ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations

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 people 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 and XX, 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-A/PGT-SR techniques

This paper provides detailed technical recommendations for the most applied methods for PGT-SR, fluorescence *in situ* hybridization (FISH), array-based comparative genomic hybridization (aCGH) and next generation sequencing (NGS) and for PGT-A (aCGH and NGS).

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

Training and Personnel

- Genetic testing procedures should be performed under the supervision of a (cyto)geneticist/ competent or authorized to perform clinical diagnostics

- All personnel undertaking genetic testing should be trained adequately as required in a clinical molecular cytogenetic laboratory and should follow written ***standard operating procedures (SOPs)***.
- Training for each technique should be documented.
    - Training for tubing is discussed in the paper on polar body and embryo biopsy for PGT (REFER BIOPSY)
    - For FISH, training should be at least to the standard required for routine testing in a clinical cytogenetic laboratory. It is recommended that at least 30 samples are successfully spread or fixed and subjected to FISH by each trainee during preclinical training. Supervised clinical training should include at least an additional 20 samples.
    - For aCGH and NGS, it is recommended that at least 30 samples are subjected to whole genome amplification (WGA), followed by aCGH or NGS by each trainee during preclinical training. Supervised clinical training should include at least an additional 20 samples.

Laboratory infrastructure, equipment and materials

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

Labelling and witnessing

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

Risk assessment

- ***Risk assessment*** for the patient should include figures on the potential risk of a viable unbalanced offspring due to failure to detect any of the unbalanced segregation products.
- In case only one of the two translocation segments can be detected, not all possible unbalanced segregation products can be identified. This limitation of the test may increase the risk of a viable unbalanced offspring, first trimester miscarriage and stillbirth after PGT-SR and should be mentioned in the preclinical work-up report.
- When sub-optimal samples or samples not meeting the requirements (f.x. lysed cells, nucleus not seen) are received for testing, this should be documented and a procedure on how to further process these samples should be in place.

Appropriate indications for specific tests

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

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

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

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

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

Several methods are applied to perform PGT-SR, amongst which FISH, aCGH and NGS. PGT-SR is mostly performed on embryonic biopsies taken at the cleavage stage (Day 3 post-insemination) or the blastocyst stage (Day 5-7 post-insemination).

1.1. FISH-based PGT-SR

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

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

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

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

Laboratory issues

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

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

<u>FISH protocol – structural rearrangements</u>

For structural rearrangements, it is recommended that the probe set contains at least sufficient probes to detect all expected unbalanced variants of the chromosomal rearrangement. The analysis of the predicting

segregation outcome for translocation carriers should include an assessment of the configuration of the quadrivalent (alternate, adjacent 1, adjacent 2, 3:1; 4:0 and meiosis II nondisjunction).

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

Where suitable probes are not available, it is acceptable to use probe combinations that cannot detect some unbalanced forms of a rearrangement, provided that they have been assessed to be non-viable in a recognizable pregnancy or to have a very low prevalence. It has to be mentioned in the (pre-validation) report that there are unbalanced forms that cannot be detected, and patients should be counselled to this effect. A cytogeneticist or suitably qualified person should determine which probe combination to use.

PGT diagnosis on a single mononucleate cell is acceptable for chromosomal rearrangements, provided that there are at least two informative probes for each chromosome involved in an unbalanced form of the rearrangement that is considered likely to be prevalent or viable in a recognizable pregnancy.

PGT diagnosis based on concordant results from two mononucleate cells is recommended where there is only one informative probe available for any of the chromosomes involved in an unbalanced form of the rearrangement that is considered likely to be prevalent or viable in a recognizable pregnancy.

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

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

FISH protocol – sexing in case of X-linked diseases

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

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

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

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

Turnaround time

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

Documentation

The patient's file should include relevant laboratory documentation:

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

Laboratory infrastructure, equipment and materials

Infrastructure

The following recommendations are for the laboratory space:

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

Equipment

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

Materials & reagents

- Required materials are glass slides and coverslips, and a probe set specific for the chromosomal structural rearrangement of interest.
- Daylight should be avoided during hybridization and post-hybridization steps.
- The use of commercial probes is recommended since they generally come with ***quality control (QC)*** and validation reports.
- The use of homemade probes is acceptable with appropriate preclinical ***quality assurance (QA)***/QC and validation.

