*Please send to Eurofins Genoma*

# INFORMATION NOTICE FOR PREIMPLANTATION GENETIC TESTING

## Definition and purpose of Preimplantation Genetic Testing (PGT)

Preimplantation Genetic Testing (PGT) is a genetic investigation to identify the presence of genetic diseases or chromosomal alterations in embryos produced with medically assisted reproduction (MAR) techniques by couples at high reproductive risk. PGT candidate couples shall first enter a MAR course in which female and male gametes (oocytes and spermatozoa, respectively) and in vitro fertilized gametes shall be collected. After fertilization, the DNA of the oocyte (via the collection of polar bodies - PBs) or of the embryo (by biopsy of blastomeres at the stage of cleavage or of blastocysts) shall be analysed according to a specific protocol which will differ in connection with the type of abnormality to be investigated (chromosomal, genomic or genetic) and in connection with the specific disease. PGT is performed on oocytic/embryonic DNA prior to transferring the embryo into the uterus.

There are three main types of PGT:

PGT-A: preimplantation genetic test for chromosomal aneuploidies

PGT-M: preimplantation genetic test for monogenic diseases;

PGT-SR: preimplantation genetic test for chromosomal abnormalities and structural rearrangements

## Techniques and types of sampling for Preimplantation Genetic Testing (PGT)

Preimplantation genetic testing can be performed on several samples taken at different times of embryonic development. Separate information with different performance may be obtained depending on the type of sample analysed (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group et al. 2020). Specifically, the cells to be subjected to genetic testing can be obtained from the embryo at the stage of cleavage (third day after fertilization) or of blastocysts (days 5-6-7 from fertilization).

To date, the most commonly used technique is embryo biopsy at the blastocyst stage, which is the stage reached starting from day 5 of embryonic development after fertilization. Each blastocyst consists of several dozen cells (from 100 to 300 or more) divided into two zones: the trophectoderm, the portion of the most peripheral cells that will form the adnexa (placenta and amniotic membrane) and the inner cell mass (ICM), the innermost portion of cells that will form the embryonic structures proper. Blastocyst biopsy is performed on the trophectoderm, without touching the ICM, and allows collecting an adequate number of cells for subsequent analysis (approximately 5-10 cells).

More rarely, the cells to be analysed are taken on the third day (day 3) after fertilization, when the embryo, called the Cleavage Stage Embryo, consists of approximately 6 – 8 cells. At this stage the cells are totipotent, non-compacted and 1-2 blastomeres (cells) can be collected for genetic testing.

Further details on embryo biopsy techniques can be obtained when talking with the staff at the MAR site of reference.

## Preimplantation Genetic Testing for Aneuploidies (PGT-A)

### Purpose and advantages of the PGT-A test

Preimplantation Genetic Testing for Aneuploidies (PGT-A) is aimed at assessing the embryonic chromosomal arrangement prior to transfer into the uterus (ESHRE PGT-SR/PGT-A Working Group et al. 2020).

The chromosomal endowment in humans is normally made up of 46 chromosomes divided into 23 pairs: 22 pairs are autosomes (equal non-sex chromosomes between males and females) and one pair of sex chromosomes consisting of two XX chromosomes in females and one X chromosome and one Y chromosome in males. Changes in the number and structure of these chromosomes may have immediate effects on the embryos resulting in a cessation of development or unsuccessful implantation. Sometimes an embryo carrying severe chromosomal abnormalities is able to implant in the uterus and lead to a pregnancy that gets terminated later (miscarriage). Some chromosomal abnormalities, on the other hand, may be compatible with foetal development and lead to the birth of an individual that may have varying degrees of clinical manifestations (e.g. malformation and/or cognitive impairment).

The purpose of PGT-A is to identify embryos that, as euploids, are most likely to result in a term pregnancy and with less risk of clinical manifestations in pregnancy or at birth once transferred.

The transfer of euploid embryos defined by PGT-A increases the probability of implantation, but cannot guarantee that this will be successful. In addition, PGT-A may shorten the time it takes to obtain a pregnancy, while not overall increasing the likelihood of pregnancy occurring.

### Purpose and advantages of the “Ploidy Panel”

The most common chromosomal copy number anomalies (aneuploidy) are represented by alterations (monosomies and trisomies) of a single chromosome (simple aneuploidy) or of more than one chromosome (complex aneuploidy). Sometimes an embryo may present anomalies in all the chromosomes: when all the chromosomes are present in a single copy it is called haploidy, while when multiple copies are present it is called polyploidy (e.g. three copies = triploidy, four copies = tetraploidy).

In haploid embryos the chromosome set present is that of a single parent, while in polyploidies there are multiple sets of chromosomes from (at least) one parent.

Ploidy anomalies have a serious impact on embryo-foetal development which is prevented (haploidy) or severely compromised (polyploidy) and for this reason it is important to be able to verify whether an embryo presents one of these alterations.

Although ploidy abnormalities represent a small percentage of the overall aneuploidies observed during conception, their detection is important to increase the success of IVF. For example, triploidy represents approximately 2% of natural pregnancies and triploidy is found in approximately 15% of spontaneous pregnancy losses with chromosomal anomaly (Marin D et. al). Furthermore, sometimes the oocytes present an "anomalous fertilization" (abnormally fertilized oocyte - AFO) defined by the presence of an anomalous number of pronuclei, i.e. different from 2. Embryos deriving from AFO are generally excluded from subsequent use as they present an increased risk of triploidy, however in the literature there are reports of cases of children born alive following the transfer of embryos resulting from AFO (Bredbacka et al.). Therefore, ploidy assessment is a useful tool in assessing the actual number of nuclei in the oocyte and can increase the number of embryos available for transfer.

