ESHRE PGT Consortium good practice recommendations for the detection of monogenic disorders

AUTHORS

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General introduction

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

Methods

A working group was composed of geneticists with hands-on expertise on the described techniques, aiming at a representation of different settings and nationalities. The working group members assessed the previous guidelines (Harton *et al.*, 2011) and deducted an outline for the current paper. All group members according to their expertise, wrote a section that was later discussed in depth with the entire group until consensus. Eleven online meetings were organised for discussion. The final draft of the paper was checked for consistency with the other papers of the series. The draft was then submitted for stakeholder review; it was published on the ESHRE website between XX June and XX July 2019, and ESHRE members were invited to send in comments. All comments were checked by the working group, and incorporated in the final version where relevant. A review report is published on the ESHRE website.

Introduction to PGT-M

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

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

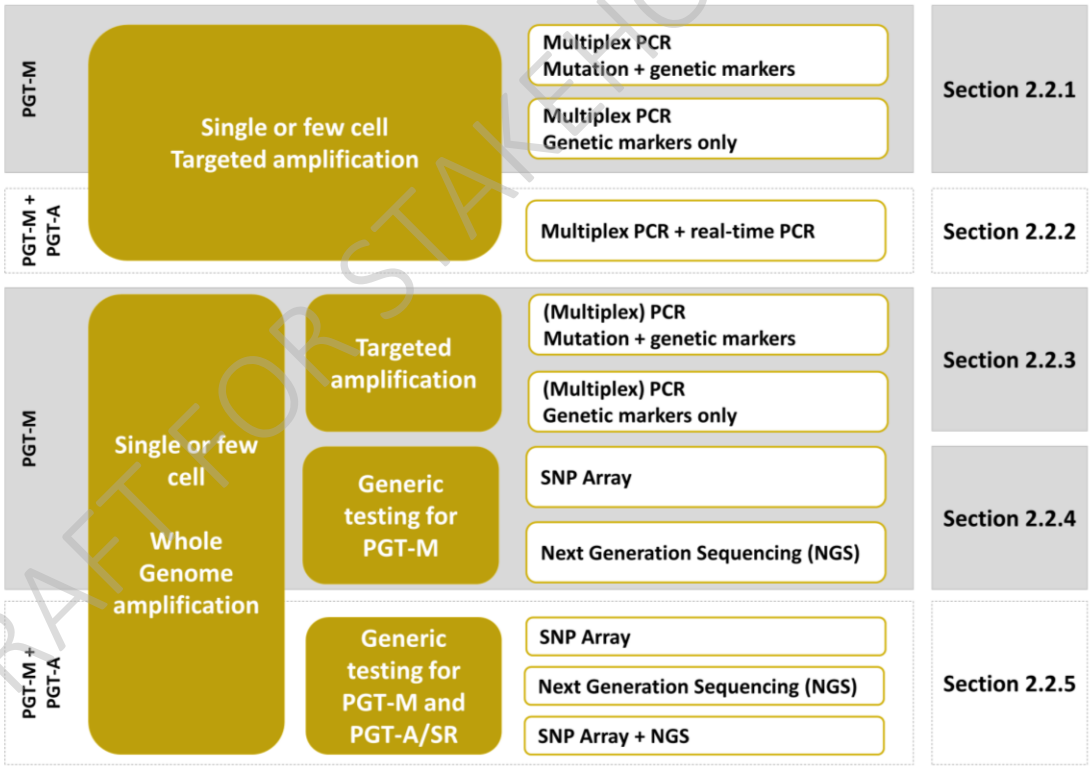
One of the greatest challenges for PGT-M is the low amount of input DNA for which sensitive DNA amplification techniques are needed. Biopsied single (after polar body or single blastomere biopsy) or few cells (i.e. 5-10 trophectoderm [TE] cells) undergo either a targeted amplification reaction via multiplex PCR or a whole genome amplification (WGA) step followed by downstream applications (targeted or genome wide) such as PCR, single nucleotide polymorphism (SNP) array or next generation sequencing (NGS) (figure 2). Each method has its advantages and its limitations. The principle of most

of these methods is based on haplotyping (i.e. determination of the group of alleles within a genetic segment on a single chromosome being inherited together). Therefore, genetic markers located close to the gene of interest are genotyped in DNA samples of the couple and relevant family members with known genetic status during preclinical work-up. Genetic markers that are informative, flank the locus of interest and allow discrimination of the parental haplotypes, are selected for use in the clinical test. The haplotype which is common in the family members with the familial mutation is referred to as the mutant or the high-risk haplotype, while the haplotype without familial mutation is referred to as the wildtype or low-risk haplotype. The clinical test can be either direct, when mutation plus linked genetic markers are assessed, or indirect when testing is based on haplotyping only.

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

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

Figure 2 : Overview of the testing strategies that can be applied for PGT-M



Training and Personnel

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

Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials are covered in the paper on organisation of PGT (refer to ORG paper).

