duplication is different from the one based on the karyotypes in the parents, and therefore they may remain undetected by the NGS protocol (if they are below the resolution of the test);
- risk of misinterpretation of the actual embryo karyotype due to the presence of chromosomal mosaicism, either at cleavage-stage or at blastocyst stage.

2.4.7. Limitations of the test

Limitations of the standard NGS protocols for PGT-SR without genotyping consist in the fact that the analysis cannot:

- detect whole ploidy changes;
- discriminate balanced from normal results;
- detect low level chromosomal mosaicism;
- detect abnormalities below the predefined resolution.

3. Strengths and limitations

Technical strengths and limitations of FISH, aCGH and NGS (without genotyping) are outlined in Table D.I.
The most important limitations include:

- Based on the embryo biopsy alone, FISH, aCGH and NGS cannot discriminate between samples carrying the rearrangement (i.e. balanced) and those that are not (i.e. normal) and this should be clearly stated in the report. Although there is no expected difference in the phenotype of embryos with a 'normal 'or a 'balanced' karyotype, many couples wish to know whether the structural rearrangement is being transferred to their offspring to be aware of possible future reproductive problems related to the rearrangement. When polar bodies are used for PGT analysis, discrimination between oocytes carrying the rearrangement and those that are not, is feasible.
- FISH and aCGH cannot, but NGS can analyse aneuploidy and gene defects simultaneously in the same diagnostic sample.
- Based on the embryo biopsy alone, FISH, aCGH and NGS without genotyping cannot identify the nature (meiotic or mitotic) nor the parental origin of aneuploidies. When polar bodies are used for PGT analysis, inferred errors in the oocyte are always of maternal, meiotic origin.
- Based on the embryo biopsy alone, FISH, aCGH and NGS without genotyping cannot detect UPD.

Table D.I. Overview of the strengths and limitations of the methods applied for PGT-SR (and PGT-A)

	PGT-SR	PGT-SR / PGT-A	
	FISH	aCGH	NGS (without genotyping)
NUMBER OF CHROMOSOMES	Information is limited to chromosomes and/or targeted loci for which probes are used.	All 24 chromosomes analysed.	All 24 chromosomes analysed.
MINIMAL RESOLUTION	Limited by the availability of (commercial) probes. Commercial probes are available for only a limited number of loci, which may complicate the selection of probes for the analysis of rare chromosomal rearrangements.	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software
WHOLE PLOIDY CHANGES	Inferred from the number of hybridisation signals from multiple probes.	Not all variants of ***polyploidy*** and haploidy can be detected.	Not all variants of polyploidy and haploidy can be detected.
NO CONCLUSIVE RESULTS	As a result of improper fixation, overlapping cells or signals. Rebiopsy is an option.	As a result of cell lysis during tubing, cells with degraded DNA, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.	As a result of cell lysis during tubing, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.

Table D.I. (continued)

	PGT-SR	PGT-SR / PGT-A	
	FISH	aCGH	NGS (without genotyping)
ABNORMALITIES NOT DIAGNOSED	FISH-based PGT-SR diagnosis of biopsied material from cleavage stage or blastocyst embryos does not allow for a distinction between embryos with a normal or a balanced karyotype.	aCGH-based PGT-SR diagnosis of biopsied material from cleavage stage or blastocyst embryos does not allow for a distinction between embryos with a normal or a balanced karyotype.	NGS-based PGT-SR diagnosis of biopsied material from cleavage stage or blastocyst embryos does not allow for a distinction between embryos with a normal or a balanced karyotype.
MOSAICISM RELATED ISSUES	Chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.
UNIPARENTAL DISOMY (UPD)	FISH analysis does not allow for the detection of UPD.	aCGH analysis does not allow for the detection of UPD.	NGS analysis does not allows for the detection of UPD.
RISK OF MISDIAGNOSIS	Contamination with cumulus cells. Visual inspection allows for the identification of sperm cells, Incomplete nucleus, or presence of nuclear fragments	Contamination with remaining cumulus cells after ICSI.	Contamination with remaining cumulus cells after ICSI.
IMPACT OF BIOPSY ON TEST RESULTS	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is less favourable compared to a single cell biopsy.	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.
SIMULTANEOUS DETECTION OF CHROMOSOME COPY NUMBER AND SINGLE GENE DISORDER(S)	Not feasible.	Not feasible.	Feasible.
ORIGIN OF ANEUPLOIDY	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy when based on the analysis of biopsied material from cleavage stage or blastocyst embryos	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy when based on the analysis of biopsied material from cleavage stage or blastocyst embryos	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy when based on the analysis of biopsied material from cleavage stage or blastocyst embryos

4. Examination process

Clinical testing protocols should include scoring criteria and reporting procedures as well as a framework for counselling patients in the presence of diagnostic results.

General recommendations on the PGT examination process as included in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020):

- The examination process and reporting of results, must comply with local guidelines or law, or with the ISO 15189 standard.
- Before starting a clinical PGT cycle, relevant documents should be available, labelling of samples should be checked, and genetic counselling provided to the couple.
- It is recommended that the PGT laboratory has clearly documented procedures for all steps of the examination process (explicit instructions and a summary of validation results) and release of results (diagnosis, reporting, embryos transfer policy). These procedures are preferably covered in a service-level agreement between the PGT and IVF centres.
- Many different methods have been published and all appropriately validated methods are acceptable for clinical cases. The method used should have been previously implemented, tested and validated in the PGT centre.

4.1. Scoring of clinical results

- It is recommended that results are reviewed and signed or electronically validated by a suitably qualified person (name, qualification, date).

4.1.1. FISH results

- FISH signals should be scored according to brightness, size and distance. The signals should have approximately the same brightness and size (depending on the probes used) and should be at least one signal in diameter apart. Two signals that are in close proximity and have approximately the same size, but are not connected by a visible link, are considered as two signals. A diffuse signal should be scored as one if the signal is continuous and of expected size. Two small signals connected by a visible link are counted as one signal.

- Signal scoring criteria should be established in a written protocol and adhered to for the interpretation of signals.

- It is recommended that signals are analysed by two independent observers and that discrepancies adjudicated (where possible) by a third observer. If no consensus is reached the embryo should not be recommended for transfer, i.e. should be given the diagnosis of uninterpretable or inconclusive result.

- It is acceptable to score signals from probes labelled with fluorochromes not detectable to the human eye using an image capture system.

- All fluorescent images should be captured and filed for QC purposes. If possible, the position and coordinates of the embryonic cells on the slide can be recorded.

- 'No result rescue'" for embryos without a clear diagnosis is acceptable. An additional hybridisation round should be performed with probes indicative of the same chromosome(s) but a different region or, if not available, at least with probes in a different colour scheme. A second biopsy can also be performed, followed by the full FISH protocol.

- When there is a combination of chromatid gain/loss in the first polar body, which is balanced by the second polar body, a normal chromosome copy number in the corresponding oocyte is predicted and reported, and the resulting embryo can be considered for transfer after discussion with the patient.

4.1.2. aCGH and NGS results

- Software analysis and copy number scoring criteria should be established in a written protocol and adhered to for the interpretation of whole chromosome and segmental-chromosome gains and/or losses.
- Interpretation of raw data or profiles resulting after specific software analysis by a single observer is acceptable. Additional confirmation by an independent observer is recommended.
- All files resulting from the scanning and sequencing, as well as profiles after specific software analysis should be stored and filed for QC purposes.
- 'No result rescue' for embryos without a clear diagnosis is acceptable. This could imply a second analysis of the existing WGA as well as a second biopsy followed by WGA, full aCGH/NGS processing and analysis.
- When there is a combination of chromatid gain/loss in the first polar body which is balanced by the second polar body, a normal chromosome copy number in the corresponding oocyte is predicted and reported, and the resulting embryo can be recommended for transfer.

4.2. Issuing a PGT report

General items required in PGT preclinical work-up or clinical cycle reports as listed in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) include:

The PGT clinical cycle report contains interpretation of the clinical results and guidance on which embryos are genetically transferable. The same recommendations apply as specified for the preclinical work-up report (see section "Preclinical work-up"), together with the following items:

- unique cycle/treatment code;
- date of oocyte retrieval;
- date of biopsy;
- date of biopsy sample arrival in the laboratory;
- information on the sample type (including number of samples and controls);
- unique ID number for each cycle and/or biopsy sample tested;
- indication for PGT.

- When scoring results from PB testing, it is recommended to report what was detected in each polar body and then infer the oocyte diagnosis. It is recommended to test both PBs.
- When scoring results from blastomere/TE testing, it is recommended to report what was detected in the sample and then infer the embryo diagnosis.
- When results are reported from 'pooling' of embryos, it is advisable to refer to each oocyte and sample collection date and clearly differentiate the embryo number between cycle/treatment.
- Reporting of clinical results to the IVF centre must follow local regulations or international accreditation guidelines, including GDPR.

- The embryo transfer policy should be agreed upon between stakeholders (IVF centre, genetic centre, genetic counsellors, clinicians and patients). In PGT-M and PGT-SR cases, embryos with no or inconclusive results are not recommended for transfer. Depending on local rules and following adequate counselling of the prospective parents, the carrier status of embryos (for autosomal recessive or X-linked recessive disorders) may be taken into consideration for embryo selection. In case of PGT-A in addition to PGT-M or PGT-SR, it is crucial that the centre has a clear policy on embryo (ranking and) transfer.
- A written or electronic report should be securely transmitted to the IVF centre to ensure transfer and/or cryopreservation of the correct embryos. Results should not be communicated orally.
- Reporting time should be kept as short as possible, and when fresh transfer is intended, reporting time should be adapted to allow the IVF centre to organise the embryo transfer.
- It is recommended that the report is clear, concise, accurate and easily understandable by non-geneticists.
- It is recommended that the overall result and interpretation (including transfer recommendation) are presented per embryo, preferably in tabulated form. Sufficient information for genetic counselling should be included, such as the chromosome(s) involved, chromosome band(s)/nucleotides, the size of the chromosomal aberration in Mb, and the correct identification of the genetic variant. Where applicable, the latest version of the international system for human cytogenetic nomenclature (ISCN)/Human genome variation society (HGVS) nomenclature can be used.
- In case of no diagnosis and re-biopsy to try and obtain a result, this should be included in the report.
- The final clinical cycle report must be signed by appropriately qualified (authorised) personnel (name, qualification, date).
- It is recommended that the clinical cycle results are discussed with the couple before embryo transfer.
- It is recommended that the report is stored in the patient file in the PGT centre, according to local regulations.
- It is recommended to include a disclaimer in the report to address limitations of the test and any other information that may be of significance to the addressee.
- It is acceptable to indicate in the report the need for prenatal testing to confirm the result in case of pregnancy.