Finally, the Ploidy Panel can perform a contamination assessment, i.e. verify that the analyzed DNA belongs completely to the embryo and has not been contaminated by maternal cells.

The Ploidy Panel is a supplementary test to PGT-A and PGT-SR and does not require a second embryo biopsy as it is performed on the same DNA used for the other techniques.

DNA fingerprinting (Sibling QC):

The Ploidy Panel can also compare the genetic variants of the DNA of different embryos to evaluate "brotherhood", i.e. belonging to the same parental pair. This approach increases the guarantees of reliability on the traceability of the embryos subjected to analysis.

## Indications for PGT-A

PGT-A may be considered for all patients undergoing a cycle of MAR.

In some circumstances the application of PGT-A has a chosen indication, in particular:

1. Advanced maternal age (AMA): couples in which the female partner is conventionally older than 35 years of age. This specific indication is suggested by numerous studies performed on blastocysts analysed in groups of women of different ages undergoing a cycle of MAR with PGT-A, which demonstrated the correlation between increased maternal age and number of aneuploid embryos (Franasiak et al. 2014; Munné et al. 2017; Rubio et al. 2017).
2. Repeated implantation failure (RIF): couples that had 3 or more morphologically good-quality embryos transferred without obtaining implantation (no ultrasound gestational sac at 5 or more weeks after embryo transfer).

3. Recurrent abortions: couples who have experienced repeated (3 or more) first-trimester terminations of pregnancy in the presence of a normal couple karyotype and no "mechanical" causes such as uterine diseases or other recognized causative factors.
4. Spermiogram abnormalities: couples in which the male partner has severe oligo-asthenoteratospermia, cryptospermia or non-obstructive azoospermia, which involve resorting to MESA (*Microsurgical Epididymal Sperm Aspiration*) and TESE (*TEsticular Sperm Extraction*) microsurgical techniques for collecting spermatozoa from the seminal tract.

PGT-A can only be performed on an embryo biopsy at the cleavage stage (day 3) and blastocyst stage (day 5, 6 and 7).

## Indications to the Ploidy Panel:

1- Couples in which miscarriages occur due to triploid pregnancies.
2- Embryos deriving from oocytes with anomalous fertilization (1PN, 2,1PN and 3PN).
3- History of molar pregnancies.

## PGT-A outcomes

PGT-A may result in the following outcomes:

- Embryos with normal or **euploid** chromosomal arrangement: the analysis has highlighted a euploid (normal) chromosomal arrangement. These embryos are eligible for transfer without any further specialist evaluation. The probability of obtaining a euploid embryo varies with the age of the female partner and other specific risk factors.
- Embryos with an altered or **aneuploid** chromosomal arrangement: the analysis has highlighted an aneuploid/abnormal chromosomal arrangement due to the presence of one or more abnormalities involving one or more chromosomes fully (aneuploidy proper) or partially (segmental chromosomal abnormality). If the polyploidy panel has been performed, this result will also include the possible result of haploidy or polyploidy (e.g. triploidy). The transfer of these embryos is strongly discouraged as it would result in implantation failure or early abortion, but could also lead to the development of a pregnancy with a chromosomal disease.
- **"Mosaic"** embryos: A mosaic embryo condition indicates the simultaneous presence of euploid cells (with correct chromosome numbers) and cells with altered chromosome numbers within the analysed specimen (embryo biopsy). The finding of mosaicism may mean that the embryo is experiencing a phenomenon known as chromosomal rescue as a result of which the foetus may have a normal chromosomal arrangement. This is confirmed by evidence that the transfer of embryos with mosaic chromosomal abnormalities may result in a foetus with a correct chromosomal arrangement (Greco,

Minasi, e Fiorentino 2015; Viotti 2020). Transferring such embryos is possible after genetic counseling (Leigh et al. 2022).

- No diagnosis: this result is obtained if the Whole Genome Amplification reaction fails (see below). In the event of a non-diagnosis, it is advisable to perform a new embryo biopsy.
- Inconclusive diagnosis: this result is obtained when the laboratory procedure has led to results of doubtful interpretation. In the case of an inconclusive diagnosis, it is advisable to perform a new embryo biopsy.

If the polyploidy panel has been performed, the outcome of the evaluation of the "brotherhood" between embryos will be added to the above results:

- Sibling QC Value Attributed: All embryos of the same couple are genetically related to each other.

- Sibling QC value Excluded: One or more embryos of the same couple have genetic characteristics that differ from the other embryos of the couple.

It is not possible to define a priori which results will be obtained from the PGT-A and the Ploidy Panel; specifically, the execution of these tests does not guarantee the availability of transferable embryos and, if there are multiple candidate embryos for transfer, this may occur at different times for different embryos.

PGT-A does not exclude conducting genetic investigations during pregnancy, which will be discussed in specific prenatal genetic counseling.

### Reporting times

The test results will be available within 7-10 business days of acceptance of the sample. These terms, however, are not mandatory and may be extended in the event of repeating the test, suboptimal or inconclusive results, in-depth diagnostic analysis or interpretative doubts. For the PGT-A test to be carried out, the test request sheet and informed consent must be duly completed and signed. In the event of missing information, the laboratory will contact the forwarding doctor/clinic or the persons concerned to obtain this information. This may change the time for processing the sample and issuing the report.

## Preimplantation Genetic Testing for structural chromosome rearrangements (PGT-SR)

Preimplantation Genetic Testing for structural chromosome rearrangements (PGT-SR) is aimed at evaluating the embryonic chromosomal arrangement prior to transfer in cases where one or, more rarely, both partners are carriers of a chromosomal rearrangement that exposes to the risk of chromosomal abnormality in conception (ESHRE PGT-SR/PGT-A Working Group et al. 2020).

