ESHRE PGD consortium best practice guidelines for amplification-based PGD†

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ABSTRACT: In 2005, the European Society for Human Reproduction and Embryology (ESHRE) PGS Consortium published a set of Guidelines for Best Practice PGS to give information, support and guidance to potential, existing and fledgling PGS programmes. The subsequent years have seen the introduction of a number of new technologies as well as the evolution of current techniques. Additionally, in light of recent advice from ESHRE on how practice guidelines should be written and formulated, the Consortium believed it was timely to revise and update the PGS guidelines. Rather than one document that covers all of PGS, as in the original publication, these guidelines are separated into four new documents that apply to different aspects of a PGS programme, i.e. Organization of a PGS centre, fluorescence in situ hybridization-based testing, Amplification-based testing and Polar Body and Embryo Biopsy for PGS/preimplantation genetic screening. Here, we have updated the sections that pertain to amplification-based PGS. Topics covered in this guideline include inclusion/exclusion criteria for amplification-based PGS testing, preclinical validation of tests, amplification-based testing methods, tubing of cells for analysis, set-up of local IVF centre and Transport PGS centres, quality control/quality assurance and diagnostic confirmation of untransferred embryos.

Key words: European Society for Human Reproduction and Embryology / PCR amplification / allele drop-out / quality control/quality assurance / single cell

Introduction

The rapidly changing nature of PGS, specifically the technologies associated with its use and increasing patient access, has necessitated review and revision of the original the European Society for Human Reproduction and Embryology (ESHRE) PGS Consortium guidelines (Thornhill et al., 2005). As a result, the ESHRE PGS Consortium (hereafter referred to as the Consortium) has prepared four sets of guidelines (Harton et al., 2010a,b,c, submitted): one relating to the organization of the PGS centre and three relating to the methods used: DNA amplification, fluorescence in situ hybridization and biopsy/embryology. The method guidelines should be read in conjunction with the organization of the PGS centre guidelines which contains information on personnel, inclusion/exclusion criteria, genetic counselling and informed consent, setting up an IVF centre, Transport PGS, quality assurance/quality control (QA/QC) and accreditation (which is also further discussed in the paper by Harper et al., 2010). In this document, the laboratory performing the diagnosis will be referred to as the PGS/preimplantation genetic screening (PGS) centre and the centre performing the IVF as the IVF centre. Topics covered in this guideline include general uses of amplification-based PGS testing, laboratory issues relating to amplification-based testing, pre-examination validation, examination process and post-examination process.

PGS has been developed for patients at high risk of transmitting a genetic abnormality to their children which includes all monogenic defects (autosomal recessive, autosomal dominant and X-linked disorders). More recently, DNA amplification-based PGS applications have broadened and include sibling-donor selection through HLA-matching (Van de Velde et al., 2009), and the analysis of familial chromosomal rearrangements (Fiorentino et al., 2010). PGS is still relatively unregulated and lacks standardization compared with other forms of diagnostic testing, however, more federal, state and local governments...
are beginning to take an interest in PGD, and some have begun accrediting laboratories that offer PGD (Harper et al., 2010). This is a logical step considering the comparative difficulty in achieving the highest levels of accuracy and reliability when analysing single cells as part of PGD versus more routine genetic testing. Many regulations, laws and voluntary networks exist in the mainstream diagnostic community to maintain the highest quality in diagnostic testing. For example, the European Quality Molecular Network has attempted to improve and standardise molecular diagnostic testing across Europe (Dequeker et al., 2001). One step towards higher quality overall and standardization for PGD is to build consensus opinion on best practices within the PGD community; a component of the mission of the Consortium (ESHRE PGD Consortium Steering Committee, 1999, 2000, 2002).