The ISCN reporting is acceptable for PGT-SR and PGT-A. It is recommended to add the following technical or interpretation items to the clinical report.

- If the profile is noisy or QCs are not sufficient, re-analysis is acceptable to try and obtain a result and this should be included in the report to the IVF centre.

- In the absence of any amplification or when contamination is suspected, rebiopsy is acceptable to try and obtain a result and this should be included in the report to the IVF centre.

- Each centre should decide whether or not to report mosaicism based on internal validation and recent literature.

- The clinical significance of transferring mosaic embryos is currently unknown. The centre's policy about the identification and transfer of embryos with mosaicism or

segmental aneuploidy should be documented and shared with the patient during genetic counselling.

- In case of an embryo with chromosomal mosaicism or segmental aneuploidy, genetic counselling should be offered to the couple and if transfer is decided and pregnancy occurs, it should receive appropriate follow-up ((ESHRE PGT Consortium Steering committee. *et al.*, 2020) section Follow-up of PGT pregnancies and children).

5. Post-examination process

Recommendations on PGT follow-up, baseline IVF live birth rates for PGT and misdiagnosis as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) .

5.1. PGT cycle follow-up

For quality purposes, it is recommended to confirm the PGT diagnosis on a subset of embryos not transferred or cryopreserved following diagnosis, in line with local regulations. Such confirmation aims to provide internal ***quality assurance (QA)*** as well as accurate and up-to-date misdiagnosis rates to prospective PGT patients. It is recommended that this is performed on as many embryos as is practicable. It is acceptable to perform this periodically.

When a pregnancy ensues following PGT testing, it is recommended that parents are (again) made aware of the chance and risks of a misdiagnosis and be informed on the possibilities for prenatal testing. PGT and IVF centres should make special efforts to follow-up with the parents following prenatal testing or birth, especially if confirmatory testing is not possible.

Follow-up data should be used for both internal ***quality control (QC)*** and QA purposes and documented in the ESHRE PGT Consortium online database for international data collection.

It is recommended that laboratories follow local regulations or accreditation schemes on storage of clinical samples and patient records. If no local regulations or guidelines exists on storage of clinical samples and patient records, it is recommended as follows.

- If embryos have been transferred and/or frozen, all relevant material (e.g. FISH slides, DNA amplification products) from the case should be retained and appropriately stored. Samples should be stored for at least 1 year. Prolonged sample storage could be considered, taking into account the availability of information on delivery and the duration of embryo cryopreservation.
- If there is no genetically suitable embryo for transfer or cryopreservation, it is not necessary to keep the samples.
- If there is no pregnancy after transfer of all genetically suitable embryos, samples can be discarded.

5.2. Misdiagnosis rate

- It is recommended that each PGT centre performs a prospective risk analysis in order to prevent and/or eliminate possible causes of ***misdiagnosis***.
- It is recommended that misdiagnosis rates should be calculated for each type of method and for all methods from a particular centre. Misdiagnosis rates include those clinical cases in which affected pregnancies arose and cases for which re-analysis results were discordant with the biopsy result.

- It is recommended that confirmatory testing should be performed at least periodically as a QA.
- It is recommended that the published and in-house estimates of misdiagnosis rates should be available on request to prospective patients along with pregnancy rates and live birth rates, to allow informed consent for PGT.
- Following a misdiagnosis, the IVF/PGT centre should investigate the possible causes of the misdiagnosis and make changes to protocols to eliminate the risk in the future. Many of the causes of misdiagnosis are avoidable by taking preventive actions and following the principles of quality management.
- Misdiagnosis should be reported, for instance through the ESHRE PGT Consortium online database.

5.3. Baseline IVF live birth rates for PGT

- Setting appropriate baseline live birth rates should be left up to the individual centres. However, it is recommended that each IVF centre should compare PGT live birth rates and matched non-PGT [routine IVF or ***intracytoplasmic sperm injection (ICSI)***] live birth rates within that IVF centre.
- Comparison of live birth rates with those reported by the ESHRE PGT Consortium or comparable peers can also be carried out to set benchmarks for continual improvement of the PGT centre.

SECTION E: DETECTION OF NUMERICAL CHROMOSOMAL ABERRATIONS

This section is entirely based on the paper:

ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations" with additions from the paper "ESHRE PGT Consortium good practice recommendations for the organisation of preimplantation genetic testing

1. Introduction to PGT-A techniques

This paper provides detailed technical recommendations for the most applied methods for PGT-A including whole genome amplification (WGA)-based array-based comparative genomic hybridisation (aCGH) and next-generation sequencing (NGS).

General recommendations for PGT-A are formulated, independent of the testing method applied.

1.1. Training and personnel

- Genetic testing procedures should be performed under the supervision of a (cyto)geneticist, competent or authorized to perform clinical diagnostics.
- All personnel undertaking genetic testing should be trained adequately as required in a clinical molecular cytogenetic laboratory and should follow written ***standard operating procedures (SOPs)***.
- Training for each technique should be documented.
    - Training for tubing is discussed in the paper on polar body and embryo biopsy for PGT (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
    - For aCGH and NGS, it is recommended that at least 30 samples are subjected to WGA, followed by aCGH or NGS by each trainee during preclinical training. Supervised clinical training should include at least an additional 20 samples.

1.2. Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020):

1.2.1. Laboratory infrastructure

Oocyte and/or embryo biopsy should be performed in a specifically designated laboratory setting. Collection of the biopsied samples and initial steps of genetic testing procedures should be carried out in laboratory settings dedicated for processing single and/or few cells. Appropriate precautions should be taken both to prevent contamination of samples by physical isolation, and to detect any such contamination. Licenses for offering embryo biopsy procedures and/or genetic testing by the centre may be obtained, according to local regulations.

1.2.2 Equipment

- All clinical equipment should meet the criteria set for the intended application, be appropriately calibrated, maintained and serviced, with all aspects supported by written ***standard operating procedures (SOPs).*** Equipment used for critical steps should have uninterrupted power supply (UPS).
- For areas within the IVF centre, whether it is a dedicated area or a room, all equipment should comply with "Revised Guidelines for good practice in IVF laboratories (2015)", section 3 "laboratory safety" (ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). Prior to the biopsy procedure, work surfaces, equipment and hoods should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory.
- For areas within the PGT centre, prior to each use, work surfaces and equipment should be cleaned and decontaminated with DNA ***decontamination*** solutions or 10% bleach, or by UV-C irradiation or autoclaving (when applicable, for example

tube racks). It is not recommended to use 70% ethanol solution only, as it does not decontaminate DNA.

- Multichannel pipettes or automated systems may be useful in the PGT laboratory to minimise the risks of mislabelling or misallocation of samples during the post-amplification steps, but they are not recommended in the pre-amplification steps.

1.2.3 Materials

- To prevent contamination, protective clothing for DNA amplification of a single and/or few cells 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). For areas within the IVF centre, protective clothing, preferably with low particle-shedding and non-powdered gloves and masks should be considered.
- The pre-amplification materials and reagents should be kept away from any DNA source and preferably stored in the pre-amplification area.
- 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 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 internal 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 all reagents.
- Reagents and solutions can be DNA decontaminated by UV-C irradiation. Alternatively, reagents and solutions made in-house can be autoclaved, preferably using a PGT-dedicated autoclave.
- Careful handling of all reagents employed must be ensured with regards to storage temperature and working conditions, following manufacturer's recommendations. Vortexing and quick temperature changes should be avoided for the most sensitive components.

Specific issues for handling of reaction tubes to reduce cross-contamination:

- It is recommended to avoid touching the inside or the lid of the tubes with your fingers.
- It is recommended to avoid touching the outside or the cap of the tubes with the tip of the pipette. If this happens, the pipette tip should be changed immediately.
- It is recommended to keep the reaction tubes open not longer than necessary.

1.2.4.Laboratory documentation

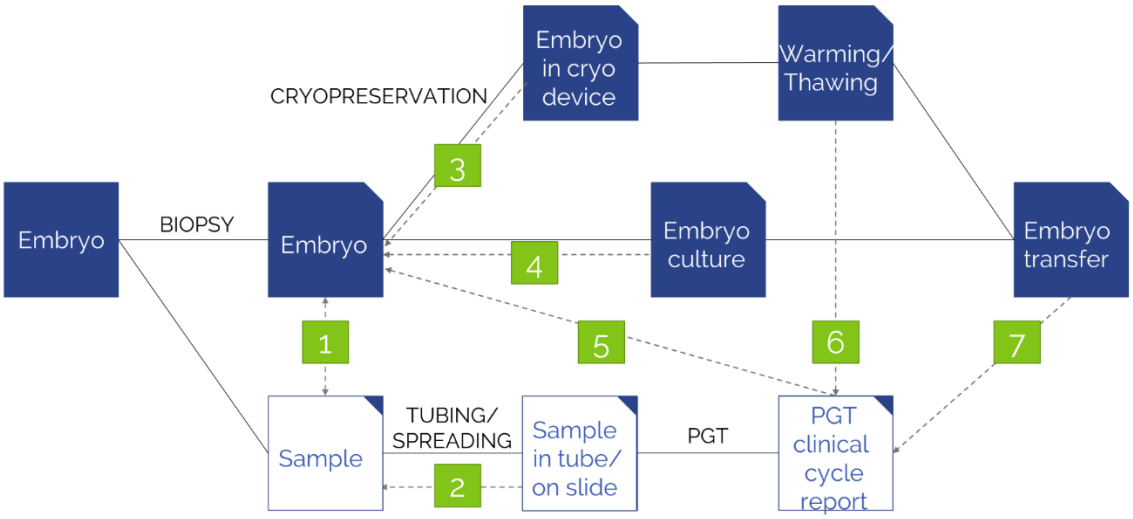
Well-structured (electronic and/or paper) laboratory forms should be available for recording wet-laboratory details of work-up and PGT cycle procedures.