The chromosomal endowment in humans is normally made up of 46 chromosomes divided into 23 pairs: 22 pairs are autosomes (equal non-sex chromosomes between males and females) and one pair of sex chromosomes consisting of two XX chromosomes in females and one X chromosome and one Y chromosome

in males. Changes in the number and structure of these chromosomes may have immediate effects on the embryos resulting in a cessation of development or unsuccessful implantation. Sometimes an embryo carrying severe chromosomal abnormalities is able to implant in the uterus and lead to a pregnancy that gets terminated later (miscarriage). Finally, some chromosomal abnormalities may be compatible with foetal development and lead to the birth of an individual that may have varying degrees of clinical manifestations (e.g. malformation and/or cognitive impairment).

However, it is known that many individuals have karyotype alterations that may have little or no effect on their health, such as balanced chromosomal abnormalities or mosaic chromosomal abnormalities. These abnormalities, however, can be transmitted in conception resulting in variable manifestations.

The purpose of PGT-SR is to identify embryos that, as euploids, are most likely to result in a term pregnancy and with less risk of clinical manifestations in pregnancy or at birth once transferred. PGT-SR may assess both the presence of any chromosomal abnormality resulting from the parental chromosomal abnormality and the presence of independent chromosomal abnormalities that may also be present in the conceptions of couples with normal karyotype.

The transfer of euploid embryos defined by PGT-SR increases the probability of implantation, but cannot guarantee that the implantation will be successful. Performing PGT-SR shortens the time it takes to obtain a pregnancy, but it does not overall increase the likelihood of obtaining a pregnancy.

It is possible to associate the Ploidy Panel with the PGT-SR analysis, for details of which please refer to the dedicated paragraphs in the previous section of this document.

## Indications for PGT-SR

PGT-SR is indicated when one or both have:

- A balanced (or apparently balanced) chromosomal abnormality: examples include reciprocal translocations between non-homologous chromosomes, Robertsonian translocations, pericentric and paracentric inversions, complex translocations.
- An unbalanced chromosomal abnormality with a loss/acquisition of genetic material: examples include deletions and chromosomal insertions of dimensions in the resolution range of classical cytogenetics, sex chromosome mosaicisms, supernumerary marker chromosomes composed of euchromatin.

In view of the fact that via PGT-SR it is also possible to identify chromosomal abnormalities that can be identified by PGT-A, any indication in PGT-A may be a secondary indication of PGT-SR.

PGT-SR can only be performed on an embryo biopsy at the cleavage stage (day 3) and blastocyst stage (day 5, 6 and 7).

## Genetic counseling and technical feasibility assessment

Each PGT-SR candidate should have a genetic counseling with the following aims: a) inform the couple about the genetic condition under examination (natural history, therapeutic possibilities) and the percentage of recurrence risk; b) verify the possibility of diagnosing the genetic defect in question by means of PGT (technical feasibility); c) discuss the available methodologies and approaches for genetic testing with their levels of accuracy; and d) describe the results of the centre's case reports obtained in relation to the indication and diagnostic methodology used.

## PGT-SR outcomes

PGT-SR may result in the following outcomes:

- Embryos with normal or balanced chromosomal arrangement: the analysis has highlighted an euploid (normal) or balanced chromosomal arrangement. No chromosomal abnormalities related to the parental chromosome abnormality or other abnormalities unrelated to this have been identified. If the parental chromosome abnormality is balanced, it is not possible to define whether or not an embryo is a carrier of the same balanced abnormality.
- Embryos with an altered or unbalanced chromosomal arrangement: the analysis has highlighted an aneuploid/abnormal chromosomal arrangement due to the presence of one or more abnormalities involving one or more chromosomes fully (aneuploidy proper) or partially (structural chromosomal abnormality). If the polyploidy panel has been performed, this result will also include the possible result of haploidy or polyploidy (e.g. triploidy). The chromosomal abnormalities found may be the result of imbalance or transmission of the parental chromosome abnormality or may be random abnormalities. The transfer of these embryos is strongly discouraged as it would result in implantation failure or early abortion, but could also lead to the development of a pregnancy with a chromosomal disease.
- "Mosaic" embryos: A mosaic embryo condition indicates the simultaneous presence of euploid cells (with correct chromosome numbers) and cells with altered chromosome numbers within the analysed specimen (embryo biopsy). The finding of mosaicism may mean that the embryo is experiencing a phenomenon known as chromosomal rescue as a result of which the foetus may have a normal chromosomal arrangement. This is confirmed by evidence that the transfer of embryos with mosaic chromosomal abnormalities may result in a foetus with a correct chromosomal arrangement (Greco, Minasi, e Fiorentino 2015; Viotti 2020). Transferring such embryos is possible after genetic counseling (Leigh et al. 2022).
- No diagnosis: this result is obtained if the Whole Genome Amplification reaction fails (see below). In the event of a non-diagnosis, it is advisable to perform a new embryo biopsy.

- Inconclusive diagnosis: this result is obtained when the laboratory procedure has led to results of doubtful interpretation. In the case of an inconclusive diagnosis, it is advisable to perform a new embryo biopsy.

It is not possible to define a priori what results will be obtained from PGT-SR; specifically, performing PGT-SR does not ensure getting transferable embryos and, if there are several candidate embryos for transfer, this may take place at different times for the different embryos.

PGT-SR does not exclude conducting genetic investigations during pregnancy, which will be discussed in specific prenatal genetic counseling.