Labelling and witnessing

General guidance on labelling and witnessing is covered in the paper on organisation of PGT (refer to ORG paper).

1. Single or few cell methods

PGT-M can be subdivided in the pre-examination process and the clinical cycle (examination process). The pre-examination process includes preclinical work-up with informativity/segregation analysis and eventually test development followed by validation. For informativity/segregation analysis, short tandem repeat markers (STR) or SNP marker genotyping is performed on DNA samples of the couple and related family member(s) to identify informative markers and to establish which combination of marker alleles (haplotype) segregates with the mutation. If the high-risk haplotype is determined during workup, an indirect testing method can be applied. Alternatively, a direct method is chosen where the detection of the mutation is combined with the genetic markers for haplotype confirmation. For some cases, it will not be possible to determine the high-risk haplotype during work-up, for instance when a *de novo* mutation is present or when no relevant family DNA samples can be obtained (see also section 3.1). In these cases, it may be determined during the clinical cycle based on the results from the biopsied embryo cells.

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

1.1 Mutation and genetic marker loci

Mutation loci can be nuclear or mitochondrial and involve germline genetic variant(s) proven to be disease causing (termed mutation). Whether the mutation loci are incorporated in the clinical test depends on multiple factors, including the nature of the mutation (familial or *de novo*), the availability of relevant family DNA samples, the mutation type, and the preclinical work-up results.

STR markers are short tandemly repeated DNA sequences (dinucleotides are the most common) which are highly polymorphic and quite abundant in the human genome (one STR per 2,000-10,000 bp). Useful STR markers are taken from published papers or *in silico* selected from public databases and usually involve many alleles (high heterozygosity). STR loci are targeted with fluorescently labelled primers and coamplified in a multiplex PCR reaction.

A fully informative STR marker will allow to discriminate all possible embryo genotypes and to detect problems of contamination, ADO, recombination and copy number aberrations. A partially-informative (semi- or limited-informative) STR marker indicates that not all embryo genotypes can be distinguished and is less powerful in detecting additional problems. A non-informative STR marker is a marker that cannot distinguish between an affected and an unaffected embryo. This is illustrated below in an example for an autosomal dominant disorder (table 1).

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

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

A SNP marker is informative when it is heterozygous in one parent and homozygous in the other. An informative SNP marker in which the wildtype allele is unique, is the most powerful, as unaffected embryos are then distinguished by heterozygous SNPs, limiting the misdiagnosis risk due to ADO. This is illustrated below in an example for an autosomal dominant disorder (table 2).

Informativity results are first evaluated for each marker separately, afterwards the overall effectiveness of the selected set of markers to be used in the clinical test is assessed for its ability to detect the monogenic disorder, and other aspects (ADO, monosomy, trisomy, and parental [mostly maternal] contamination).

Table 1: Example of STR informativity results for an autosomal dominant disorder (the mutant allele is indicated with *after validation of segregation analysis with a suitable reference)

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

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

Table 2: Example of SNP informativity results for an autosomal dominant disorder (the mutant allele is indicated with *after validation of segregation with a suitable reference).

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

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

1.2 Basics methods for allele discrimination

Mutation and marker loci are amplified with primer pairs in which one primer is fluorescently labelled, allowing sensitive detection of the amplification products afterwards. The method is designed as such that wildtype and mutant allele discrimination is part of the amplification itself (e.g. D-ARMS), or allele discrimination is carried out in a post-amplification step (e.g. mini-sequencing). In some cases, a DNA purification step may be required to remove primers and buffer components of the amplification reaction, before starting the post-amplification reaction.

Fragment length analysis

Principle of the test

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

Limitations of the test

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

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

Restriction enzyme digestion

Principle of the test

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

Limitations of the test

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

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

Double amplification refractory mutation system (D-ARMS)

Principle of the test

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

Limitations of the test

D-ARMS is not recommended when the mutation is part of a nucleotide stretch since the difference in amplification specificity between mutant and wildtype alleles may be insufficient.

Real-time PCR for mutation detection and genotyping

Principle of the test

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

Limitations of the test

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

Mini-sequencing

Principle of the test

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

Limitations of the test

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

1.3 Single or few cell targeted amplification

Following embryo biopsy, biopsied cell samples are washed, transferred to reaction tubes and lysed. Amplification reaction components are then added directly to the lysed cell(s) without prior DNA purification. Samples undergo either targeted amplification by means of multiplex PCR or WGA (see section 1.4). The prevention for external DNA contamination is a requisite, together with accurate and strict sample processing. This requires a specialized laboratory environment and working attitude.

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

Laboratory infrastructure, equipment and materials

General aspects on infrastructure, equipment and materials are covered in the paper on organisation of PGT (refer org paper). For targeted amplification-based PGT specifically, the following recommendations are made:

Infrastructure

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

Equipment

Equipment required for amplification-based analysis of samples includes:

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

Materials.

