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# Preimplantation genetic testing guidelines of International Society of Reproductive Genetics

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## Abstract

The International Society of Reproductive Genetics (ISRG) assembled a workgroup made up of clinicians, clinical laboratory directors, and scientists for the purpose of creating the guidelines for preimplantation genetic testing (PGT). The most up-to-date information and clinical insights for the optimal PGT practice were incorporated in these guidelines. Recommendations are provided for embryologists, medical geneticists, clinical laboratorians, and other healthcare providers to improve the wellbeing of patients seeking assisted reproductive treatment and their offspring.

**Keywords:** Preimplantation genetic testing, Guidelines, International Society of Reproductive Genetics (ISRG)

Preimplantation genetic testing (PGT) guidelines represent the views of the International Society of Reproductive Genetics (ISRG). These views are based on published literature and the latest findings on PGT research. Adherence to these guidelines is voluntary. The final decision regarding specific clinical management should be jointly reached by the healthcare provider and patients, considering the patients' clinical and family history, as well as disease characteristics. These guidelines reflect the best data and clinical insights currently available. Future studies may need to provide revisions to recommendations in these guidelines.

PGT, conventionally called preimplantation genetic diagnosis (PGD) and screening (PGS), is used to examine DNA from polar bodies or embryos to detect monogenic defects, human leukocyte antigen (HLA) typing (PGT-M), chromosomal structural

rearrangements (PGT-SR), or aneuploidy (PGT-A)<sup>[1–3]</sup>. PGT-M is primarily utilized to prevent the transmission of monogenic diseases from parents to their offspring. PGT-SR is used to help patients with chromosomal rearrangements select euploid embryos. With recent technical advances, the translocation carrier state can be resolved from *in vitro* fertilized embryos by PGT-SR<sup>[4,5]</sup>. PGT-A is used for aneuploidy testing, which may afford clinical benefits such as increased pregnancy rates per embryo transfer, decreased miscarriage rates, and reduced time-to-pregnancy in a subgroup of patients undergoing *in vitro* fertilization (IVF) (see “Indications for PGT-A”)<sup>[3]</sup>. Various PGT technologies have markedly improved the success rate of assisted reproduction treatment and reduced congenital birth defects; however, further technical advances or scientific discoveries are needed to address unresolved challenges. For instance,

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the mechanism underlying embryo mosaicism and self-repair of chromosomal abnormalities during embryo development remain poorly understood, hindering the interpretation of PGT-A results. In PGT-M, inevitable allele dropout (ADO) during whole-genome amplification (WGA) of a few embryo biopsy cells induces the risk of misdiagnosis by directly examining the disease-causing locus. Therefore, haplotype linkage analysis is required in such cases, which renders PGT-M challenging for families without an available proband or those affected by *de novo* mutations. Additionally, exclusion from PGT should be considered if ovarian stimulation, oocyte retrieval, or pregnancy pose health risks to female patients<sup>[1]</sup>.

## Part I: Clinical procedures and quality control of PGT

### Indications and contraindications

#### Indications for PGT-M/PGT-SR/PGT-A

PGT-M should be considered in couples at high risk of transmitting monogenic diseases to the next-generation, including autosomal dominant, autosomal recessive, X-linked recessive, X-linked dominant, and Y-linked disorders. Pathogenic variants and pathogenic gene linkage markers should be analyzed prior to initiating IVF treatment.

PGT-M can be provided to couples when either one or both gamete providers carry pathogenic variants that cause significant genetic susceptibility to severe diseases, such as *BRCA1* and *BRCA2* pathogenic variants associated with hereditary breast and ovarian cancer.

PGT-M can be used for HLA matching. For couples with a previous child with a severe hematopoietic disease warranting bone marrow transplantation, PGT-M can be employed to select a suitable embryo with HLA type matched to the sick child, who can be subsequently treated by harvesting and transplanting hematopoietic stem cells from the umbilical cord blood of the newborn.

PGT-M can be provided to patients with mitochondrial diseases for selecting embryos with minimal mitochondrial DNA mutation load or performing mitochondrial replacement therapy for embryos if such procedures are in compliance with regulations.

#### Indications for PGT-SR

PGT-SR could be employed to test either one or both members of a couple with a chromosomal structural abnormality. Chromosomal structural abnormalities include reciprocal translocation, Robertsonian translocation, inversion, complex translocation, and pathogenic microdeletions or microduplications. To improve the likelihood of successful pregnancy and reduce miscarriages due to aneuploidy, conventional PGT-SR can be used to identify embryos with a balanced or normal karyotype for implantation. However, these embryos have a significant probability of being balanced translocation carriers. Recent advances in molecular diagnostic techniques have enabled the identification of balanced translocation carriers<sup>[4–8]</sup>. It is recommended to test the carrier status of embryos and avoid transmission to future generations when possible. However, euploid embryos without balanced translocation are not always available for every patient during each treatment cycle. Under such circumstances, embryos with balanced translocations are acceptable for transfer upon sufficient genetic counseling and informed consent.