1.3. Labelling and witnessing

General aspects on labelling and witnessing as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) :

- It is recommended that an adequate labelling system, written or barcoded (electronic), with two unique patient identifiers plus the embryo/cell(s) number is used to match the sample's diagnostic result with the embryo from which that sample was taken. This should ensure traceability throughout the IVF and PGT process until reporting of the final results.
- The labelling system should be comprehensible and practical for both the IVF and the PGT centres. Printed sticker labelling may be superior to pens, as labelling should be legible and uneditable.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer and signed off (Figure E1). These critical steps are detailed in the technical papers for the various methods (ESHRE PGT-M Working Group *et al.*, 2020, ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020, ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020).
- After biopsy, the sample may be analysed in house, or sent for genetic testing in another centre (see " Transport PGT").

Figure E1: Outline of the biopsy and genetic testing procedure with indications of the 7 critical steps where labelling and sample identification should be confirmed.



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) In case of cryopreservation, immediately after biopsy before acquiring the genetic analysis results, at placing and labelling the embryo into the cryopreservation device; (4) For further embryo culture, at placing and labelling the embryo into the culture dish; (5) When diagnostic results are issued to ensure accuracy and correlation with the correct sample identification; (6) During the thawing/warming procedure to ensure accuracy and correlation with the correct embryo diagnostic result; and (7) At the time of selecting the embryo(s) for embryo transfer.

1.4. Risk assessment

- When sub-optimal samples or samples not meeting the internal requirements (f.ex. lysed cells, nucleus not seen) are received for testing, this should be documented and a procedure on how to further process these samples should be in place.

1.5. Appropriate indications for specific tests

It is recommended that specific indications for PGT should remain within the scope of individual clinics.

- FISH is not recommended for PGT-A as only a subset of chromosomes can be tested, and better comprehensive molecular approaches to detect aneuploidy for all 24 chromosomes are available.
- Selection of embryos based on sex for social reasons is not acceptable.

2. Preimplantation testing for numerical aberrations

Applications of PGT-A comprise former PGS in IVF couples with normal karyotype and PGT-A in couples with chromosomal numerical aberrations such as Klinefelter syndrome and other sex chromosome abnormalities. Both types share the same techniques, but reporting may be different.

FISH is not recommended for PGT-A, as only a subset of chromosomes can be tested and better comprehensive molecular approaches to detect aneuploidy for all 24 chromosomes are available.

Real-time qPCR is used for PGT-A, but the limits of the technique, such as the low resolution in the detection of chromosomal mosaicism, have led to its disuse in favour of techniques such as NGS. For this reason, real-time qPCR will not be addressed in this paper.

2.1. Array-based and NGS-based PGT-A

aCGH was clinically applied for PGT of whole chromosome abnormalities and has revolutionised the field by providing accurate identification of comprehensive chromosome copy numbers and rapid analysis.

aCGH platforms utilizing ***bacterial artificial chromosomes*** (BACs), chromosome-specific libraries, oligonucleotides and SNPs have been clinically applied and all succeed in detecting aneuploidies in polar bodies, single blastomeres and TE samples.

The use of NGS for the detection of copy number variation differs from aCGH by direct reads of genomic sequencing fragments and their quantitation according to sequence read numbers instead of signal intensity comparison of fluorescently labelled test and reference DNA samples. NGS has been extensively validated using cells of a known genotype and is now used for detecting aneuploidies in polar bodies, single blastomeres and TE samples.

2.1.1. Laboratory issues

Information on protocols, turnaround time and documents for aCGH and NGS is presented as in the sections “Array-based PGT-SR” and “Next generation sequencing”, respectively.

2.1.1.a. aCGH

The aCGH workflow involves: (i) sample cell lysis and whole genome amplification (WGA); (ii) labelling of sample and reference DNA with different fluorochromes (e.g. green and red); (iii) purification of labelled DNA; (iv) microarray processing (hybridisation of biopsied and reference DNA samples followed by washing of microarray slides); (v) scanning; and (vi) analysis of scanned microarray tiff images where data is extracted to fluorescence ratio. The resulting ***log2 of fluorescence ratios*** is computed by specific software to identify structural and numerical chromosome copy number aberrations.

aCGH protocol

- It is recommended that wet-laboratory experimental conditions be established for all steps in the aCGH workflow followed by a preclinical assessment of the accuracy of the test to detect a chromosome aberration.
- It is acceptable to perform aCGH-based PGT on polar body biopsies, provided that both polar bodies can be analysed, and all unbalanced products of meiotic segregation can be detected so that it is possible to know the contents of the oocyte.
- It is acceptable to perform aCGH-based PGT on single cell biopsies, although they present with an overall increased noise and step change chromosome artefacts in the aCGH profile. Acceptance criteria for noise level should be part of the QA/QC parameters.
- Blastocyst biopsy for an aCGH-based PGT diagnosis allows for a more reliable diagnosis as on average a TE sample contains 5-10 cells.
- It is recommended to use a WGA protocol which is compatible with the specific aCGH platform that has been used for validation.

Turnaround time

The net aCGH turnaround time from sample processing to comprehensive chromosome analysis is 24 h, although results can be obtained within 8-12 h. However, each laboratory needs to validate whether shorter hybridisation times affect hybridisation efficiency.

Documentation

Relevant laboratory documentation should include:

- a patients' karyotype, preferably at high resolution (550-800 bands)
- a report on any previous unbalanced products of conception;
- genetic counselling report with possibly a recommendation for PGT, an indication of the testing method and the benefits and the limitations of the test;
- the informed consent of the couple with risk assessment and indication of test limitation.

2.1.1.b. NGS

NGS protocol

The sequencing by NGS protocol comprises five steps: (i) sample processing; (ii) initial quality analysis; (iii) library preparation; (iv) sequencing; and (v) data analysis.

The sample processing and sequencing generally includes any or all of the following processes: handling of biopsy samples (PB, single blastomere or TE cells); cell lysis; barcoding (molecular indexing) of samples; adapter ligation; amplification; library preparation; flow cell loading; and generation of sequence reads. It is recommended to perform initial quality analysis of DNA; contamination of starting material can lead to poor sequencing data quality.

DNA sequence generation by NGS platforms is almost entirely automated and the output consists of millions to billions of short sequence-reads. Raw data produced after sequencing are further processed by computational analyses and bioinformatics using a variety of algorithms to map and align the short sequence reads to a linear reference human genome sequence.

As these processes may vary depending on the platform, it is recommended to optimise and validate each step individually (including the entire wet-bench process as well as the bioinformatic analyses) to empirically determine optimal assay conditions and analysis settings.

For each platform, the ***genome coverage, average read depth*** and minimum number of reads should be defined with validation experiments (see pre-examination process section).

Turnaround time

The turnaround time of NGS (from DNA amplification to reporting) can vary according to the platform, but currently it is at least 12 h. Turnaround time is expected to significantly decrease in the future.

With the aim of accumulating samples for an NGS run, biopsy samples can be stored short-term (weeks), and WGA samples can be stored long-term (years) at -20˚C or -80˚C.

Documentation

Relevant laboratory documentation should include:

- a patients' karyotype, preferably at high resolution (550-800 bands), if available with verified breakpoints from an accredited/certified cytogenetics laboratory; often, the rearrangement breakpoints are defined based on GTG-banded chromosomes and as the resolution of this technique is quite low, there is a potential risk that the actual translocation segments are (much) smaller than expected and hence the probability of detection of all the unbalanced segregation products of the structural rearrangement (much) lower;
- a report on any previous unbalanced products of conception;
- genetic counselling report with possibly recommendations for PGT, an indication of the testing method and the benefits and the limitations of the test;
- the informed consent of the couple with risk assessment and indication of test limitation.

2.1.2. Laboratory infrastructure, equipment and materials

Information on infrastructure, equipment and materials for aCGH and NGS as presented in the sections "Array-based PGT-SR" and "Next generation sequencing", respectively.

2.1.2.a. aCGH

Infrastructure

- To prevent carry-over of amplified DNA, the laboratory space should be divided into pre- and post- amplification rooms that are physically separated.
- Preferably the pre- and post-amplification rooms/areas should be equipped with UV-C light for DNA ***decontamination***.
- Positive air-pressure is recommended for the pre-amplification room. When positive and negative pressure rooms are present, they are preferably enclosed by a lock chamber.
- A dedicated set of equipment, consumables and laboratory coats should be used for each designated area and not be exchanged between the pre- and post-amplification rooms.
- Pre-amplification steps should be carried out in a laminar downflow cabinet. The workflow between the pre- and post-amplification areas should be unidirectional, from the pre-amplification room (clean room) to the post-amplification room.
- Constant regulation of environmental conditions (ozone, temperature and humidity) is recommended during all steps to ensure efficient hybridisation results.