Specific materials required for targeted-amplification of samples include:

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

Tubing of samples

General recommendations about biopsy and transfer of samples to tubes (referred to as tubing) is provided in the paper on polar body and embryo biopsy for PGT (refer biopsy paper).

Work practice controls

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

- As a positive control sample, diluted and/or undiluted genomic DNA from the couple is recommended. DNA samples from other family members may also be included. In addition, single or few cell samples can be used. Positive control cell samples can be lymphocytes, buccal cells or cultured cells. If the test includes the mutation detection, it is recommended to use:
    - For dominant diseases: DNA samples with high-risk and low-risk genotypes.
    - For X-linked diseases: DNA samples with high-risk, low-risk, male and female genotypes.
    - For recessive diseases: DNA samples with heterozygous mutation carrier, homozygous normal, and (if available) homozygous or compound heterozygous genotypes.
- Negative controls should be included to confirm that there was no contamination introduced from the procedure of sample collection or from the amplification reactions.
    - A minimum of one negative control per buffer (sample collection buffer, biopsy media, or washing media, depending on the protocols of the PGT centre) is recommended to control for contamination during each step of cell sample collection (i.e. the IVF laboratory negative control); e.g. collection on two different timepoints for a specific cohort of embryos should yield minimum 2 negative controls of this type. As the contamination risk is substantially higher when working with single cells in comparison to few cells, the number of negative controls should preferably be increased.
    - A minimum of one negative control with amplification mixture only is recommended to control for contamination during setting up of amplification reactions (i.e. the genetic laboratory negative control).

1.4 Single or few cell whole genome amplification

Following embryo biopsy, cell samples are washed and transferred to reaction tubes. After cell lysis, WGA reaction components are added without prior DNA purification. WGA allows providing sufficient DNA template from minute DNA samples to carry out subsequent DNA amplifications or to be used with other downstream techniques like multiple standard PCR testing, array-based comparative genomic hybridization (aCGH), SNP array or high-throughput assays like NGS. Moreover, WGA products can be stored for years (-20°C) and used later in time to reconfirm results/diagnosis or carry out new tests.

Several methods for WGA have been developed over time and are available as commercial kits. Any WGA technique should be evaluated with regards to genomic coverage, high fidelity of the sequence, reliable quantification of copy number variation and technical errors of ADO and allele drop in (ADI). A WGA method should be selected in function of the downstream application, taking into account advantages and disadvantages. Currently, multiple displacement amplification (MDA) is recommended

for haplotyping, whereas displacement degenerate oligonucleotide-primed PCR (DOP-PCR) (marketed Picoplex/Sureplex) is the method of choice for the detection of chromosomal copy number variation.

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

Laboratory infrastructure, equipment and materials

In general, follow the recommendations as stated in section 1.3.

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

Infrastructure

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

Equipment

Additional equipment includes:

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

Materials

Specific materials required for WGA of samples include:

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

Tubing of samples

General recommendations about biopsy and tubing are provided in the paper on polar body and embryo biopsy for PGT (refer biopsy paper).

Work practice controls

Positive and negative controls should be included to monitor the WGA reaction, as described in section 1.3.

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

2. Pre-examination process

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

2.1. Informativity/segregation analysis

It is recommended to perform a preclinical work-up to assess PGT-M feasibility, identify informative genetic markers, establish parental haplotypes (when possible) and work on a clinical testing strategy. It is recommended to perform the informativity/segregation analysis for STR markers as well as for SNP markers. The results allow evaluating the expected genotypes in the embryos.

- A geneticist experienced in pedigree and linkage analysis should determine which familial DNA samples are needed for a reliable and accurate diagnosis.
- For all diseases, samples should be collected from the prospective parents and close relatives with known disease status (proven via genetic reports) to establish the high-risk and low-risk haplotypes:
    - For dominant diseases, it is recommended that these samples include DNA from at least one affected (ideally two) and/or one unaffected individual as a reference.
    - For recessive diseases, these would include at least a homozygous or compound heterozygous affected individual and one non-carrier individual as a reference, but a proven carrier would be recommended.
    - For X-linked diseases, an affected individual must be used as a reference and/or one unaffected individual. A proven carrier would also be recommended.
- It is recommended that the original molecular genetic reports including the description of identified variants together with the reference sequence are obtained from an accredited laboratory. It is advisable to confirm the mutation whenever possible.