### Reporting times

The test results will be available within 7-10 business days of acceptance of the sample. These terms, however, are not mandatory and may be extended in the event of repeating the test, suboptimal or inconclusive results, in-depth diagnostic analysis or interpretative doubts. For the PGT-SR test to be carried out, the test request sheet and informed consent must be duly completed and signed. In the event of missing information, the laboratory will contact the forwarding doctor/clinic or the persons concerned to obtain this information. This may change the time for processing the sample and issuing the report.

## Preimplantation Genetic Testing for monogenic diseases (PGT-M)

The purpose of Preimplantation Genetic Testing for Monogenic disease (PGT-M) is to verify the transmission to embryos of one or more familial genetic diseases (ESHRE PGT-M Working Group et al. 2020). This investigation has the purpose of investigating only the genetic diseases known in the nuclear family, but may be combined with PGT-A and/or PGT-SR for assessing the embryonic chromosomal arrangement prior to transfer (see specific subsection).

### Indications for PGT-M

PGT-M may be offered as a reproductive option to all couples at risk of having a child suffering from monogenic disease. PGT-M can be performed regardless of the mode of transmission of the disease (autosomal dominant, autosomal recessive and X-linked) when the disease gene and causative pathogenetic variant are known.

For late-onset diseases (e.g. Huntington's disease), in the case of an informative nuclear family, it is possible to make a diagnosis of exclusion, thus avoiding using the pre-symptomatic test on the partner at risk.

The exclusion analysis involves the execution of a linkage analysis aimed only at ascertaining the transmission of the haplotype inherited by the affected member of the family, without knowing whether this haplotype is in fact associated with the pathology. This survey is applicable only in cases where the family unit is found to be informative and the result is expressed in terms of "at risk" and "not at risk". The exclusion approach can

be associated, at the couple's choice, with PGT-A analysis already described above, generally only on non-at-risk embryos.

In the case of paternal X-linked dominant diseases or X-linked diseases whose mode of transmission is known, but not the specific familial pathogenetic variant, only the determination of the embryonic chromosomal sex is possible.

In the case of a genetic disease due to "de novo" mutation it is possible to perform the PGT-M test through direct analysis, however this can only be proposed for some types of genetic alterations. In the event of a "de novo" mutation it is always advisable to contact the laboratory to verify the technical feasibility of the analysis.

PGT-M may be performed on embryo biopsy at the cleavage stage (day 3) and blastocyst stage (day 5, 6 and 7).

## Genetic counseling, technical feasibility assessment and pre-clinic setup

Each PGT-M candidate should have a genetic counseling with the following aims: a) inform the couple about the genetic condition under examination (natural history, therapeutic possibilities) and the percentage of recurrence risk; b) verify the possibility of diagnosing the genetic defect in question by means of PGT (technical feasibility); c) discuss the available methodologies and approaches for genetic testing with their levels of accuracy; and d) describe the results of the centre's case reports obtained in relation to the indication and diagnostic methodology used.

At the end of the counseling, the geneticist will initiate the pre-clinical setup phase, which involves the design and optimization of the diagnostic protocol of genetic testing from a single cell or a few cells, adapted to the specific genetic disease and the related familial genetic variants. The set-up activity is performed on the genomic DNA of the two partners that can be obtained from blood sampling or buccal swab. Sometimes it may be necessary to obtain DNA from other individuals in the nuclear family of one or both partners. When the results produced are in line with the recommended parameters of the international guidelines (ESHRE PGT-M Working Group, 2020), the protocol may be applied clinically.

After completion of the pre-clinical setup phase, embryo testing may be carried out.

## Embryo testing

Embryo testing to verify the transmission of the monogenic disease for which the couple is at risk (PGT-M proper) will use two different methods.

### *Direct testing:*

performed by searching directly for the familial gene variant(s). This investigation therefore allows assessing the presence/absence of the gene variant in the test sample. For technical reasons, direct

searching might not be applicable for a specific gene variant. In this case, only indirect testing will be carried out.

*Indirect testing:*

also called linkage analysis, it is performed by analysing some repeated sequences of the genome known as STRs (short tandem repeats). In the preliminary set-up phase, STRs are identified in the chromosomal region in which the relevant gene is located and the haplotype (combination of variants) linked to each specific gene variant is reconstructed, as well as the wild haplotype (not linked to a gene variant) if present.

## PGT-M outcomes

The outcomes of PGT-M will be distinguished in relation to the mode of transmission of the genetic condition being examined:

- Affected/ At risk/ Not transferrable: the embryo is at risk of developing the genetic disease investigated.
- Unaffected/ Not at risk/ Transferrable: the embryo is not at risk of developing the condition or transmitting it to the following generations
- Carrier: the embryo is not at risk of developing the genetic disease investigated, but is however at risk of transmitting it.
- No diagnosis: this result is obtained if the Whole Genome Amplification reaction fails (see below). In the event of a non-diagnosis, it is advisable to perform a new embryo biopsy.
- Inconclusive diagnosis: this result is obtained when the laboratory procedure has led to results of doubtful interpretation. In the case of an inconclusive diagnosis, it is advisable to perform a new embryo biopsy.

The different results will need to be interpreted in the context of the specific mode of transmission of the disease under investigation:

Autosomal dominant diseases:

The term affected refers to the presence of the causative familial pathogenetic variant of the disease and/or of the haplotype at risk. The term unaffected refers to the absence of the familial pathogenetic variant and/or of the haplotype at risk.

Autosomal recessive diseases:

The term affected refers to the presence in homozygosis or heterozygosis composed of the familial variant(s) and/or the haplotypes at risk. The term unaffected refers to the absence of the familial variant(s) and/or of

the haplotypes at risk. The term carrier refers to the presence in simple heterozygosis of the familial variant and/or of only one of the haplotypes at risk.