Equipment

- Equipment required for WGA and aCGH analysis of biopsied samples includes:
    - a class II safety cabinet, preferably equipped with UV-C light, to prevent contamination of samples at the pre-amplification stage;
    - thermal cyclers with heated lids (one for the pre- and one for the post-amplification room);
    - microcentrifuges (one for pre-amplification, one for all the following stages) and a benchtop swingout centrifuge;
    - a magnetic stirrer, fume cabinet, hybridisation oven/incubator, water bath, gel electrophoresis equipment to check successful amplification and a vortex mixer;
    - a scanner, equipped with the corresponding lasers, to excite the hybridised fluorophores to read and store the resulting images of the hybridisations, placed in the post-amplification room in an atmosphere with low ozone parameters, regulated temperature and protected from daylight and validated and adjusted to the required resolution for the specific PGT protocols.
- The use of a DNA quantification system (to determine the amount of amplified DNA after WGA) and a vacuum concentrator (to reduce the time required to process high numbers of samples) is optional.
- Associated servers should be also allocated in proper conditions and instruments used in critical steps should be UPS-connected.
- It is recommended that prior to each step of the protocol, the temperature ranges and/or pH values of equipment and solution are verified. Specific temperature and thermocycler programmes should be validated in individual PGT centres for all

equipment, and instruments should be serviced and calibrated regularly to ensure accuracy.

- Software for automatic calling of structural aberrations is not always available and therefore segmental aneuploidies need to be manually called by the operator.

Materials

Materials required for WGA and aCGH analysis of biopsied samples include:

- cell lysis, pre-amplification, amplification enzymes and buffers specific to each amplification method used;
- DNA labelling reaction buffers, enzymes, dNTPs and fluorophore-marked dUTP that should be used under minimal light exposure since they are light sensitive;
- hybridisation and washing buffers, human Cot-1 DNA, and DNase/RNase-free distilled water;
- microarray slides.

2.1.2.b. NGS

Infrastructure

General aspects on infrastructure are covered in the introduction of this section (E.1.2.) and in section aCGH (2.1.2.a.).

Equipment

NGS platforms differ, among others, in price, capacity, chemistry and read length. Initial set-up of an NGS system should follow manufacturer's instructions and it is recommended to collaborate with the manufacturer to ensure that the laboratory space has been optimised to meet the requirements. In addition, it is recommended to involve informaticians with relevant expertise to make sure all required elements (hardware, servers, data storage, internet) are in place.

NGS-based PGT requires the following equipment:

- A DNA quantitation instrument; it is crucial to accurately determine the amount of starting DNA for library preparation. There are several options that give highly accurate quantitation of low amounts of DNA. Amongst those is the Qubit high-sensitivity double-stranded DNA (HS dsDNA) fluorometer, which measures dsDNA. HS dsDNA has been found to give a much more accurate estimation of the amount of DNA present in the sample, compared with standard spectrophotometry. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. It is recommended to use DNA with absorbance ratio values ranging from 1.8 to 2.0.
- Thermocyclers–DNA amplification and labelling are necessary steps during the library preparation, therefore requiring the use of a thermocycler.
- Pipettors or pipetting robots–dedicated multi-channel and single-channel pipettes are a necessity for NGS.
- Multichannel pipette or automated systems are recommended to minimise the risks of mislabelling or misallocation of samples during the different steps of the protocol.
- Sequencers should be allocated in a specifically designed room, with modulated light exposure and regulated temperature according to manufacturers´

instructions. Associated servers should also be kept under proper conditions and instruments used in critical steps should be UPS-connected.

- Sequencers should be validated for the specific PGT protocols and incorporate the latest version of the specified software, allowing proper performance of the PGT protocol.
- It is recommended that prior to each step of the protocol, the temperature ranges and or pH values of equipment and solution are verified. Specific temperature and thermocycler programmes should be validated in individual PGT centres for all equipment, and instruments serviced and calibrated regularly to ensure accuracy.
- Software for automatic calling of structural aberrations is not always available and therefore segmental aneuploidies need to be manually called by the operator.

Materials

For all reagents employed in the different steps of the protocol, the lot numbers and expiration dates should be recorded.

Depending on the manufacturer, NGS kits may include one or more of the following constituents:

- cell lysis and DNA extraction media; lysis buffer and specific enzymes for DNA extraction;
- DNA amplification media: some WGA protocols are PCR-based whereas others are not, and it is recommended to use a WGA protocol which is compatible with the specific NGS platform has been validated;
- library preparation media: although many methods are available, some preparation procedures are specific for a particular NGS platform, and therefore, it is recommended to pay attention to the compatibility of the libraries with the sequencing platforms.

2.1.3. Work practice controls

Information on identification and witnessing for aCGH and NGS is as it is presented in the sections "Array-based PGT-SR" and "Next generation sequencing", respectively.

2.1.3.a. aCGH

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell (s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifiers plus the embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing (see also the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020). Witnessing is also indicated at the following steps of the aCGH procedure:
    - at the start of the WGA procedure to ensure that the correct volume of PCR master mixture is loaded into each tube;
    - at the start of the labelling procedure to ensure that the correct volume of labelling mixture is loaded into each tube;

- at loading of the labelled DNA samples on array slides to ensure that each sample matches the sample identifier on the slide;
- and when recording aCGH results to ensure that aCGH files correspond to the correct cell and/or embryo.

Use of intra-assay controls for aCGH

Information on using intra-assay controls for aCGH as it is presented in section "Array-based PGT-SR"

- Suitable positive controls are not readily available (i.e. unbalanced single human blastomeres, TE cells or other cell types to represent unbalanced human blastomeres or TE cells).
- Negative controls serve to confirm that no contamination is present in the 'no-template' tube, which does not confirm the absence of contamination for the rest of reaction tubes carrying the biopsied samples.
- Diluted genomic DNA is recommended for positive intra-assay controls to check successful amplification of single or few cells and a successful reaction, respectively.
- Negative controls with sample collection buffer, biopsy media, or washing media (based on the protocols of the PGT centre) are recommended to control for contamination for each biopsy sample cohort (i.e. the IVF laboratory negative control).
- A minimum of one negative control with amplification mixture only is recommended to control for contamination during setting up of amplification reactions. (i.e. the genetic laboratory negative control).

2.1.3.b. NGS

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell(s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifier and embryo/cell number be witnessed and signed off by two operators during biopsy, sample collection and genetic testing (see above). Witnessing is also indicated at the following steps of the WGA/NGS procedure:
    - at the start of the WGA procedure to ensure that the correct volume of PCR master mixture is loaded into each reaction tube;
    - at the start of the library preparation to ensure that embryo identification corresponds with a dedicated barcode or index primers;
    - at pooling, to make sure that all barcoded libraries are included in the pool before the start of the NGS run;
    - during NGS run preparation; data input for each sample should be checked to ensure that samples match their identifier on the plate.

Use of intra-assay controls for NGS

- For intra-assay control in each routine test, it is recommended to use negative and positive controls in the same NGS run with separate barcodes with the aim to monitor if the section has contamination or amplification failure.
- It is recommended to perform intra-assay control using isolated samples composed with single cells containing known whole-chromosome aneuploidies diagnosed with a previously validated technique.

2.1.4. Pre-examination process

Information on test efficiency materials for aCGH and NGS is as it is presented in the sections "Array-based PGT-SR" and "Next generation sequencing", respectively.

2.1.4.a. Internal quality control

- Effective resolution of the aCGH and NGS platform and protocol should be internally validated in each laboratory prior to clinical application for PGT-A.
- It is recommended to validate aCGH and NGS for aneuploidy testing with a series of positive controls that should include DNA from:
  - single cells from cell lines with established numerical copy number changes (aneuploidy);
  - previous aneuploid pregnancies, when available;
  - blastomeres or TE biopsies isolated from donated embryos from previously performed PGT-A cases analysed with an established technique, when available. Pre-clinical testing on polar bodies is not straightforward, as it would deprive the couple of valuable embryos that could also be used for clinical treatment.
- It is recommended to determine false-negative, false-positive, specificity and sensitivity rates of the specific platform to be used.
- When using aCGH and NGS for aneuploidy testing in TE biopsy samples, the possibility of misdiagnosis due to chromosomal mosaicism represents the main issue relating to copy number variation (CNV) and log2 ratio value threshold detection by NGS and aCGH, respectively.
- It is recommended to perform validation studies with true aneuploid and euploid cell lines and mosaic models by using cell mixtures (ratios from 10% to 90%) to establish thresholds for chromosomal mosaicism detection rates (i.e. the minimum ratio of aneuploid to euploid cells that is needed to detect a chromosomal copy number variation) and quantification of mosaicism levels. After statistical analysis, the results of these experiments can be used as a reference to determine the mosaicism level of analysed samples. In the first step of the validation process, it is recommended to analyse a wide number of euploid samples (including six to eight cells from euploid cell lines), in order to determine the standard deviation from the euploidy baseline value (two chromosome copy number and log2 ratio for NGS and aCGH, respectively) and thus define the 'euploidy'-threshold values. Similarly, threshold values should be defined for ***trisomy*** and ***monosomy***.
- It is recommended to test replicates of the same DNA sample to perform accuracy and variability assessment in independent aCGH experiments and NGS runs.
- To mimic a blastocyst biopsy, a sample size of 8-10 cells is recommended for all mosaicism cell mixture models. Although validation experiments will set euploid/aneuploid parameters, it is important to mention that limitations still exist when analysing biopsy samples with few cells, where it will be almost impossible to detect changes that represent less than 20-30% of the biopsy.