2.2 Testing strategies and test development

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

The three main testing strategies for PGT-M are:

1) targeted amplification of informative markers with or without the mutation(s) in a single/few cell multiplex PCR *(sections 2.2.1 and 2.2.2)*

2) WGA followed by targeted amplification of informative markers with or without the mutation(s) *(section 2.2.3)*

3) WGA followed by a generic method such as SNP array or NGS-based sequencing *(sections 2.2.4 and 2.2.5)*

Targeted amplification is mostly coupled with day 3 biopsy (but not only) which leaves sufficient time for analysis and fresh embryo transfer. The major disadvantage of this approach is that development and validation of the multiplex PCR to the single/few cell level has to be repeated with every new gene/locus of interest. The first strategy including the development/validation of a new test is more time consuming and labor intensive than the WGA-based strategies and turnaround time between referral and clinical cycle is significantly increased. The second strategy is a step towards a more generic method because the adaptation/validation of PCR reactions to the single cell level can be omitted from the preclinical work-up. Locus-specific information is available in both cases in the form of either

genotypes (mutation detection, SNP) or allele length (STR). Nevertheless, due to their targeted nature, the majority of these tests do not provide a comprehensive view of the genome. The third approach, the development of genome-wide generic methods, tackled this issue. SNP array, as well as sequencing-based approaches, allow on the one hand genome-wide haplotyping, and on the other hand copy number typing. The extent to which the whole genome is analysed depends on the platform and/or approach. SNP array-based methods are restricted by the fixed number of probes included on the platform of choice. Sequencing-based approaches can be more or less comprehensive depending on the genome coverage, and the depth of sequencing. Additionally, sequencing-based approaches are high-throughput and allow automation, reducing hands-on time and minimising the possibility of human errors. The second and third strategies are mostly coupled with Day 5-7 biopsy which leaves often insufficient time for fresh embryo transfer. This is overcome by cryopreservation and embryo transfer in a deferred cycle.

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

2.2.1 Targeted amplification for PGT-M

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

Recommendations for single or few cell targeted amplification concerning infrastructure, equipment, materials, tubing and work practice controls are described in section 1.3. At the preclinical work-up, informativity/segregation analysis is required, together with the development of a locus-specific test at the level of single or few cells. Based on the results of informativity/segregation analysis, suitable STR markers close to the locus of interest are selected for co-amplification in a multiplex PCR, alone or in combination with the mutation.

The adaptation of PCR reaction conditions is usually carried out in several steps. The amplicons are preferably first multiplexed on genomic DNA samples. Further fine-tuning is then carried out with single or few cell samples. For test development, processing of at least one negative control with amplification mixture only for each amplification reaction is recommended. When working with single or few cells, negative controls with sample collection buffer only should be added as well, to control for contamination during sample collection. The optimised single/few cell PCR protocol is then validated on a series of single or few cells along with positive and negative controls *(see also section 2.3)*.

<u>Familial mutation + genetic markers (STRs and/or SNPs)</u>

When developing mutation and STR and/or SNPs analysis for single or few cells, the following recommendations are made:

- Amplicons should be designed ideally to be sized between 100 and 500bp using combinations of fluorochromes allowing loci discrimination.
- Single-round multiplex PCR is preferred compared to nested or semi-nested PCR as it is less error prone. When available, the use of STRs with tri-, tetra- or penta-nucleotide repeats is preferable to reduce the phenomenon of stutter patterns and improve allele discrimination.

- It is recommended to avoid STRs with a very wide range of alleles since the ADO risk of the large alleles is increased even at the genomic DNA level leading to false homozygous genotypes during pre-clinical work-up and PGT-M.
- Before moving on to single-cell validation, it is recommended to establish a correct discrimination of mutation/wildtype or marker alleles of the test at hand. It is recommended to test various genotypes concerning the mutation or marker of interest using the following DNA samples:
    - affected (autosomal dominant) DNA samples,
    - carrier (autosomal recessive, X-linked diseases) DNA samples,
    - unaffected DNA samples for the mutation to be tested, and
    - DNA samples with heterozygous markers for indirect tests.
- When a protocol is employed for PGT-M, it is recommended to apply the specific test to DNA or single cells from each particular couple to discover any unexpected test results which could render future blastomere results questionable (for example, a polymorphism which may exist under a primer used in the single cell assay but not in the routine laboratory assay).
- Polymorphic markers should have a high degree of heterozygosity and produce a clearly interpretable peak pattern, and preferably be intragenic.
- When using extragenic markers, it is recommended to stay within 1 Mb distance from the mutation of interest to reduce the misdiagnosis risk due to recombination events (on average, loci 1cM apart are expected to show 1% recombination). If no suitable markers are available within 1Mb, markers within 2Mb are acceptable. This may be adapted in case of large genes or duplications.
- The risk of misdiagnosis due to recombination should be considered for every marker and is especially important in case of large genes and genes with recombination hot spots.
- Defining the minimum number of informative markers required in the single/few cell test: assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended to include at least one STR or 3 SNPs proximal and one STR or 3 SNPs distal to the region of interest together with the mutation locus (choose markers with rank 1 or 2 in table 1 and rank 1 in table 2). In case of AF and ADO rates between 5 -10%, either the test should be improved, or a higher number of markers should be included. In case of insufficient markers of the highest rank, markers of lower rank can be selected for test development, but the number of markers should then be increased.
- More markers will make the test more robust; analysis of at least two loci closely linked to the gene will reduce the risk of unacceptable misdiagnosis owing to ADO. Also, the risk for no diagnosis due to AF of a single amplicon in the multiplex will decrease.

