Approaches to Preimplantation Diagnosis

2

Introduced only in 1990 as an experimental procedure, preimplantation genetic diagnosis (PGD) is now becoming an established clinical option in reproductive medicine [1–3]. Thousands of apparently healthy children have been born after PGD, validating that there is no ostensible evidence of any incurred adverse effect. Over 100,000 PGD cases have presently been performed in more than 100 centers around the world, allowing at-risk couples not only to avoid producing offspring with genetic disorders but, more importantly, to have unaffected healthy babies of their own without facing the risk of pregnancy termination after traditional prenatal diagnosis. Without PGD it is likely that a few of these children would have been born.

Applied first for preexisting Mendelian diseases [4, 5], such as cystic fibrosis (CF) and X-linked disorders, PGD initially did not seem to be practical. Only a few babies were born during the first 3 years of work, and several misdiagnoses were reported [6, 7]. After the introduction of fluorescent in situ hybridization (FISH) analysis in 1993–1994 for PGD of chromosomal disorders [8–13] (Chap. 5); however, the number of PGD cycles began to double annually, yielding more than 100 unaffected children by the year 1996 [14, 15] (Fig. 2.1).

Application of PGD increased further when the ability to detect translocations became possible in 1996, first using locus-specific FISH probes, then more widely available subtelomeric probes [16, 17], (Chap. 5), and presently by haplotyping and also by microarray technology [18–29] (see below). Because many carriers of balanced translocations have a poor chance of having an unaffected pregnancy, PGD has a clear advantage over the traditional prenatal diagnosis in assisting these couples to establish an unaffected pregnancy and deliver a child free from unbalanced translocation [1, 30–33]. Of course, there are differences in the reproductive outcomes depending on the origin and type of translocation, with the majority resulting in early fetal loss and rarely in an affected birth. However, it may take years until the translocation carriers could be lucky enough to get at last an unaffected offspring, so the current recommendations of PGD International Society (PGDIS), European Society of Human Reproduction and Embryology (ESHRE), and American Society for Reproductive Medicine (ASRM) Practice Committee include chromosomal rearrangements as one of the main indications for PGD [34]. The experience of over 3,000 PGD cycles for translocations accumulated by the present time demonstrates at least a six fold reduction of spontaneous abortions in these couples, compared to their experience before PGD [32–37] (see Chap. 6).

The natural extension of PGD's ability to allow transfer of euploid embryos should have positive impact on the liveborn pregnancy outcome. This is especially applicable to poor prognosis in vitro fertilization (IVF) patients (prior IVF failures, maternal age over 37, repeated miscarriages). Introduction of commercially available 5-color probes in 1998–1999, and currently also 24 chromosome testing by microarray analysis, has led to the accumulated experience of more than

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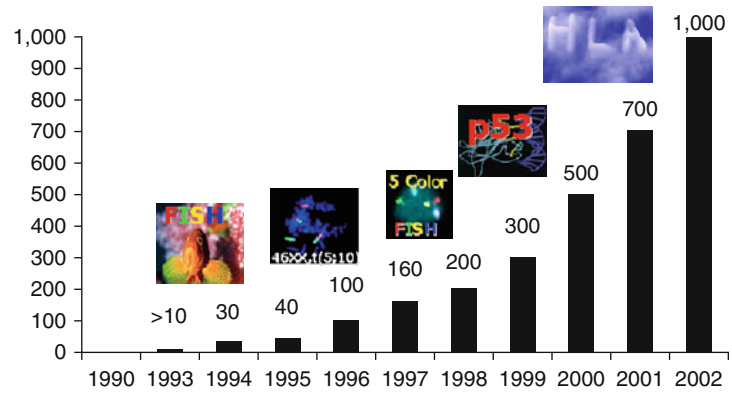

Fig. 2.1 Babies born after (PGD): 1990–2002. *Bar graphs* showing the number of children born each year after application of PGD. Application of each novel technique for PGD is shown above the *bar graphs*, leading to the expanding indications, such as chromosomal aneuploidies in 1993–1994 (shown as FISH), translocations in 1996 (shown by example of translocation t (5; 10), five-color FISH in 1998, application to late-onset disease with genetic predisposition in 1999 (shown as an example of cancer predisposition caused by mutation in p53 gene mutations), and t application to non-disease testing in 2000 (shown as an example of preimplantation HLA typing)

50,000 clinical cycles worldwide for the aneuploidy testing [2, 35, 37–41] (Chap. 5). This has resulted in the birth of over 10,000 children, including a few with misdiagnosis, suggesting the continued need for the improvement of accuracy of aneuploidy testing. According to the experience of the majority of centers, the overall pregnancy rate per transfer is higher than that in non-PGD IVF patients of comparable age group (average age over 39 years), although this is still a controversial issue (see Chap. 6). Available data indicate that the current IVF practice of transferring embryos based solely on morphological criteria is inefficient and needs a revision, given that half of these embryos are chromosomally abnormal and would compromise the reproductive outcome (Sect. 6). The current introduction of 24 chromosome testing combined with polar body (PB) or blastocyst biopsy shows further improvement of reproductive outcomes in poor prognosis IVF patients, confirming the need for preselection of euploid embryos for transfer [18–29].

The application of PGD has further expanded with its introduction to late-onset diseases with genetic predisposition [24, 42] (Chap. 3), a novel indication never previously considered for the traditional prenatal diagnosis. For the patients with inherited pathological predisposition PGD provides a realistic reason for undertaking pregnancy, with a reasonable chance of having an unaffected offspring. Prospective parents at such risk should be aware of this emerging option, especially when there is no opportunity to diagnose the disease until it is fully realized, such as in cases of inherited cardiac diseases leading to premature or sudden death (Chap. 3).

Another unique option that can presently be considered, although involving ethical debate (Chap. 8), is HLA typing as part of PGD, which has never been considered in traditional prenatal diagnosis either [43] (Chap. 4). In this application PGD offers not only preventative technology to avoid affected offspring, but also a new method for treating (older) siblings with congenital or acquired bone marrow diseases, for which there is still no available therapy. This may in future be applied for any condition that can be treated by embryonic stem cell transplantation.

Preimplantation HLA typing was first applied to couples desiring an unaffected (younger) child free from the genetic disorder in the older sibling. In addition to diagnosis to assure a genetically normal embryo, HLA-matched, unaffected embryos were replaced. At delivery, cord blood (otherwise to be discarded) was gathered for stem cell transplantation. As will be described, this approach has been also used without testing of the causative gene, with the sole purpose of

finding a matching HLA progeny for a source of stem cell transplantation for affected siblings with congenital or acquired bone marrow disease or cancer [44] (see Chap. 4).

As will be described in this book, the over 20 years PGD experience demonstrates considerable progress. As mentioned, over 100,000 PGD attempts worldwide have resulted in the birth of dozens of thousands of apparently unaffected children, with no detrimental effect on embryo development, as demonstrated by no significant differences in the overall congenital malformation rate after PGD from the population prevalence [45–47]. With the highly improved accuracy of genetic analysis and indications expanding well beyond those for prenatal diagnosis, thousands of PGD cycles are now performed annually. As seen in Fig. 2.1, already in the year 2006, only the annual experience has resulted in the birth of nearly the same number of children as during the entire preceding decade since the introduction of PGD. This is clearly because PGD offers a special attraction not possible with traditional prenatal diagnosis, such as avoiding clinical pregnancy termination. This is extremely attractive for translocation carriers, couples at risk for producing offspring with common diseases of autosomal-dominant or autosomal-recessive etiology, as well as for couples wishing to have not only an unaffected child but an HLA-compatible cord blood donor for treatment of an older moribund sibling with a congenital disorder. Yet the greatest numerical impact of PGD may be expected in standard assisted reproduction practices (Chap. 6), where improved IVF efficiency through aneuploidy testing will surely evolve to become standard, despite the recent controversy, which will be considered in detail throughout Chaps. 5 and 6.

2.1 Obtaining Biopsy Material

At present, biopsy material for performing preimplantation genetic diagnosis (PGD) may be obtained from three major sources:

1. Matured and fertilized oocytes, from which the first and second polar bodies (PB1 and PB2) are removed
2. Eight-cell cleavage-stage embryo, from which one or two blastomeres are biopsied
3. Blastocyst-stage embryos, from which up to a dozen cells may be removed

The biopsied material is tested for single-gene disorders using PCR analysis, or used for PGD for chromosomal abnormalities by fluorescent in-situ hybridization (FISH), or microarray analysis (see below).

Each of these PGD methods has advantages and disadvantages, and their choice depends on circumstances; however, in some cases, a combination of two or three methods might be required. Despite a possible embryo cell number reduction, which might have a potential influence on the embryo viability, blastomere biopsy allows detecting paternally derived abnormalities. Removal of PB1 and PB2, on the other hand, should not have any effect on the embryo viability as they are naturally extruded from oocytes as a result of maturation and fertilization, but they provide no information on the paternally derived anomalies, even if this constitutes less than 5% of chromosomal errors in preimplantation embryos. Approaches for preconception testing of the paternally derived mutations are being developed, although are still at a very initial stage with not yet considerable progress, as will be described in Sect. 2.1.3.

To perform PGD for chromosomal aneuploidies both PB1 and PB2 are removed simultaneously, next day after insemination of the matured oocytes or ICSI, and analyzed by FISH or microarray technology, as described in Sect. 2.2. PB1 is the by-product of the first meiotic division and normally contains a double signal for each chroSmosome, each representing a single chromatid (see below). Accordingly, in case of meiosis I error, instead of a double signal, four different patterns might be observed, ranging from no or one signal to three or four signals, suggesting either chromosomal nondisjunction, evidenced by no or four signals, or chromatid missegregation, represented by one or three signals [48]. The genotype of the oocytes will, accordingly, be opposite to PB1 genotype, that is, missing signals will suggest an extra chromosome material in the corresponding oocyte, while an extra signal (or signals) will indicate monosomy or nullisomy status of the

tested chromosome. In contrast to PB1, the normal FISH pattern of PB2 is represented by one signal for each chromosome (chromatid), so any deviation from this, such as no or two signals instead of one, will suggest a meiosis II error. Microarray technology also appeared to be extremely reliable in detecting the chromosomal status of polar bodies, as will be described in Sects. 2.2 and (Chap. 5), distinguishing also between chromosome and chromatid errors in PB1, not previously detectable by traditional CGH studies [24–27].

The method of blastomere biopsy was extensively used in PGD, despite its limitation due to the high mosaicism rate in cleaving embryos (see Chap. 5). The FISH pattern of blastomeres is represented by two signals for each chromosome tested, so any deviation from this number will suggest chromosomal abnormality. The same pattern applies to blastocyst analysis, which has an advantage of analyzing not one but a group of cells, obviating the problem of mosaicism at least to some extent. The aneuploidy testing in blastocyst biopsy was further improved by the application of microarray technology, which may pick up mosaicism of 10% and higher [23, 28, 29]. It is also expected that the microarray approach may improve the results in blastomere aneuploidy testing, possibly by avoiding the problems of artifactual loss of chromatid material during the slides preparation involved in the procedure of FISH analysis.

Although more data have to be collected to exclude completely the short-term and long-term side effects, the data available show no evidence for any detrimental effect of the PB, blastomere, or blastocyst biopsy. Overall, these methods were used now in a total of almost 100,000 clinical cycles, and resulted in the birth of dozens of thousands of unaffected children, showing a comparable prevalence of congenital abnormalities to that in the general population, which suggests no detrimental effect of any of the above biopsy procedures mentioned [41, 45–47].

2.1.1 Polar Body Diagnosis

Introduced 22 years ago [2, 5], PB biopsy has become one of the established approaches for PGD. The idea of performing PB PGD is based on the fact that polar bodies are the by-products of female meiosis, which allow predicting the resulting genotype of the maternal contribution to the embryos. Neither PB1, which is extruded as a result of the first meiotic division, nor PB2, extruded following the second meiotic division, has any known biological value for pre- and postimplantation development of the embryo. Initially, only PB1 was tested, based on the fact that in the absence of crossing over, PB1 will be homozygous for the allele not contained in the oocyte and PB2 [49]. However, the PB1 approach was not applicable for predicting the eventual genotype of the oocytes, if crossing over occurred, because the primary oocyte in this case will be heterozygous for the abnormal gene. As the frequency of crossing over varies with the distance between the locus and the centromere, approaching as much as 50% for telomeric genes, the PB1 approach appears to be of a limited value, unless the oocytes can be tested further on. So, the analysis of PB2 has been introduced to detect hemizygous normal oocytes resulting after the second meiotic division. As will be described below, this PGD technique presently involves a two-step oocyte analysis, which requires a sequential testing of PB1 and PB2 [50].

PB1 and PB2 are removed following stimulation and oocyte retrieval using a standard IVF protocol. Following extrusion of PB1, the zona pellucida (ZP) is opened mechanically using a microneedle, and PB1 aspirated into a blunt micropipette (see micromanipulation setup and procedure steps in Figs. 2.2, 2.3, and 2.4). The oocytes are then inseminated with motile sperm or by using introcytoplasmic sperm injection (ICSI), and examined for the presence of pronuclei and extrusion of PB2s, which are removed in the same manner as PB1 (Fig. 2.5). To avoid an additional invasive procedure, both PB1 and PB2 are removed simultaneously for FISH analysis (Fig. 2.6), and are fixed and analyzed on the same slide, while sequential PB1 and PB2 sampling procedure is used for microarray analysis and PGD for monogenic disorders. The biopsied oocytes are then returned to culture, checked for cleavage, and transferred, depending on the genotype of the corresponding PB1 and PB2 [48].