- In order to define the detection threshold, the quality (intrinsic DNA sample quality, QC) of the experiments, the noise and technical artefacts should also be considered.
- As different chromosomes might have a different resolution, the series of aneuploid samples should represent the range of aneuploidies that the test is required to detect.
- Sensitivity and specificity of the mosaicism detection specifically apply for each aCGH and NGS platform (hardware and protocol for WGA or library preparation for NGS) and software or bioinformatics paradigm used to analyse the data. These cannot be exchanged among platforms.
- During the validation of aCGH and NGS for PGT-A, ***de novo segmental chromosome aberrations*** are also encountered.
- It is recommended to establish the true resolution and specificity of the aCGH and NGS platform to detect segmental aneuploidy through a validation study

2.1.4.b. Test efficiency for aCGH

- To check for amplification efficiency, it is recommended that samples and intra-assay controls (if used) be put on an agarose gel and/or quantified by Qubit Fluorometer.
- The use of male and female reference DNA is recommended to assess hybridisation efficiency and interpret the results. Marked X/Y chromosome separation is indicative of a successful experiment in gender-mismatched samples, and the corresponding levels of gain for the X chromosome and loss for the Y chromosome are used as a reference to evaluate aneuploidy events for the autosomes.
- Gender-matched samples must show consistently no change on chromosome X or Y and none of the probes in the array should report a change.
- Negative amplification, negative intra-assay control or failed hybridisation should show a consistent noisy profile where no significant pattern is observed.
- Storage time and temperature have an impact on the integrity of cells, DNA and/or solutions and laboratories should validate that the conditions used in their protocols are fit for purpose. Furthermore, it is not recommended to use repeatedly frozen-thawed solutions containing DNA or enzymes.
- Hybridisation bias due to drying out of the microarray surface could lead to signal loss, degradation of fluorophore-marked dUTP and suboptimal scanned images.
- It is recommended to stringently wash the aCGH slides with minimum light exposure and under controlled ozone concentration, temperature and humidity. The use of laboratory carbon-loaded non-woven filters is recommended in case of high ozone levels.
- It is recommended to avoid the use of detergents to clean the wash equipment, as this may interfere with signal intensity.
- Washing and scanning of slides in small batches (2-3 slides) is recommended to minimise the exposure of slides and of labelling dyes to air.
- It is critical that slides are dried by centrifugation shortly after the final washing step, to avoid drying through evaporation.
- Scan images should have defined features with red and green images well registered and the colours evenly balanced.
- The assay signal to background noise ratio (SBR) should be sufficiently high for the log2 ratio change to be observed. In case of low SBR, additional washing of the slides and rescanning are acceptable.
- It is recommended to calculate the acceptable and optimum ranges of QCs for every array experiment. The QC measures of array data for every experiment are extrapolated by specific software and are indicative for the successful calling of all

target probes. The QC measures will vary between array types and different scanners.

2.1.4.c. Test efficiency for NGS

For amplification efficiency checking, gel electrophoresis is recommended for samples and intra-assay controls using proper standards.

- It is recommended that the WGA procedure be performed in the same tube that the sample was collected in.
- After preparation the library should be quantified and normalised for each sample before creating the library pool.
- It is recommended to have high coverage for the region of interest and ascertain that the expected translocation is covered by a sufficient number of sequenced fragments.
- Sequencing by NGS comprises a series of steps that uniquely contribute to the overall quality of the data set. Thus, each individual step needs to be controlled to ensure high-quality results.
- NGS run parameters (coverage, number of reads, noise) should be monitored before the analysis of raw sequencing data to ascertain that the overall and individual run parameters for each sample correspond to the platform-specific required criteria. These sequencing quality metrics can provide important information about the accuracy of each step in this process, including library preparation, base calling, and read alignment.
- From the total number of reads, 70-80% should align to the genome. Lower percentages indicate contamination in the DNA sample, degraded DNA or suboptimal WGA.
- Each run should have an acceptable, previously established level of noise. It is recommended to perform an internal validation to establish a test-specific threshold for the overall noise value.
- Various amplification protocols are in use, which may be affected by single cell artefacts, such as allele drop out (ADO), amplification bias or allele drop in (ADI), that might affect the accuracy of the diagnostic test, and therefore extensive validation of WGA is required.
- It is recommended to calculate the acceptable and optimum ranges of QCs for every NGS experiment. The QC measures of NGS data for every experiment are extrapolated by specific software and are indicative for the successful calling of all target DNA sequencing. The QC measures will vary between NGS platforms and different software versions.

2.1.5. Preclinical work-up and report

Information on preclinical work-up and report related to aCGH and NGS is presented as in the sections “Array-based PGT-SR” and “Next generation sequencing”, respectively.

2.1.5.a. Preclinical work-up

Case-specific preclinical work-up or specific genetic documentation is not required when performing aCGH and NGS for aneuploidy testing (high-risk and low-risk).

Preclinical work-up aCGH

- Karyotype reports should be obtained for both partners from an accredited/certified cytogenetics laboratory.
- A case-specific work-up is not required when performing aCGH for structural rearrangements, unless the carrier has an unbalanced karyotype.

- It is recommended to upfront ensure that all unbalanced products of the specific rearrangement can be identified with the platform used. The ability to detect an unbalanced product depends on the effective resolution and the coverage of the array used. This needs to be established prior to clinical application by using DNA from cell lines with well-established segmental aneuploidy to validate the presence and the number of all (consecutive) clones/probes representing the respective chromosome regions.
- It is acceptable that three out of four segments for two-way reciprocal translocations are detected to reliably identify unbalanced segregation products

Preclinical work-up for NGS

- It is recommended to check whether the chromosomal segments involved in the rearrangement are adequately covered, in terms of the number of sequence reads.
- Parental karyotypes may facilitate testing and genetic counselling.
- It is acceptable that at least three out of four segments for two-way reciprocal translocations can be detected to reliably identify unbalanced segregation products.
- It is acceptable to adjust the lower detection limit provided by the platform's manufacturer, based on a feasibility study using DNA from previous unbalanced products of conception.

2.1.5.b. Preclinical work-up report

A case-specific preclinical wet-laboratory work-up report is not required for aCGH and NGS.

2.1.6. Risk assessment

Information on risk assessment related to aCGH and NGS is presented as in the sections "Array-based PGT-SR" and "Next generation sequencing", respectively.

2.1.6.a. Risk assessment aCGH

Risk assessment should cover:

- risks caused by errors in sample tracking,
- risks caused by handling biopsy samples prior to aCGH analysis (tubing, washing) that, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA;
- risk of inconclusive or false results due to suboptimal experimental conditions,
- risk of inconclusive or false results due to biological reasons: (i) unbalanced segregations may arise from crossing-over during meiosis I in the gametes of the carrier of the rearrangement; (ii) chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype; (iii) embryos of poor morphology are at risk of containing cells with degraded DNA;
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is below the resolution of the test, whether biological or caused by a technical error.

2.1.6.b Risk assessment NGS

Risk assessment should cover:

- risks caused by errors in sample tracking,

- risks caused by handling biopsy samples prior to NGS analysis (tubing, washing) which, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA;
- risk of inconclusive or false results due to suboptimal experimental conditions at WGA or due to high background noise or low coverage;
- risk that the size of the deletion or duplication is different from the one based on the karyotypes in the parents, and therefore they may remain undetected by the NGS protocol (if they are below the resolution of the test);
- risk of misinterpretation of the actual embryo karyotype due to the presence of chromosomal mosaicism, either at cleavage-stage or at blastocyst stage.

Additional issues related to aCGH and NGS for PGT-A :

- The clinical significance of transferring embryos with mosaicism and/or *de novo* segmental abnormalities (full or in mosaic state) is under current investigation and therefore unknown. The transfer of such embryos could potentially carry a risk of first-trimester miscarriage or of a viable unbalanced offspring.
- aCGH and NGS can detect chromosomal mosaicism and segmental aneuploidies. However, both biological limitations and technical artefacts may affect the accuracy of the test and this should be discussed during patient counselling.
    - Biological limitations may include non-specific chromosome gain or loss due to cells being in S-phase, the biopsy being non-representative of the embryo, failure to detect chromosomal mosaicism due to non-disjunction, and apoptotic or dead cells in the biopsy sample that can generate profiles resembling mosaicism.
    - Technical artefacts may include WGA artefacts, contamination, cells damaged during biopsy and cell lysed during tubing.

2.1.7. Limitations of the test

- aCGH and standard NGS cannot reliably detect all variants of polyploidy (they can detect polyploidy with unbalanced sex chromosome ratios as 69,XXY and 69,XYY) and haploidy.
- The currently used aCGH platforms for PGT-A are unable to detect small microdeletions or microduplications, such as the 22q11.2 microdeletion syndrome (DiGeorge/velocardiofacial syndrome).
- Due to the intrinsic nature of chromosomal mosaicism, the chromosomal make-up achieved from a biopsy only may represent a picture of a small part of the embryo and may not necessarily reflect the chromosomal content of the entire embryo. Also, the mosaicism level inferred from a multi-cell TE biopsy might not unequivocally represent the exact chromosomal mosaicism percentage of the TE cells or the ***inner cell mass*** constitution.
- NGS and aCGH are currently able to detect mosaicism down to 20%-30% when no noise is present in the sample and after proper validation. Array-based PGT analysis is less sensitive to detect mosaicism than NGS.
- As the number of cells in a TE biopsy is unknown, the exact level of mosaicism in the sample cannot be determined.
- aCGH cannot analyse aneuploidy and gene defects simultaneously, whereas NGS can.

- Based on the embryo biopsy, aCGH cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy whereas genotyping-based NGS can provided phasing references are available.
- Noisy profiles are difficult to evaluate and to appropriately score the chromosome copy number.

3. Strengths and limitations

Technical strengths and limitations of aCGH and NGS (without genotyping) are outlined in Table E.I.