<u>Genetic Markers only (STRs and/or SNPs)</u>

Targeted indirect analysis of single or few cell(s) is applied for (1) exclusion testing, (2) HLA typing, (3) in case of unknown mutation but the region of interest is proven causative, (4) triplet repeat expansion (e.g. the FMR1 CGG repeat expansion reluctant to single cell amplification), (5) large deletions/insertions with unknown breakpoints, or (6) in case direct mutation testing is not successful [presence of pseudogene(s), GC rich sequences refractory to single-cell amplification]. An indirect testing strategy is only applicable when high-risk and low-risk haplotypes have been established during preclinical work-up (exception, *see section 3.1 de novo mutations).*

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

- Assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended to include at least 2 STRs or 6 SNPs proximal and 2 STRs or 6 SNPs distal to the locus of interest (choose markers according to table 1 and 2). Here too, more markers are required in case higher AF and ADO rates are obtained and more markers will make the test more robust.
- In cases where the region of interest is located close to a centromere or telomere, flanking markers may not be possible. It is then recommended to include the mutation in the test strategy and to combine the test with TE biopsy to limit the risk of allele dropout at the mutation locus. The risk of misdiagnosis due to recombination should be reconsidered. In exceptional cases where flanking markers are not possible, and the mutation locus cannot be included, the test strategy will be linked with a higher risk of misdiagnosis. Such exceptional cases should be counselled in depth and the need for prenatal testing should be explained.

2.2.2 Targeted amplification for combined PGT-M and PGT-A

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

2.2.3 WGA followed by targeted amplification for PGT-M

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

Recommendations for single or few cell WGA concerning infrastructure, equipment, materials, tubing and work practices are described in section 1.4.
The following recommendations are made:

- It is recommended to use an MDA-based WGA protocol for haplotyping applications because of better genome coverage and low genotyping error rates.
- At the preclinical work-up, informativity/segregation analysis is required, together with the development of a locus-specific test, using WGA products as template DNA.

- It is recommended to carry out a validation assay for the WGA protocol and the specific downstream test(s) in function of the number of biopsied cells, to determine the rate of AF, ADO and preferential amplification.

Familial mutation + genetic markers (STRs and/or SNPs)

In general, when developing a test with WGA of single or few cells followed by familial mutation + STR and/or SNPs analysis, follow the recommendations as stated in section 2.2.1.

- ADO rates for WGA plus multiplex PCR at the single cell level are higher (20-30%) than for single cell multiplex PCR. Biopsy of few cells is recommended for WGA application as ADO rates will be lower.
- Single cell biopsy is acceptable, but the higher ADO risk should be taken into account when defining the number of markers required in the downstream test.
- Defining the minimum number of fully informative markers required in the few cell test; assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended to include at least 1 STRs or 3 SNPs proximal and 1 STRs or 3 SNPs distal to the locus of interest together with the mutation locus (choose markers according to table 1 and 2). Again, more markers are required in case higher AF and ADO rates are obtained.

Genetic Markers only (STRs and/or SNPs)

In general, when developing a test with WGA of single or few cells followed by indirect STR and/or SNPs analysis, follow the recommendations as stated in section 2.2.1 and in the previous paragraph (Familial mutation + genetic markers (STRs and/or SNPs) after WGA)

- ADO rates for WGA plus multiplex PCR at the single cell level may be higher than for single cell multiplex PCR and this should be taken into account when defining the number of markers required in the downstream test.
- Defining the minimum number of fully informative markers required in the few cell test; assuming validation data of AF and ADO rates remain below 5%, it is recommended to include at least 2 STRs or 6 SNPs proximal and 2 STRs or 6 SNPs distal to the locus of interest (choose markers according to table 1 and 2).
- Here too, more markers are required in case higher ADO rates are obtained.

2.2.4 WGA followed by generic testing for PGT-M

SNP Array for PGT-M only

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

The following recommendations are made:

- A relatively high DNA input is necessary for SNP arrays, so that a prior WGA step is required.

- It is recommended to use an MDA-based WGA protocol for haplotyping applications because of better genome coverage and low genotyping error rates.
- As SNP arrays are generic platforms, preclinical work-up only requires informativity/ segregation analysis for the locus of interest; the locus-specific development can be omitted.
- It is recommended to carry out a validation assay for the WGA protocol and the SNP array in function of the number of biopsied cells. No-call-rates and ADO rates for WGA plus SNP array at the single cell level will be higher than for few cells and this should be taken into account when defining the minimum number of informative SNPs required in the region of interest.
- When using commercially available SNP array protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet and dry-laboratory workflow prior to clinical use. For specific recommendation regarding the implementation validation, see also section 2.3.
- The turnaround time from sample processing to data analysis can vary from 24 hours to several days, depending on the setting and the platform of choice. It is recommended that each laboratory validates in-house whether the implementation of shortened protocols has an effect on hybridization efficiency and data quality.