- It is recommended that all probe vials be tested before clinical application, to confirm that they contain the correct chromosome-specific probe and are labelled with the correct fluorochrome or hapten. Furthermore, that they are informative for the intended PGT-SR couple, and meet documented acceptance levels for signal specificity, brightness and discreteness. Batch numbers should be recorded to ensure continuous traceability.
- It is recommended that only appropriately qualified personnel (as documented in written competency lists) authorize selection of probes for clinical use.
- In case of a Robertsonian translocation, fluorescent probes for any locus on the long arm of the two acrocentric chromosomes involved in the rearrangement can be used. For reciprocal translocations, alpha-satellite probes, locus-specific probes, or sub-telomere probes indicative of the translocated regions may be used. For inversions, mostly locus-specific probes for the short and the long arm of the intended chromosome are used, possibly combined with alpha-satellite repeat probes. For the detection of deletions or duplications it is preferable to use locus-specific probes indicative of the target chromosomal region combined with a control probe (alpha-satellite or subtelomere probe) to discriminate between a true deletion/duplication and a whole chromosome copy number change.
- It is recommended that for each round of FISH all probes be labelled with a different fluorochrome or combination of fluorochromes so that the colour of different probe signals can be distinguished from one another. The signals should be 1 domain apart.
- When using prehybridization steps, such as pepsin and paraformaldehyde, it is recommended that measures should be taken to ensure appropriate QC for these solutions. Creation dates of solutions for all steps should be recorded and the solutions should be checked for possible cellular contamination prior to use.
- Mounting medium containing antifade (with or without DAPI - depending on the probe combination) is recommended to allow maintenance of fluorescent signals.
- It is recommended that prior to each FISH procedure, denaturation, hybridization and wash temperatures are verified.

Work practice controls

Identification and witnessing

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

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

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

Pre-examination process

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

Preclinical work-up and test development

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

Pre-examination validation

- It is acceptable to perform the validation only on the partner who carries the rearrangement.
- It is acceptable to perform the validation on blastomeres and TE cells from embryos donated to research prior to clinical PGT-SR testing. It is also acceptable to perform the validation on other cell types such as peripheral blood lymphocytes and fibroblasts.
- It is recommended that at least 10 metaphase spreads are examined: (1) to ensure that the probes are specific for the correct chromosomes, (2) to assess chromosome ***polymorphism*** and signal cross-hybridization and (3) with respect to carriers of a chromosome rearrangement, to ensure that the probes hybridize to the expected segments of the rearrangement.
- It is recommended that at least 100 interphase nuclei are scored using appropriate scoring criteria (signal specificity, brightness and discreteness)
- Acceptable ranges of FISH hybridization efficiency should be determined in each laboratory for each FISH probe and combined probe set. Validation tests should at least confirm that the probes

hybridize as expected, that they are informative for the rearrangement and that >95 % of the cells shows the expected number of signals for each of the probes used.

- It is recommended that scoring criteria are determined ahead of time (published or 'in-house') and should be adhered to as per written procedure.

Preclinical work-up report

General guidance and recommendations on administration and patient information for the preclinical work-up report is given in the paper on organisation of PGT (refer ORG paper). A preclinical work-up report should also include a summary of the PGT-SR work-up with details on the protocol and validation steps. It should further describe the FISH probes used and the hybridization efficiency, the false positive and the false negative rate of the probe set. Reporting may rely on the international system for human cytogenetic nomenclature (ISCN). Finally, the report should include potential limitations of the test.

Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking
- risks caused by handling biopsy samples prior to FISH analysis that, if not performed with care, may compromise DNA integrity.
- risk of inconclusive or false results due to sub-optimal experimental conditions; the reliability of the FISH diagnosis may be negatively influenced by the inability to accurately interpret signals, inconsistent fixation or suboptimal hybridization. Signal overlap may lead to an underestimation of the actual chromosome (region) copy number. In addition, locus-specific and sub-telomere probes produce less bright signals when compared to alpha-satellite probes and show a higher rate of split signals, which compromises correct signal scoring.
- risk of inconclusive or false results due to biological reasons: (1) Unbalanced segregations may arise from crossing-over during meiosis I in the gametes of the carrier of the rearrangement, (2) chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is below the resolution of the test, whether biological or caused by a technical error.