Diseases related to the recessive X-linked chromosome:

The term affected refers to the presence of the familial pathogenetic variant in hemizygosis and/or of the haplotype at risk in embryos with an XY sex complement (males). The term unaffected refers to the absence of the familial variant and/or of the haplotype at risk in embryos regardless of the XX or XY sex complement. The term carrier refers to the presence in simple heterozygosis of the familial variant and/or of the haplotype at risk in embryos with an XX sex complement (females).

Diseases related to the dominant X-linked chromosome:

The term affected refers to the presence of the familial pathogenetic variant in hemizygosis or heterozygosis and/or of the haplotype at risk in embryos regardless of the XX or XY sex complement. The term unaffected refers to the absence of the familial variant and/or of the haplotype at risk in embryos regardless of the XX or XY sex complement.

### Reporting times

The set-up prior to PGT-M will be concluded within 30-40 business days of the acceptance of the samples necessary for this investigation. The results of the PGT-M test on the embryos will be available within 7-10 business days of acceptance of the sample. The terms defined for completing the preliminary setup and for reporting the PGT-M testing, however, are not mandatory and may be extended in the event of repeating the test, suboptimal or inconclusive results, in-depth diagnostic analysis or interpretative doubts. For the PGT-M test to be carried out, the test request sheet and informed consent must be duly completed and signed. In the event of missing information, the laboratory will contact the forwarding doctor/clinic or the persons concerned to obtain this information. This may change the time for processing the sample and issuing the report.

## Diagnostic methodology

### Whole Genome Amplification

All PGT applications require lysis of the biopsied embryonic cells and the isolation of the nuclear (genomic) DNA. The next step is the whole genome amplification (WGA) process that permits amplifying the embryonic genome millions of times to obtain a suitable amount of DNA for subsequent analysis. For PGT applications WGA is performed using the Ion SingleSeq Kit (Thermo Fisher Scientific).

## PGT-A and PGT-SR

### *Massive Parallel sequencing*

The analysis of the entire chromosomal copy number of the embryo is performed by massively parallel sequencing (MPS) using the Ion GeneStudio S5 Plus instrument (Thermo Fisher Scientific) with the Ion Reproseq protocol (Thermo Fisher Scientific). The chromosome sequences obtained by MPS are then quantified through bioinformatics analysis that permits aneuploidy screening on all 24 chromosomes and the data obtained are analyzed using the Ion Reporter Software platform (Thermo Fisher Scientific). Chromosomal assessment by PGT-A/SR can be applied either on a single cell (blastomere biopsy) or on a few cells (blastocyst biopsy) of an embryo. The method has a resolution of 8 Mb and is therefore able to identify chromosomal imbalances greater than 8 Mb.

The system is capable of detecting the presence of a chromosomal mosaicism mix, in particular of highlighting the presence of an euploid-aneuploid mosaicism for the coexistence of a euploid cell line and one or more aneuploid/altered cell lines.

The ReproSeq PGS – Ion GeneStudio S5 Plus is able to determine the presence of mosaicism in low percentages, in particular the minimum detection threshold is 30% as suggested by the international guidelines (ESHRE, 2022). Validation for the determination of the threshold of chromosomal mosaicism was also carried out internally in the PGT laboratory of Eurofins Genoma and the results were published in 2021 (Biricik et al. 2021).

### *Reporting criteria*

The analysis is capable of detecting mosaicisms between 30% and 70%. Chromosomal mosaicism will be reported with incremental values of 10% above the 30% detection threshold. The transfer of a mosaic embryo must be preceded by a specific genetic counseling.

If both mosaic chromosomal aneuploidies and homogeneous aneuploidies are identified in the same embryo, the embryo will in any case be considered aneuploid.

### *Limitations of the procedure and risk of diagnostic error for PGT-A/PGT-SR*

The assessment of the embryonic chromosomal arrangement by PGT-A/SR as described above is capable of producing results in 95% of the samples analysed. In the remaining cases, the analysis may produce no results (no outcome) due to WGA failure or may produce inconclusive results if there is any doubt as to the interpretation of the results.

The technical/biological reasons for non-diagnosis could be:

1. No Result:
    a. No nuclei of the sampled embryonic cells
    b. Insufficient sampled embryonic material for the required analysis

c. Technical problems during the biopsy or tubing procedure (inserting embryonic cells into the test tube) of the sampled embryonic cells, resulting in the loss of the biological material to be tested.

2. Inconclusive result:
   a. Genetic profile cannot be interpreted due to poor embryo quality and/or DNA degradation in the sampled embryonic cells
   b. Presence of external DNA contamination
   c. Doubt over a polyploid genotype (PGT-A) or disagreement between the mutation and markers linked with it (PGT-M).

In the event of non-diagnosis for the reasons described above, a new embryo biopsy is recommended

Contamination of the analysed sample by non-embryonic material (of maternal or external origin) could lead to failure of the analysis and to a misdiagnosis if such contamination is not detected. A blank sample consisting of the last drop of the medium in which the biopsy was contained prior to transfer to the tube intended for the laboratory will be required to determine any potential contamination of the biopsied embryonic cells. The "blank" samples will be amplified in parallel with the corresponding samples to rule out any contamination.

Due to the phenomenon of mosaicism, an embryo may have both chromosomally normal and altered cells. As a result of this phenomenon, the analysed sample, and therefore the pertinent embryo, could be misdiagnosed as normal in the case of aneuploidy or as abnormal in the case of euploidy.

The PGT-A/SR analysis might not identify any euploid or balanced embryos.