Limitations of the test

SNP array haplotyping requires at least one close relative for phase determination. As an indirect testing strategy is only applicable when high-risk and low-risk haplotypes have been established during preclinical work-up (exception, *see section 3.1 de novo mutations*).

NGS for PGT-M only

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

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

The following recommendations are made:

- A relatively high DNA input is necessary for NGS, so that a prior WGA step is required.
- If long read sequencing is applied, it is recommended to use a suitable WGA to ensure amplification of high molecular weight DNA.
- Given that sequencing-based analysis is a generic approach, preclinical work-up only requires informativity/segregation testing; the locus-specific development can be omitted.
- It is recommended to carry out a validation assay for the WGA protocol and the NGS protocol in function of the number of biopsied cells.
- When using commercially available NGS-based protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet and dry-laboratory workflow prior to clinical use. Specific recommendations regarding the implementation validation are provided in section 2.3.
- Each step in the NGS protocol will contribute to the overall quality of the data set. QC metrics should be established throughout the procedure, among others including analysis of the

fragment length before and after adapter incorporation as well as quantification of the prepared library before and after possible size selection, to ensure optimal sample quality and DNA fragment representation in the multiplexed library samples. QC metrics should be established regarding the quality of the final sequencing data.

- Optimal indexing of the samples with respect to the upcoming sample combination should be used to ensure that different samples can be efficiently distinguished from each other during demultiplexing of the sequencing data.
- The turnaround time from sample processing to data analysis can vary from 24 hours to several days, depending on the setting and the platform of choice. Consequently, an embryo transfer can be planned in the current or a subsequent cycle.

Further general recommendations on NGS are covered in the paper on detection of structural and numerical chromosomal aberrations (refer PGT-A/SR paper).

Limitations of the test

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

Generic haplotyping-based approaches require at least one close relative for phase determination. As an indirect test, it is not applicable in case of *de novo* mutations for couples without previous pregnancies (see also *section 3.1 - de novo mutations*)

Analysis software is only available for some of the developed approaches. If not, the availability of skilled bioinformaticians needs to be guaranteed and the software requires validation.

2.2.5 WGA followed by generic testing for combined PGT-M and PGT-A

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

The following recommendations are made:

- When combined PGT-M and PGT-A is offered, it is recommended that the couple receives comprehensive counselling regarding the possible findings and the consequences on the transfer policy, according to the method used.
- Regardless of the centre-specific transfer policy, it is recommended that if, following analysis, unaffected embryos free of aneuploidies are available, they are given priority for transfer.
- The preclinical work-up for PGT-M should be performed, as described in 2.2.4.
- These approaches can also be used for inherited chromosomal structural variants in PGT-SR. Depending on the size of the involved segments, aberrant intensity ratios may or may not be detectable for the region(s) of interest. If detectable, it is recommended that the diagnosis is supported by Log R ratio and B allele frequency values.

- Additionally, even if a commercially available platform is used, an implementation validation to determine or confirm the lower size limit for the detection of segmental aneuploidies is recommended. These values may differ between platforms. It is recommended to perform the validation assay with WGA products from single or few cell samples of known karyotype and/or WGA products from embryonic cell(s) diagnosed with a formerly validated method.

Further recommendations specific to PGT-A are covered in the paper on detection of structural and numerical chromosomal aberrations (refer PGT-A/SR paper).

Limitations of the test

- As these tests require the presence of phasing reference(s), it is not applicable to all PGT-A indications.
- Ploidy changes cannot be detected by all approaches.: Methods based on aCGH or NGS cannot reliably detect all types of polyploidy and haploidy (see also Table 1 In the paper on detection of structural and numerical chromosomal aberrations (refer PGT-A/SR paper); SNP array and NGS-based haplotyping can identify polyploidy and haploidy.
- Meiotic errors cannot be distinguished from mitotic in all cases and by all approaches.
- Defining mosaicism detection threshold is recommended.

2.3 Pre-examination validation

For PGT-M

- Validation criteria are dependent on the number of cells biopsied (single cell at cleavage stage, or few cells at blastocyst stage) and on the type of strategy used for PGT-M. It is acceptable to perform the validation on cell(s) from embryos donated to research or on other cell types such as peripheral blood lymphocytes.
- Misdiagnosis risk needs to be established.
- The following criteria apply for targeted STR-based testing, with or without prior WGA:
    - Validation assays will determine amplification efficiency, accuracy, and ADO rate. Accuracy should be >99% for single or few cell samples of known genotype.
    - The amplification efficiency per locus should be >95%. An amplification efficiency of >90% is acceptable, but more markers need to be included.
    - The ADO rate per locus should be <5%. ADO rate less than 10% is acceptable, but more markers need to be included.
- Every new test based on targeted amplification should be validated.
- For targeted amplification, validation assays should be performed in 50 single or few cells samples, in two or three separate runs prior to clinical use. It is acceptable to validate updated protocols (i.e. adaptations of existing protocols) with less samples.
- No validation is needed on few cells samples when the protocol has been previously validated on single cells.
- The following criteria apply for generic strategies, such as SNP array with prior WGA:
    - When using commercially available SNP array or NGS-based protocols, which already have been validated by the manufacturer, it is still recommended to carry out an implementation validation of the complete wet and dry-laboratory workflow prior to clinical use.