Although PB1 and PB2 have no any known biological significance in pre- and postimplantation

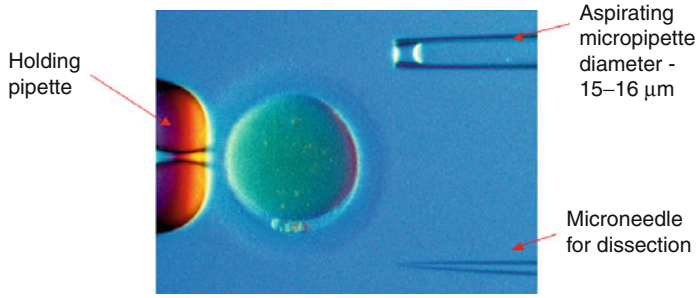

Fig. 2.2 Required microtools for first polar body removal. Image of a metaphase II oocyte along with the three microtools that are required to perform first polar body (PB1) removal. A holding pipette is used (*left*) to hold the oocyte in position by gentle suction created by a hydraulic microsyringe system. On the right, a microneedle for partial zona dissection (*bottom*) and a micropipette (inner diameter 15 µm) for polar body removal are placed into a double tool holder. The micropipette is also attached to a hydraulic microsyringe (100 µl) system for fine control during the procedure. The same tools are required for embryo biopsy with the exception of the micropipette, which has a larger inner diameter of 30–35 µm

development, follow-up studies have been carried out to investigate possible detrimental effect. Following PGD and ICSI, zygotes with two pronuclei were observed in 1,192 (81.8%) of 1,458 oocytes, compared to 30,972 (77.3%) of 40,092 in a routine non-PGD cycle, which suggested no difference in fertilization rate observed after PB1 in comparison to nonbiopsied oocytes. Also there was no difference in blastocyst formation of the embryos resulting from the biopsied oocytes. We compared the blastocyst formation of embryos resulting from biopsied oocytes observed in 1,653 (50.2%) of 3,293 embryos, which was not different from 49.8% (9,726 of 19,529 nonbiopsied embryos) observed in routine IVF. Similarly, no detrimental effect was noted after PB2 removal, which was evident from cleavage rate, blastocyst formation, and the number of cells in the respective blastocysts [51]. As will be seen below, there was no difference either after sequential PB1, PB2, and blastomere sampling.

The PB approach will have an increasing impact on those ethnic groups in which PGD may be done only before fertilization, such as in Austria, Germany, Switzerland, and Malta. In these countries, the testing may be limited to PB1, which, as mentioned above, might not be sufficient to predict embryo genotype, unless PB2 is tested before fusion of pronuclei, which may be combined with freezing of the oocytes at the pronuclear stage. After the analysis, in a subsequent menstrual cycle, only the oocytes predicted as having the normal maternal allele may be thawed and cultured to allow fusion of the pronuclei, embryo development, and the embryo transfer.

In fact, it is presently possible to complete the testing of PB2 in approximately 9 h after removal, so there is no need for freezing the oocytes free of mutation or aneuploidy, which may be cultured as usual and replaced on day 3 or day 5, while the abnormal oocytes are frozen at the pronuclear stage [52]. Since zygotes are not considered to be embryos until pronuclear fusion, and no abnormal oocytes may be thawed and cultured, obviating the establishment of the affected embryo, this technique may be ethically more acceptable to many couples. Therefore, the technique may allow creating a new class of genetic diagnosis, which may be called pre-embryonic genetic diagnosis, pushing the frontier of genotyping to an even earlier stage, as shown in pre-embryonic diagnosis of sickle cell and Sandhoff disease (SHD) presented below.

2.1.2 Pre-embryonic Genetic Diagnosis (PEGD)

2.1.2.1 PEGD with Freezing at Pronuclear Stage

A 33-year old woman and her spouse at risk for producing a child with sickle cell disease referred for PGD to avoid a possible termination of a pregnancy following prenatal diagnosis. A standard

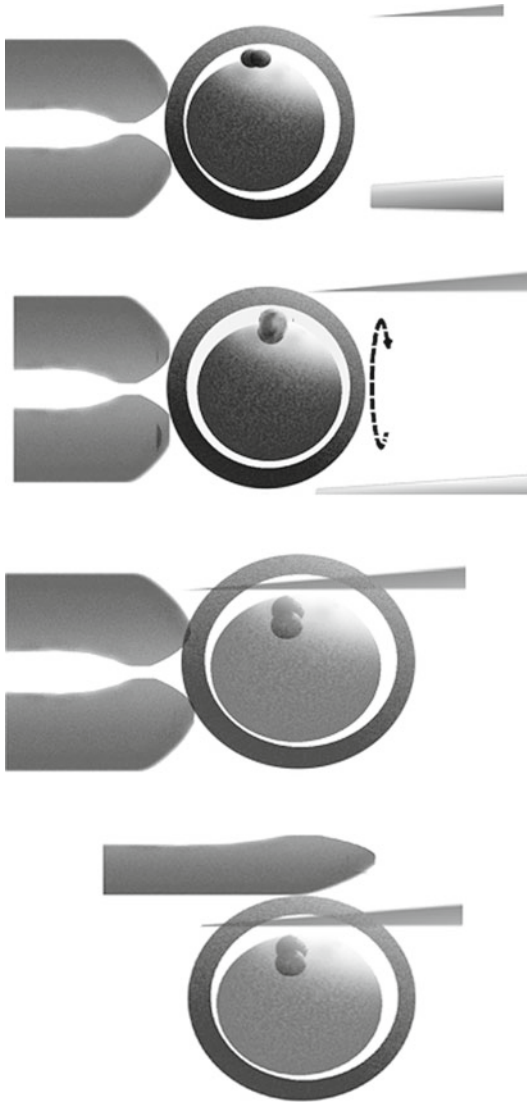

Fig. 2.3 First polar body removal prior to ICSI. (*Step 1*) The oocyte is secured by gentle suction from the holding pipette and the oocyte is rotated so that the PB1 is visualized at 12 o'clock. (*Step 2*) The oocyte is rotated horizontally, slightly forward, so that it faces the operator. (*Step 3*) The microneedle is passed through the zona pellucida at the 1–2 o'clock position and passed tangentially through the perivitelline space and out at the 10–11 o'clock position. (*Step 4*) The oocyte is released from the holding pipette and held by the microneedle. The microneedle is brought to the bottom of the holding pipette and pressed to it, pinching a portion of the zona pellucida. By gently rubbing against the holding pipette with a sawing motion, the cut is accomplished and the oocyte is released

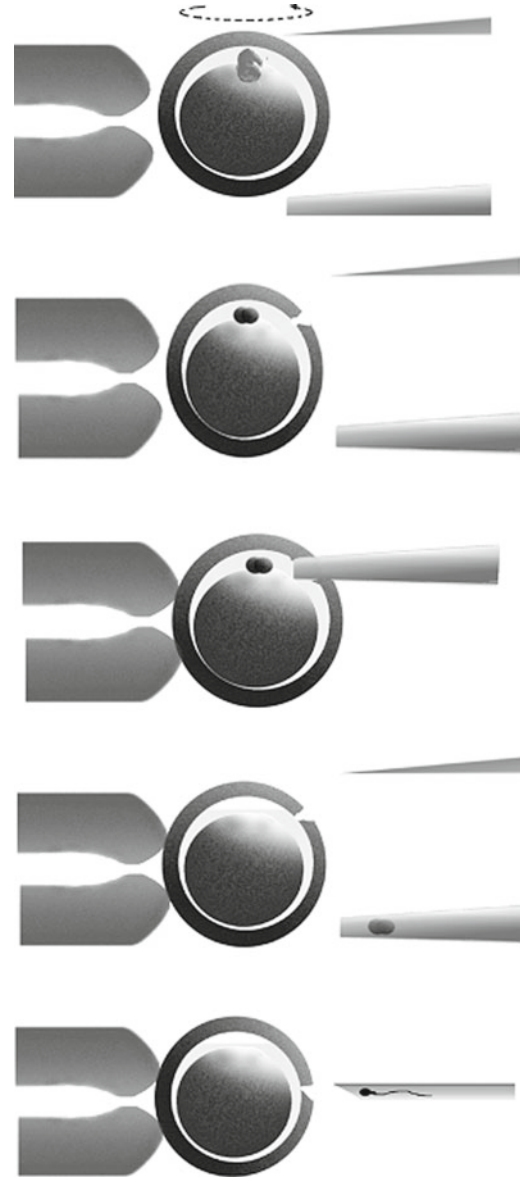

Fig. 2.4 First polar body removal continued (see Fig. 2.3). (*Step 5*) The oocyte is brought to the holding pipette by moving the microscope stage and is rotated vertically using the microneedle until the slit opening is visualized at the 2 o'clock position. (*Step 6*) Once again it is held in place by gentle suction from the holding pipette, making sure the slit opening is at the 2 o'clock position and is in focus. (*Step 7*) The micropipette is brought into the same focal plane as the slit opening and PB1. The micropipette is passed under the zona pellucida to PB1. (*Step 8*) Using gentle suction created by the hydraulic microsyringe system, PB1 is aspirated into the micropipette and then deposited in a separate microdrop of medium. (*Step 9*) The oocyte is transferred to another micromanipulation dish for intracytoplasmic sperm injection (ICSI), which is performed by passing the microtool through the slit opening

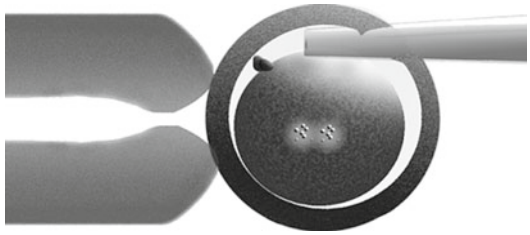

Fig. 2.5 Second polar body removal on day 1 after fertilization assessment. The second polar body (PB2) is removed following fertilization assessment. The same microtools are required as with PB1 removal. The zygote is held in place by gentle suction from the holding pipette and is rotated using the microneedle. The same slit opening can be used or if PB2 has been extruded away from the opening, a second intersecting slit (3D-PZD) (see Fig. 2.12) can be made in order to have easier access to the second polar body

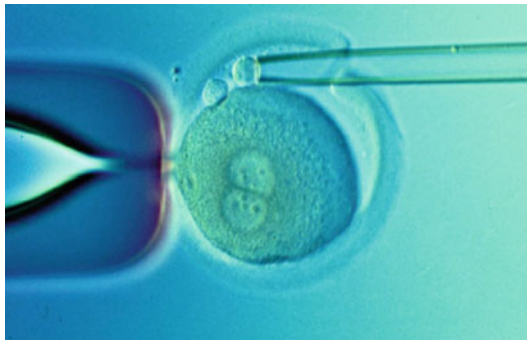

Fig. 2.6 Simultaneous removal of the first and second polar bodies. Both PB1 and PB2 are removed following fertilization assessment, which is used for aneuploidy testing. The same microtools are required as with PB1 removal. The zygote is held in place by gentle suction from the holding pipette and is rotated using the microneedle. The same slit opening can be used as above (see Fig. 2.5), allowing sufficient opening to remove both PB1 and PB2

IVF protocol was initiated but the patient suffered from hyperstimulation syndrome, which precluded transfer of embryos in that cycle. Twenty-eight mature oocytes were aspirated and placed in culture medium. Of the 28 aspirated oocytes, 14 extruded PB1 that were removed. The oocytes were then fertilized by intracytoplasmic sperm injection. As soon as the PB2s were removed and prior to the fusion of the male and female pronuclei, all embryos were frozen. The PB1 and PB2 were analyzed by multiplex nested PCR to avoid allele dropout (ADO), which occurs in approximately 5–10% of PB analyses. This involved nested, multiplex PCR with primer sets for the sickle cell mutation and two linked short tandem repeat (STR) markers: one located at the 5 end of the beta-globin gene (5 STR) and the other in the human thyrosin hydroxylase gene (THO-STR), for both of which the mother was heterozygous.

To detect potential contamination with extraneous DNA and identify the embryo that implanted and established pregnancy, three additional non-linked STRs were amplified, including STR at the 5 untranslated region of human coagulation factor A subunit gene (HUMF13A01), STR for von Willebrand disease (vWD), and an STR for chromosome 21 (D21S11). The list of primer sequences, reaction conditions, and details of the nested PCR were described earlier [48, 53].

The pronuclear-stage oocytes predicted to be normal were thawed, cultured to develop into the cleaving embryos of the 6–8-cell stage, and transferred back to the patient in the two subsequent clinical cycles. The oocytes predicted to contain the mutant maternal gene were not thawed, but analyzed directly at the pronuclear stage for the confirmation of PB diagnosis. Of 28 aspirated oocytes, 14 extruded their PB1 and were studied for the presence of sickle cell mutation. Following intracytoplasmic sperm injection PB2s were extruded from 13 of them and studied. Results of both PB1 and PB2 were available in 12 of these 13 oocytes. Overall, 6 oocytes were predicted to contain a normal allele, based on the heterozygous status of PB1 and the hemizygous mutant status of PB2.