The Consortium recognizes that owing to variations in local or national regulations and specific laboratory practices, there will remain differences in the ways in which PGD is practiced (from initial referral through IVF treatment, single-cell analysis to follow-up of pregnancies, births and children). However, this does not preclude a series of consensus opinions on best practice based upon experience and available evidence. Indeed, the American Society for Reproductive Medicine (ASRM) published a practice committee report for PGD in 2008 (American Society of Reproductive Medicine and Society for Assisted Reproductive Technology. Practice Committee Report, 2008) essentially reviewing PGD practice in the USA. The PGD International Society (PGDIS) has also drafted guidelines which were recently updated and are more in-depth than the ASRM report. These guidelines are concise and remain so in their recent revised edition (Preimplantation Genetic Diagnosis International Society, 2004, 2008). The consensus opinions provided in this document and the accompanying guidelines, not only reflect current use of PGD but also offer consensus-based specific guidance regarding how best to practice clinical PGD based upon clinical experience, and data, both published and unpublished.

The Consortium hopes that a minimum standard might be achieved across all centres actively providing clinical PGD. Achieving this goal could ultimately have the net effect that patients receive the best care possible regardless of the centre at which they are treated. Rather than a drift towards the lowest common denominator, established and fledgling centres alike can learn from global experiences and be guided by consensus opinion.

These opinions are not intended as rules or fixed protocols that must be followed, nor are they legally binding. The unique needs of individual patients may justify deviation from these opinions, and they must be applied according to individual patient needs using professional judgement. However, guidelines and opinions may be incorporated into laws and regulations and practitioners should check the status of clinical practice guidelines in their own countries to determine the status of this document.

1. General uses of DNA amplification-based tests

1.1. Amplification-based tests can be used for the diagnosis of monogenic defects at the DNA level (Sermon et al., 2002; Thornhill and Snow, 2002). This includes specific diagnosis for X-linked disease, as well as diagnosis of autosomal recessive and dominant diseases.

1.2. Owing to the risk of contamination and allele drop-out (ADO), it is recommended that DNA amplification protocols include the use of linked or unlinked markers in addition to the disease locus (Sermon et al., 2002; Thornhill and Snow, 2002).

1.3. For X-linked diseases, analysis of the mutation and linked markers allows for the transfer of unaffected males as well as the exclusion of carrier females, if the patient is so inclined.

1.4. When sexing only is performed for X-linked diseases by DNA amplification, it is recommended that several loci are included to monitor contamination and preclude misdiagnosis owing to ADO (Renwick et al., 2006; Renwick and Ogilve, 2007).

2. Laboratory issues relating to single-cell DNA amplification

2.1. Laboratory infrastructure, equipment and materials

The following recommendations are made:

2.1.1. There should be physical separation of pre-amplification (preferentially a positive pressure room), amplification and post-amplification (preferentially a negative pressure room) laboratories and the biopsy laboratory.

2.1.2. When positive and negative pressure rooms are present, they are enclosed by a lock chamber.

2.1.3. It is recommended that primary amplification reactions are set-up in a laminar flow hood in the pre-amplification area.

2.1.4. When performing two rounds of PCR, the area for setting up the first round should still be separated from the area for the second round of PCR.

2.1.5. Each area has its dedicated materials and equipment.

2.1.5.1. The pre-amplification reagents and materials should be kept away from any DNA source.

2.1.5.2. An appropriate unidirectional workflow should be in place, avoiding any backfire of amplified products to the pre-amplification area.

2.1.6. All clinical equipment should meet the criteria set for the intended application, be appropriately maintained and serviced, with all aspects supported by written standard operating procedures (SOPs).

2.1.7. Protective clothing for single-cell DNA amplification work should be worn, including full surgical gown (clean, not sterile and changed after each case), hair cover/hat and face mask (covering nose and mouth) and shoe covers. Gloves should be worn at all times and changed frequently. These should be well-fitting (e.g. latex or nitrile, but not vinyl examination gloves).

2.1.8. All plastic-ware used, including filter tips, should be certified DNA, DNAase free.

2.1.9. Work surfaces, equipment, etc. should be cleaned with DNA decontamination solutions or 20% bleach prior to each case. All tube racks should be either one-use or, if re-used for PGD, be autoclaved/cleaned in 20% bleach after each use.