Limitations of the test

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

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

- Commercial probes are available for only a limited number of loci, which may complicate the selection of probes for the analysis of rare chromosomal rearrangements.

1.2. Array-based PGT-SR

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

Arrays are considered a more reliable approach for PGT-SR when compared to FISH since they provide multiple points of measure for each translocation segment. Furthermore, they allow for simultaneous copy number assessment of the chromosomes not involved in the rearrangement. This information could be used to rank embryos for transfer.

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

Laboratory issues

The aCGH workflow involves: (1) sample cell lysis and whole genome amplification (WGA) (2) labelling of sample and reference DNA with different fluorochromes (e.g. green and red) (3) purification of labelled DNA (4) microarray processing (hybridisation of biopsied and reference DNA samples followed by washing of microarray slides), (5) scanning and (6) analysis of scanned microarray tiff images where data is extracted to fluorescence ratio. The resulting ***log2 of fluorescence ratios*** is computed by specific software to identify structural and numerical chromosome copy number aberrations.

<u>aCGH protocol</u>

- It is recommended that wet-laboratory experimental conditions be established for all steps in the aCGH workflow followed by a preclinical assessment of the accuracy of the test to detect a chromosome aberration.
- It is acceptable to perform aCGH-based PGT-SR on polar body biopsies, provided that both polar body I and II are analysed to achieve a diagnosis. The presence of cumulus cells attached to the ***zona pellucida (ZP)*** could heavily affect the result of the PGT-SR analysis. PGT-SR performed on polar bodies carries a high risk of ***misdiagnosis*** for the carriers of structural rearrangements due to an uneven number of crossovers that occur in meiosis I which may be undetectable through aCGH.
- It is acceptable to perform aCGH-based PGT-SR on single cell biopsies, although they present with an overall increased noise and step change chromosome artefacts in the aCGH profile. Acceptance criteria for noise level should be part of the QA/QC parameters.
- It is recommended to use a WGA protocol which is compatible with the specific aCGH platform that has been validated with.

Turnaround time

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

Documentation

Relevant laboratory documentation should include:

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

Laboratory infrastructure, equipment and materials

Infrastructure

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

Equipment

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

temperature and protected from daylight. Scanners should be validated and adjusted to the required resolution for the specific PGT protocols.

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

Materials

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

- Cell lysis, pre-amplification, amplification enzymes and buffers specific to each amplification method used.
- DNA Labelling reaction buffers, enzymes and dNTPs. Cyanine-3-UTP and cyanine -5-UTP fluorophores that should be used under minimal light exposure since they are light sensitive,
- hybridization and washing buffers, human Cot-1 DNA, and DNase/RNase-free distilled water, and
- microarray slides.

Work practice controls

Identification and witnessing

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

Intra-assay controls

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

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

Pre-examination process

Quality control

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

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

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

Test efficiency

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

loss for the Y chromosome are used as a reference to evaluate aneuploidy events for the autosomes.

- Gender-matched samples must show consistently no change on chromosome X or Y and none of the probes in the array should report a change.
- Negative amplification, negative intra-assay control or failed hybridisation should show a consistent noisy profile where no significant pattern is observed.
- It is not recommended to store non-amplified biopsied material for extended periods of time, to store samples and solutions at suboptimal temperature or use repeatedly frozen-thawed solutions containing DNA or enzymes.
- Hybridization bias due to drying out of the microarray surface could lead to signal loss, degradation of Cy5 and suboptimal scanned images.
- It is recommended to stringently wash the aCGH slides with minimum light exposure and under controlled ozone concentration, temperature and humidity. The use of lab carbon-loaded non-woven filters is recommended in case of high ozone levels.
- It is recommended to avoid the use of detergents to clean the wash equipment, as this may interfere with signal intensity.
- Washing and scanning of slides in small batches (2-3 slides) is recommended to minimize the exposure of slides and labelling dyes to air.
- It is critical that slides are dried by centrifugation shortly after the final washing step to avoid drying through evaporation.
- Scan images should have defined features with red and green images well registered and the colours evenly balanced.
- The assay signal to background noise ratio (SBR) should be sufficiently high for the log2 ratio change to be observed. In case of low SBR, additional washing of the slides and rescanning is acceptable.
- It is recommended to calculate the acceptable and optimum range of QCs for every array experiment. The QC measures of array data for every experiment is extrapolated by specific software and is indicative for the successful calling of all target probes. The QC measures will vary between array types and different scanners.