In one of the oocytes, although the sickle cell analysis of PB1 showed only the normal allele in agreement with the 5 -STR, it was heterozygous for the THO-STR, suggesting that this is a case of ADO. Therefore, without simultaneous amplification of linked STRs, a misdiagnosis of the heterozygous oocyte as homozygous due to ADO would have occurred leading to a misdiagnosis of the maternal contribution to the zygote. In this particular instance, the error would have caused an unaffected zygote to be misdiagnosed as affected, but the reverse could also occur.

Subsequently, the patient was prepared for a frozen embryo transfer. In the first frozen cycle, four zygotes determined to have the maternal

unaffected allele were thawed and cultured. Three developed into cleaving embryos of acceptable quality, and were transferred, resulting in a singleton pregnancy, which was spontaneously aborted [53]. In the second frozen cycle, two unaffected embryos were transferred and resulted in a singleton pregnancy and birth of an unaffected child, following confirmation of PB diagnosis by chorionic villus sampling (CVS). The results of the application of non-linked markers allowed not only the exclusion of a possible DNA contamination but also the identification of the embryo that was implanted yielding a clinical pregnancy. All the remaining oocytes predicted to contain an abnormal gene were not further cultured, but exposed directly to PCR analysis at the pronuclear stage for confirmation of diagnosis as frozen sample, and shown to be abnormal as predicted by PB analysis.

2.1.2.2 PEGD Without Pronuclear-Stage Freezing

A 32-year old woman and her spouse at risk for producing a child with SHD requested PGD to be performed without any possible discard of embryos even if affected [52]. As seen from the pedigree shown in Fig. 2.7, the couple had one affected son with classical features of SHD, who died at the age of 1 year and 3 months despite bone marrow transplantation.

SHD results from the defect in the beta chain of the hexaminidase B gene (HEXB) on chromosome 5, which consists of 14 exons distributed over 40 Kb of DNA (MIM 268800; 606873). Mutation in this gene causes beta-hexaminidase deficiency, resulting in the lysosomal storage disease GM2-gangliosidosis. The same condition is caused also by Tay–Sachs disease resulting from the defect of the hexaminidase A gene (HEXA).

The child inherited two different mutations from his parents: the paternally derived I 270 V mutation in exon 5 of HEXB gene, resulting from ATT to GTT substitution, and a large maternal 16Kb deletion (16Kb Del), involving as many as 5 exons, from exon 1 to exon 5 (Fig. 2.8). The paternal mutation was identified by the *Hinf* I restriction digestion, which cuts the normal allele into two fragments of 32 and 25 bp, leaving the mutant allele uncut, and the maternal 16 Kb Del detected by a fragment size analysis. Five closely linked polymorphic markers, D5S1982, D5S1988, D5S2003, D5S349, and D5S1404, were tested simultaneously with the HEXB gene in a multiplex heminested PCR system. The maternal and paternal haplotypes, established by family studies and PB analysis, are presented in Fig. 2.7, while primer sequences are listed in Table 2.1.

A single PGD cycle was initiated, which was performed according to the following modification of the timetable of the applied procedures of sequential PB1 and PB2 analysis described above. PB1 was removed as usual 3.5 h after aspiration, followed by ICSI. PB2 was removed soon after it was extruded, approximately within 6.5 h after ICSI, to allow sufficient time for completion of the DNA analysis before pronuclei fusion (see Fig. 2.9). DNA analysis is currently done in less than 9 h overall (see procedure description below), making it realistic to freeze the oocytes predicted to contain the deleted HEXB allele before syngamy (within 24 h after aspiration or 12 h after PB2 removal), and culture the HEXB deletion-free oocytes to blastocyst and transfer at day 5, following confirmation of the maternal mutation-free status of the embryos by the day 3 blastomere biopsy.

Of 18 oocytes available for testing in a single PGD cycle, 16 were with conclusive PB1 and PB2 results, of which 8 contained the maternal 16Kb deletion and were frozen at the pronuclear stage (Fig. 2.7). Four of these oocytes contained heterozygous PB1 and normal PB2 (oocytes #3, #9, #11, and #14), and four homozygous normal PB1 and mutant PB2 (Fig. 2.7b).

The remaining eight oocytes were free of the deletion, two originating from the oocytes with heterozygous PB1 and mutant PB2 (oocytes #1 and #5) and the others from the oocytes with homozygous mutant PB1 and normal PB2. As the predicted genotypes in these oocytes may erroneously appear opposite, due to a possible undetected ADO of one of the alleles in the actually heterozygous PB1, similar to the 4 mutant oocytes predicted on the basis of homozygous normal PB1 and mutant PB2, the testing for five closely linked polymorphic markers was essential, confirming all the predicted oocyte genotypes mentioned.

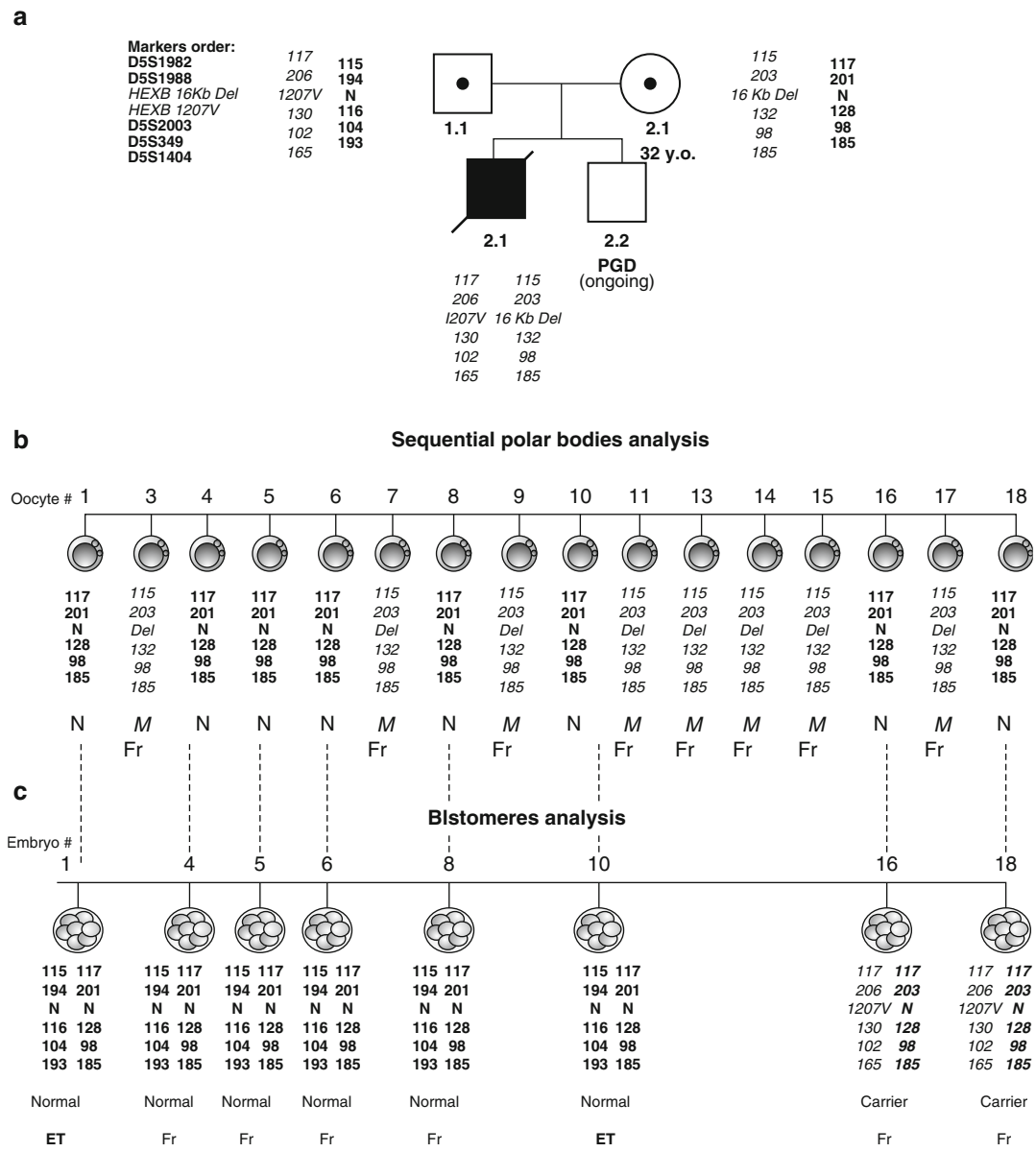

Fig. 2.7 Pre-embryonic diagnosis of Sandhoff disease. (**a**) Family pedigree with mutation and haplotype analysis based on parental (1.1 and 1.2) and affected child's (2.1) genomic DNA testing. The markers' order is presented on the *upper left* for the father and *upper right* for the mother. Maternal and paternal mutations and the linked markers are shown in *non-bold*, while normal alleles and their linked markers are shown in *bold*. (**b**) Results of sequential first and second polar body analysis of 16 oocytes, showing 8 normal (*bold*) and 8 mutant oocytes (*non-bold*) which were frozen prior to syngamy. (**c**) Blastomere analysis of embryos, resulting from the mutation-free oocytes, which confirms the polar body diagnosis. Two of these embryos, #1 and #10, free of both maternal and paternal mutations, were transferred, resulting in the birth of an unaffected child. The remaining 6 embryos, two of which were heterozygous, were frozen for future use by the couple

A follow-up blastomere analysis of the embryos deriving from the oocytes predicted to be free of maternal deletion showed the complete correspondence to the PB diagnosis. Six of these embryos appeared to contain also a normal paternal allele (embryos 1, 4, 5, 6, 8, and 10), while only 2

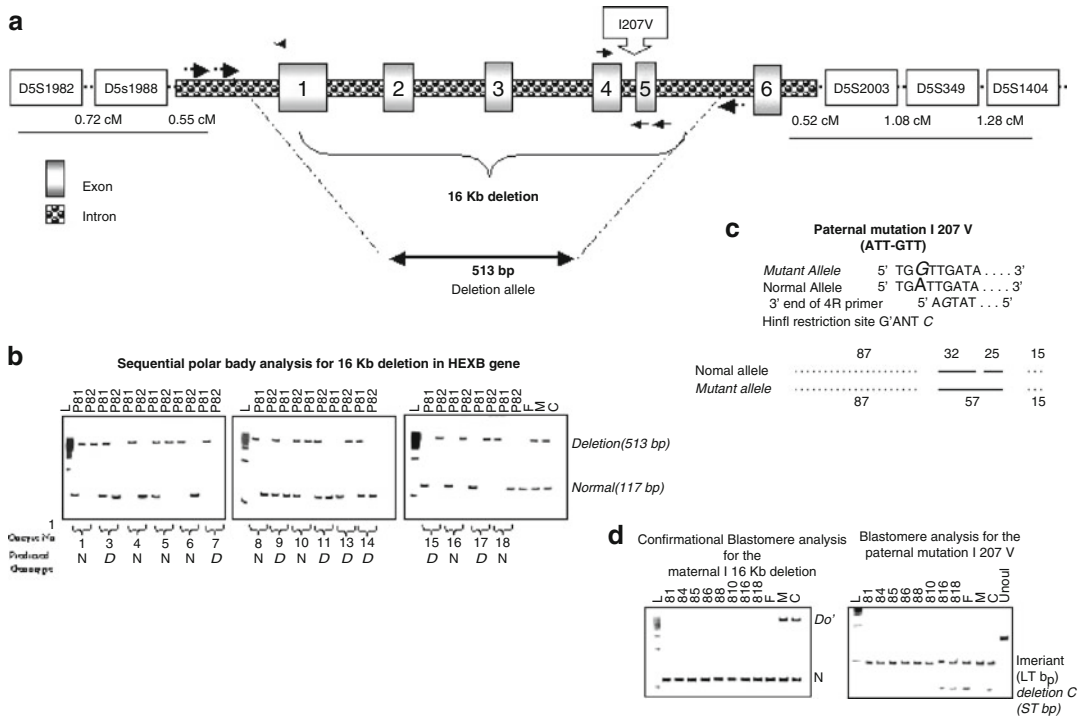

Fig. 2.8 Map of human HEXB gene and results of maternal and paternal mutation testing in polar bodies and blastomeres. (**a**) Schematic presentation of maternal and paternal mutations and linked polymorphic markers. (**b**) Polar body analysis of the maternal 16 kb deletion (*N* normal, *D* deletion). (**c**) Restriction map: HhaI enzyme created two fragments in normal gene, leaving the paternal mutation I 207 V uncut. (**d**) Blastomere analysis for maternal deletion and paternal mutation, confirming the PB diagnosis

(embryos 16 and 18) inherited the paternally derived mutant allele, confirmed by all five linked polymorphic markers tested (Figs. 2.7 and 2.8).