The most important limitations include:

- Based on the embryo biopsy alone, aCGH and NGS cannot discriminate between samples carrying the rearrangement (i.e. balanced) and those that are not (i.e. normal) and this should be clearly stated in the report. Although there is no expected difference in the phenotype of embryos with a 'normal 'or a 'balanced' karyotype, many couples wish to know whether the structural rearrangement is being transferred to their offspring to be aware of possible future reproductive problems related to the rearrangement. When polar bodies are used for PGT analysis, discrimination between oocytes carrying the rearrangement and those that are not, is feasible.
- aCGH cannot, but NGS can analyse aneuploidy and gene defects simultaneously in the same diagnostic sample.
- Based on the embryo biopsy alone, aCGH and NGS without genotyping cannot identify the nature (meiotic or mitotic) nor the parental origin of aneuploidies. When polar bodies are used for PGT analysis, inferred errors in the oocyte are always of maternal, meiotic origin.
- Based on the embryo biopsy alone, , aCGH and NGS without genotyping cannot detect UPD.

Table E.I. Overview of the strengths and limitations of the methods applied for PGT-A

	PGT-A	
	aCGH	NGS (without genotyping)
NUMBER OF CHROMOSOMES	All 24 chromosomes analysed.	All 24 chromosomes analysed.
MINIMAL RESOLUTION	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software
WHOLE PLOIDY CHANGES	Not all variants of ***polyploidy*** and haploidy can be detected.	Not all variants of polyploidy and haploidy can be detected.
NO CONCLUSIVE RESULTS	As a result of cell lysis during tubing, cells with degraded DNA, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.	As a result of cell lysis during tubing, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.
ABNORMALITIES NOT DIAGNOSED	aCGH-based PGT-SR diagnosis of biopsied material from cleavage stage or blastocyst embryos does not allow for a distinction between embryos with a normal or a balanced karyotype.	NGS-based PGT-SR diagnosis of biopsied material from cleavage stage or blastocyst embryos does not allow for a distinction between embryos with a normal or a balanced karyotype.
MOSAICISM RELATED ISSUES	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.
UNIPARENTAL DISOMY (UPD)	aCGH analysis does not allow for the detection of UPD.	NGS analysis does not allows for the detection of UPD.
RISK OF MISDIAGNOSIS	Contamination with remaining cumulus cells after ICSI.	Contamination with remaining cumulus cells after ICSI.
IMPACT OF BIOPSY ON TEST RESULTS	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.
SIMULTANEOUS DETECTION OF CHROMOSOME COPY NUMBER AND SINGLE GENE DISORDER(S)	Not feasible.	Feasible.
ORIGIN OF ANEUPLOIDY	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy when based on the analysis of biopsied material from cleavage stage or blastocyst embryos	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy when based on the analysis of biopsied material from cleavage stage or blastocyst embryos

4. Examination process

Clinical testing protocols should include scoring criteria and reporting procedures as well as a framework for counselling patients in the presence of diagnostic results.

General recommendations on the PGT examination process as included in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020):

- The examination process and reporting of results, must comply with local guidelines or law, or with the ISO 15189 standard.
- Before starting a clinical PGT cycle, relevant documents should be available, labelling of samples should be checked, and genetic counselling provided to the couple.
- It is recommended that the PGT laboratory has clearly documented procedures for all steps of the examination process (explicit instructions and a summary of validation results) and release of results (diagnosis, reporting, embryos transfer policy). These procedures are preferably covered in a service-level agreement between the PGT and IVF centres.
- Many different methods have been published and all appropriately validated methods are acceptable for clinical cases. The method used should have been previously implemented, tested and validated in the PGT centre.

4.1. Scoring of clinical results

- It is recommended that results are reviewed and signed or electronically validated by a suitably qualified person (name, qualification, date).

4.1.1. aCGH and NGS results

- Software analysis and copy number scoring criteria should be established in a written protocol and adhered to for the interpretation of whole chromosome and segmental-chromosome gains and/or losses.
- Interpretation of raw data or profiles resulting after specific software analysis by a single observer is acceptable. Additional confirmation by an independent observer is recommended.
- All files resulting from the scanning and sequencing, as well as profiles after specific software analysis should be stored and filed for QC purposes.
- 'No result rescue' for embryos without a clear diagnosis is acceptable. This could imply a second analysis of the existing WGA as well as a second biopsy followed by WGA, full aCGH/NGS processing and analysis.
- When there is a combination of chromatid gain/loss in the first polar body which is balanced by the second polar body, a normal chromosome copy number in the corresponding oocyte is predicted and reported, and the resulting embryo can be recommended for transfer.

4.2. Issuing a PGT report

General items required in PGT preclinical work-up or clinical cycle reports as listed in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020) include:

The PGT clinical cycle report contains interpretation of the clinical results and guidance on which embryos are genetically transferable. The same recommendations apply as specified for the preclinical work-up report (see section "Preclinical work-up"), together with the following items:

  - unique cycle/treatment code;
  - date of oocyte retrieval;
  - date of biopsy;
  - date of biopsy sample arrival in the laboratory;
  - information on the sample type (including number of samples and controls);
  - unique ID number for each cycle and/or biopsy sample tested;
  - indication for PGT.
- When scoring results from PB testing, it is recommended to report what was detected in each polar body and then infer the oocyte diagnosis. It is recommended to test both PBs.
- When scoring results from blastomere/TE testing, it is recommended to report what was detected in the sample and then infer the embryo diagnosis.
- When results are reported from 'pooling' of embryos, it is advisable to refer to each oocyte and sample collection date and clearly differentiate the embryo number between cycle/treatment.
- Reporting of clinical results to the IVF centre must follow local regulations or international accreditation guidelines, including GDPR.
- The embryo transfer policy should be agreed upon between stakeholders (IVF centre, genetic centre, genetic counsellors, clinicians and patients). In case of PGT-A in addition to PGT-M or PGT-SR, it is crucial that the centre has a clear policy on embryo (ranking and) transfer.
- A written or electronic report should be securely transmitted to the IVF centre to ensure transfer and/or cryopreservation of the correct embryos. Results should not be communicated orally.
- Reporting time should be kept as short as possible, and when fresh transfer is intended, reporting time should be adapted to allow the IVF centre to organise the embryo transfer.
- It is recommended that the report is clear, concise, accurate and easily understandable by non-geneticists.
- It is recommended that the overall result and interpretation (including transfer recommendation) are presented per embryo, preferably in tabulated form. Sufficient information for genetic counselling should be included, such as the chromosome(s) involved, chromosome band(s)/nucleotides, the size of the chromosomal aberration in Mb, and the correct identification of the genetic variant. Where applicable, the latest version of the international system for human cytogenetic nomenclature (ISCN)/Human genome variation society (HGVS) nomenclature can be used.
- In case of no diagnosis and re-biopsy to try and obtain a result, this should be included in the report.
- The final clinical cycle report must be signed by appropriately qualified (authorised) personnel (name, qualification, date).
- It is recommended that the clinical cycle results are discussed with the couple before embryo transfer.
- It is recommended that the report is stored in the patient file in the PGT centre, according to local regulations.
- It is recommended to include a disclaimer in the report to address limitations of the test and any other information that may be of significance to the addressee.
- It is acceptable to indicate in the report the need for prenatal testing to confirm the result in case of pregnancy.

The ISCN reporting is acceptable for PGT-A. It is recommended to add the following technical or interpretation items to the clinical report.

- If the profile is noisy or QCs are not sufficient, re-analysis is acceptable to try and obtain a result and this should be included in the report to the IVF centre.
- In the absence of any amplification or when contamination is suspected, rebiopsy is acceptable to try and obtain a result and this should be included in the report to the IVF centre.
- Each centre should decide whether or not to report mosaicism based on internal validation and recent literature.
- The clinical significance of transferring mosaic embryos is currently unknown. The centre's policy about the identification and transfer of embryos with mosaicism or segmental aneuploidy should be documented and shared with the patient during genetic counselling.
- In case of an embryo with chromosomal mosaicism or segmental aneuploidy, genetic counselling should be offered to the couple and if transfer is decided and pregnancy occurs, it should receive appropriate follow-up (ESHRE PGT Consortium Steering committee. *et al.*, 2020 section Follow-up of PGT pregnancies and children).

5. Post-examination process

Recommendations on PGT follow-up, baseline IVF live birth rates for PGT and misdiagnosis as covered in the paper on organisation of PGT (ESHRE PGT Consortium Steering committee. *et al.*, 2020):

5.1. PGT cycle follow-up

For quality purposes, it is recommended to confirm the PGT diagnosis on a subset of embryos not transferred or cryopreserved following diagnosis, in line with local regulations. Such confirmation aims to provide internal ***quality assurance (QA)*** as well as accurate and up-to-date misdiagnosis rates to prospective PGT patients. It is recommended that this is performed on as many embryos as is practicable. It is acceptable to perform this periodically.

When a pregnancy ensues following PGT testing, it is recommended that parents are (again) made aware of the chance and risks of a misdiagnosis and be informed on the possibilities for prenatal testing. PGT and IVF centres should make special efforts to follow-up with the parents following prenatal testing or birth, especially if confirmatory testing is not possible.

Follow-up data should be used for both internal ***quality control (QC)*** and QA purposes and documented in the ESHRE PGT Consortium online database for international data collection.

It is recommended that laboratories follow local regulations or accreditation schemes on storage of clinical samples and patient records. If no local regulations or guidelines exists on storage of clinical samples and patient records, it is recommended as follows.

- If embryos have been transferred and/or frozen, all relevant material (e.g. DNA amplification products) from the case should be retained and appropriately stored. Samples should be stored for at least 1 year. Prolonged sample storage could be considered, taking into account the availability of information on delivery and the duration of embryo cryopreservation.

- If there is no genetically suitable embryo for transfer or cryopreservation, it is not necessary to keep the samples.

- If there is no pregnancy after transfer of all genetically suitable embryos, samples can be discarded.

