

171 **Double amplification refractory mutation system (D-ARMS)**

172 **Principle of the test**

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

182 **Limitations of the test**

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

185 **Real-time PCR for mutation detection and genotyping**

186 **Principle of the test**

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

192 **Limitations of the test**

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

195 **Mini-sequencing**

196 **Principle of the test**

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

205 **Limitations of the test**

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

209 1.3 Single or few cell targeted amplification

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

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

216 Laboratory infrastructure, equipment and materials

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

220 Infrastructure

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

237 Equipment

238 Equipment required for amplification-based analysis of samples includes:

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

245 Materials.

246 Specific materials required for targeted-amplification of samples include:

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

250 Tubing of samples

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

253 Work practice controls

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

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

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278 1.4 Single or few cell whole genome amplification

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

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

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

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

295 **Laboratory infrastructure, equipment and materials**

296 In general, follow the recommendations as stated in [section 1.3](#).

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

298 **Infrastructure**

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

303 **Equipment**

304 Additional equipment includes:

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

309 **Materials**

310 Specific materials required for WGA of samples include:

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

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315 **Tubing of samples**

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

318 **Work practice controls**

319 Positive and negative controls should be included to monitor the WGA reaction, as described in section
320 [1.3](#).

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

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324 2. Pre-examination process

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

327 2.1. Informativity/segregation analysis

328 It is recommended to perform a preclinical work-up to assess PGT-M feasibility, identify informative
329 genetic markers, establish parental haplotypes (when possible) and work on a clinical testing strategy.

330 It is recommended to perform the informativity/segregation analysis for STR markers as well as for SNP
331 markers. The results allow evaluating the expected genotypes in the embryos.

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

347 2.2 Testing strategies and test development

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

350 The three main testing strategies for PGT-M are:

- 351 1) targeted amplification of informative markers with or without the mutation(s) in a single/few
352 cell multiplex PCR (*sections 2.2.1 and 2.2.2*)
- 353 2) WGA followed by targeted amplification of informative markers with or without the
354 mutation(s) (*section 2.2.3*)
- 355 3) WGA followed by a generic method such as SNP array or NGS-based sequencing (*sections*
356 *2.2.4 and 2.2.5*)

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

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

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

378 2.2.1 Targeted amplification for PGT-M

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

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

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

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

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

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

- 404 • It is recommended to avoid STRs with a very wide range of alleles since the ADO risk of the large
 405 alleles is increased even at the genomic DNA level leading to false homozygous genotypes
 406 during pre-clinical work-up and PGT-M.
- 407 • Before moving on to single-cell validation, it is recommended to establish a correct
 408 discrimination of mutation/wildtype or marker alleles of the test at hand. It is recommended to
 409 test various genotypes concerning the mutation or marker of interest using the following DNA
 410 samples:
- 411 - affected (autosomal dominant) DNA samples,
 - 412 - carrier (autosomal recessive, X-linked diseases) DNA samples,
 - 413 - unaffected DNA samples for the mutation to be tested, and
 - 414 - DNA samples with heterozygous markers for indirect tests.
- 415 • When a protocol is employed for PGT-M, it is recommended to apply the specific test to DNA
 416 or single cells from each particular couple to discover any unexpected test results which could
 417 render future blastomere results questionable (for example, a polymorphism which may exist
 418 under a primer used in the single cell assay but not in the routine laboratory assay).
- 419 • Polymorphic markers should have a high degree of heterozygosity and produce a clearly
 420 interpretable peak pattern, and preferably be intragenic.
- 421 • When using extragenic markers, it is recommended to stay within 1 Mb distance from the
 422 mutation of interest to reduce the misdiagnosis risk due to recombination events (on average,
 423 loci 1cM apart are expected to show 1% recombination). If no suitable markers are available
 424 within 1Mb, markers within 2Mb are acceptable. This may be adapted in case of large genes or
 425 duplications.
- 426 • The risk of misdiagnosis due to recombination should be considered for every marker and is
 427 especially important in case of large genes and genes with recombination hot spots.
- 428 • Defining the minimum number of informative markers required in the single/few cell test:
 429 assuming validation data of AF and ADO rates per locus remain below 5%, it is recommended
 430 to include at least one STR or 3 SNPs proximal and one STR or 3 SNPs distal to the region of
 431 interest together with the mutation locus (choose markers with rank 1 or 2 in table 1 and rank
 432 1 in table 2). In case of AF and ADO rates between 5 -10%, either the test should be improved,
 433 or a higher number of markers should be included. In case of insufficient markers of the highest
 434 rank, markers of lower rank can be selected for test development, but the number of markers
 435 should then be increased.
- 436 • More markers will make the test more robust; analysis of at least two loci closely linked to the
 437 gene will reduce the risk of unacceptable misdiagnosis owing to ADO. Also, the risk for no
 438 diagnosis due to AF of a single amplicon in the multiplex will decrease.