Preclinical work-up and report

Preclinical work-up

- A case specific work-up is not required when performing aCGH for structural rearrangements, unless the carrier has an unbalanced karyotype.
- It is recommended to upfront ensure that all unbalanced products of the specific rearrangement can be identified with the platform used. The ability to detect an unbalanced product depends on the effective resolution and the coverage of the array used. This needs to be established prior to clinical application by using DNA from cell lines with well-established segmental aneuploidy to validate the presence and the number of all (consecutive) clones/probes representing the respective chromosome regions.
- It is acceptable that 3 out of 4 translocation segments are detected to reliably identify unbalanced segregation products.
- It is not acceptable to perform a clinical PGT-SR test if the size of the translocation segments, inferred from the karyotype, is below the threshold of resolution of the platform used.

- It is acceptable to forego any additional work-up when performing aCGH for structural rearrangements.

Preclinical work-up report

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

Risk assessment

Risk assessment should cover:

- risks caused by errors in sample tracking,
- risks caused by handling biopsy samples prior to aCGH analysis that, if not performed with care, may compromise DNA integrity and lead to failed or poor WGA,
- risks that the size of the structural rearrangement is different from the one expected based on parental karyotypes and therefore may remain undetected by the aCGH protocol (if they are below the resolution of the platform used),
- risk of inconclusive or false results due to suboptimal experimental conditions,
- risk of inconclusive or false results due to biological reasons: (1) unbalanced segregations may arise from crossing-over during meiosis I in the gametes of the carrier of the rearrangement, (2) chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype, (3) embryos of poor morphology are at risk of containing cells with degraded DNA.
- patient's risk of miscarriage, stillbirth, (viable) unbalanced offspring, mosaic offspring or offspring with a chromosomal imbalance that is below the resolution of the test, whether biological or caused by a technical error.

Limitations of the test

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

1.3. SNP array

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

SNP array-based PGT-SR requires a preclinical work-up to phase the imbalance. Phasing is performed using DNA from the couple and one reference (a balanced reference is recommended, but an unbalanced is acceptable). If no reference is available, diagnosis can be performed during the clinical cycle and requires at least an unbalanced embryo or well-defined breakpoints to distinguish unbalanced embryos.

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

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

1.4. Next generation sequencing (NGS)

NGS allows for direct reading of sequenced DNA fragments and their quantification based on sequence read numbers. Depending on the sequencing read depth, NGS can be applied in different assays– from whole chromosome aneuploidy to medium size deletions or insertions in chromosomes and detection of single gene disorders. Compared to aCGH, chromosomal copy number assessment based on NGS may offer several advantages including: (1) reduced DNA sequencing cost made possible by high throughput sequencing technologies and the larger number of samples that can be simultaneously sequenced during a single experiment (the latter requires adding a unique tag); (2) enhanced detection of deletions and duplications because of the potential increase in resolution (as assessed in the pre-examination validation); (3) increased dynamic range enabling enhanced detection of chromosomal mosaicism in TE samples; (4) the potential automation of the sequencing library preparation to minimize human errors, reduce hands-on time and enable higher throughput and consistency.

Laboratory issues

NGS protocol

The sequencing by NGS protocol comprises five steps: (1) sample processing (2) initial quality analysis (3) library preparation, (4) sequencing, and (5) data analysis.