Two of these embryos (embryos #1 and #10), with both maternal and paternal normal alleles, were transferred, resulting in a singleton pregnancy and the birth of an unaffected child. The remaining six unaffected embryos were frozen to be available for the couple in the future, while eight mutant oocytes were frozen at the pronuclear stage.

The presented results show that PEGD is a realistic option for those couples that cannot accept traditional PGD, because of their objection to any micromanipulation and potential discard of the tested embryos. In contrast to the previous PEGD described above, involving the freezing of all the tested oocytes at the pronuclear stage immediately after ICSI and extrusion of PB2, the presented case is realized without freezing the mutation-free oocytes, which were detected well before the pronuclei fusion, after which the embryo discard could not be avoided. Although all the oocytes could have been frozen irrespective of the DNA diagnosis, as described in the previous case, not all frozen pronuclear-stage oocytes could potentially be successfully recovered, which may incidentally include also a few preselected unaffected embryos for transfer and could have affected the PEGD outcome. The realization of PEGD in the same clinical cycle is clearly an important practical step, which has become realistic because of DNA analysis being completed within less than 9 h.

This approach may currently be applied to autosomal-recessive, X-linked, and maternally derived dominant and chromosomal mutations, detectable by sequential PB1 and PB2 analysis. To perform PEGD for paternally derived dominant and chromosomal mutations, a technique for

Table 2.1 Primers for the detection of the 16 Kb deletion and I 207 V mutation in the hexaminidase B gene (HEXB) causing Sandhoff disease, and the linked markers in heminested PCR analysis

Gene/polymorphism	Accession no.	Heterozygosity index	No alleles	Upper primer	Lower primer	Annealing temperature (°C)
16 kb deletion (heminested) amplifies only deleted sequence	ENST00000261416 (Ensembl)	NA	NA	Outside: 5 ACCTCTTTA TGGCTGGCTCC 3	5 AATTATGGGATG ACTGCCTATT 3	62–45
				Inside: 5 AGACACGGCA AGATTAGAGTAATAT 3	5 AATTATGGGATG ACTGCCTATT 3	55
Exon 4-Exon 5 amplifies only normal sequence	ENST00000261416 (Ensembl)	NA	NA	Outside: 5 TAGAGACCTT TAGCCAGTTAGTTTA 3	5 GCTAAGACAAAT ATCTGGGGAAA 3	62–45
				Inside: 5 TAGAGACCTTTA GCCAGTTAGTTTA 3	5 CTAAACAGGTTAC ATTTTTTTCTAT 3	55
I 207 V (heminested) (Hinf I cuts normal sequence)	ENST00000261416 (Ensembl)	NA	NA	Outside: 5 AATAGATTTAG TCTTCATTGAGTTC 3	5 ATTACTTACCAGA GTTTTAAGAATA 3	62–45
				Inside: 5 AATAGATTTAGT CTTCATTGAGTTC 3	5 GCAGATAATGTCT GGATGTATGA 3	55
D5S1982 (heminested)	Z52566	0.70	7	Outside: 5 AGAGTTTGGG CAAGGCGTA 3	5 GGAAAGACATTTA ACCCTTTCTCT 3	62–45
				Inside: 5 GATGAGAATGA AGGTTAAAAAGTCC 3	5 GGAAAGACATTTA ACCCTTTCTCT 3	55
D5S1988 (heminested)	Z52691	0.87	14	Outside: 5 AGCTTACTTCA CTTGGCATAA 3	5 AAGAAATGGAAG CAACCTAAG 3	62–45
				Inside: 5 AGCTTACTTCA CTTGGCATAA 3	5 GTCCACCGATGG ATGAATG 3	55
D5S2003 (heminested)	Z52980	0.81	9	Outside: 5 AGCCTAAGTGA CAAAGTGAGACA 3	5 CTCACAGAGGGT GTGTTATAATAGA 3	62–45
				Inside: 5 AGCCTAAGTGA CAAAGTGAGACA 3	5 TAGAGTCCTTTTC ATTGCCAA 3	55
D5S349 (heminested)	M87741	0.81	8	Outside: 5 ATATTTGGTTT CCATAGAATCTGAG 3	5 CCACCAGATTAAG CGTGAATC 3	62–45
				Inside: 5 ATATTTGGTTT CCATAGAATCTGAG 3	5 CCTCTAGAAAATG GTAGTTGGG 3	55
D5S1404 (heminested)	L30346	0.81	10	Outside: 5 GCCAATTTCT TGTCTATTCCTTAG 3	5 TAATTTACCCACTG TATCAGTCAGG 3	62–45
				Inside: 5 GCCAATTTCTT GTCTATTCCTTAG 3	5 GGTTCCATGAGAA GTAAGAGATCTA 3	55

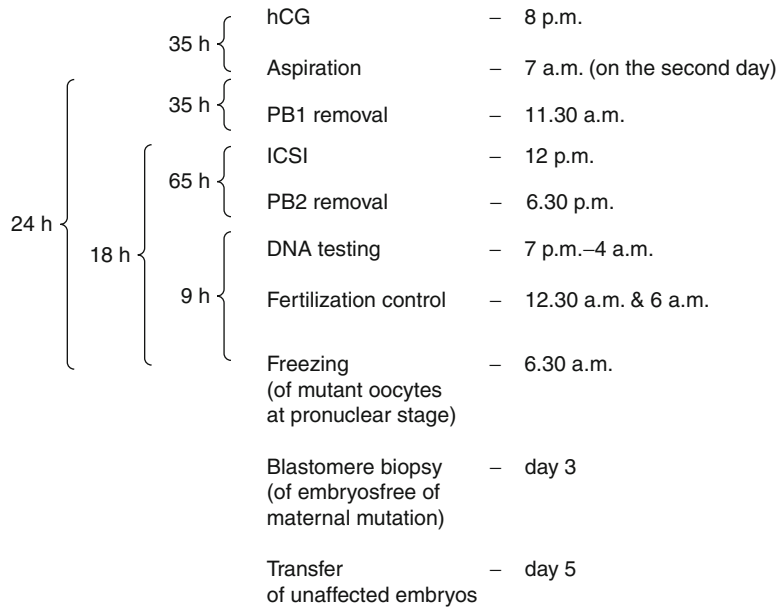

Fig. 2.9 Timeframe for preembryonic diagnosis of Sandhoff disease (see explanation in the figure)

sperm duplication prior to genetic analysis may be required, shown below to be feasible through sperm nuclear transfer into anucleated metaphase II oocytes. The technique will allow genetic analysis of one of the sperm duplicates, using the other one for fertilization and transfer of the resulting embryos if the corresponding duplicate shows normal genotype. In this way, the establishment and discard of any embryo containing a paternal mutation may be avoided. However, more data might be necessary to work out special conditions supporting the faithful replication of human sperm genome, to ensure that the haploid cell pairs obtained from sperm duplication are identical.

As mentioned, PEGD may be applied for aneuploidy testing, as the majority of chromosomal disorders deriving from the female meiosis can be tested by PB analysis. Available experience is presently limited to translocation or aneuploidy testing by PB1 analysis, which, as mentioned, leaves meiosis II errors undetected. As seen from the presented results, the detection of the second meiosis errors is currently feasible within the time available prior to pronuclei fusion, so PEGD for chromosomal disorders may in future be also applied in those countries where PGD is still not acceptable because of the potential discard of the affected embryos with the currently used methods.

With the addition of the PEGD approach, the presently available techniques allow offering a greater variety of methods for predicting and avoiding not only the birth, but also conception or implantation, of the affected embryos. This provides the at-risk couples with any possible option for avoiding the offspring with genetic and chromosomal disorders, independent of their attitudes to oocyte or embryo micromanipulation and testing.

Presented data demonstrate feasibility of performing PEGD for single-gene disorders, which resulted in obtaining unaffected pregnancies and birth of healthy children. Of course PGD for single-gene disorders may be performed by the use of PB1 analysis alone, as described in the first case of PGD by PB1 [5]. Although this allowed preselection of a few mutation-free oocytes inferred from the homozygous abnormal status of PB1, the majority of oocytes were heterozygous after the first meiotic division, so the genotype of the resulting embryos could not be predicted, thus limiting the number of normal embryos for transfer.

The data also show that to avoid discard of the preimplantation embryos reaching the cleavage stage by the time the PB genotyping results were obtained, freezing of oocytes may be used immediately after fertilization and extrusion of PB2 and prior to fusion of the male and female pronuclei – the actual point which is considered

to be the beginning of the embryonic period of development [54]. In fact, freezing presently may be omitted entirely, as the recent developments in PCR analysis allow completing the genetic diagnosis before pronuclei fusion. This opens a possibility for the application of PGD for couples unable to accept any intervention and discard of the human embryos.

2.1.3 Preconception Testing for Paternally Derived Mutations by Sperm Duplication

As seen above, the genetic composition of oocytes may reliably be tested through removal and testing of PB1 and PB2. On the other hand, no method has yet been available for testing the outcome of male meiosis, as genetic analysis destroys the sperm, making it useless for fertilization. To overcome this problem, a new technique has been introduced, allowing duplicating a sperm before genetic analysis, so one of the duplicated sperms can be used for testing and the other for fertilization and consequent transfer of the resulting embryos, provided that the genetic analysis of the corresponding duplicate shows normal genotype [55]. To demonstrate the reliability of the technique, over 100 human sperms from chromosomally normal donors, as well as from translocation carriers, were injected into the enucleated mouse oocytes, and the duplicated cells resulting from an overnight culture were tested by FISH to compare the chromosomal status of both daughter cells. All but 3% of the haploid cell pairs derived from the normal donors were identical for the chromosomes tested, while, as expected, a high proportion of the paired nuclei derived from sperm of translocation carriers were chromosomally unbalanced, suggesting that ooplasm from mature mouse eggs can support the faithful replication of any human sperm genome, irrespective of the genotype.

A similar technique was developed to duplicate human sperm using human oocytes (Fig. 2.10), however, showing that the duplication of sperm may be done faithfully in only half of the cases, in contrast to the use of murine oocytes, so the technique has still to be tested further before applying clinically, with the expected important practical implications for PGD of paternally derived

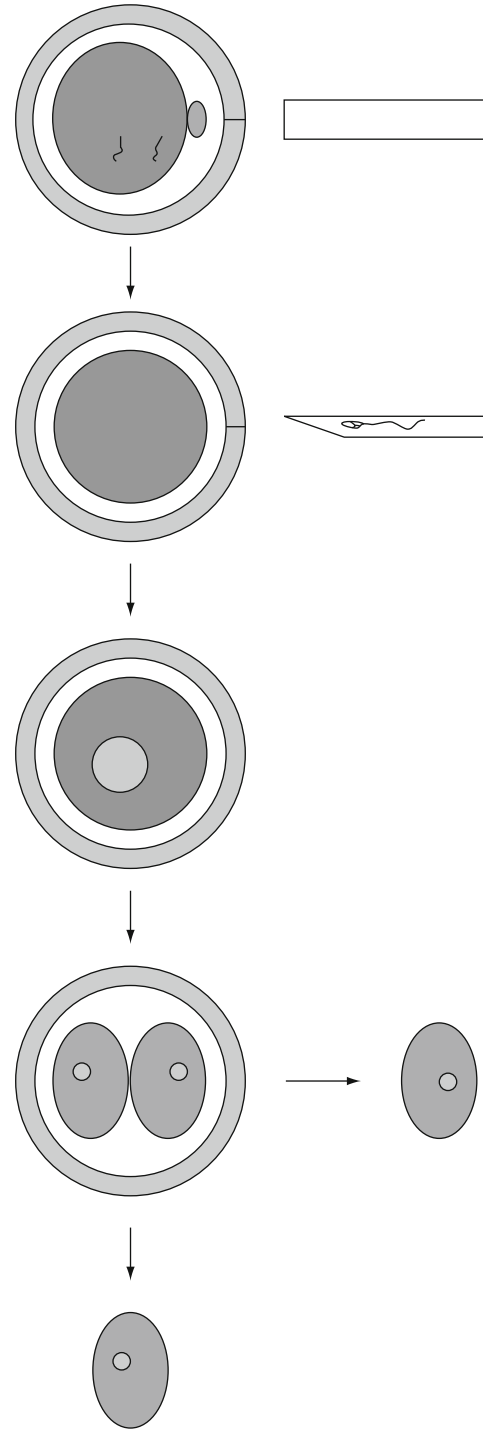

Fig. 2.10 Flowchart of sperm duplication in human MII cytoplast: (*Step1*) enucleation of human metaphase II oocyte; (*Step 2*) injection of single human sperm into the cytoplast; (*Step 3*) reconstructed androgenic embryo with one pronucleus; (*Step 4*) development of this pronucleus into 2-cell embryos; (*Step 5*) testing of one of the cells, with the other one available for further zygote construction of known male contribution

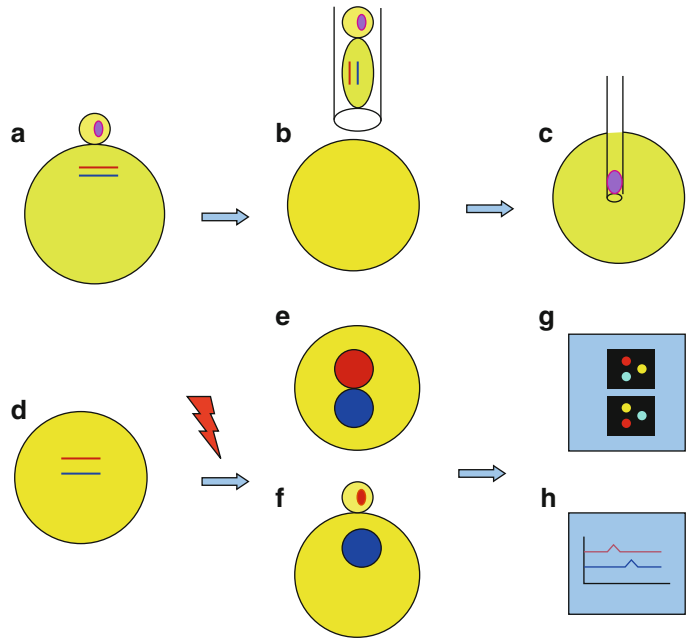

Fig. 2.11 Flowchart of haploidization procedure for obtaining gametes from somatic cells: (**a**) in vitro matured MII oocyte with extruded first polar body (PB1); (**b**) both PB1 and metaphase II chromosomes are removed (*shown in pipette*), to prepare a recipient enucleated ooplast; (**c**) single diploid cumulus cells were prepared as a nuclei donor, from which a nucleus was mechanically isolated, for the introduction into ooplast; (**d**) reconstructed oocyte converts somatic cell nucleus into metaphase chromosomes; (**e**) two pronuclei; or (**f**) pronucleus and PB1 obtained after electrostimulation using electrofusion device RGI-4 (Chicago, IL); (**g**) fluorescent in situ hybridization analysis; or (**h**) PCR-based chromosomal analysis, confirming the formation of two haploid sets of chromosomes in both (**e**) and (**f**) scenarios

conditions, such as translocations, known to produce as much as 70% of abnormal sperm on an average.