### Indications for PGT-A

The development of high-throughput molecular assays has enabled comprehensive chromosome screening (CCS) of preimplantation embryos (PGS 2.0). In recent years, a growing number of clinical studies have revealed that PGT-A with CCS enhances clinical and sustained implantation rates, improves live birth rates, and reduces time-to-pregnancy, particularly in patients with normal ovarian reserve<sup>[9]</sup>. A meta-analysis evaluating three randomized clinical trials (RCTs) compared PGS 2.0- and morphology-based embryo selection ( $n = 659$ ) and revealed that PGS 2.0 was associated with significantly higher clinical and sustained implantation rates. Another meta-analysis assessing eight observational studies demonstrated improved clinical and sustained implantation rates when compared with conventional IVF<sup>[10]</sup>.

The clinical benefits of PGT-A are restricted to one subgroup of the IVF population. An RCT has shown that the ongoing pregnancy rate (OPR) per embryo transferred was significantly higher in the PGT-A group than that in the morphology group only for patients aged 35 to 40 years, but not for those aged <35 years<sup>[11]</sup>. A more recent RCT study has revealed that for an IVF population aged between 20 and 37, the cumulative live births within 1 year in the PGT-A group were even lower than those in the morphology group<sup>[12]</sup>. Considering that the current PGT procedure relies on invasive embryo biopsy, which may pose a risk to the embryo, the indications for PGT-A should be strictly followed. Currently, PGT-A is recommended for use in the following patient groups.

Advanced maternal age (AMA) is 38 years old and above.

Recurrent miscarriage (RM) owing to unknown causes (two or more times), namely, pregnancy loss, repeatedly occurred between gestational weeks 4 and 12.

Recurrent implantation failure (RIF) occurs with three or more attempted embryo transfers, either transfer of cleavage stage embryos or blastocysts with a high morphological score.

Unexplained severe teratozoospermia with more than 96% of sperms exhibiting abnormal morphology caused by defects in the head, midpiece, and/or tail.

### Contraindications for PGT

PGT should not be offered under the following conditions:

Without a definitive genetic disease diagnosis or positioning information for the disease-causing locus.

Selection of non-disease traits, such as appearance, height, and skin color.

Other circumstances where PGT is restricted under local laws, regulations, or ethics.

### Some special considerations

Certain numerical aberrations of the sex chromosomes, such as 47,XXX, 47,YYY, have a low risk of transmission; therefore, PGT is not recommended for these conditions. However, the risk of chromosomal abnormalities increases in the offspring of parents with 47,XXY<sup>[13]</sup>. Therefore, the implementation of PGT may be considered appropriate in these cases.

PGT is not recommended for common chromosomal polymorphisms such as lqh+, 9qh+, inv(9) (p12q13), inv(Y) (p11q11), and Yqh+.

### Technical limitations of PGT

Biological and technical factors may reduce the accuracy of PGT. For instance, cells undergoing DNA replication may

possess imbalanced DNA content, which may cause false-positive results in PGT. Apoptotic or dead cells in the biopsy sample may generate mosaicism profiles. Improper embryo biopsy techniques may increase the rate of mosaicism. Given the intrinsic nature of embryo mosaicism, testing results from trophoctoderm (TE) biopsies may only represent a small part of the embryo and may fail to reflect the chromosomal content of the entire embryo, especially the inner cell mass.

In PGT-M, pathogenic variants associated with long repeat sequences or homologous pseudogenes are not amenable to the assay; therefore, these disease-causative variants may not be directly detectable in embryos. Accordingly, haplotype phasing should be performed for embryo selection, but rare misdiagnoses may occur in these cases because of homologous recombination.

### **Genetic counseling and informed consent**

Before PGT, genetic counseling should be provided to patients to comprehensively clarify the scope, limitations, and implications of the test. Patients need to have appropriate expectations for PGT, especially in cases where infertility is not the underlying reason for afforded care. Prenatal testing is imperative during follow-up care, considering the test limitations for PGT. Patients with well-documented counseling records and consent should voluntarily select the test.

The patients' family disease, fertility history, specialist examination, and health assessment results should be collected for personal and pedigree analyses. In PGT-HLA, the patient's clinical diagnosis, disease severity, and treatment history should be carefully evaluated to determine whether the test is appropriate, as it can be a lengthy process.

A comprehensive assessment should be performed to determine the risk of reproduction of the couple, based on the results of family surveys, genetic testing, and the etiology and epidemiology of the specific disease.