The analysis is also unable to detect:

- balanced chromosomal rearrangements
- unbalanced chromosomal rearrangements where the pseudoautosomal regions of the X and Y chromosomes or the heterochromatic regions are involved (e.g. pericentromeric regions, short arm of the acrocentric chromosomes, etc.)
- chromosome regions not represented on the platform
- chromosomal rearrangements of any type if smaller than the resolution (8 Mb)
- Sequence variants (points) of DNA
- Defects of methylation,
- Polyploidy (example triploidy 69,XXX). This limitation is eliminated when the polyploidy panel is applied to the ET biopsy samples under analysis.
- Chromosomal mosaicisms less than 30%

The diagnostic error of PGT-A/PGT-SR is estimated to be less than 1% (ESHRE PGT-SR/PGT-A Working Group et al. 2020).

## Ploidy Panel

The ploidy panel is based on the detection of single nucleotide variants (SNPs) by next generation sequencing (NGS) with the Ion AmpliSeq Polyploidy Panel Kit (Thermo Fisher Scientific). A total of 360 amplicons covering approximately 590 SNPs common in the general population are included in the kit.

The limits of the Ploidy Panel:

- The Ploidy Panel cannot be applied alone, it is a supplementary test for standard PGT-A and PGT-SR.

- The Ploidy Panel requires an additional testing protocol even if it will be applied on the same embryo biopsy sample. This may require a longer reporting time than standard PGT-A and PGT-SR.

- The Ploidy Panel cannot be reported if the standard PGT is uninformative for the same embryo.

- The Ploidy Panel can only be applied on embryonic cells taken at the blastocyst level (days 5-6-7 after fertilization), and not on embryonic cells taken at the segmentation stage (day 3 after fertilization).

- DNA Fingerprinting (sibling QC) assessment can only be performed for cohorts of 2 or more diploid embryos.

## PGT-M

*Direct and indirect analysis*

Direct analysis for searching for familial variants is performed with different selection methodologies based on the characteristics of the specific gene variant to be investigated. The reference techniques are Minisequencing, fluorescent PCR and NGS deep sequencing for PGT set-up applications. The PGT-M test on embryonic cells will be performed either by direct analysis of the familial mutation using the Minisequencing and/or fluorescent PCR methodology or by indirect analysis (linkage) using short tandem repeat (STR) markers. Both strategies have been validated and published by Eurofins Genoma (F. Fiorentino et al. 2006; F. Fiorentino 2003) and have been accepted as a reference method for the preimplantation diagnosis of monogenic diseases by international bodies such as ESHRE PGT Consortium and GenQA (Deans Z. et. al., 2022, ESHRE PGT-M Working Group 2020).

### *Limitations of the procedure and risk of diagnostic error for PGT-M*

The assessment of the presence of one or more monogenic diseases in embryos is capable of producing results in 95% of the samples analysed. In the remaining cases, the analysis may produce no results (no outcome) due to WGA failure or may produce inconclusive results if there is any doubt as to the interpretation of the results.

The technical/biological reasons for non-diagnosis could be:

1. No Result**:**
    a. No nuclei of the sampled embryonic cells
    b. Insufficient sampled embryonic material for the required analysis
    c. Technical problems during the biopsy or tubing procedure (inserting embryonic cells into the test tube) of the sampled embryonic cells, resulting in the loss of the biological material to be tested.
2. Inconclusive result:
    a. Genetic profile cannot be interpreted due to poor embryo quality and/or DNA degradation in the sampled embryonic cells
    b. Presence of external DNA contamination
    c. Doubt over a polyploid genotype (PGT-A) or disagreement between the mutation and markers linked with it (PGT-M).

In the event of non-diagnosis for the reasons described above, a new embryo biopsy is recommended

Contamination of the analysed sample by non-embryonic material (of maternal or external origin) could lead to failure of the analysis and to a misdiagnosis if such contamination is not detected. A blank sample consisting of the last drop of the medium in which the biopsy was contained prior to transfer to the tube intended for the laboratory will be required to determine any potential contamination of the biopsied embryonic cells. The "blank" samples will be amplified in parallel with the corresponding samples to rule out any contamination.

PGT-M may be subject, more frequently than other molecular analyses, to Allele Drop Out (ADO), a phenomenon consisting of the failed amplification of one of the two alleles, caused by technical reasons that are typical of single-cell genetic diagnosis, the incidence of which may vary due to several factors (e.g. region of analysis, single or multicellular starting sample, etc.) and it can reach 5%. Because of this phenomenon, one of the alleles searched for (mutated or wild type) might not be identified, resulting in erroneous results. In particular, in the case of non-amplification of the normal allele, the embryo would be classified as affected while actually being unaffected (false positive). On the contrary, in the case of non-amplification of the altered allele, there would be an erroneous attribution of the embryo as unaffected while actually being affected (false negative).

Recombination is the mechanism of exchanging genetic material from both parents. This mechanism involves the exchange of homologous portions of genetic material, which occurs between two chromatids belonging to two different chromosomes of an homologous pair. Depending on its location on the genome, the gene under examination may be affected by an event of recombination between the homologous chromosomes,

which may result in replacement of the affected and normal alleles. In indirect PGT-M analysis, markers as close as possible to the familial disease-gene will be used to reduce the risk of error related to recombination events.

Direct analysis by sequencing and linkage analysis cannot detect:

- Chromosomal rearrangements
- DNA sequence variants (points) except those specifically searched for
- Defects of methylation
- Mosaicisms

The diagnostic error of PGT-M is estimated to be less than 1% (ESHRE PGT-M Working Group et al. 2020)

## Confirmation of preimplantation test results by prenatal testing

Preimplantation genetic testing should have diagnostic confirmation during pregnancy. In particular, in couples who have done PGT-M and/or PGT-A/SR, invasive investigations (amniocentesis and villocentesis) should be considered as the reference options. The non-invasive prenatal test (NIPT), performed on circulating foetal DNA (cfDNA), may be proposed for a reassessment of the risk of chromosomal abnormalities for the PGT-A/SR chromosomes prior to a final decision on whether or not to undergo confirmatory invasive prenatal testing.