The technique has also potential for research purposes, as shown in preliminary work devoted to the study of mosaicism nature [56]. Following duplication of human sperm in cow oocytes, a series of 31 resulting embryos were cultured up to the 8-cell stage, and tested by probes specific to chromosomes 13, 16, 18, 21, and 22. As many as 16% of the resulting sperm duplicates appeared to be not identical, which may further be related to the genetic differences between the donors involved. In fact, one of the three sperm donors for the above experiment produced mostly mosaic embryos in two PGD cycles. However, the rate of mosaicism in sperm duplicates of the three donors involved in this small series was similar, indicating that the generation of mosaic embryos, at least in the patients previously tested by PGD, may not be related to the sperm genotype, but to the sperm centrosome [56].

The genotype of the sperm may also be tested following the testicular biopsy culture and promotion of the developmental progression of spermatocytes through meiosis in vitro, providing the possibility of meiosis outcome analysis to infer the genotype of the resulting sperm to be used for fertilization. However, this is still a theoretical possibility, which has not been realized.

Preconception diagnosis may be realized also in the future by the development of human gametes, using the techniques of haploidization, which is described below.

2.1.4 Development of Artificial Human Gametes In Vitro

Attempts were undertaken for creating both female and male gametes, both demonstrating a strong morphological evidence for haploidization [57, 58]. The technique is based on inducing nuclei of mitotic somatic cells to skip the S-phase of the cell cycle and undergo haploidization when introduced into oocytes, which allows obtaining artificial gametes from somatic cells through the process of haploidization. We showed that the efficiency of haploidization of donor cumulus cell nuclei differs depending on the stage of development of the enucleated recipient oocyte [59, 60]. This may be tested using the extruded PBs, or generated pronuclei, which also allow investigating the correctness of chromosomal segregation. As seen from the flowchart in Fig. 2.11, the first step involves enucleation of in vitro matured MII oocytes under the

control of UV luminescence, which is important to ensure the accuracy of chromosome analysis of the resulting haploid nuclei. Then the cumulus cell nuclei, which are at the G0 of the cell cycle, are introduced into ooplasts by injection and the oocytes are activated by electrostimulation delivered by the electrofusion device (XRONOS, RGI-4 (Chicago, IL)). Following the oocyte activation, the chromosomes of the transferred nuclei segregate with the extrusion of polar bodies, or form two pronuclei both evidencing the formation of artificial gametes through somatic cell haploidization [59, 60]. The FISH analysis and DNA fingerprinting of PB1 and pronuclei resulting from the haploidization procedure showed the haploid chromosomal set, with the resulting DNA originating from the donor nuclei, so the extruded PB1 may be used to investigate the genetic contents of the corresponding pronucleus. However, preliminary data showed that as many as 90% of these haploid nuclei appeared to be with chromosomal aneuploidies. This suggests that the use of the resulting haploid nuclei in the gamete reconstruction procedure may not be acceptable at the present time.

To determine if an incubation time of nuclei in ooplast improves chromosomal segregation, two groups of a total of 122 reconstructed MII oocytes were studied, one activated 5–7 h after the nuclear transfer, and the other after 12–21 h. However, an aneuploidy rate as high as approximately 90% was observed irrespective of incubation time, with the majority being of a complex nature, suggesting no improvement of the accuracy of chromosomal segregation with the prolonged incubation time [59, 60]. So, although haploidization of somatic cells may be achieved using MII oocyte cytoplasm, the aneuploidy rate is much higher than in normal meiosis, which currently makes the techniques not acceptable for clinical practice.

2.1.5 Embryo Biopsy

Embryo biopsy is performed as soon as the embryo reaches a minimum of six cells or more so as not to cause a considerable decrease in cell number at the later stages of development. A mechanical opening of zona pellucida has been developed, called 3D-PZD, allowing the creation

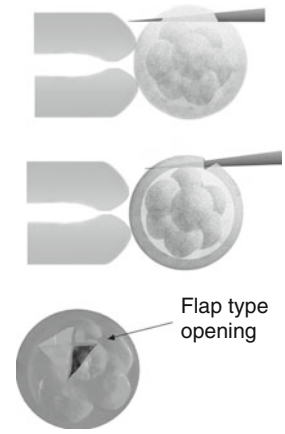

Fig. 2.12 Three-dimensional partial zona dissection (3D-PZD) for embryo biopsy. (*Step 1*) The embryo is held in position by gentle suction from the holding pipette. The embryo is rotated using the microneedle so that a blastomere with a well-visualized nucleus is present at 12 o'clock. The embryo is then rotated horizontally so that the blastomere of choice is facing the operator. The microneedle is passed tangentially under the zona pellucida through the perivitelline space moving from the 1–2 o'clock position to the 10–11 o'clock position. A first slit is created as described in Fig. 2.3. (*Step 2*) The embryo is rotated so that the slit opening is at the 1–2 o'clock position and a second intersecting slit is made by passing the microneedle through the first slit opening and out at the 10–11 o'clock position. The cut is accomplished as with all partial zona dissection previously described in Fig. 2.3. This second intersecting slit creates a larger flap-type opening for the purpose of accessing PB2 as mentioned in Fig. 2.5 or to utilize the required larger micropipette for embryo biopsy

of a V-shaped triangular flap or square flap opening, sufficient in size for a micropipette to pass through in order to remove a blastomere(s) [48]. Micromanipulation dishes are prepared the same way as for PB1 removal, except for sucrose use, which is eliminated. The micromanipulation setup is the same as for PB1 removal with one exception – the micropipette has a larger diameter of 25–30 μm. The embryo biopsy procedure is shown and described in Fig. 2.12.

With the current tendency for blastocyst transfer, there has been a renewed interest in the development of methods for blastocyst biopsy, which has resulted in successful PGD cycles performed by blastocyst biopsy for genetic and chromosomal disorders, yielding unaffected clinical pregnancies and births of healthy children [61]. Blastocyst biopsy may be performed by mechanical methods,

as the trophectoderm is beginning to herniate through the zona pellucida [48]. Several trophectoderm cells are removed at this stage by smooth aspiration with a biopsy pipette with an internal diameter of 30 µm. However, most centers are currently using a microlaser procedure, which is applied to break down the tight junctions between trophectoderm cells, followed by the aspiration of 5–6 trophectoderm cells.

Gradually, blastocyst biopsy is becoming a method of choice in many centers, and has currently a growing potential with the improvement of freezing techniques, and particularly vitrification. It has also special implication with the introduction of microarray technology, which has much more accurate and reliable results when performed on blastocyst biopsy [23, 28, 29]. In addition to using blastocyst biopsy as the method of choice, it is also used as additional testing required for confirming PB or blastomere diagnosis.

The follow-up studies of embryos after blastomere biopsy did not show any detrimental effect. No increase in congenital malformation has been reported among thousands children born following PB or blastomere biopsy, although further systematic study will be needed to monitor the clinical outcomes of PGD using PB1 and PB2 sampling or embryo biopsy and to collect further data on the safety of the procedures used in PGD for single-gene and chromosomal disorders. However, according to the data presented in Fig. 2.13, there seems to be no difference in the developmental potential of embryos following single, double, or triple biopsy procedures, compared to the rate of blastocyst formation following ICSI (control group). As can be seen from Fig. 2.13, no significant differences were observed in blastocyst formation between the embryos with the embryo biopsy procedure of a single blastomere biopsy and the embryos with two biopsy procedures, including simultaneous PB1 and PB2 removal on day 1, followed by the removal of a single blastomere on day 3. There was no decrease, but even an increase in the rate of blastocyst formation when compared to the control group, for the group of embryos in which three biopsy procedures were performed, including PB1 removal prior to ICSI, PB2 removal at the time of fertilization assessment, and a single blastomere removal on day 3. This is in agreement with the pregnancy

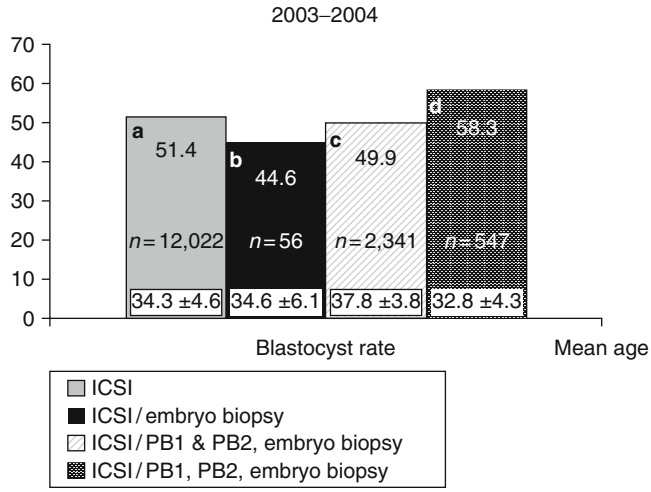

Fig. 2.13 Effect of micromanipulations on embryo development. Bar graph demonstrating nondetrimental effect of biopsy procedures on the rate of blastocyst formation following intracytoplasmic sperm injection (control group). No significant difference was seen between embryos after (1) embryo biopsy procedure of a single blastomere and also embryos in which (2) biopsy procedures were performed in which the first and second polar bodies were removed simultaneously on day 1 (pronuclear stage) followed by the removal of a single blastomere on day 3, when compared to the control group. There was no decrease, but a significant increase in the rate of blastocyst formation when compared to the control group, for the group of embryos in which three biopsy procedures were performed. The first polar body was removed prior to ICSI, the second polar body was removed at the time of fertilization assessment, and a single blastomere was removed on day 3

outcome data, which will be presented in (Chap. 3), showing a 40% pregnancy rate in approximately 1,000 PGD cycles performed for single-gene disorders by sequential PB1 and PB2 analysis, followed by blastomere biopsy.

Therefore, accumulated experience allows concluding that the biopsy procedures involved in PGD do not seem to have significant detrimental effect on embryo viability, although further randomized control studies are needed to obtain more detailed information. At present, preliminary data of available randomized control studies are controversial. In one of them, the effect was so significant that it was probably caused by the lack of sufficient experience in biopsy procedures [62].

2.2 Single-Cell Genetic Analysis

Single-cell genetic analysis includes single-cell DNA and FISH analysis, which have become important tools for the application of PGD in assisted reproduction and genetic services, providing an important option for couples at genetic risk to avoid the birth of an affected offspring and have a healthy child of their own.

2.2.1 DNA Analysis

Because PGD for single-gene disorders is based on single-cell genetic analysis, its accuracy depends largely on the limitations of single-cell DNA analysis, which may potentially cause misdiagnosis. One of the key contributors to misdiagnosis is the phenomenon of preferential amplification, also known as allele-specific amplification failure (allele drop out, ADO), requiring the application of special protocols to ensure the highest ADO detection rate [48, 63, 64]. A few previously reported misdiagnoses, involving PGD for beta-thalassemia, myotonic dystrophy (DM), fragile-X syndrome (XMR1), and cystic fibrosis (CF), might have been due to this phenomenon, which has not initially been fully realized [39–41, 46, 65].

It has been demonstrated that ADO rates in single cells might be different for different types of heterozygous cells [66]. The ADO rate may exceed 20% in blastomeres compared to the ADO rate in single fibroblasts and PB1, which was shown to be under10%. A high rate of ADO in blastomeres may lead to an obvious misdiagnosis, especially in compound heterozygous embryos. As mentioned, most misdiagnoses, especially those at the initial stage of application of PGD for single-gene disorders, were in the cases of blastomere biopsy from apparently compound heterozygous embryos.