- It is recommended to perform the validation assay with WGA products from single or few cell samples of known genotype and/or WGA products from embryonic cell(s) diagnosed with a formerly validated method.
- The validation assay should be performed with a minimum of 50 WGA samples, ideally covering various indications.
- Validation assays will determine - amplification efficiency, accuracy and minimum of genetic markers in the region of interest required for diagnosis.
- The - amplification efficiency should be >95% for good quality samples (this may not be achievable for biopsy samples from embryos donated for research/training). Accuracy should be >99% for WGA samples from single or few cells of known genotype. Similarly, for WGA products from embryonic cell(s) formerly diagnosed concordance with another validated method should be >99%.
- If both single and few cell analyses are to be performed clinically, it is necessary to validate each separately.

For combined PGT-M and PGT-A

- It is necessary to validate both indications. Again, validation criteria are dependent on the number of cells biopsied (single cell at cleavage stage, or few cells at blastocyst stage) and on the type of strategy used. For PGT-M, the above-mentioned recommendations apply. For PGT-A recommendations for validation are described in the paper on detection of structural and numerical chromosomal aberrations (refer PGT-A/SR paper).
- Once validated, preclinical work-up and testing of PGT-M conditions on 5 WGA products is sufficient.

2.4 Risk assessment

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

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

Risk assessment should also cover:

- risks caused by errors in sample tracking
- risks caused by handling biopsy samples prior to DNA analysis that, if not performed with care, may compromise DNA integrity.

- risk of inconclusive or false results due to sub-optimal experimental conditions (contamination, ADO, ADI) or due to biological reasons (recombination, double recombination, meiotic or mitotic chromosomal aberrations);

2.5 Preclinical work-up report

General guidance and recommendations on administration and patient information for the preclinical work-up report are provided in the paper on organisation of PGT (refer org paper). For PGT-M, the preclinical work-up report should also include a summary of the work-up and specify the test strategy for the clinical cycle.

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

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

3. Special cases

3.1 *De novo* mutations

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

de novo mutation in a prospective parent

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

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

during the PGT cycle(s). In case only DNA samples of the prospective parents are available, establishing the haplotypes and phasing needs to be based on the genotypes of the embryos.

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

- It is mandatory to include mutation detection in the test strategy.
- TE biopsy is recommended to limit the risk for ADO at the mutation locus. If cleavage-stage biopsy is performed, two independent cells should be tested.
- When too few embryos are available for biopsy, it is recommended to biopsy and analyse unfertilised oocytes (if mutation in the female partner) and/or embryos which are non-suitable for biopsy, to support phasing.
- Ideally, at least one affected embryo and one unaffected embryo are needed to establish the correct phase and detect recombination events. The mutation should be consistently detected in the presence of the same parental haplotype. If this is not possible, it is recommended to cryopreserve the embryos and to wait for the analysis of embryos from next cycle(s). Couples should be counselled upfront about this possibility. Alternatively, it is acceptable to transfer embryos after extended counselling and strongly recommend confirmation by prenatal diagnosis.
- As germline mosaicism cannot be excluded, it is not recommended to use an unaffected child/prenatal/embryo sample as phasing reference. If mosaicism has been detected, the transmission risk has to be evaluated.
- Germline mosaicism detected in the prospective parents can be an indicator of somatic mosaicism. In the single cell validation of the PGT protocol for the ADO rate of the mutation tested, using single cells of such an individual can lead to increased ADO rates of the mutant allele, depending of the degree of mosaicism.
- Following single cell analysis, it is not recommended to transfer embryos with a high-risk haplotype but wildtype allele at the mutation locus because of the ADO risk. It is acceptable to transfer such embryos following analysis of TE samples. Prenatal diagnosis is then strongly recommended.

de novo mutation in an affected child

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

3.2 Consanguineous relationships

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

Consanguineous grandparents

A prospective parent may have two identical haplotypes in the region of interest because of a consanguineous relationship between his/her parents and it may be difficult to find informative genetic markers within 1-2 Mb flanking region. In case of autosomal dominant disease, the mutation analysis should be included in the test strategy and ADO rates for the mutation locus after validation should be low (TE sample analysis is preferable to a two-cell analysis at day 3). In case of autosomal recessive disease, diagnosis should be based on the low-risk haplotype in the other partner.

Consanguineous couple

In case the prospective parents share the high-risk haplotype for an autosomal recessive disorder, parental contamination (most often maternal) in a homozygous affected embryo cannot be distinguished from a carrier embryo and this may lead to adverse misdiagnosis with transfer of an affected embryo. It is recommended to adapt the testing strategy by either including analysis of unlinked informative polymorphic marker(s)or by performing the analysis on 2 independent biopsy samples. If this is not done, it is acceptable to prioritize the transfer of healthy embryos compared to carrier embryos.