Based on the reproductive risk assessment, advantages and disadvantages of the potential medical intervention, including, but not limited to, prenatal diagnosis, PGT, and oocyte/sperm donation, should be presented to the patient couple to help facilitate informed decisions. Before selecting to undergo PGT, couples should be fully aware of the variable potential risks during the entire process, including the conventional procedure of IVF, embryo biopsy, embryo cryopreservation and thawing, the possibility of unclear or no diagnosis in one or more embryos, embryo ineligibility for transfer based on test results, uncertain developmental potential of mosaic embryos, inability to routinely identify carriers of chromosomal structural abnormalities, risk of possible misdiagnosis owing to the intrinsic biological nature of the embryo, limitations of detection technology, and the necessity of prenatal diagnosis when sustained pregnancy is achieved.

### **Embryo transfer**

Following PGT, single embryo transfer is recommended. With improvements in molecular techniques, mosaicism of up to 20% to 30% is detectable by microarray and next-generation sequencing (NGS)-based PGT<sup>13</sup>. Consequently, the reporting of mosaicism in PGT has become increasingly common. Most clinics could identify mosaic embryos at an incidence of 5% to 20%<sup>14–16</sup>, with the highest report reaching up to 40%<sup>17</sup>. A growing number of clinical observations have also demonstrated that transferring embryos with mosaicism may result

in an acceptable live birth rate<sup>18</sup>. Typically, when transferring embryos with mosaicism <40%, a fair implantation rate can be achieved, whereas viable pregnancy is less likely when embryo mosaicism ranges between 40% and 80%<sup>19</sup>. Therefore, when limited euploid embryos are available following PGT, embryos with mosaicism may be considered for transfer with proper genetic counseling and informed consent<sup>18,19</sup>. The decision to report or transfer mosaic embryos should be made by each clinical center based on internal test validation<sup>19</sup>. Genetic counseling should be provided to any patient considering the transfer of mosaic embryos. Preferential selection may be primarily based on the level of mosaicism identified and the specific chromosomes involved. Once a decision is reached, the implications for follow-up procedures such as prenatal diagnosis need to be discussed. Patients should consider how to approach an abnormal finding during prenatal testing, specifically pregnancy termination.

Transfer of embryos with chromosomal or segmental aberrations associated with pathogenic uniparental disomy, severe intrauterine growth retardation, or syndromic diseases should be avoided where possible<sup>17</sup>.

Either fresh cycle transfer or freeze-thaw cycle transfer may be selected, although the latter strategy is more widely applied. A notable improvement in NGS techniques has been documented, enabling the accomplishment of PGT-A overnight. The short turnaround time allows embryos to undergo PGT-A prior to fresh transfer, while clinical studies have demonstrated that clinical pregnancy, early miscarriage, and live birth rates of PGT-A-based fresh embryo transfers were similar to those of freeze-thaw embryo transfers<sup>20</sup>.

### **Follow-up**

Prenatal genetic counseling and a discussion of potential prenatal diagnostic testing options should be offered to patients when PGT is pursued<sup>18</sup>. Invasive prenatal diagnosis is recommended after PGT, particularly in high-risk pregnancies. Noninvasive prenatal screening is not recommended for high-risk patients, as test results are not diagnostic. An agreement between patients and the clinic for pursuing PGT should be in place after the patients are fully informed of the risks and implications of PGT.

### **Clinical quality control**

Couples must receive at least one genetic counseling session before entering the PGT cycle. Complete counseling records, informed consent, and medical records should be well documented.

Appropriate and sufficient clinical indications to perform PGT cycles in a patient couple should be ensured.

To calculate the success rate of PGT, the live birth per cycle started is recommended.

## **Part II: Micromanipulation and quality control in embryo laboratory**

### **Selection of fertilization methods**

Intracytoplasmic sperm injection (ICSI) is the most common fertilization method currently used for PGT cycles. ICSI is used considering the interference of maternal cumulus/granulosa cells and sperm on the accuracy of downstream genetic testing.

## **In vitro fertilization**

Traditional IVF insemination of oocytes can be performed, followed by trophoctoderm biopsy (blastocyst biopsy) and NGS. Contamination from sperm or granulosa cells should be carefully ruled out for each embryo before implementing IVF-based PGT.

### **Timing of biopsy**

#### **Polar body biopsy**

For PGT using polar body biopsy, the genetic information of the maternal source can be analyzed and determined.

#### **First polar body (PB1) biopsy**

This can be performed after harvesting oocytes or 0.5 to 2 hours post-ICSI.

#### **Second polar body (PB2) biopsy**

This can be performed when the PB2 has been discharged, approximately 8 to 14 hours post-ICSI.

The PB1 and PB2 can be obtained and tested separately by biopsy within 8 to 14 hours post-ICSI fertilization.