The reliability of PGD depends on the controlling ADO. We have previously demonstrated that ADO rates in single-cell PCR may vary with different lysis procedures, cell types, and loci analyzed [48, 63, 67]. Therefore, reliable methods are needed to detect potential ADO, avoiding misdiagnosis in PGD for single-gene disorders. Our experience demonstrated feasibility of detection of ADO by a sequential analysis of oocytes, using PB1 and PB2, and by simultaneous amplification of mutant genes with linked polymorphic markers [48, 64, 67], which is described in brief below.

The biopsied single cells are placed directly into a lysis solution, consisting of 0.5 mcl 10×PCR buffer, 0.5 mcl 1% Tween 20, 0.5mcl 1% Triton×100, 3.5 mcl H2O, and 0.05 mcl Proteinase K (20 mg/ml in a 0.5 ml PCR tube). After spinning down at a low speed in a microfuge for a few seconds, the samples are covered with one drop of mineral oil and incubated at 45°C for 15 min in a thermal cycler. Proteinase K was then inactivated at 96°C for 20 min, which is also the beginning of the hot start of the first-round PCR. Lower stringency and longer annealing time are used in the first-round PCR, with the introduction of the mixture of all outside primers for both mutant genes and polymorphic markers. Following the first-round PCR, separate aliquots are amplified in the second-round PCR with specific inside primers for each site, using a higher stringency. Such a dual or multiple amplification reaction allows detection of most of the ADO cases. If there are pseudogenes, to eliminate false priming, the first-round primers are designed to anneal to the regions of nonidentity with pseudogenes [48]. In addition to short tandem repeats (STR) linked to the genes studied, STRs located on other chromosomes are also studied for testing of a possible contamination by

extraneous DNA, and identification of the origin of individual embryos in the established pregnancies. A list of STRs, their sequences, and PCR reaction conditions for their analysis are presented in each respective section.

Fluorescent PCR (F-PCR) is used for a direct fragment-size analysis of a PCR product [68]. F-PCR is useful also for a direct sequencing of the PCR product in the detection of point mutations and for distinguishing preferential amplification from ADO. A considerable proportion of ADO is detected by sequential analysis of PB1 and PB2, which may be demonstrated by data on sequential PCR analysis of 26 alleles in PB1 and PB2 obtained from 1,047 oocytes, which showed that 32 of 53 of all ADOs in mutation analysis are detectable simply by sequential analysis of PB1 and PB2, avoiding misdiagnosis due to ADO when no informative polymorphic markers are available. As mentioned, a high rate of ADO is observed especially in blastomeres, leading to an obvious misdiagnosis in compound heterozygous embryos, which was the case at the initial stage of application of PGD for single-gene disorders based on blastomere biopsy obtained from apparently compound heterozygous embryos [39, 46]. This is now avoided by testing two or more linked polymorphic markers if available, making DNA testing in PB or single blastomeres a highly reliable procedure. Contrary to expectation, the application of F-PCR does not sufficiently improve detection of potential misdiagnoses in PGD of single-gene disorders. Testing of 148 single fibroblasts by both conventional and F-PCR provided minor contribution to ADO detection rates. Based on this observation, it was postulated that simultaneous amplification of single cells for any causative gene, together with one linked polymorphic marker, reduces the ADO rate by more than half, irrespective of the use of conventional or F-PCR. With the additional second marker in multiplex PCR, the ADO rate may further be reduced by half, being completely absent if three or more linked markers are simultaneously amplified, as shown above.

We previously reported the results of 114 PGD cycles for couples at high risk for having children with single-gene disorders, resulting in preselection and transfer of a sufficient number of mutation-free oocytes in almost all cycles. Of 1,047 oocytes with DNA results, 672 (64.1%) had heterozygous PB1, that is, with both normal and mutant genes amplified, which is therefore ideal for further testing, although their potential transfer depended entirely on the identification of mutant genes in the sequential analysis of PB2. Thus, priority in preselection of embryos for transfer was given to the embryos resulting from the oocytes with heterozygous PB1 because in the absence of DNA contamination this indicates the absence of ADO of either the normal or mutant allele. Although most of the transferred embryos were preselected using this particular strategy, some preselected embryos still originated from homozygous normal oocytes, inferred from the homozygous mutant status of PB1 and hemizygous normal status of PB2. These embryos were accepted for transfer only if ADO could have been excluded using linked polymorphic marker analysis. Otherwise, such embryos were excluded from transfer and exposed to follow-up confirmation analysis of the resulting embryos.

The follow-up analysis of the embryos excluded from transfer either because they were affected or because there was insufficient information to preselect them for transfer provided the data for evaluating the proportion of undetected ADO. Overall, 82 (7.8%) ADOs were observed, which included 75 detected and 7 undetected ones, suggesting that not 970 but actually 1,052 oocytes were heterozygous. The genotype of six embryos appeared to be different from that predicted by mutation analysis, and one by STR, due to ADO in PB1, which were diagnosed as homozygous instead of their actual heterozygous status. The data indicated to 98% accuracy, which is quite acceptable for clinical use of PGD for single-gene disorders.

As can be seen from these data, to avoid a misdiagnosis due to preferential amplification, a simultaneous detection of the mutant gene together with up to three or more highly polymorphic markers, closely linked to the gene tested, may be required [48, 63]. Each additional linked marker may reduce the misdiagnosis rate by half, so with one linked marker amplified together with mutation, a misdiagnosis risk in blastomere analysis may be reduced from 20% to 10%, with two

from 10% to 5%, and with three from 5% to practically zero. So a *multiplex nested PCR* analysis is performed, with the initial PCR reaction containing all the pairs of outside primers, so that following the first-round PCR, separate aliquots of the resulting PCR product may be amplified using the inside primers specific for each site. Only when the polymorphic sites and the mutation agree are embryos transferred. So multiplex amplification allows detecting ADO and prevents the transfer of misdiagnosed affected embryos.

Our data show that one of the most efficient approaches for avoiding misdiagnosis is a sequential genetic analysis of the PB1 and PB2 in PGD for maternally derived mutations. Detection of both mutant and normal alleles in the heterozygous PB1, together with the mutant allele in the corresponding PB2, leaves no doubt that the resulting maternal contribution to the embryo is normal, even without testing for the linked markers as a control. However, it will be ideal to test simultaneously at least for one linked marker to confirm the diagnosis. Alternatively, the mutation-free oocytes is also predicted when the corresponding PB1 is a homozygous mutant, in which scenario the corresponding PB2 should be a hemizygous normal, similar to the resulting maternal pronucleus. However, the genotype of the resulting maternal contribution may be quite opposite, that is, mutant, if the corresponding PB1 is in fact heterozygous, but erroneously diagnosed as homozygous normal because of ADO of the normal allele. In the above scenario, the extrusion of the normal allele with PB2 would lead to the mutant allele left in the resulting oocyte. Therefore, the embryos resulting from the oocytes with homozygous mutant PB1 cannot be acceptable for transfer, unless the heterozygous status of PB1 is excluded by the use of linked markers as described. The example of misdiagnosis, due to ADO of the normal allele in PB1, has been described earlier in a PGD cycle performed for FMR1 [65]. To completely avoid misdiagnosis, a sequential PB1 and PB2 may be required to combine with multiplex PCR to exclude the possibility of an undetected ADO in heterozygous PB1. As described above, the analysis of more than 1,000 oocytes tested by sequential PB1 and PB2 analysis showed that more than half of the ADOs were detected by sequential analysis of PB1 and PB2, with the remaining cases detected by multiplex PCR. The accuracy of this approach may be demonstrated by the reports of PGD for thalassemia and familial dysautonomia (FD) (see below), resulting in the transfer of three unaffected embryos in each case, which were confirmed by the birth of two sets of triplets free from thalassemia and FD [69, 70].

The other method with the proved potential for detecting and avoiding misdiagnosis due to preferential amplification is fluorescence PCR (F-PCR), which may allow detection of some of the heterozygous PB1 or blastomeres misdiagnosed as homozygous in conventional PCR, and therefore having the potential of reducing the ADO rates at least to some extent [64]. In addition, the method also allows a simultaneous gender determination, DNA fingerprinting, and detection of common aneuploidies. F-PCR combined with a multiplex system and sequential PB1 and PB2 analysis in cases of maternally derived mutations allows excluding almost completely the risk for misdiagnosis due to preferential amplification.

The accuracy of PGD has been further improved with the application of fluorescent PCR with the expand long template (ELT) kit, which enabled reducing the ADO rate from as high as 30–35% in both conventional and fluorescent PCR to as low as 5% in testing for DM [71]. Another development in improving the accuracy of single-cell PCR analysis involves the application of real-time PCR, which was found to reduce the ADO rate almost by half, compared to conventional or fluorescent PCR (Fig. 2.14). The application of these approaches together with simultaneous testing for the causative mutation along with at least one or two linked markers allows avoiding reliably the risk for misdiagnosis.

Finally, because of the high rate of mosaicism at the cleavage stage, testing for the chromosome, in which the gene in question is mapped, is of an obvious value, to exclude the lack of mutant allele due to monosomy of this chromosome in the biopsied blastomere. As mentioned, aneuploidy testing is technically feasible and is done by adding primers for chromosome-specific microsatellite

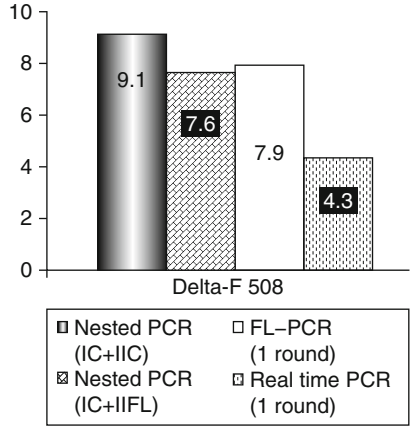

Fig. 2.14 Reduction of allele dropout (ADO) rates by real time PCR. ADO rates are shown for nested conventional PCR (*left bar*), nested combined PCR (first-round conventional, second-round fluorescent: *middle–left bar*), one-round fluorescent PCR (*middle right bar*), and one-round real-time PCR (*right bar*) with single human fibroblasts heterozygous for deltaF508 mutation in the CFTR gene. Comparable ADO rates are seen in the first three types of PCR, while application of one round of real-time PCR reduces ADO in single fibroblasts almost by half, demonstrating that real-time PCR is the most sensitive for detection of ADO, although it cannot completely prevent misdiagnosis

markers to the multiplex PCR protocols worked out for specific genetic disorder [72]. The development of multiplex nested PCR systems also allows performing PGD for different conditions simultaneously, as attempted with PGD for CFTR mutation together with XMR1 or gender determination [73, 74], and will be also described in Sect. 4. The PCR-based strategy for aneuploidy testing is shown in Fig. 2.15, listing polymorphic markers used for identification of the copy number of chromosomes 13, 15, 16, 17, 18, 21, 22, and X. Y. However, this may currently be performed by microarray analysis in the same whole-genome amplification product, combining testing for mutations and polymorphic markers with 24 chromosome aneuploidy testing [18–29].

Due to the need for the development of a custom-made PGD design for each mutation and each couple, preparatory work has become an integral part of PGD for single-gene disorders to ensure avoiding potential misdiagnosis. For example, in some cases, a particular set of outside primers has to be designed to eliminate false priming to the pseudogene, as described in PGD for long-chain 3-hydroxyacyl-Coa dehydrogenase deficiency [75]. Also, the preparatory work may frequently involve a single sperm typing needed for establishing paternal haplotypes, so that linked marker analysis could be performed in addition to mutation testing, especially in cases of paternally derived dominant conditions or PGD combined with preimplantation HLA matching (see below). The use of haplotyping for PGD without direct mutation testing is presently used as a more universal procedure, called preimplantation genetic haplotyping (PGH) [76]. This also improves the accuracy of PGD, as the availability of the parental haplotypes, irrespective of whether the mother or the father is a carrier, allows not only confirming the absence of the mutant gene but also the presence of both maternal and paternal wild alleles in PGD by blastomere analysis, especially when only one informative marker is available (Chap. 3).

2.2.2 FISH Analysis

PGD for the age-related aneuploidies is currently done by FISH analysis, using commercially available chromosome-specific probes (Vysis, Downers Groves, IL, USA). It was first applied in 1991 for gender determination using DNA probes specific either for the X or Y chromosome [8]. Since testing for only one of the sex chromosomes could lead to misdiagnosis of gender due to a possible failure of hybridization, a dual FISH was introduced, involving the simultaneous detection of X and Y, each in a different color [9]. Further, the dual FISH analysis was combined with a ploidy assessment to improve the accuracy, by adding a centromeric probe specific for chromosome-18 [10, 11]. Testing was then extended up to five autosomes, including chromosomes 13, 16, 21, and 22 [48, 77, 78], although it is currently possible to analyze up to a dozen chromosomes, using additional rounds of re-hybridization.