2.1.10. All batches of reagents should be recorded so that they may be traceable to specific assays.
2.1.11. Whenever possible, all solutions should be purchased ‘ready to use’ and should be of ‘molecular biology’ grade or equivalent.
2.1.12. All reagent-solutions should be split into single-use aliquots and no aliquot should be re-used for a clinical PGD case.
2.1.13. Solutions made in-house may be autoclaved (using a dedicated autoclave) or if this is not possible, passed through 0.2 \( \mu \)M filters under stringent conditions.
2.1.14. A DNA amplification master mix may be pre-treated either by UV irradiation or restriction enzyme digestion for decontamination purposes.

2.2. Work practice controls
The following recommendations are made:

2.2.1. It is essential that an adequate labelling system is used to match the cell diagnostic result with the embryo from which that cell was biopsied. Comprehensive and robust labelling is required; printed ticker labelling may be superior to pens as labelling should be legible and indelible.
2.2.2. Labelling and sample identification should be confirmed for critical and high risk steps. It is recommended that the unique patient identifier and embryo/cell number should be witnessed and signed by two members of the PGD team at the following steps:

(a) at cell tubing to confirm that the cell identification matches the labelling on the relevant tube;
(b) when diagnostic results are recorded to ensure accuracy and correlation with the correct cell and/or embryo identification.
2.2.3. All personnel undertaking single-cell tubing and testing should be adequately trained and shown competent for single-cell procedures, prior to working on clinical specimens. This training should be documented and competencies should be retested regularly.

2.3. Tubing cells
2.3.1. Tubing should be carried out under stringent precautions to minimize contamination. This implies that materials and reagents should be prepared in a dedicated pre-amplification laminar flow hood [UV light to be turned on before (and after) each cycle and regular cleaning of working areas with DNA degrading detergents is required].
2.3.2. Specific measures to avoid contamination are:

2.3.2.1. Granulosa cells are meticulously removed from the oocytes to avoid maternal contamination and fertilization is achieved through ICSI to avoid contamination with sperm. The embryo biopsy procedure should preclude operator contamination.
2.3.2.2. Notably, ill-fitting gloves can be a source of carry-over contamination.
2.3.2.3. For each cell assessed (either in the validation phase or during clinical PGD cases), a blank containing medium collected from the last wash droplet and all amplification components except DNA should be run. However, a contaminated blank is only an indication of a more general problem and does not mean that the corresponding sample is contaminated.
2.3.3. Single cells should be washed at least twice using a sterile transfer pipette before transfer into amplification tubes. The amount of medium co-transferred with the cell in the tube should be minimal.
2.3.4. It is acceptable to transfer embryonic cells to amplification tubes with or without microscopic visualization.
2.3.5. If the embryonic cells are lysed during washing or transfer, the pipette is possibly contaminated and has to be discarded. For cleavage-stage biopsy, another blastomere should be sampled.
2.3.6. If accurate and reliable amplification methods are in place, it is recommended to biopsy only one cell from cleavage-stage embryos as it has been shown that the biopsy of two blastomeres from 8-cell embryos results in a 40% lower live birth rate when compared with 8-cell embryos having lost only one blastomere (Goossens et al., 2008; De Vos et al., 2009).
2.3.7. Efficient cell lysis and adequate DNA denaturation are required. Both alkaline lysis and proteinase K/sodium dodecyl sulphate are accepted as the best cell lysis methods (Thornhill et al., 2005).

3. Single-cell amplification characteristics: specificity, efficiency, ADO and contamination
Many variations in DNA amplification methods have been published. Both PCR-based and non-PCR-based amplification methods can be used, aiming at the amplification of specific region(s) or the whole human genome. Each method has its merits and its limitations. The methods used should have been previously implemented, tested and validated in the PGD centre.

3.1. PCR-based amplification
Fluorescent PCR amplification followed by post-PCR methods for allele discrimination is regarded as the gold standard. Analysis of fluorescent PCR fragments on an automated sequencer is much more sensitive and yields a more accurate fragment size determination than analysis of conventional PCR fragments on ethidium bromide stained gels (Hattori et al., 1992; Lissens and Sermon, 1997).