#### **Cleavage stage biopsy**

Biopsy at the cleavage stage is typically performed approximately 66 to 70 hours post-fertilization. At this stage, the embryo has developed into six to eight cells with debris, implying that <30% may be biopsied. Only one blastomere should be biopsied to avoid damaging the embryo. After cleavage biopsy, the embryo can continue to grow for 2 to 3 days to form blastocysts. If embryonic genetic testing can be accomplished within this period, cleavage stage biopsy-based PGT followed by fresh embryo transfer may be preferred in cases where blastocyst biopsy can be challenging to achieve, such as in ovarian insufficiency, resulting in fewer embryo numbers or reduced chance of blastocyst formation in embryo culture.

#### **Blastocyst biopsy**

Blastocyst biopsy minimally impacts embryo development and is currently the primary biopsy method for PGT. During the blastocyst stage, the biopsy is performed between days 5 and 6 post-fertilization when full blastocyst expansion occurs. It is recommended that biopsied blastocysts exhibit a morphological score  $\geq 4BC$ , while the number of biopsied cells should range between 5 and 10. Generally, after blastocyst biopsy, embryos undergo vitrification immediately and are stored until the genetic analysis is completed. Embryos with normal PGT results should be thawed and transferred.

### **Embryo biopsy**

#### **Breaching of the zona pellucida**

Methods for breaching the zona pellucida include mechanical, chemical (acidified Tyrodes), and laser ablation. Currently, the laser method is the most commonly employed. It should also be noted that thermal damage to cells should be minimized during the biopsy.

Mechanical and laser breaching can be used for polar body biopsies prior to fertilization. Exposure to acidified Tyrode's solution at this stage may negatively impact spindle formation.

Biopsy at the cleavage stage may be performed using mechanical, chemical (acidified Tyrodes), or laser methods.

Biopsy at the blastocyst stage may be performed using mechanical or laser methods.

Breaching of the zona pellucida for blastocyst biopsy can be performed on days 3 and 5 post-fertilization, 4 hours before biopsy, or at the time of biopsy. A recent report has revealed that breaching the zona pellucida 2 days before the biopsy results in a significantly higher mosaic rate in subsequent PGT-A than when breaching is performed on the day of biopsy<sup>[21]</sup>.

Poor biopsy techniques may negatively influence subsequent PGT analyses. Studies have shown that excessive laser shots and cell debris retrieved from embryo biopsies may impact the mosaic detection rate or chaotic copy number variation (CNV) results. Appropriate biopsy procedures should be optimized and validated for each clinic<sup>[22]</sup>.

#### **Number of biopsied cells**

The PB1 and PB2 should be removed sequentially or simultaneously, according to genetic testing requirements. One cell should be removed from the cleavage stage embryo. The number of trophoblast cells collected from blastocyst biopsies should range between 5 and 10 cells.

#### **Second or repeated biopsy (re-biopsy)**

Re-biopsy affects the developmental potential of embryos. However, when the first biopsy affords an unclear diagnosis, such as obtaining no WGA product or chaotic CNV results, a second biopsy (including cleavage or blastocyst stage embryos) may be considered<sup>[23]</sup>.

#### **Selection of cells for biopsy**

Mononuclear cells should be selected and ideally removed during biopsy at the cleavage stage. At the blastocyst stage, trophoctodermal cells, far from the inner cell mass, should be selected for biopsy.

#### **Biopsied cell tubing**

It is recommended that the biopsied cells be transferred into the sample tube under microscopic visualization. The addition of a buffer carrier to the sample tube should be minimal (<1  $\mu$ L). Brief centrifugation can be performed after cell tubing<sup>[22]</sup>. Vigorous shaking or agitation should be avoided to prevent the loss of biopsied cells due to sticking to the upper wall of the sample tube. If long-distance transportation is needed prior to PGT, the sample should be frozen solidly from biopsy cell tubing to the initiation of the WGA reaction.

#### **Storage of biopsied cells**

Biopsied cells in phosphate-buffered saline (PBS) should be processed for WGA as soon as possible (within days), even when stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . Biopsy samples in a preservation buffer with nuclease-inhibiting components can be stored for several weeks at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . WGA product samples can be stored at  $-20$  or  $-80^{\circ}\text{C}$  for several years<sup>[3]</sup>.

### **Cryopreservation of biopsied embryo**

Cryopreservation of biopsied embryos is required while obtaining PGT results. It is recommended that the vitrification freezing technique is adopted. The freezing method for embryos at the cleavage or blastocyst stage post-biopsy was the same as that performed for conventional embryo freezing.

### **Pretreatment of biopsy samples**

For the downstream WGA process, biopsy-removed cells should be washed in PBS before placing them into an appropriate buffer, according to the requirements of the specific WGA protocol. A small amount of washing buffer is sampled from every operational batch as a blank control in subsequent tests.