The prenatal testing options will in any case be discussed during a specific genetic counseling in which the couple must receive the information necessary to understand the characteristics of the test (biological sources, foetal fraction) and its limitations (specificity and sensitivity, foetal-placental mosaicisms), also in relation to the other available prenatal testing techniques.

If the couple decide not to undergo prenatal testing, the possibility of confirming the PGT outcome in the post-natal period by DNA analysis of the baby must be discussed.

Preimplantation testing does not detect any other malformations or defects not specifically investigated. It is therefore advantageous that following PGT, regardless of the type and outcome of the test, the pregnancy be monitored by ultrasound and other investigations which the attending obstetrician gynaecologist will consider advantageous as per good clinical practice.

## References

1. Biricik, Anil, Ettore Cotroneo, Maria Giulia Minasi, Pier Francesco Greco, Sara Bono, Matteo Surdo, Federica Lecciso, et al. 2021. «Cross-Validation of Next-Generation Sequencing Technologies for Diagnosis of Chromosomal Mosaicism and Segmental Aneuploidies in Preimplantation Embryos Model». Life 11 (4): 340. https://doi.org/10.3390/life11040340.

2. Bredbacka, Peter , Antonio Capalbo , Kirsi Kananen , Ludovica Picchetta , Candido Tomás; Healthy live birth following embryo transfer of a blastocyst of tetrapronuclear (4PN) origin: a case report; Hum Reprod 2023 Sep 5;38(9):1700-1704

3. Deans, Zandra C., Anil Biricik, Martine De Rycke, Gary L. Harton, Miroslav Hornak, Farrah Khawaja, Céline Moutou, Jan Traeger-Synodinos, Pamela Renwick, "Twelve years of assessing the quality of preimplantation genetic testing for monogenic disorders"; Prenatal Diagnosis, November 2022, https://doi.org/10.1002/pd.6263.

*4. ESHRE Working Group on Chromosomal Mosaicism, Martine De Rycke , Antonio Capalbo , Edith Coonen, Giovanni Coticchio , Francesco Fiorentino , Veerle Goossens , Saria Mcheik , Carmen Rubio, Karen Sermon, Ioannis Sfontouris , Claudia Spits , Joris Robert Vermeesch , Nathalie Vermeulen , Dagan Wells , Filippo Zambelli and Georgia Kakourou; ESHRE survey results and good practice recommendations on managing chromosomal mosaicism; Human Reproduction Open, pp. 1–18, 2022.*

5. ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group, Georgia Kokkali, Giovanni Coticchio, Fernando Bronet, Catherine Celebi, Danilo Cimadomo, Veerle Goossens, et al. 2020. «ESHRE PGT Consortium and SIG Embryology Good Practice Recommendations for Polar Body and Embryo Biopsy for PGT†». *Human Reproduction Open* 2020 (3): hoaa020. https://doi.org/10.1093/hropen/hoaa020.
6. ESHRE PGT-M Working Group, Filipa Carvalho, Céline Moutou, Eftychia Dimitriadou, Jos Dreesen, Carles Giménez, Veerle Goossens, et al. 2020. «ESHRE PGT Consortium Good Practice Recommendations for the Detection of Monogenic Disorders†». *Human Reproduction Open* 2020 (3): hoaa018. https://doi.org/10.1093/hropen/hoaa018.
7. ESHRE PGT-SR/PGT-A Working Group, Edith Coonen, Carmen Rubio, Dimitra Christopikou, Eftychia Dimitriadou, Julia Gontar, Veerle Goossens, et al. 2020. «ESHRE PGT Consortium Good Practice Recommendations for the Detection of Structural and Numerical Chromosomal Aberrations†». *Human Reproduction Open* 2020 (3): hoaa017. https://doi.org/10.1093/hropen/hoaa017.
8. Fiorentino, F. 2003. «The Minisequencing Method: An Alternative Strategy for Preimplantation Genetic Diagnosis of Single Gene Disorders». *Molecular Human Reproduction* 9 (7): 399–410. https://doi.org/10.1093/molehr/gag046.
9. Fiorentino, F., A. Biricik, A. Nuccitelli, R. De Palma, S. Kahraman, M. Iacobelli, V. Trengia, et al. 2006. «Strategies and Clinical Outcome of 250 Cycles of Preimplantation Genetic Diagnosis for Single Gene Disorders». *Human Reproduction* 21 (3): 670–84. https://doi.org/10.1093/humrep/dei382.
10. Franasiak, Jason M., Eric J. Forman, Kathleen H. Hong, Marie D. Werner, Kathleen M. Upham, Nathan R. Treff, e Richard T. Scott. 2014. «The Nature of Aneuploidy with Increasing Age of the Female Partner: A Review of 15,169 Consecutive Trophectoderm Biopsies Evaluated with Comprehensive Chromosomal Screening». *Fertility and Sterility* 101 (3): 656-663.e1. https://doi.org/10.1016/j.fertnstert.2013.11.004.
11. Greco, Ermanno, Maria Giulia Minasi, e Francesco Fiorentino. 2015. «Healthy Babies after Intrauterine Transfer of Mosaic Aneuploid Blastocysts». *New England Journal of Medicine* 373 (21): 2089–90. https://doi.org/10.1056/NEJMc1500421.
12. Leigh, D., D.S. Cram, S. Rechitsky, A. Handyside, D. Wells, S. Munne, S. Kahraman, et al. 2022. «PGDIS Position Statement on the Transfer of Mosaic Embryos 2021». *Reproductive BioMedicine Online* 45 (1): 19–25. https://doi.org/10.1016/j.rbmo.2022.03.013.