3.1.1. Fluorescent PCR is also an efficient way to significantly reduce ADO (Sermon et al., 1998).
3.1.2. Other PCR design factors leading to better specificity, higher PCR efficiency and low ADO rates are smaller amplicon size (<350 bp) and appropriate primer design using primer software tools together with BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches and single nucleotide polymorphism detection to ensure specificity (Piyamongkol et al., 2003).
3.1.3. DNA degradation and the choice of DNA polymerases also have an impact on efficiency and ADO of single-cell PCR (Piyamongkol et al., 2003).
3.1.4. The co-amplification of polymorphic marker(s), linked or unlinked, with the mutation of interest in a multiplex PCR is
3.3. Mutation detection strategies

3.3.1. Many mutation detection strategies exist. The following recommendations are made:

3.3.1.1. All methods require validation on suitable genomic DNA controls.

3.3.1.2. Strategies should allow the ability to distinguish between mutant alleles, wild-type alleles and amplification failure.

3.3.1.2.1. For instance it is not recommended to only amplify the normal allele in case of a large deletion/insertion as the mutant allele cannot be distinguished from amplification failure.

3.3.1.3. It is recommended in case of triplet repeat expansions that strategies based on amplification of the normal alleles are only applied for fully informative couples.

3.3.1.4. For small deletions or insertions, direct fragment size determination can be performed.

3.3.1.5. Detection of single nucleotide substitution via restriction enzyme digestion should include a strategy to control the digestion reaction.

3.3.1.6. Indirect mutation detection is applied for exclusion testing, for HLA typing, in case of unknown mutation or large deletions/insertions with unknown breakpoints, as general strategy for genes with high numbers of (private) mutations or in case direct mutation testing is not successful [presence of pseudogene(s), GC rich sequences refractory to single-cell amplification].

3.3.1.7. Indirect protocols are not applicable in case of de novo mutations for couples without previous pregnancies.

3.3.2. It is recommended to include at least two flanking markers in indirect mutation PCR-multiplex protocols.

3.3.2.1. Analysis of at least two loci closely linked to the gene underlying the disease will reduce the risk of unacceptable misdiagnosis (i.e. transfer of an affected embryo) owing to ADO (presumed to be around 5%) to a minimum (<1%).

3.3.2.2. More than two markers will make the test more robust: an assay with just one marker at each side of the mutation will yield ‘no diagnosis’ when one marker fails to amplify. Therefore, two upstream and two downstream markers are preferably applied.

3.3.3. Ideally the polymorphic marker should be intragenic with a high degree of heterozygosity and produce a clearly interpretable peak pattern.

3.3.3.1. The risk of recombination should be considered for every marker and is especially important in case of large genes and genes with recombination hot spots.

3.3.3.2. It is recommended to choose extragenic markers within 1 Mb of the mutation of interest to reduce the risk of recombination events. Even though loci 1 cM apart are expected to show only ~1% recombination, crossover events between markers and a specific mutation cannot be completely ruled out even for very closely linked markers (Altarescu et al., 2008).

3.3.3.3. The recombinition risk is important for the risk assessment of accuracy and reliability of the results.

3.3.4. Specifically for indirect HLA typing, a sufficient number of microsatellites should be chosen to be able to detect recombination within the HLA region:

3.3.4.1. A minimum one marker located upstream of HLA-A, minimum one marker between HLA-A and
3.3.5. When linked markers are used, the same minimum haplotyping standards exist for PGD testing as for regular prenatal diagnosis.

3.3.5.1. A molecular biologist experienced in pedigree and linkage analysis should determine which samples are needed as well as the number of samples required for reliable and accurate diagnosis.

3.3.5.2. More than one meiosis is preferentially used in building accurate haplotypes.

3.3.5.3. When no additional family members are available for analysis in cases of paternally linked disorders, single sperm should be used for haplotype construction.

4. Pre-examination validation

4.1. Confirmatory testing of the mutation on DNA or segregation results (using standard tests) is recommended. It is acceptable to outsource confirmatory testing to outside laboratories if the PGD centre is not able to offer appropriate testing of the disease-causing mutation.