For fluorescence *in situ* hybridization (FISH) testing, a few conventional cell fixation protocols may obtain satisfactory results. Given that most fixation reagents are toxic or carcinogenic, strict caution regarding personal protection measures during the process is warranted.

### **Quality control of the embryo biopsy laboratory**

#### **Quality control of embryo biopsy environment**

Embryo biopsy must be performed in sterile medium droplets covered with sterile mineral oil under a laminar flow hood maintained at a constant temperature of 37°C, which is consistent with ICSI *in vitro* embryo operation procedures.

To minimize cross-contamination between embryos, it is crucial to maintain only one embryo per droplet. The biopsy needle and transfer pipette must be free of residual biopsy cell components.

The technical personnel performing embryo biopsy should have adequate operational experience regarding embryological manipulation and should strictly follow the aseptic protocol during the entire procedure to avoid exogenous contamination.

Witnessing is recommended to ensure traceability through the PGT procedure.

During the biopsy, the witness should confirm matching the embryo and biopsy sample.

During biopsy sample tubing, the witness should confirm matching the sample identification labeling on the tube.

When placing and labeling the oocyte/embryo into the culture dish during subsequent cultures.

When placing and labeling the oocyte/embryo into the cryopreservation device.

When placing and labeling the embryo into the culture dish for further embryo culture.

When issuing the diagnostic results, the witness should ensure accuracy and correlation with the correct sample and/or embryo identification.

During the thawing/warming procedure and at the time of selecting the embryo(s) for transfer<sup>[22]</sup>.

### **Part III: Genetic testing and quality control in the genetic laboratory**

Based on the objectives of embryo genetic testing, PGT can be categorized at the genetic or chromosomal level.

#### **Gene level test**

##### **Scope of the test**

Following in-depth clinical consultation, PGT-M may apply to the following situations: couples with a high risk of transmitting

a specific monogenic disease to their offspring, either one/both parties carry specific genetic variants that cause significant genetic susceptibility to serious diseases, and selection of HLA matching.

### **Testing strategy and general requirements**

To avoid misdiagnosis or unclear diagnosis induced by amplification failure, biased amplification, ADO, and sample contamination, it is recommended that embryo gene testing by PGT-M should include the direct analysis of mutation sites and linkage analysis of genetic polymorphic sites<sup>[24,25]</sup>.

The genetic polymorphic site in linkage analysis may be a short tandem repeat (STR) or a single nucleotide polymorphism (SNP).

It is recommended to select at least three polymorphic sites<sup>[26]</sup> to potentially provide genetic information within a range of 1 Mb upstream and 1 Mb downstream of the pathogenic mutation site. In addition, the selection of SNP sites in regions of high homology, as well as adjacent sequences with high GC content or polynucleotide sequences, should be avoided.

For sex-linked genetic diseases, it is recommended to include the gender indicator locus test.

For HLA matching, upstream and downstream regions of HLA-A, HLA-B, HLA-DRA, and HLA-DKB1 must be covered with genetic polymorphic sites for linkage analysis. The selection of at least five polymorphic sites is recommended to provide genetic information for each region.

When performing linkage analysis of genetic polymorphic sites, it is important to pay additional attention to genomic recombination around the mutation site.

### **Detection methods**

For the nested polymerase chain reaction (PCR) method, the first round of multiplex PCR should amplify multiple target sites (including mutation and polymorphic sites for linkage analysis).

The amplification products of WGA can be used to identify mutation sites and polymorphic linkage sites using various methods, including fluorescent PCR, Sanger sequencing, SNP arrays, NGS, and a combination of the previously mentioned methods.

### **Preclinical work-up for PGT-M**

Known pathogenic mutations need to be verified in familial samples before performing PGT-M.

Polymorphic sites upstream and downstream of the mutation site can be selected for linkage analysis of familial samples. A haplotype with linkage to the mutation site is constructed using informative polymorphic sites that were examined.

The efficacy of planning the PGT-M strategy should be validated at the single-cell level.

Validating the protocol on a single-cell basis is required to evaluate the effectiveness of target site detection and the ADO rate prior to performing the PGT-M assay.

Samples used for the validation test can include, but are not limited to, lymphocytes, granulosa cells, luminal mucosal cells, buccal cells, and embryonic cells. However, the use of variable cells may affect the efficiency of amplification and ADO rate.

For cleavage stage embryo biopsy, one cell per validation sample is recommended. For blastocyst biopsies, it is recommended that each validation sample contain 5 to 10 cells.

If direct PCR amplification is performed, it is recommended that 50 known samples should be subjected to a pre-validation test, including, where possible, cells harboring the causative mutation, as well as normal cells, prior to the first clinical application of a specific assay<sup>[27]</sup>.

For WGA amplification, it is recommended that the validation test be performed on at least 10 known samples.