As mentioned, the overall experience of preimplantation FISH analysis currently involves more than 50,000 clinical cycles, resulting in an improved pregnancy rate in poor-prognosis IVF patients [2, 3, 79–82], despite the present

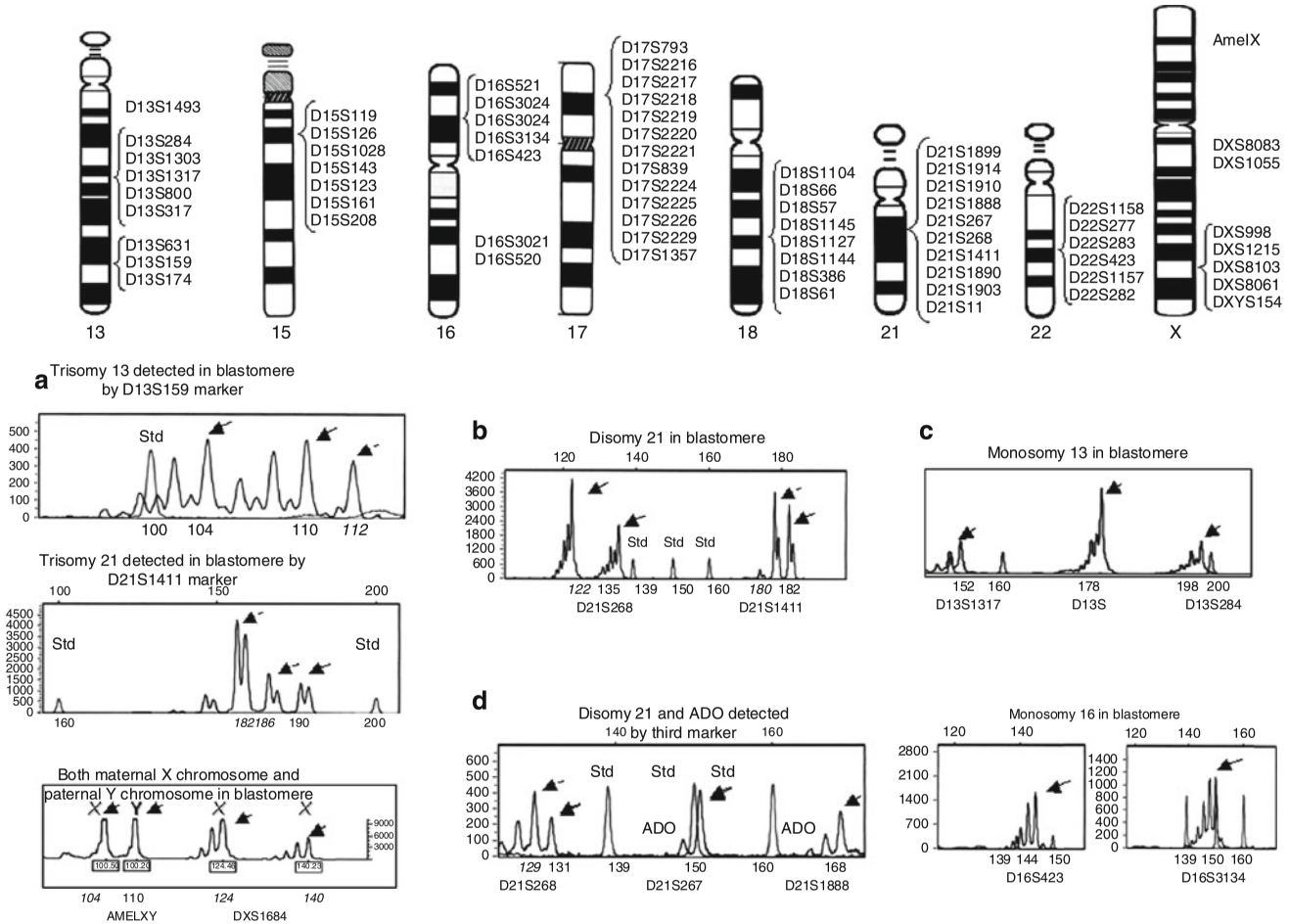

Fig. 2.15 PCR-based aneuploidy testing. (*Top*) Positions of polymorphic markers applied for the PCR-based aneuploidy testing for selected chromosomes. To differentiate between allele dropout (ADO) and the absence of the whole chromosome (monosomy), at least three informative polymorphic markers for each chromosome are tested. (*Bottom*) Examples of single-blastomere PCR results with (**a**) trisomy13, 21, and XXY; (**b**, **d**) normal for chromosome 21; (**c**) monosomy 13 and 16; and (**d**) ADO, distinguished from monosomy 21 by the use of three linked markers

controversy on this issue. The majority of these cycles were performed by FISH analysis of blastomeres, while approximately one-fifth was done by FISH analysis of PB1 and PB2, resulting in thousands of unaffected pregnancies and healthy children born at the present time. The follow-up confirmation studies of the preselected abnormal embryos, and the babies born following the procedure, demonstrated an acceptable accuracy of the FISH analysis, which is described below.

The reliability of the FISH technique for aneuploidy detection in blastomeres has been extensively studied [1, 13, 31, 35, 83, 84]. By comparing the FISH results in the cleaving embryos to morphological abnormalities and maternal age, it was established that the observed chromosomal abnormalities were indeed not related to the limitations of the FISH technique, but were due to the embryo variables [13, 83]. However, a high rate of mosaicism was observed at the cleavage stage [12, 13, 83, 84], which was particularly high in slow embryos, exhibiting an arrested development. Initially, an overall 12% mosaicism rate was suggested in cleaving embryos [15], but it has now been shown to occur in as many as half of preimplantation embryos, representing one of the major limitations of the FISH analysis for aneuploidies, performed at this stage [1, 35]. Clearly mosaicism will affect the accuracy of the diagnosis, except for those cases when PGD detects the abnormal cell from a mosaic embryo, which will not be transferred. It was also shown that mosaicism may present diagnostic problems at the blastocyst stage [85], despite the initial prediction that the abnormal cells are deviated mainly to trophectoderm.

The first attempt to use FISH analysis for testing PB1 was undertaken in 1994. In this work, 130 unfertilized MII oocytes were tested simultaneously with their PB1, using X-chromosome and chromosome 18 specific probes. It was demonstrated that PB1 FISH data allow an exact prediction of the chromosome set in the corresponding oocytes [86, 87]. Each chromosome in PB1 was represented by double dots (signals), corresponding to two chromatids in each univalent (Fig. 2.16). The data suggested that the number of signals (chromatids) in PB1 reliably predicts the corresponding number of signals (chromatids) in the MII oocytes, therefore, providing an excellent tool for the genetic preselection of oocytes. It was also of interest that, in addition to a normal distribution of signals in PB1 and the corresponding MII oocytes, meiotic

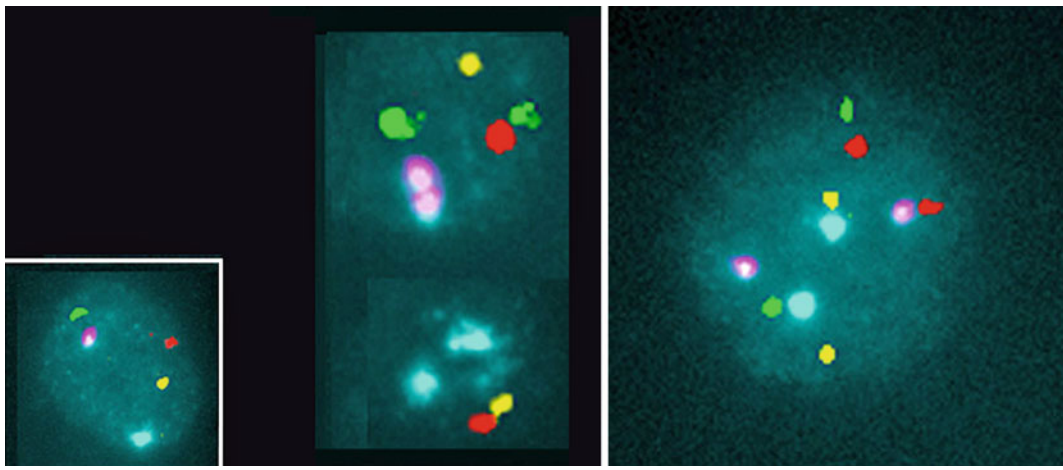

Fig. 2.16 Normal pattern of FISH images of PB1 and PB2 and blastomeres after a 3-h hybridization with MultiVysion PB panel probe for autosomes 13 (*red*), 16 (*aqua*), 18 (*violet blue*), 21 (*green*), and 22 (*gold*). PB1 and PB2 were removed simultaneously on day 1 at the pronuclear stage of development following fertilization assessment. (*Middle panel*) PB1 chromosomes showing a normal number of single dot signals (two per chromosome, representing each chromatid). (*Lower left*) A normal number of signals (one per chromosome (chromatid)) are present in the PB2 interphase nucleus inferring a normal chromosome complement in the oocyte and resulting embryo. (*Right panel*) Normal FISH images of interphase nuclei isolated from blastomeres, resulting from the oocytes shown on the left, after embryo biopsy on day 3 of embryo development (two signals for each of the autosomes tested)

errors were also detected, confirming the accuracy of PB1 diagnosis for predicting the genotype of the corresponding oocyte. For example, in one PB1 four signals for chromosome 18 were detected, perfectly in accordance with the lack of the chromosome 18 signals in the corresponding MII oocyte (chromosome 18 non-disjunction). This suggested that the chromosomal complements of the oocyte could be inferred from the testing of PB1, which can be removed following its extrusion from the mature oocyte, with no potential influence on the embryo viability. Another interesting phenomenon was the observation of chromatid malsegregation as a possible cause of chromosomal aneuploidy in the resulting mature oocytes. In four oocytes, instead of the expected two signals, three were found in the MII oocytes, which perfectly corresponded to a single signal in the corresponding PB1. Similar results were reported by another group, confirming diagnostic significance of the PB1 FISH analysis for predicting the genotype of the preimplantation embryo [88].

PB1 testing was one of the first approaches used for PGD of translocations (16), based on the fact that PB1 never forms an interphase nucleus and consists of metaphase chromosomes. It is known that PB1 chromosomes are recognizable when isolated 2–3 h after *in vitro* culture, with degeneration beginning 6–7 h after extrusion. Therefore, whole-chromosome painting or the chromosome segment-specific probes could be applied for testing of maternally derived chromosomal translocations in PB1. Although the method resulted in a significant reduction of spontaneous abortions in the patients carrying translocations, yielding unaffected pregnancies and births of healthy children, it has shown to be sensitive to malsegregation and/or recombination between chromatids, requiring a further follow-up analysis of PB2, in order to accurately predict the meiotic outcome following the second meiotic division (Chap. 5).

In contrast to PB1, PB2 is the by-product of the second meiotic division, extruded following fertilization of an oocyte. The need for FISH analysis of PB2 for PGD of aneuploidies was first proposed in 1995, when it was demonstrated that PB1 testing alone does not allow predicting the resulting genotype of the oocyte [89]. It was shown that in contrast to the paired dots in PB1, each chromosome in PB2 was represented by a single dot (see normal pattern of FISH signals in Fig. 2.16), so the lack of or addition of a signal for a particular chromosome provided evidence of a second meiotic division error. Although only 19 of 55 oocytes in this first study were tested by both PB1 and PB2, evidence for errors was observed not only in meiosis I, detected by PB1 analysis, but also in meiosis II, which may be detected by PB2 testing. These data suggested that some oocytes selected as normal, based on the PB1 FISH analysis, still could have been abnormal following nondisjunction in the second meiotic division. Therefore, FISH analysis for both PB1 and PB2 has become the basic requirement for PGD of aneuploidies, which allows detecting errors in both the first and second meiotic divisions. Currently, more than 25,000 oocytes have been analyzed by FISH analysis, showing the accuracy and reliability of PB1 and PB2 testing for predicting the karyotype of the embryo, resulting from the corresponding oocyte [24]. As will be described below, more than 50% of oocytes from IVF patients of advanced reproductive age are abnormal, resulting from the errors in both the first and second meiotic divisions, in contrast to the previously believed concept that aneuploidies mainly originate from meiosis I.

As mentioned, PB2 testing is also an important component of PGD for maternally derived translocations. However, this is still done on interphase as, despite the progress in transforming PB2 into metaphase chromosomes via electrofusion of PB2 nucleus with foreign one-cell human embryo, the proportion of metaphase plates obtained was not sufficient to be useful in clinical practice [90]. Much higher efficiency was observed in conversion of interphase nuclei of blastomeres, which is described below.

Visualization of chromosomes of individual blastomere nuclei requires the application of nuclear transfer technique, which was initially attempted for the conversion of interphase nuclei of PB2 into metaphase [91]. The original design was to fuse individual blastomeres with enucleated human oocytes. Although metaphases were obtained from two-thirds of blastomeres treated by this method, its efficiency was not high enough to be applicable to PGD. This was due to the inability

of a replicating nucleus to form metaphase chromosomes after the induction of premature chromosome condensation (PCC). However, because biopsied blastomeres may be at any stage of the cell cycle at the time of biopsy, there was a need to control the timing of mitosis of blastomere nuclei, which can be achieved by the introduction of a blastomere into the cytoplasm of a cell at a known cell cycle [92]. To achieve such reprogramming, the individual blastomeres were fused with intact or enucleated mouse zygotes at pronuclear stage, known to be at the S-phase of the cell cycle.