439 **Genetic Markers only (STRs and/or SNPs)**

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

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

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

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462 2.2.2 Targeted amplification for combined PGT-M and PGT-A

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

473 2.2.3 WGA followed by targeted amplification for PGT-M

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

480 Recommendations for single or few cell WGA concerning infrastructure, equipment, materials, tubing
481 and work practices are described in section 1.4.

482 The following recommendations are made:

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

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

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

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

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

504 **Genetic Markers only (STRs and/or SNPs)**

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

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

516 **2.2.4 WGA followed by generic testing for PGT-M**

517 **SNP Array for PGT-M only**

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

525 The following recommendations are made:

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

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

543 ***Limitations of the test***

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

547 **NGS for PGT-M only**

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

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

555 The following recommendations are made:

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

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

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

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

581 *Limitations of the test*

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

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

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

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590 **2.2.5 WGA followed by generic testing for combined PGT-M and PGT-A**

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

597 The following recommendations are made:

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

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

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

615 *Limitations of the test*

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

624 2.3 Pre-examination validation

625 *For PGT-M*

- 626 • Validation criteria are dependent on the number of cells biopsied (single cell at cleavage stage,
627 or few cells at blastocyst stage) and on the type of strategy used for PGT-M. It is acceptable to
628 perform the validation on cell(s) from embryos donated to research or on other cell types such
629 as peripheral blood lymphocytes.
- 630 • Misdiagnosis risk needs to be established.
- 631 • The following criteria apply for targeted STR-based testing, with or without prior WGA:
 - 632 ○ Validation assays will determine amplification efficiency, accuracy, and ADO rate.
633 Accuracy should be >99% for single or few cell samples of known genotype.
 - 634 ○ The amplification efficiency per locus should be >95%. An amplification efficiency of
635 >90% is acceptable, but more markers need to be included.
 - 636 ○ The ADO rate per locus should be <5%. ADO rate less than 10% is acceptable, but more
637 markers need to be included.
- 638 • Every new test based on targeted amplification should be validated.
- 639 • For targeted amplification, validation assays should be performed in 50 single or few cells
640 samples, in two or three separate runs prior to clinical use. It is acceptable to validate updated
641 protocols (i.e. adaptations of existing protocols) with less samples.
- 642 • No validation is needed on few cells samples when the protocol has been previously validated
643 on single cells.
- 644 • The following criteria apply for generic strategies, such as SNP array with prior WGA:
 - 645 ○ When using commercially available SNP array or NGS-based protocols, which already
646 have been validated by the manufacturer, it is still recommended to carry out an
647 implementation validation of the complete wet and dry-laboratory workflow prior to
648 clinical use.

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

663 For combined PGT-M and PGT-A

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

671 2.4 Risk assessment

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

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

685 Risk assessment should also cover:

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

- 689 - risk of inconclusive or false results due to sub-optimal experimental conditions
 690 (contamination, ADO, ADI) or due to biological reasons (recombination, double
 691 recombination, meiotic or mitotic chromosomal aberrations);
 692

693 2.5 Preclinical work-up report

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

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

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

706 3. Special cases

707 3.1 *De novo* mutations

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

711 *de novo* mutation in a prospective parent

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

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

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

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

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

755 *de novo* mutation in an affected child

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

769

770 3.2 Consanguineous relationships

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

773 Consanguineous grandparents

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

780 Consanguineous couple

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

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

789

790 3.3 HLA typing

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

798 Test strategy

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

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

823

824 3.4 Exclusion testing

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

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

836 3.5 Mitochondrial DNA Disorders

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

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

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

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

867 4. Examination process

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

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

871 4.1 Scoring of clinical results

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

886 4.2 Clinical cycle report

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

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

- 890 - unaffected carrier or affected when reporting for monogenic disease,

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

898 5. Post-examination process

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

901 References

902 Harton GL, De Rycke M, Fiorentino F, Moutou C, SenGupta S, Traeger-Synodinos J, Harper JC. ESHRE PGD
903 consortium best practice guidelines for amplification-based PGD. *Hum Reprod* 2011;**26**: 33-40.

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