The amplification efficiency of the WGA should exceed 90%, and the ADO rate should be <10%. If the ADO rate is >10%, it is recommended to increase the upstream and downstream genetic polymorphic sites for linkage analysis<sup>[28]</sup>.

### PGT-M strategies

Linkage analysis using extragenic markers is recommended to focus on a distance of 1 Mb (~1 cM) from the target variant. This can minimize misdiagnosis due to recombination incidences, given that a 1% recombination rate is expected for loci within the 1 cM distance. When suitable extragenic markers are unavailable within 1 Mb, extending the range to 2 Mb is acceptable. Although this approach is not desirable, it can be adopted in the presence of large genes or duplications<sup>[29]</sup>.

Number of informative genetic markers needed. Generally, the more informative the genetic markers (eg, STRs and SNPs) used in the test, the more robust the results obtained. In practice, the inclusion of one STR or three informative SNPs, proximal and distal to the gene of interest, is recommended. Meanwhile, the risk of misdiagnosis due to ADO and/or recombination is reduced to an acceptable level on analyzing two informative SNP markers that are closely linked to and flanking the target gene. In addition, a high number of informative genetic markers should be used if the ADO rate is >10%<sup>[29]</sup>.

Under the following circumstances, PGT-M results may be obtained *via* an indirect haplotype-only approach if the target pathogenic variant cannot be directly detected:

- A. For exclusion testing,
- B. For HLA typing,
- C. Tandem repeats (such as FMR1 CCG repeats) or GC-rich regions that are refractory to WGA,
- D. Direct mutation detection, which is inapplicable owing to pseudogenes (eg, PKD1), and
- E. For large deletion or insertion fragments without determining breakpoints.

In the case of the indirect haplotype-only approach, at least two informative STRs or six SNPs proximal and distal to the region of interest should be included.

It may be markedly challenging to obtain sufficient informative markers (STRs/SNPs) flanking the target region when the target site is located in the proximity of a centromere or telomere (eg, HBA, F8). In this case, the concordance of direct target detection and haplotype phasing results is critical to avoid misdiagnosis due to recombination events.

In extremely difficult cases, only proximal or distal markers (STRs/SNPs) of the target gene are available, and the mutation site cannot be assessed directly (eg, facioscapulohumeral muscular dystrophy [FSHD]). There may be an increased risk of misdiagnosis under these circumstances. Therefore, in-depth consultation, informed consent, and prenatal testing should be performed to manage expectations or avoid unexpected adverse outcomes.

Generic testing for the combined PGT-M and PGT-SR/PGT-A can be performed in parallel using the same WGA product with

two different approaches: one aimed at PGT-M and the second at PGT-A. Alternatively, genome-wide approaches that enable concurrent haplotyping and copy number change detection can be undertaken to allow simultaneous PGT-M and PGT-A within the same test. These generic approaches can be SNP array- or sequencing-based, or a combination of the two.

Multiple displacement amplification (MDA)-based WGA yields better genomic coverage than alternate WGA approaches in 5 to 10 cells. Thus, MDA is generally recommended for target site detection and haplotyping analysis. However, MDA followed by the NGS approach may occasionally generate sub-optimal results for CNV analysis when compared with other WGA methods, such as multiple annealing and looping-based amplification cycles (MALBAC), especially when the biopsy cell number is <3. Therefore, the selection of WGA methods may be specifically optimized for the given cases.

For SNP array and NGS-based PGT, preclinical testing only requires informativity/segregation analysis; the locus-specific development can be omitted.

Typically, at least one first-degree relative is essential to determine haplotype phasing. When applying the indirect test in the case of *de novo* pathogenic variant(s) in couples without affected parents and with available offspring, additional single sperm or polar body analysis may be included in the preclinical work-up for phasing establishment.

Long-read sequencing (so-called third-generation sequencing) can assist in the determination of haplotype phasing from the proband and parents.

Given that germline mosaicism due to post-zygotic *de novo* pathogenic variant(s) in the prospective parent cannot be excluded, the use of an unaffected child/prenatal/embryo sample as a phasing reference is not recommended<sup>[29]</sup>.

### Detection at the chromosome level

#### Scope of application

PGT at the chromosome level, namely PGT-SR or PGT-A, may be applicable for the following purposes: couples with one or both parties carrying a known chromosomal abnormality, aneuploidy screening before embryo implantation, or sex selection for medical purposes. PGT-A should be performed in an exclusion testing manner, in which the test results rank the embryos according to their implantation potential score. The subsequent embryo transfer may follow the ranking score sequentially, from high to low, to reduce the early miscarriage rate and time-to-pregnancy and improve the live birth rate, while decreasing the number of embryo transfers needed to achieve a live birth per cycle.