The sample processing and sequencing generally include any or all of the following processes: handling of biopsy samples (PB, single blastomere or TE cells), cell lysis, barcoding (molecular indexing) of samples, adapter ligation, amplification, library preparation, flow cell loading, and generation of sequence reads. It is recommended to perform initial quality analysis of DNA. Contamination of starting material can lead to poor sequencing data quality. The ancillary DNA quantitation equipment listed in 1.3 section are crucial to ensure the starting material will be sufficient to continue through the sequencing process. Laboratories should also consider whether they use any robotic or high-throughput protocols for DNA isolation and ensure that these protocols are optimized and have proper quality assurance. DNA sequence generation by NGS platforms is almost entirely automated and the output consists of millions to billions of short sequence-reads. Raw data produced after sequencing are further processed by computational analyses and bioinformatics using a variety of algorithms to map and align the short sequence reads to a linear reference human genome sequence.

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

For each platform, the ***genome coverage*** and minimum number of reads needs to be established.

Turnaround time

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

With the aim of accumulating samples for an NGS run, biopsy samples can be stored short-term, while WGA samples can be stored long-term at -20°C.

Documentation

Relevant laboratory documentation should include:

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

Laboratory infrastructure, equipment and materials

Infrastructure

General aspects on infrastructure are covered in the paper on organisation of PGT (refer ORG paper), and in section 2.1 aCGH.

Equipment

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

NGS-based PGT requires the following equipment:

- A DNA quantitation instrument; it is crucial to accurately determine the amount of starting DNA for library preparation. There are several options that give highly accurate quantitation of low amounts of DNA. Amongst those is the Qubit high sensitivity double stranded DNA (HS dsDNA) fluorometer, which measures dsDNA. HS dsDNA has been found to give a much more accurate estimation of the amount of DNA present in the sample, compared to standard spectrophotometry. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. It is recommended to use DNA with absorbance ratio values ranging from 1.8 to 2.0.

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

Materials

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

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

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

Work practice controls

Identification and witnessing

- An adequate labelling system with two unique patient identifiers and embryo/cell(s) number is recommended.
- Labelling and sample identification should be confirmed for critical and high-risk steps by an independent observer, preferably one who is trained in molecular genetics. It is recommended that the unique patient identifier and embryo/cell number be witnessed and signed off by two operators at the following steps:
  - after biopsy to confirm that the embryo and the biopsy sample match,
  - at tubing to confirm that labelling of the reaction tubes with the biopsied samples matches the information on biopsy/cell loading sheet,

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

Intra-assay controls

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

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

Pre-examination process

Quality control

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

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

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

- In general, poor quality or failed WGA products should be excluded from further analysis as these samples may affect the sequencing read distribution per sample after library pooling and sequencing.
- Following accuracy assessment tests, it is recommended to calculate the performance (sensitivity, specificity, positive predictive value and negative predictive value) of the protocol.
- As the presence of chromosomal mosaicism is an issue when analysing TE biopsy samples, it is recommended to include mosaic samples (i.e. a mixture of cells with known segmental aneuploidies and euploid cells) in the validation study (see also 2.1).

Test efficiency

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

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

Preclinical work-up and report

Preclinical work-up

- It is recommended to check whether the chromosomal segments involved in the rearrangement are adequately covered, in terms of the number of sequence-reads.
- Parental karyotypes may facilitate testing and genetic counselling.

- It is acceptable that at least 3 out of 4 translocation segments can be detected to reliably identify unbalanced segregation products.
- It is not acceptable to perform a clinical PGT-SR test if the size of the translocation segments, inferred from the karyotype, is below the threshold of resolution of the platform used.
- It is acceptable to adjust the lower detection limit provided by the platform's manufacturer based on a feasibility study using DNA from previous unbalanced products of conception.
- It is acceptable to forego any additional work-up when performing NGS for structural rearrangements.

Preclinical work-up report

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

Risk assessment

Risk assessment should cover:

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

Limitations of the test

Limitation of NGS consist in the fact that the analysis cannot:

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

2. Preimplantation testing for numerical aberrations

Applications of PGT-A comprises low risk PGT-A (former PGS) and high risk PGT-A (patients seeking PGT for numerical aberrations such as Klinefelter and other sex chromosome abnormalities). Both types share the same techniques, but protocols for aCGH and NGS applied for PGT-A may be different.

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

Real-time qPCR has been used for PGT-A, but the limits of the technique, such as the possibility to process only very small numbers of samples and the low resolution in the detection of chromosomal mosaicism, have led to its disuse in favour of techniques such as NGS. For this reason, real-time qPCR will not be addressed in this paper.