When SNP markers are used after WGA, parental contamination can be detected.

3.3 HLA typing

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

Test strategy

- The preferred PGT methodology is indirect HLA haplotyping, which involves linkage analysis of polymorphic markers flanking the HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ regions to identify matching haplotypes between the tested embryos and the affected child.
- The PGT protocol must include a minimum of one fully informative marker located at each of the following regions: telomeric to the HLA-A, between HLA-A and HLA-B, between HLA-B and HLA-DRA, between HLA-DRA and HLA-DQB1 and downstream to HLA-DQB1. It must be noted that some difficulty in finding markers between HLA-DRA and DQB1 has been encountered. In this case, two fully informative markers flanking the HLA-DQB1 must be included.
- If a fully informative marker is not available for each of the regions above, a combination of partially informative markers must be included to provide adequate information on the parental HLA haplotypes.

- A highly multiplexed protocol must be preferred where possible (i.e. more than one marker per region) to make the test more robust and to assist in detecting potential recombination that can occur throughout the whole Major Histocompatibility Complex (MHC) region.
- As HLA haplotyping may be compromised by genetic recombination, it is recommended that during preclinical PGT work-up, protocol testing includes testing of any additional available first-degree family members, aside from the parents and the affected child, to be able to detect recombination having occurred in the affected child.
- In case recombination is detected in the affected child or in case recombination is detected in embryos during the PGT cycle, any decision on PGT and selection of embryos for transfer must be carefully discussed with the haematologist and transplantation experts as it may be that a certain mismatch is permissive of hematopoietic stem cell transplantation. The location of the recombination event is of major significance for this purpose. This further highlights the importance of a highly multiplexed protocol.

3.4 Exclusion testing

In families with a history of late-onset diseases, individuals at risk who want to avoid pre-symptomatic testing but wish for their own biological children to be free of the disease, may opt for PGT. It is not recommended to offer direct testing with non-disclosure of the results as this obligates extreme confidentiality and brings along moral and ethical issues. Exclusion testing is the recommended option.

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

3.5 Mitochondrial DNA Disorders

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

Next to the recommendations for targeted amplification of nuclear monogenic disorders, the following recommendations are relevant for the quantitative analysis of monogenic mtDNA disorders by restriction enzyme digestion (see also section 1.2):

- PCR master mix should be decontaminated by restriction enzyme treatment prior to PCR amplification to eliminate external mtDNA contamination.

- Complete restriction enzyme digestion should be checked by spiking the amplification product with an amplicon containing the restriction site of interest if no control site is present in the amplification product.
- Reproducibility of the mtDNA mutation protocol based on restriction enzyme digestion should be validated preclinically, using replicates of mixes of wildtype and mutant mtDNA molecules in a broad range from 0 to 100% mutant and especially for the mutation load around the expression threshold.
- In addition to the controls described in section 1.3, control samples with a known mutation load (preferably around the expression threshold) should be used.
- Biopsy at the cleavage stage is recommended. It is acceptable to biopsy one blastomere. Biopsy at the preconception stage (1st and 2nd PB) is not recommended. For biopsy at the blastocyst stage, sufficient data on the representability of the TE biopsy for the embryo as a whole is currently lacking to make recommendations at this point (refer to ORG paper).
- It should be taken into account that the cytoplasm of lysed blastomeres may no longer fully represent the embryo mutation load.

4. Examination process

General recommendations on the PGT examination process are described in the paper on organisation of PGT (refer to ORG paper).

The sections below highlight specific issues relevant to PGT-M.

4.1 Scoring of clinical results

- When suboptimal samples or samples not meeting the requirements (namely lysed cells or nucleus not seen) are received for testing, this should be documented and a procedure how to further process and interpret these samples should be in place.
- It is recommended that results are analysed by two independent observers and discrepancies adjudicated by a third observer (where possible). If no consensus is reached the embryo should not be recommended for transfer and should therefore be given the diagnosis of uninterpretable or inconclusive.
- Haplotyping scoring criteria should be established in a written protocol and adhered to for the interpretation of the results.
- "No result rescue" for embryos without a clear diagnosis is acceptable. In case of targeted amplification at the cleavage biopsy stage, a second biopsy may be performed, followed by a second analysis. In case of genome wide testing, this could imply a second analysis of the existing WGA as well as a second biopsy followed by WGA and a second analysis.

4.2 Clinical cycle report

General items required on PGT work-up and clinical cycle reports are included in the paper on organisation of PGT (refer to ORG paper).

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

- unaffected carrier or affected when reporting for monogenic disease,

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

5. Post-examination process

Recommendations on PGT follow-up, Baseline IVF pregnancy rates for PGT and misdiagnosis are covered in the paper on organisation of PGT (refer to ORG paper)

References

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