#### PGT-SR and PGT-A strategies

For couples with one or both parties carrying abnormal chromosomes, PGT-SR can be performed to analyze specific chromosomes only or numerical abnormalities in all chromosomes simultaneously.

Gender selection can be used for sex chromosome-linked diseases involving unknown genes. However, mutation gene analysis is recommended for families with clear genetic diagnoses, and sex selection is not recommended.

For Y-linked monogenic disease, only gender selection can be performed.

Conventional PGT-SR testing may fail to identify the carrier embryo for chromosomal translocations. Recent progress in

molecular technologies has facilitated the implementation of a few strategies, such as Mapping Allele with Resolved Carrier State Test (MaReCs<sup>®</sup>), microdissection with NGS, preimplantation genetic haplotyping, haplarhythmism, and karyomapping, to resolve translocation carrier states of embryos, thus ensuring the implantation of a normal embryo. Although qualified laboratories may provide carrier screening, the technology used for testing must be fully evaluated.

For PGT-A, methods that can analyze all chromosomes simultaneously are typically recommended, namely CCS approaches, such as array comparative genomic hybridization (aCGH), SNP array, or NGS. However, FISH is not recommended for PGT-A.

### Testing techniques

For the nested PCR method, the first round of multiplex PCR should simultaneously amplify the specific sites of the target chromosome, whereas the second round of quantitative PCR should include a copy number analysis of each chromosome target site<sup>[30]</sup>.

The chromosome copy number can be detected by combining WGA and high-throughput genetic testing techniques, such as aCGH<sup>[31,32]</sup>, SNP array<sup>[27,33]</sup>, and NGS<sup>[26,34]</sup>.

To solve the status of translocation carriers, SNP array- and NGS-based PGT-SR requires preclinical investigation for haplotype phasing of the translocation chromosomes. DNA samples from nuclear families can be employed as a phasing reference. Long-read sequencing methods can also be utilized for this purpose<sup>[35]</sup>. Alternatively, the diagnosis can be reached in the clinical cycle, in which at least one unbalanced embryo with a clear breakpoint is required as a phasing reference<sup>[3]</sup>.

For FISH analysis, a specific detection probe must be selected for the target chromosome. When embryos from a translocation carrier are employed, combined application of the FISH probe should facilitate the identification of all possible imbalanced translocations in embryos. FISH is preferred when the translocation segment is smaller than the effective resolution of high-throughput genetic assays.

System effectiveness should be verified before the clinical implementation of PGT-SR/PGT-A techniques. Compared with FISH, SNP array and NGS are considered more reliable for PGT-SR, given that these techniques can assess the entire length of the translocation fragment. Furthermore, they allow the simultaneous copy number assessment of chromosomes not involved in target translocation<sup>[3]</sup>.

Commercially available kits for technologies, including aCGH, SNP arrays, and NGS, have well-established standard operating procedures (SOPs) and quality control parameters. The local laboratory must verify the effectiveness and stability of the test platform prior to clinical application. Generally, a preclinical trial experiment is not essential for every single case.

Two types of commercial array platforms are currently used. The first is the aCGH platform, which is based on oligonucleotides with a resolution of 5 to 10 Mb. The second is the oligonucleotide-based SNP array platform with a resolution of 2.4 to 5 Mb<sup>[3]</sup>.

For chromosome copy number analysis using FISH, the karyotypes of peripheral blood metaphase spreads of the couple should be verified in advance, and the fluorescence intensity and specificity of the probe should be tested and analyzed in interphase nuclei.

NGS allows direct reading of sequenced DNA fragments and quantifies them according to the number of reads. Based on the different reading depths of sequencing, NGS can be employed for diverse analyses, ranging from whole-chromosome aneuploidy to mega-base-sized deletion or insertion and monogenic disorder detection. The turnaround time for NGS (from DNA amplification to reporting) varies from platform to platform. Currently, the turnaround time has been optimized to less than 9 hours to enable and support overnight PGT-A for fresh embryo implantation<sup>[3]</sup>.

PGT laboratories may set up in-house chromosomal copy number baselines by testing serial samples of WGA products. Values between the euploid and aneuploid ranges were considered to be mosaic. If necessary, the detection and cutoff of the mosaic level can be determined. Typical lower and upper cutoff values are 20% and 80%, respectively<sup>[17,36–40]</sup>.

Cutoff threshold for chromosomal mosaic detection (eg, the minimum ratio of aneuploid to euploid cells required for detecting chromosome CNVs) should be established using a mixture (10%–90%) of aneuploid and euploid cell lines. Furthermore, it is recommended that six to eight cells should be initially analyzed from euploid cell lines to determine the standard deviation from the true euploidy baseline, thereby defining the “true euploidy” threshold. Similarly, thresholds should be defined for trisomy and genetic monomers<sup>[3]</sup>.