4.2. Confirmatory testing of the clinical PGD assay on parental and proband blood DNA is recommended since the PGD assay could be non-informative owing to PCR failure as a result of polymorphisms or DNA sequence variations present in the population (Thornhill et al., 2005).

4.3. Before moving on to single-cell validation, it is recommended that testing of various genotypes using DNA samples be performed, including affected (autosomal dominant), carrier (autosomal recessive, X-linked diseases) and unaffected samples for the mutation to be tested or DNA samples with heterozygous markers for tests using a linkage only approach.

4.4. Single-cell analysis should include cells from affected, carrier (autosomal recessive, X-linked diseases) and unaffected individuals (Sermon et al., 2002; Thornhill and Snow, 2002).

4.4.1. The use of diluted DNA samples to mimic single cells (~6 pg of DNA is equivalent to one cell) is not recommended as stochastic variation in pipetting renders the results unreliable.

4.4.2. Acceptable cell types for single-cell analysis include lymphocytes, lymphoblastoid cell lines, fibroblasts or buccal cells. Other somatic cell types should be acceptable assuming the laboratory has validated their use internally.

4.4.3. Testing of embryonic cells would be beneficial since they are the target cell type for clinical testing, however, their use will depend on availability.

4.4.4. The type of cell used for validation may influence the amplification efficiency and ADO rate (Glentis et al., 2009).

4.4.5. It is recommended that at least 50 single cells are used for validation of any amplification-based test and these cells should be run in multiple experiments of 10–20 cells/experiment to assess amplification efficiency, ADO rate and run-to-run variability.

4.4.6. The same number of cell wash blanks should be run along with the single cells to assess contamination.

4.4.7. If embryonic cells are analysed, a minimum of 10 samples is recommended, and where possible, several embryonic cells from the same embryo should be tested to determine the expected genotype for the mutation and linked markers.

4.5. Assessing DNA amplification efficiency and ADO rates

4.5.1. Assessing amplification efficiency is an important part of pre-clinical validation. An amplification efficiency of at least 90% for each marker is recommended.

4.5.2. Assessing ADO rates is an important part of pre-clinical validation. ADO rates should be determined using cells that either carry the mutation of interest or, for linkage only tests, wild-type cells that are heterozygous for the markers being used.

4.5.3. ADO rates should be as low as possible, preferably <10%. It should be noted that a higher ADO rate can be tolerated when dealing with autosomal recessive diseases when compared with autosomal dominant or compound heterozygous diseases. Higher ADO rates can also be tolerated when dealing with WGA-based protocols, however, in this case an increased number of linked markers and/or a 2-cell biopsy strategy can be employed in the protocol.

4.6. Reporting pre-examination validation

A report has to be written detailing the protocol and validation steps of the PGD protocol workup (Harper et al., 2010).

5. Examination process

5.1. Existence of and adherence to clinical testing protocol

The following recommendations are made (Thornhill et al., 2005; Harper et al., 2010):

5.1.1. Clinical testing protocols should include explicit instructions, including a summary of results from the validation steps of assay development, scoring criteria, reporting procedures as well as a framework for counselling patients in the presence of diagnostic results.

5.1.2. SOPs are required for all protocols, all equipment and all processes that take place in the PGD centre and should include selection and validation of examination procedures, clinical relevance, purpose of examination, specimen requirements and means of identification, equipment and special supplies, reagents, standards or calibrations and internal control materials, instructions for performance of the examination, limitations of the examinations, recording and calculation of results, internal QC procedures and criteria against which examination processes are judged, reporting reference limits, responsibilities of personnel in
5.3. Scoring clinical DNA amplification results

The following recommendations are made:

5.3.1. Scoring criteria should be established in written protocol and adhered to for the interpretation of results.

5.3.2. Results should be analysed by two independent observers and discrepancies adjudicated by a third observer (where possible). If no resolution is reached the embryo should not be recommended for transfer, i.e. should be given the diagnosis of uninterpretable or inconclusive.