5.2. Misdiagnosis rate

- It is recommended that each PGT centre performs a prospective risk analysis in order to prevent and/or eliminate possible causes of ***misdiagnosis***.
- It is recommended that misdiagnosis rates should be calculated for each type of method and for all methods from a particular centre. Misdiagnosis rates include those clinical cases in which affected pregnancies arose and cases for which re-analysis results were discordant with the biopsy result.
- It is recommended that confirmatory testing should be performed at least periodically as a QA.
- It is recommended that the published and in-house estimates of misdiagnosis rates should be available on request to prospective patients along with pregnancy rates and live birth rates, to allow informed consent for PGT.
- Following a misdiagnosis, the IVF/PGT centre should investigate the possible causes of the misdiagnosis and make changes to protocols to eliminate the risk in the future. Many of the causes of misdiagnosis are avoidable by taking preventive actions and following the principles of quality management.
- Misdiagnosis should be reported, for instance through the ESHRE PGT Consortium online database.

5.3. Baseline IVF live birth rates for PGT

- Setting appropriate baseline live birth rates should be left up to the individual centres. However, it is recommended that each IVF centre should compare PGT live birth rates and matched non-PGT [routine IVF or ***intracytoplasmic sperm injection (ICSI)***] live birth rates within that IVF centre.
- Comparison of live birth rates with those reported by the ESHRE PGT Consortium or comparable peers can also be carried out to set benchmarks for continual improvement of the PGT centre.

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ANNEXES

ANNEX 1: WORKING GROUP MEMBERS

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Declaration of interest

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.

Dr. Zuccarello reports personal fees from Igenomix Italia, outside the submitted work.

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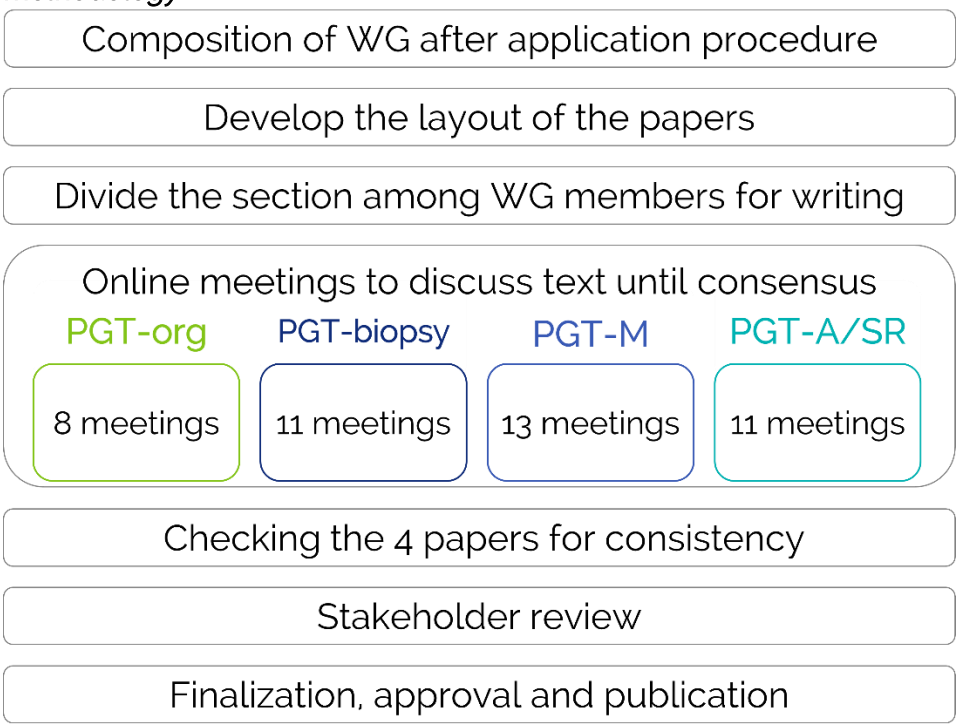
ANNEX 2: METHODS

The current papers were developed according to the published methodology for ESHRE Recommendations for good practice papers (Vermeulen *et al.*, 2019). The PGT-Steering Committee assessed the previous guidelines (Harton *et al.*, 2011a) and deducted an outline for the current papers. All members of the working group according to their expertise, wrote a section that was later discussed in depth with the entire working group until consensus was reached. 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. Several online meetings were organised for discussion. The final draft of the papers was checked for consistency with the other papers of the series. The draft were then submitted for stakeholder review; they were published on the ESHRE website between 10 June and 11 July 2019, and ESHRE members were invited to send in comments. All comments were checked by the PGT-Steering Committee and/or working groups, discussed in an online meeting, and incorporated in the final version where relevant. A review report is published on the ESHRE website. The list of experts that participated in the stakeholder review is available in Annex 5.

For easier use of the recommendations, terms in bold and italic are explained in a glossary (Annex 3: Supplementary table 1) and abbreviations are listed (Annex 4: Supplementary table 2).

Overview methodology



ANNEX 3:
SUPPLEMENTARY TABLE I. GLOSSARY

This glossary defines the most important terms used in the PGT papers and was developed facilitate understanding of the recommendations. Where appropriate, definitions were used as previously published, sometimes with adaptations to the context of PGT. Other definitions were derived from explanations within the context of the recommendations papers.

Term	Definition	Reference
Accuracy	a conclusive result on the status of the cell, even with the presence of ADO/ADI	
Advanced maternal age (AMA)	Maternal age above 35 years	
Amplification bias	Artefactual duplications or deletions due to local concentration differences in guanine and cytosine bases (% G+C bias), as well as the prevalence of chimeric DNA molecules, allele drop-outs (ADOs), preferential allelic amplifications and nucleotide misincorporations, during amplification varying substantially between different WGA approaches. A major challenge in single-cell genome analysis is discriminating such WGA artefacts from genuine genetic variants present in the cell before WGA.	(Vermeesch *et al.*, 2016)
Allele drop out (ADO)	The failure to detect an allele in a sample or the failure to amplify an allele.	(Vermeesch *et al.*, 2016)
Allele drop in (ADI)	An allele which is detected but is not actually part of the genotype.	
Assisted hatching	An ART procedure in which the zona pellucida of an embryo is either thinned or perforated by chemical, mechanical or laser methods.	(Zegers-Hochschild *et al.*, 2017)
B allele frequency (BAF)	The signal intensity of the B allele over the total signal intensity (A+B) for an SNP	
Blastocoele	Fluid-filled inner cavity of the blastocyst	Adapted from (Zegers-Hochschild *et al.*, 2017)
Blastocyst	The blastocyst contains a fluid filled inner cavity (blastocoele), an outer layer of cells (trophectoderm) and an inner group of cells (inner cell mass). This stage of preimplantation embryo development occurs around Day 5–6, and occasionally Day 7, after ICSI	Adapted from (Zegers-Hochschild *et al.*, 2017)
Blastomere	A cell from a cleavage-stage embryo	Adapted from (Zegers-Hochschild *et al.*, 2017)
Cleavage-stage embryos	Embryos beginning with the two-cell stage and up to, but not including, the morula stage	(Zegers-Hochschild *et al.*, 2017)
Cryopreservation	The process of slow freezing or vitrification to preserve biological material (e.g. gametes, zygotes, cleavage-stage embryos, blastocysts or gonadal tissue) at extreme low temperature	(Zegers-Hochschild *et al.*, 2017)
***De novo* segmental chromosome aberration in embryo**	A segmental or partial chromosome deletion/ duplication arising in embryos of couples with normal karyotypes	
Decontamination	The process of removing or neutralising contaminants	(European Directorate for the Quality of Medicines and

		HealthCare (EDQM), 2017)
Denudation	The removal or stripping of the cumulus and corona cells from the oocyte	Adapted from (European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Sequencing read depth	A crucial parameter in sequencing referring to the number of times a particular read was found independently at a given genomic position	
Electronic witnessing system	Any automated electronic system that assists the traceability of the gametes/embryos in an IVF centre	
Embryo / oocyte biopsy	The removal of polar bodies, blastomeres or trophectoderm cells from the embryo for the purpose of genetic analysis	Adapted from (European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Diploidy/euploidy	The condition in which a cell has two haploid sets of chromosomes. Each chromosome in one set is paired with its counterpart in the other set. A diploid embryo has 22 pairs of autosomes and two sex chromosomes, the normal condition	(Zegers-Hochschild *et al.*, 2017)
Exclusion testing	An optional add-on to genetic testing, where DNA from parents and grandparents is compared with the DNA of the embryo or foetus. Exclusion testing means that the at-risk parent does not have to have a presymptomatic genetic test to have disease-free children	Adapted from https://huntingtonstudygroup.org/glossary/exclusion-testing/
Freeze-all cycle	An ART cycle in which, after oocyte aspiration, all oocytes and/or embryos are cryopreserved, and no oocytes and/or embryos are transferred to the uterus of a woman in that cycle.	(Zegers-Hochschild *et al.*, 2017)
Genome coverage	The percentage of the target genome covered (in amplification or in sequencing)	
Hatching	The process by which an embryo at the blastocyst stage extrudes out of, and ultimately separates from, the zona pellucida	(Zegers-Hochschild *et al.*, 2017)
High-order multiple gestation	A pregnancy with three or more embryos or foetuses	(Zegers-Hochschild *et al.*, 2017)
Homoplasmy	A basic genetic state of mitochondria, in which all of the hundreds to thousands of mitochondrial (mt)DNA copies within a cell or an individual have the same nucleotide-sequence	(Shibata and Ling, 2007)
Informativity testing	Genotyping of different loci within or flanking the region of interest using DNA from the couple and other relatives prior to segregation analysis for PGT-M preclinical process	
Informed consent	A person's voluntary agreement based upon adequate knowledge and understanding of relevant information, to donate, to participate in research or to undergo a diagnostic, therapeutic or preventive procedure	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Inner cell mass (ICM)	A group of cells in the blastocyst that give rise to the embryonic structures and the foetus, the yolk sac, the allantois and the amnion	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Intracytoplasmic sperm injection (ICSI)	A procedure in which a single spermatozoon is injected into the oocyte cytoplasm.	(Zegers-Hochschild *et al.*, 2017)