To simulate a blastocyst biopsy, a sample size of 8 to 10 cells is recommended for all mosaic cell mixture models. Although the validation experiment will set parameters for euploid and aneuploid cell lines, it should be noted that a small number of cells in biopsy samples induces process limitations. Moreover, it is almost impossible to detect changes that account for <20% to 30% of biopsy samples<sup>[3]</sup>.

### Quality control

While examination error rates may vary for PGT-M, PGT-SR, PGT-A, and various detection platforms, most IVF/PGT centers currently achieve an overall error rate (leading to misdiagnosis) of 1% to 3%<sup>[41]</sup>. Each clinical PGT laboratory shall choose its testing platforms according to its conditions and specialties, and all types of testing methods shall be established based on an SOP. Quality control parameters in key experimental steps of different testing platforms should be established according to the process requirements. It should be noted that this guide only provides suggestions for general quality control measures in PGT laboratories.

Biopsied cells of embryos should be given a clear and unique identification marker during the entire testing process, corresponding to the original embryo.

When using the nucleic acid amplification method in PGT, whether nested PCR or WGA, it is necessary to set a blank control of the biopsied cell washing solution and a blank control of the amplification reagent to evaluate the potential risk of contamination.

SOP documents should be established for all testing technologies, which need to be strictly followed, evaluated, and updated periodically.

For detection technologies with available commercial kits, such as array CGH, SNP array, and NGS, SOP procedures and quality control measures appropriate for the local laboratory should be established.

Test results shall be analyzed and interpreted by two independent operators. If these two operators fail to reach a consensus, a third independent observer will provide the final judgment. Embryo test results without consensus should be interpreted as



undiagnosed. Embryos that are undiagnosed with PGT-M/SR are not recommended for transfer. Embryos undiagnosed after PGT-A can be transferred following appropriate counseling related to age-adjusted estimates of aneuploidy risk.

For embryos with unclear diagnoses, “no rescue” was performed. Either the second array/NGS analysis of the existing WGA product or the second biopsy followed by WGA and array/NGS analysis is acceptable<sup>[3]</sup>. In the absence of any amplification or suspected contamination, an attempt can be made to obtain results by re-biopsy, which should be included in the report submitted to clinicians<sup>[3]</sup>.

Internal quality control and external quality assessment shall be performed regularly, and records shall be logged.

### Test report

The PGT report should include the couple’s name, age, indications, embryo number, embryo biopsy stage, date of biopsy, testing methods, test results, operator, witness, reviewer, date of the report, and remarks. Reporting the sex of the embryos should be governed by local laws, regulations, and/or ethical traditions, as well as the medical necessity of sex determination.

### Part IV: Prospects on PGT

With the rapid advancement of high-throughput molecular technology, PGT applications have had profound impacts (eg, comprehensive chromosomal screening of PGT-A), and progress remains ongoing and continuous. Accumulating genetic knowledge from genome-wide association studies has allowed the prediction of polygenic disease risk on an individual basis, such as coronary artery disease and diabetes. Once the prediction algorithm is applied to PGT for comparing relative disease risk among sibling embryos, the selected embryo can be transferred based on the score of reasonably low risk for screened polygenic diseases. This introduces a new concept of PGT, namely PGT-P, wherein the “P” indicates polygenic diseases<sup>[42]</sup>. In the future, PGT-P could be potentially performed with a noninvasive embryo testing method; the method was under development when these guidelines were written. A combination of these new techniques may greatly expand the indications of PGT application, potentially resulting in healthier offspring.

The development of high-throughput technology has facilitated the emergence of methods that allow simultaneous PGT-A, PGT-M, and PGT-SR using an integrated workflow<sup>[25,43–46]</sup>. No biological mechanism excludes the simultaneous occurrence of variable PGT indications in any given case. Therefore, the co-occurrence of multiple genetic deficiencies in the same patient/embryo is not rare in clinical circumstances, whereas the conventional application of PGT variants exclusively was due to the inability to apply multiple diverse testing strategies to one biopsy sample of embryos. The breakthrough of new technologies integrating distinctive assays can enable a comprehensive evaluation of embryos from multiple dimensions, thereby ensuring the transfer of a rigorously optimal embryo among its siblings.

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### Author contributions

C.X. and H.F.H. designed the study for the development of these guidelines. S.L., S.C., J.Z., C.X., Y.G., Y.S., Y.C., L.W., F.J., G.L., P.L., Y.Z., Y.W., D.Z., B.Y., V.A.K., Z.Y., J.L., Z.C., A.T., A.N.B., J.S., J.F., A.H., and H.F.H. provided scientific and clinical input for the development of these guidelines. All authors edited and approved the manuscript.

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### Conflicts of interest

S.L. is an employee of Yikon Genomics Co. Ltd. The other authors declare no conflict of interest related to this work.

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