2.1 Array-based and NGS-based PGT-A

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

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

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

Laboratory issues

Information on protocols, turnaround time and documents for aCGH and NGS are presented in sections 1.2 and 1.4, respectively.

Laboratory infrastructure, equipment and materials

Information on infrastructure, equipment and materials for aCGH and NGS are presented in sections 1.2 and 1.4, respectively.

Work practice controls

Information on identification and witnessing for aCGH and NGS are presented in sections 1.2 and 1.4, respectively.

<u>Use of intra-assay controls for aCGH</u>

Information on using intra-assay controls for aCGH is presented in section 1.2

<u>Use of intra-assay controls for NGS</u>

- For intra-assay control in each routine test it is recommended to use negative and positive controls in the same NGS run with separate barcodes with the aim to monitor if the section has contamination or amplification failure.

- It is recommended to perform intra-assay control using isolated samples composed with single cells containing known whole chromosome aneuploidies diagnosed with a previously validated technique.

Pre-examination process

Information on test efficiency materials for aCGH and NGS are presented in sections 1.2 and 1.4, respectively.

Quality control

- Effective resolution of the aCGH and NGS platform and protocol should be internally validated in each laboratory prior to clinical application for low and high risk PGT-A.
- It is recommended to validate aCGH and NGS for aneuploidy testing with a series of positive controls that should include DNA from:
  - single cells from cell lines with established numerical copy number changes (aneuploidy);
  - previous aneuploid pregnancies, when available;
  - cells or TE biopsies isolated from donated embryos from previously performed PGT-A cases analysed with an established technique, when available.
- It is recommended to determine false negative, false positive, specificity and sensitivity rates of the specific platform to be used.
- When using aCGH and NGS for aneuploidy testing in TE biopsy samples, the possibility of misdiagnosis due to chromosomal mosaicism represents the main issue relating to CNV and log2 ratio value threshold detection by NGS and aCGH, respectively.
- It is recommended to perform validation studies with mosaic models by using cell mixture samples of aneuploid and ***euploid*** cell lines (ratios from 10% to 100%) to establish thresholds for chromosomal mosaicism detection rates (i.e. the minimum ratio of aneuploid to euploid cells that is needed to detect a chromosomal copy number variation) and quantification of mosaicism levels. After statistical analysis, the results of these experiments can be used as a reference to determine the mosaicism level of analysed samples. In the first step of the validation process, it is recommended to analyse a wide number of euploid samples (including 6 to 8 cells from euploid cell lines), in order to determine the standard deviation from the euploidy baseline value (2 chromosome copy number and log2 ratio for NGS and aCGH respectively) and thus define the "euploidy'' threshold values. Similarly, threshold values should be defined for ***trisomy*** and ***monosomy***.
- It is recommended to test replicates of the same DNA sample to perform accuracy and variability assessment in independent aCGH experiments and NGS runs.
- To mimic a blastocyst biopsy, a sample size of 8-10 cells is recommended for all mosaicism cell mixture models. Although validation experiments will set euploid/aneuploid parameters, it is important to mention that limitations still exist when analysing biopsy samples with few cells, where it will be almost impossible to detect changes that represent less than 20-30% of the biopsy.
- In order to define the detection threshold, the quality (intrinsic DNA sample quality, QC) of the experiments, the noise and technical artefacts should also be considered.
- As different chromosomes might have a different resolution, the series of aneuploid samples should represent the range of aneuploidies that the test is required to detect.
- Sensitivity and specificity of the mosaicism detection specifically apply for each aCGH and NGS platform (hardware and protocol for WGA or library preparation for NGS) and software or bioinformatics paradigm used to analyse the data. These cannot be exchanged among platforms.

- During the validation of high resolution aCGH and NGS for PGT-A, ***de novo segmental chromosome aberrations*** are also encountered.
- It is recommended to establish the true resolution and specificity of the aCGH and NGS platform to detect segmental aneuploidy through a validation study that is already mentioned in sections 1.2 and 1.4 for PGT-SR.

Preclinical work-up and report

Information on preclinical work-up and report related to aCGH and NGS are presented in sections 1.2 and 1.4, respectively.