In vitro fertilisation (IVF)	A sequence of procedures that involves extracorporeal fertilisation of gametes. It includes conventional in vitro insemination and intracytoplasmic sperm injection (ICSI)	(Zegers-Hochschild *et al.*, 2017)
Log2 of fluorescence ratios	The log2 transformed value of the normalized signal intensity of a SNP. (A logR of 0 and BAF values of 0, 0.5 and 1 represent a normal copy number (n=2). A single copy yields a logR of -1 and BAF values of 0 and 1, whereas three copies yield a logR of 0.58 and BAF values of 0, 0.33, 0.66 and 1)	
Misdiagnosis	When a technical procedure has failed, is inaccurate or has been incorrectly interpreted. Misdiagnoses may be sample- or technique-specific	Adapted from (Wilton *et al.*, 2009)
Monosomy	The absence of one of the two homologous (sex) chromosomes in embryos	Adapted from (Zegers-Hochschild *et al.*, 2017)
Morula	An embryo formed after completion of compaction, typically 4 days after ICSI	Adapted from (Zegers-Hochschild *et al.*, 2017)
Chromosomal mosaicism	A state in which there is more than one karyotypically distinct cell population arising from a single embryo	(Zegers-Hochschild *et al.*, 2017)
Pathogenic variant	A permanent change in the nucleotide sequence, proven to be disease-causing (class 4-5) and usually with a frequency below 1%	(Richards *et al.*, 2015)
Negative control	The IVF laboratory negative control is a negative control with sample collection buffer, biopsy media, or washing media to control for contamination during each step of cell sample collection. The genetic laboratory negative control is a negative control with amplification mixture only to control for contamination during set-up of amplification reactions	
Non-disclosure testing	PGT with non-disclosure of the direct test results to the couple	
Perivitelline space	The space between the cytoplasmic membrane enclosing the oocyte and the innermost layer of the zona pellucida. (This space may contain the first and second polar bodies and extracellular fragments)	(Zegers-Hochschild *et al.*, 2017)
Polar bodies (PBs)	The small bodies containing chromosomes segregated from the oocyte by asymmetric division during telophase. The first polar body is extruded at telophase I and normally contains chromosomes each with 2 chromatids (2c); the second polar body is extruded in response to fertilisation or in response to parthenogenetic activation and normally only contains chromosomes each comprising of a single chromatid (1c)	(Zegers-Hochschild *et al.*, 2017)
Polyploidy	The condition in which a cell has more than two haploid sets of chromosomes (f.x. a triploid embryo has three sets of chromosomes; a tetraploid embryo has four sets)	Adapted from (Zegers-Hochschild *et al.*, 2017)
Preimplantation genetic testing (PGT)	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. These include PGT for aneuploidies (PGT-A), PGT for monogenic/single-gene defects (PGT-M), and PGT for chromosomal structural rearrangements (PGT-SR)	(Zegers-Hochschild *et al.*, 2017)
Quality assurance (QA)	The actions planned and performed to provide confidence that all systems and elements that	(European Directorate for the Quality of Medicines and

	influence the quality of the product are working as expected, both individually and collectively	HealthCare (EDQM), 2017)
Quality control (QC)	The part of quality management focused on fulfilling quality requirements. In terms of preparation, it concerns sampling specifications and testing; for an organisation, it relates to documentation and release procedures, which together ensure that the necessary and relevant tests have actually been carried out and that materials have not been released for use until their quality has been judged to be satisfactory	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Quality management system	The organisational structure, with defined responsibilities, procedures, processes and resources, for implementing quality management, including all activities that contribute to quality, directly or indirectly	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Recurrent implantation failure (RIF)	Three or more failed in vitro fertilisation-embryo transfer cycles involving high-quality embryos	Adapted from (Harper *et al.*, 2010)
Recurrent miscarriage (RM) / Recurrent pregnancy loss	Two or more pregnancy losses before 24 weeks of gestation (including chemical pregnancy)	(The ESHRE Guideline Group on RPL *et al.*, 2018)
Risk assessment	Identification of potential hazards with an estimation of the likelihood that they will cause harm and of the severity of the harm should it occur	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Segregation testing	Phasing or haplotyping (determination of the group of alleles within a genetic segment on a single chromosome being inherited together) after informativity testing to establish the high-risk and low-risk haplotypes	
Severe male factor (SMF)	Sperm sample with a combination of oligospermia (low sperm concentration), asthenozoospermia (poor sperm motility) and/or teratozoospermia (abnormal sperm morphology) or azoospermia	Adapted from (World Health Organisation, 2010)
Sexing	Selection of embryos based on sex	
Standard operating procedure (SOP)	Written instructions describing the steps in a specific process, including the materials and methods to be used and the expected result	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Sub-optimal environmental conditions	Any deviation from the theoretical physiological pH, temperature, oxygen level, or osmolarity	
Supernumerary embryos	Excess embryos after embryo transfer	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Time-lapse imaging	The photographic recording of microscope image sequences at regular intervals in ART, referring to gametes, zygotes, cleavage-stage embryos or blastocysts	(Zegers-Hochschild *et al.*, 2017)
Trisomy	An abnormal number of chromosome copies in a cell characterised by the presence of three homologous chromosomes rather than the normal two	Adapted from (Zegers-Hochschild *et al.*, 2017)
Trophectoderm	Cells forming the outer layer of a blastocyst that have the potential to develop into the placenta and amniotic membranes	(Zegers-Hochschild *et al.*, 2017)

Sample collection	The procedure in which 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	
Tubing	The procedure in which after biopsy, cell(s) are washed and collected in small reaction tubes for amplification-based testing	
Uniparental disomy (UPD)	The presence of two copies of (part of) a chromosome, from one parent and no copies from the other parent	(Vermeesch *et al.*, 2016)
Validation	Documented evidence giving a high degree of assurance that a specific process or system, including pieces of equipment or the environmental conditions, will perform consistently to deliver a product meeting its pre-determined specifications and quality attributes, based on intended use	(European Directorate for the Quality of Medicines and HealthCare (EDQM), 2017)
Vitrification	An ultra-rapid cryopreservation procedure that prevents ice formation within a cell whose aqueous phase is converted to a glass-like solid	(Zegers-Hochschild *et al.*, 2017)
Zona pellucida	The glycoprotein coat surrounding the oocyte and the developing embryo up to the blastocyst stage	Adapted from (Zegers-Hochschild *et al.*, 2017)

ANNEX 4:
SUPPLEMENTARY TABLE II. LIST OF ABBREVIATIONS

Abbreviation	Full term
aCGH	Array-based comparative genomic hybridisation
ADI	Allele drop-in
ADO	Allele drop-out
AF	Amplification failure
AMA	Advanced maternal age
BACs	Bacterial artificial chromosomes
BMI	Body mass index
bp	Base pairs
CNV	Copy number variation
DAPI	4′,6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA
D-ARMS	Double amplification refractory mutation system
dNTPs	Deoxyribonucleotide triphosphates
EQA	External quality assessment
FISH	Fluorescence in situ hybridisation
FM	Flanking marker
GDPR	General data protection regulation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (buffering agent)
HGVS	Human genome variation society
HLA	Human leukocyte antigen
HS dsDNA	High-sensitivity double-stranded DNA
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IQA	Internal quality assessment
ISCN	International system for human cytogenetic nomenclature
IVF	In vitro fertilisation
Mb	Megabases
MDA	Multiple displacement amplification
MOPS	3-(N-morpholino) propane sulphonic acid (buffer)
mtDNA	Mitochondrial DNA
NGS	Next-generation sequencing
PB	Polar body
PCR	Polymerase chain reaction
pg	Picogram
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
PGT	Preimplantation genetic testing
PGT-A	PGT for aneuploidy
PGT-M	PGT for monogenic/single-gene defects
PGT-SR	PGT for chromosomal structural rearrangements
QA	Quality assurance
QC	Quality control

QMS	Quality management system
qPCR	Quantitative real-time PCR
RIF	Recurrent implantation failure
RM	Recurrent miscarriage
SBR	Signal to background noise ratio
SMF	Severe male factor
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
STR	Short tandem repeat (markers)
TE	Trophectoderm
UPD	Uniparental disomy
UPS	Uninterrupted power supply
UV-C	Ultraviolet C
WGA	Whole-genome amplification
ZP	Zona pellucida

ANNEX 5:
SUPPLEMENTARY TABLE III. LIST OF EXPERTS THAT PARTICIPATED IN THE STAKEHOLDER REVIEW

Reviewer Name(s)	Country
Ahmet Berkız TURP	Turkey
Alan H Handyside	UK
Alessandra Alteri	Italy
Alexia Chatziparasidou	Greece
Andreas Schmutzler	Germany
Caio Graco Bruzaca	Brazil
Emmanuelle Kieffer	France
ESHRE Special Interest Group in Psychology and Counseling Steering Committee (submitted by Mariana Moura-Ramos)	na
Frank Broekmans	The Netherlands
Hans Jakob Ingerslev	Denmark
Inge Liebaers	Belgium
Italian Society of Human Genetics	Italy
Joanne Traeger-Synodinos, Thalia Mamas, Christina Vrettou	Greece
Joshua Blazek; Elizabeth Cameron; Inger Britt Carlsson; David Chrimes; Tony Gordon; Mike Large; Colleen Lynch; Beki Sanderson; Kristine McWilliams	USA and UK
Karen Sermon	Belgium
Kersti Lundin	Sweden
Laura Corti	Italy
Lauren Walters-Sen, Swaroop Aradhya, Michelle Strecker, Neha Kumar	United States
M. Cristina Magli. Luca Gianaroli	Italy
Päivi Forsblom	Germany
Raul Piña-Aguilar	USA, Mexico, UK
Ros Hastings, Katrina Rack, PGT Assessors from GenQA	na
Sandrine Chamayou	Italy
Véronique Cottin	Switzerland