5.3.3. All data from clinical testing should be kept for QC purposes and records.

5.3.4. Results should be reviewed and signed or electronically validated by a suitably qualified person.

5.3.5. A written or online electronic report should be prepared and double-checked. The checked report is given to the IVF centre to ensure transfer of the correct embryos.

5.3.6. Reporting of clinical results to the IVF centre must follow local guidelines or law, or if nothing local exists, the guidelines in ISO15189 (Harper et al., 2010).

6. Post-examination process

PGD cycle follow-up.

6.1. The following recommendations are made (ESHRE PGD Consortium Steering Committee, 1999, 2000, 2002; PGDIS, 2008).

6.1.1. Confirmation of the diagnosis should be performed on as many untransferred embryos as possible following diagnosis to provide QA as well as accurate and up to date misdiagnosis rates to prospective PGD patients. As follow-up procedures are costly and time-consuming, alternative methods of QA exist which can be combined with limited follow-up of embryos, including: collection of tissue at prenatal testing, collection of cord blood following birth and surveys of PGD patients with successful pregnancy.

6.1.2. PGD and IVF centres should make special efforts to follow-up prenatal testing or birth, especially if confirmatory testing is not possible.

6.1.3. Follow-up of pregnancies (including multiple pregnancy rate and outcome), deliveries, and the health of children at birth and beyond should be attempted and maintained along with the cycle data. These data should be used both for internal QC/QA purposes and sent to the Consortium during annual data collections.

6.1.4. Recently a pediatric follow-up working group has been formed by the Consortium for the follow-up of children born after PGD. PGD centres are encouraged to take part in this project.

6.2. Baseline IVF pregnancy rates for PGD

6.2.1. Setting appropriate baseline pregnancy rates should be left to the individual Centres. However, it is recommended that each IVF centre should compare PGD pregnancy rates and matched non-PGD (routine IVF or ICSI) pregnancy rates within that IVF centre (Thornhill et al., 2005).

6.2.2. Comparison of pregnancy rates with those reported by the annual data collections of the Consortium can also be carried out to set benchmarks for continual improvement of the PGD centre (Goossens et al., 2009).

Appropriate indications for specific tests

6.2.3. It is recommended that specific indications for PGD/PGS should remain within the purview of individual clinics (Thornhill et al., 2005; Harton et al., 2010a).

6.3. Misdiagnosis rate

6.3.1. The Consortium makes the following recommendations (Thornhill et al., 2005; PGDIS, 2008; Thornhill and Repping, 2008; Wilton et al., 2009):

6.3.1.1. It is recommended that misdiagnosis rates should be calculated for each type of assay and for all assays from a particular Centre (Lewis et al., 2001). Such rates include...
those clinical cases in which affected pregnancies arose and post-transfer confirmation of diagnosis assays that were discordant with the biopsy result.

6.3.1.2. It is recommended that confirmatory testing should be performed at least periodically as a QA.

6.3.1.3. It is recommended that the published and in-house estimates of misdiagnosis rates should be available to prospective patients along with pregnancy rates on request to allow informed consent for PGD.

6.3.1.4. Following a misdiagnosis, the laboratory should investigate the possible causes of the misdiagnosis and make changes to protocols to eliminate the risk in the future (Wilton et al., 2009).

6.3.1.5. Misdiagnosis should be reported to the Consortium each year during routine data collection (Thornhill et al., 2005, Goossens et al., 2009).

6.3.1.6. Most of the causes of misdiagnosis are avoidable by taking preventative action and following the principles of quality management present in modern accredited diagnostic testing laboratories. The Consortium has recently published an article reviewing the possible causes and adverse outcomes of misdiagnosis (Wilton et al., 2009): it is recommended that the suggestions made in this paper for the prevention of specific misdiagnosis causes should be taken into consideration by the PGD centre to eliminate the possible causes of misdiagnosis.

7. Transport PGD

7.1. For general recommendations on Transport PGD see the Organization of a PGD centre guideline (Harton et al., 2010a).

References