*13. Marin D, Zimmerman R, Tao X, Zhan Y, Scott RT Jr, Treff NR. Validation of a targeted next generation sequencing-based comprehensive chromosome screening platform for detection of triploidy in human blastocysts. Reprod Biomed Online 2018;36:388–95.*

14. Munné, S., M. Alikani, L. Ribustello, P. Colls, Pedro A. Martínez-Ortiz, Referring Physician Group, e D.H. McCulloh. 2017. «Euploidy Rates in Donor Egg Cycles Significantly Differ between Fertility Centers». *Human Reproduction* 32 (4): 743–49. https://doi.org/10.1093/humrep/dex031.
15. Rubio, Carmen, José Bellver, Lorena Rodrigo, Gema Castillón, Alfredo Guillén, Carmina Vidal, Juan Giles, et al. 2017. «In Vitro Fertilization with Preimplantation Genetic Diagnosis for Aneuploidies in Advanced Maternal Age: A Randomized, Controlled Study». *Fertility and Sterility* 107 (5): 1122–29. https://doi.org/10.1016/j.fertnstert.2017.03.011.

16. Viotti, Manuel. 2020. «Preimplantation Genetic Testing for Chromosomal Abnormalities: Aneuploidy, Mosaicism, and Structural Rearrangements». *Genes* 11 (6): 602. https://doi.org/10.3390/genes11060602.

# CONSENT TO CARRY OUT THE PREIMPLANTATION GENETIC TEST

| | |
|---|---|
| *The undersigned (male partner) | |
| *Place of birth | *Date of birth |
| *Tax Code: | |
| *Domiciled at: | *Street: |
| *Phone: | *email: |
| *Document | *Nr. |
| *Issued on | *by |
| | |
| *The undersigned (female partner) | |
| *Place of birth | |
| *Tax Code | |
| *Domiciled at: | *Street: |
| *Phone: | *email: |
| *Document | *Nr. |
| *Issued on | *by |

***Information marked with an asterisk is mandatory***

With a view to undergoing at the Centre for medically assisted reproduction (MAR) a cycle of ICSI (in vitro fertilization with intracytoplasmic sperm injection) with subsequent biopsy and genetic analysis of a single or a few embryonic cells, we declare that we have read the information form attached to this consent in its entirety and that we have fully understood its content and that we have received all the information in detail, both on the methods and on success and diagnostic error rates.

We declare that we have preliminarily had one or more meetings with staff of the MAR centre and/or of the Eurofins Genoma Group laboratory during which we were presented with all the points of the above-mentioned information notice; we were able to ask the necessary questions and we received the consequent answers.

We also declare that we have been informed about the following aspects:

**WE CONSENT** ***(in case of multiple indications, tick multiple boxes):***

execution of the following analysis(s) on embryo biopsy:

- ☐ PGT-A
- ☐ PGT-M
- ☐ PGT-SR
- ☐ PGT-A + Ploidy Panel
- ☐ PGT-SR + Ploidy Panel

We also declare that we have been informed of the following aspects:

## Definition of the roles and subject of the cooperation

The Eurofins Genoma Laboratory is a specialized laboratory of molecular genetics and biology, authorized by the Municipality of Rome, prot. No. 14965 of 19.03.2003, to perform molecular genetic diagnosis.

The Eurofins Genoma laboratory DOES NOT carry out Medically Assisted Reproduction techniques, regulated by Law 19 February 2004, No. 40.

The Eurofins Genoma laboratory is organized to conduct specialized genetics tests for third-party facilities, thus acting as a "Service" of reference.

The MAR Centre has requested the specialist support of the Eurofins Genoma laboratory for the optimization and performance of genetic testing on a single or a few embryonic cells for the diagnosis of hereditary genetic diseases, in order to provide the above generalized subjects, upon their explicit request, with information on the state of health of the embryos produced and to be transferred into the uterus, in accordance with Art. 14 subsection 5 of Law 40/2004.

## Limits of responsibility

The Eurofins Genome laboratory does not produce embryos by means of Medically Assisted Reproduction techniques. This procedure will be carried out in the laboratories of the Medically Assisted Reproduction Centre, by technicians/biologists employed by the above-mentioned centre.

The Eurofins Genoma laboratory is responsible solely for the results of the genetic test carried out on the embryonic cells and has no responsibility for the work of the Medically Assisted Reproduction Centre.

## Costs of the activity carried out

The cost of the PGT diagnostic activity are reported in the offer provided to patients by Eurofins Genoma staff or by the staff of the Medically Assisted Reproduction Centre.

The cost of the diagnostic activity related to the set-up phase prior to PGT-M, i.e. the optimization of the diagnostic protocol, is a lump sum which users undertake to pay jointly and severally even if later, for reasons not depending on the Eurofins Genoma laboratory, the PGT-M analysis will not be carried out.

The cost of the PGT-M/SR/A diagnostic activity will be paid for each case of preimplantation testing carried out in accordance with the instructions given in the agreed offer.

The cost of medically assisted reproduction techniques (MAR cycle and embryo biopsy) is to be agreed upon entirely with the Medically Assisted Reproduction Centre to which reference is hereby made.

Place and date, _____

Name of the female partner _____

Signed _____ 

Name of the male partner _____

Signed _____ 

The Specialist/Clinician who collected the consent:

(Name e Surname) _____

Tel. _____ E-Mail _____

Signature and stamp: _____