Preclinical work-up

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

Preclinical work-up report

A case-specific preclinical wet-laboratory work-up report is not required for low and high risk PGT-A with aCGH and NGS.

Risk assessment

Information on risk assessment related to aCGH and NGS are presented in sections 1.2 and 1.4, respectively and additional issues related to aCGH and NGS for PGT-A are listed here.

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

Limitations of the test

- aCGH and NGS cannot detect all variants of polyploidy and haploidy.
- The currently used aCGH platforms for PGT-A are unable to detect small microdeletions or microduplications, such as the 22q11.2 microdeletion syndrome (DiGeorge/velocardiofacial syndrome).

- Due to the intrinsic nature of chromosomal mosaicism, the chromosomal make-up achieved from a biopsy only represent a picture of a small part of the embryo and does not necessarily reflect the chromosomal content of the entire embryo. In this view, the mosaicism level inferred from a TE biopsy might not unequivocally represent the exact chromosomal mosaicism percentage of the TE cells or the ***inner cell mass*** constitution.
- aCGH will not necessarily detect low level mosaicism. NGS is able to accurately detect mosaicism down to 20% when no noise is present in the sample.
- The number of cells in a TE biopsy is unknown. Therefore, the exact mosaicism level in the biopsy sample cannot be determined.
- aCGH cannot analyse aneuploidy and gene defects simultaneously whereas NGS can.
- Based on the embryo biopsy, aCGH cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy whereas NGS can, provided phasing references are available.
- Noisy profiles are difficult to evaluate and appropriately score the chromosome copy number.

3. Strengths and limitations

Technical strengths and limitations of FISH, aCGH and NGS are outlined in table 1.

The most important limitations include:

- Based on the embryo biopsy alone, FISH, aCGH and NGS cannot discriminate between samples carrying the rearrangement (i.e. balanced) and those that are not (i.e. normal) and this should be clearly stated in the report. Although there is no expected difference in the phenotype of embryos with a 'normal 'or a 'balanced' karyotype, many couples wish to know whether the structural rearrangement is being transferred to their offspring to be aware of possible future reproductive problems related to the rearrangement.
- FISH and aCGH cannot, but NGS can analyse aneuploidy and gene defects simultaneously in the same workflow.
- Based on the embryo biopsy alone, FISH, aCGH and NGS cannot identify the nature (meiotic or mitotic) nor the parental origin of aneuploidies. However, in PGT-A parental origin is of no biological/therapeutic importance.
- Based on the embryo biopsy alone, FISH, aCGH and NGS cannot detect UPD.

932 Table 1. Overview of the strengths and limitations of the methods applied for PGT-SR and PGT-A

	PGT-SR	PGT-SR / PGT-A	
	FISH	aCGH	NGS
NUMBER OF CHROMOSOMES	Information is limited to chromosomes and/or targeted loci for which probes are used.	All 24 chromosomes analysed.	All 24 chromosomes analysed.
MINIMAL RESOLUTION	Limited by the availability of (commercial) probes. Commercial probes are available for only a limited number of loci, which may complicate the selection of probes for the analysis of rare chromosomal rearrangements.	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software	Limited by the empirical resolution of the platform established in each laboratory after proper validation of wet-laboratory protocol and analysis software
WHOLE PLOIDY CHANGES	Inferred from the number of hybridisation signals from multiple probes.	Not all variants of ***polyploidy*** and haploidy can be detected.	Not all variants of polyploidy and haploidy can be detected.
NO CONCLUSIVE RESULTS	As a result of improper fixation, overlapping cells or signals. Rebiopsy is an option.	As a result of cell lysis during tubing, cells with degraded DNA, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.	As a result of cell lysis during tubing, cell loss or poor experimental conditions. Re-analysis or rebiopsy is an option.
ABNORMALITIES NOT DIAGNOSED	FISH-based PGT-SR diagnosis does not allow for a distinction between embryos with a normal or a balanced karyotype.	aCGH-based PGT-SR diagnosis does not allow for a distinction between embryos with a normal or a balanced karyotype.	NGS-based PGT-SR diagnosis allows for a distinction between embryos with a normal or a balanced karyotype, provided ***phasing references*** are available.
MOSAICISM RELATED ISSUES	Chromosomal mosaicism, either at cleavage stage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.	Chromosomal mosaicism, either at cleavage or blastocyst stage, may lead to misinterpretation of the actual embryo karyotype.
UNIPARENTAL DISOMY (UPD)	FISH analysis does not allow for the detection of UPD.	aCGH analysis does not allow for the detection of UPD.	NGS analysis allows for the detection of UPD, provided ***phasing references*** are available.
RISK OF MISDIAGNOSIS	Contamination with cumulus cells. Visual inspection allows for the identification of sperm cells.	Contamination with remaining cumulus cells after ICSI.	Contamination with remaining cumulus cells after ICSI.
IMPACT OF BIOPSY ON TEST RESULTS	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is less favourable compared to a single cell biopsy.	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.	Cells (DNA) damaged during biopsy may have a negative impact on the reliability of the test result. Analysis of a multi-cell biopsy is more efficient than of a single cell biopsy.
SIMULTANEOUS DETECTION OF CHROMOSOME COPY NUMBER AND SINGLE GENE DISORDER(S)	Not feasible.	Not feasible.	Feasible.
ORIGIN OF ANEUPLOIDY	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy.	Cannot identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy.	Can identify the nature (meiotic or mitotic) and/or the parental origin of aneuploidy provided ***phasing references*** are available.

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4. Examination process

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

General recommendations on the PGT examination process are included in the paper on organisation of PGT (refer ORG paper). The sections below highlight specific issues relevant to PGT-S/R and PGT-A.

4.1 Scoring of clinical results

FISH results

- FISH signals should be scored according to brightness, size and distance. The signals should have approximately the same brightness and size (depending on the probes used) and should be at least one signal diameter apart. Two signals that are in close proximity and have approximately the same size, but are not connected by a visible link, are considered as two signals. A diffuse signal should be scored as one if the signal is continuous and of expected size. Two small signals connected by a visible link are counted as one signal.
- Signal scoring criteria should be established in a written protocol and adhered to for the interpretation of signals.
- It is recommended that signals are analysed by two independent observers and discrepancies adjudicated (where possible) by a third observer. If no consensus is reached the embryo should not be recommended for transfer, i.e. should be given the diagnosis of uninterpretable or inconclusive result.
- It is acceptable to score signals from probes labelled with fluorochromes not detectable to the human eye using an image capture system.
- All fluorescent images should be captured and filed for QC purposes. If possible the position and coordinates of the embryonic cells on the slide can be recorded.
- "No result rescue" for embryos without a clear diagnosis is acceptable. An additional hybridization round should be performed with probes indicative of the same chromosome(s) but a different region or, if not available, at least with probes in a different colour scheme. A second biopsy can also be performed, followed by the full FISH protocol.
- When there is a combination of chromatid gain/loss in the first polar body which is balanced by the second polar body, a normal chromosome copy number is predicted and reported, and the corresponding embryo can be recommended for transfer.

aCGH and NGS results

- Software analysis and copy number scoring criteria should be established in a written protocol and adhered to for the interpretation of whole chromosome and segmental chromosome gains and/or losses.
- Interpretation of raw data or profiles resulting after specific software by a single observer is acceptable. Additional confirmation by an independent observer is recommended. If no consensus is reached, the embryo should not be recommended for transfer, i.e. should be given the diagnosis of uninterpretable or inconclusive result.

- All files resulting from the scanning, sequencing as well as profiles after specific software analysis should be stored and filed for QC purposes.
- "No result rescue" for embryos without a clear diagnosis is acceptable. This could imply a second analysis of the existing WGA as well as a second biopsy followed by WGA, full aCGH/NGS processing and analysis.
- When there is a combination of chromatid gain/loss in the first polar body which is balanced by the second polar body, a normal chromosome copy number is predicted and reported, and the corresponding embryo can be recommended for transfer.

4.2 Issuing a PGT report

General items required in PGT preclinical work-up or clinical cycle reports have been listed in the paper on organisation of PGT (refer to ORG paper). The ISCN reporting is acceptable for PGT-A and PGT-SR. It is recommended to add the following technical or interpretation items to the clinical report:

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

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)

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