So the method involves the use of frozen mouse zygotes, which can be purchased from Charles River Laboratories (Wilmington, MA) as recipient cytoplast to induce the conversion of blastomere nucleus into metaphase. Also there is no need for enucleation of mouse oocytes, as mouse and human chromosomes may be clearly distinguished. Blastomere biopsy is performed in the same way as described above, except for choosing only intact blastomeres with clearly visible nuclei. Several precautions have also to be taken to ensure the integrity of the blastomere plasma membrane during biopsy procedure. Although intact blastomeres may be inserted microsurgically into perivitelline space, this appeared to be traumatic and was replaced by blastomere–zygote agglutination with phytohemagglutinin (Irvine Scientific, Santa Ana, CA). Before the procedure, the thawed mouse zygotes are freed of zonae pellucidae with acidic Tyrode's solution and pipetted through the flame-polished Pasteur pipettes with an internal diameter of 80 μm. Then, using the flame-polished Pasteur pipettes with an internal diameter of 100 μm, blastomere–zygote pairs are brought together and agglutinated in 300 μg/ml of phytogemagglutinin in protein-free human tubal fluid buffered with 20 mM of HEPES in a four-well plastic dish (Nunc).

Electrofusion is induced in the same way as mentioned in the above section, except for substitution of 0.5% polyvinylpirrolidone in the electrofusion medium by 0.1% with molecular weight 360.000 (kd). Blastomere–zygote pairs are oriented between electrodes by hand, with the final orientation achieved with alternating current (500 kHz; 0.2 kV/cm for 2 s). Cell fusion is induced with a single direct current pulse (1 kV/cm for 500 s), and the results are assessed in 20 min.

When human blastomeres are fused with intact mouse zygotes, the heterokaryons entering mitosis are identified under a dissecting microscope. Because of the transparency of mouse cytoplasm, the disappearance of pronuclei and the formation of the joint metaphase plate are clearly visible. The heterokaryons with persisting pronuclei are exposed for 1 h to 5 μM of OA in phosphate-buffered saline containing 3 mg/nL of bovine serum albumin and 0.5 μg/nL of cytochalasin D. After 10–15 min incubation in a hypotonic solution (0.1% sodium-citrate and 0.6% bovine serum albumin), the resulting mitotic heterokaryons are fixed in a cold 3:1 solution of methanol and acetic acid in a four-well plastic dish. When the cytoplasm clears, heterokaryons are transferred onto slides and air-dried. Chromosome plates are assessed by phase contrast and then used for standard chromosome analysis. For FISH analysis the slides are pretreated with formaldehyde and pepsin (Abbott Inc., Downers Grove, IL).

Overall success rate of the procedure is as high as 83%, with its efficiency improved with experience [36, 48]. Similar results were obtained by using bovine ooplasts for fusion with human blastomeres [92]. The data showed that some of the failures are simply due to the absence of the nucleus in biopsied blastomeres, or because the heterokaryons were fixed after they had already cleaved. It is also useful to perform blastomere biopsy not earlier than day 3 or day 4, to avoid biopsy of 2- and 4-cell embryos, leading to the accelerated heterokaryon cleavage. However, the success rate did not depend at all on whether mouse zygotes were enucleated before fusion with blastomeres. This allows simplifying the procedure by using intact mouse zygotes.

So, the procedure is quite simple and includes the following components. Mouse zygotes are thawed free of zonae pellucidae and PB2 1–2 h before electrofusion with human blastomeres. Four hours after fusion, heterokaryons are monitored for signs of the disappearance of pronuclei, and fixed at mitosis following hypotonic treatment. To avoid monitoring and a possible miss of mitosis the heterokaryons may be cultured in the presence of microtubuli inhibitors, vinblastine, or

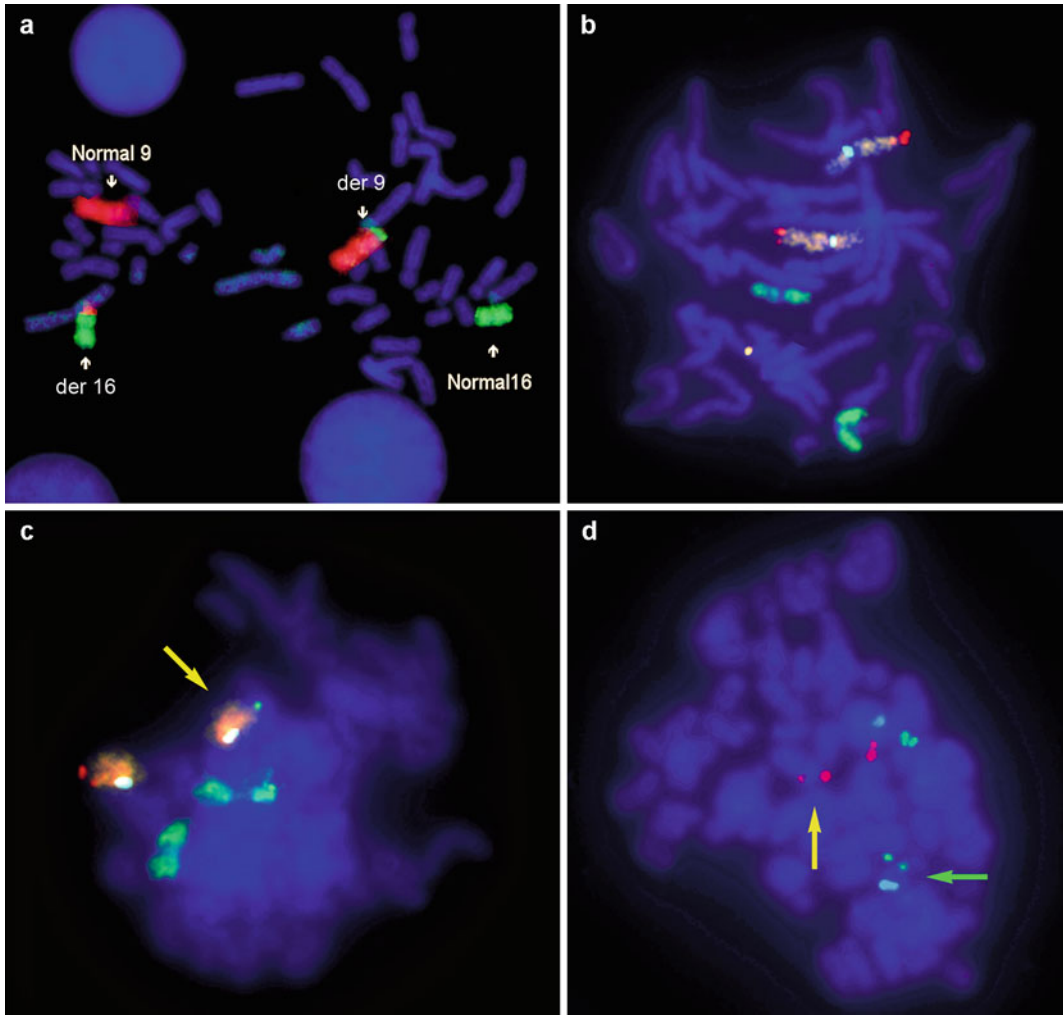

Fig. 2.17 Blastomere nuclear conversion to metaphase for PGD of a maternally derived reciprocal translocation [46,XX, t(9;16)(q34.3;p13.1)]. (**a**) FISH analysis of metaphase chromosomes of a peripheral blood lymphocyte from the carrier. Chromosome 9 is identified with WCP in orange (seen through a red single bandpass filter) in conjunction with Tel 9q in *red*. Chromosome 16 is identified with WCP in *green* in conjunction with Tel 16p in *green*. Sub-telomeric probes were added to this probe cocktail since the translocated portion of each chromosome is small. Derivatives are seen with both *red* and *green* fluorescence. (**b**) WCP in *orange* for chromosome 9 in conjunction with CEP 9 in *aqua* and Tel 9q in *red* along with WCP 16 (*green*) in conjunction with Tel 16p (*green*) on karyotypically normal, peripheral blood. (**c**) Analysis by embryo biopsy and blastomere nucleus conversion to metaphase chromosomes. Unbalanced chromosome complement [9,der (9),16,16] in which derivative (9) is present, evident by the presence of *red* and *green* fluorescence from Tel 16p (*yellow arrow*). This embryo was omitted from transfer. (**d**) Analysis by embryo biopsy and blastomere nucleus conversion to metaphase chromosomes. FISH analysis utilizing locus-specific and sub-telomeric probes revealed a balanced chromosome complement by the presence of both derivatives. Derivative 9 is distinguished from the normal chromosome 9 by the presence of a CEP signal in *aqua* in conjunction with a Tel 16p signal in *green* (*green arrow*) and derivative 16 is distinguished from normal chromosome 16 by a Tel 9 q signal (only) in *red* with the absence of a CEP 9 *aqua* signal (*yellow arrow*). This corresponding embryo was suitable for transfer barring any developmental problems

podophyllotoxin. All the embryos, left in the culture by the ninth hour after fusion, are fixed following 1 h pretreatment with OA. The example of blastomere nucleus conversion applied for PGD of reciprocal translocation 46,XX, t(9;16)(q34.3;p13.1) is shown in Fig. 2.17.

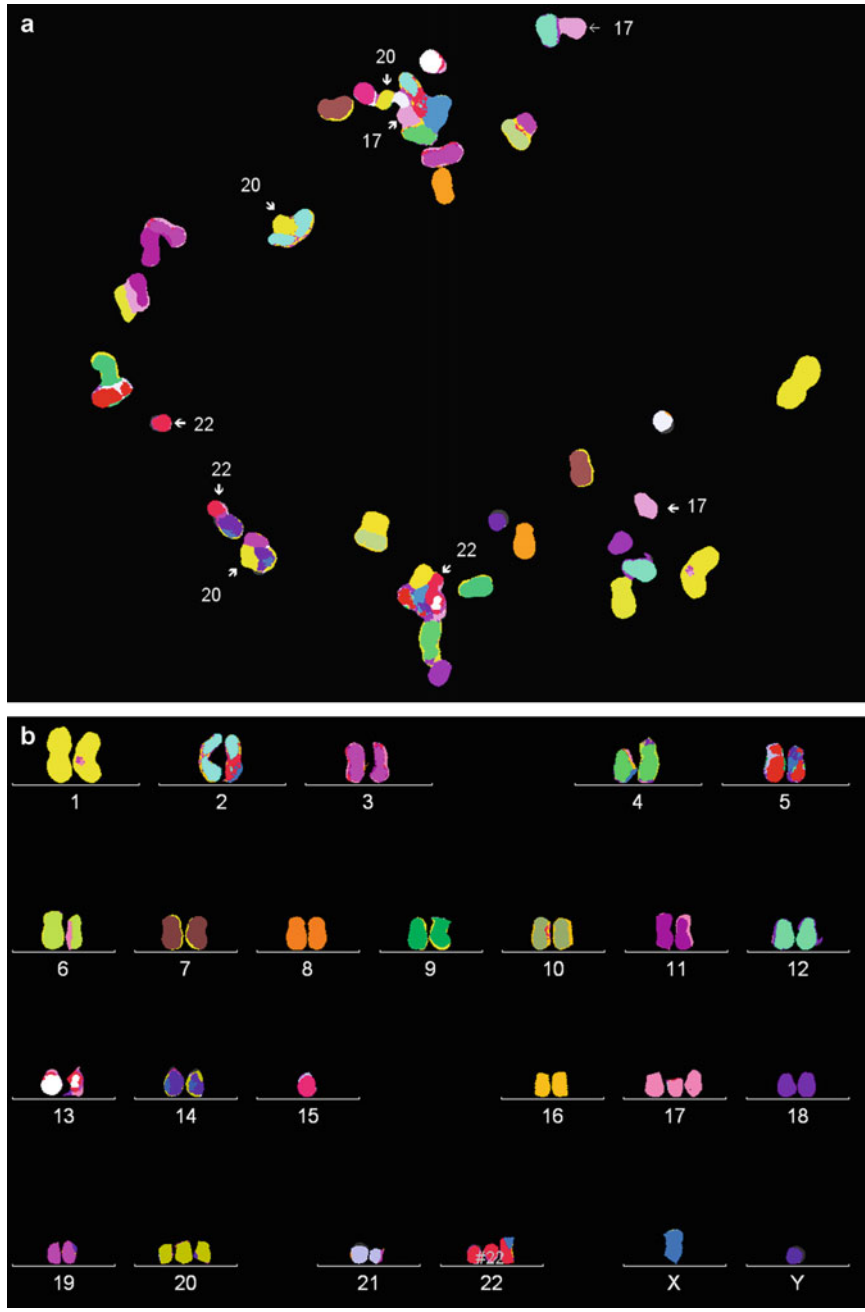

Fig. 2.18 Karyotype of blastomere obtained without conversion technique. Embryo follow-up analysis after polar body analysis revealed second meiotic division error for chromosome 22 indicating a trisomy 22 embryo. (**a**) Testing was performed by embryo biopsy and "nonconversion" techniques used to obtain metaphase chromosomes from a single blastomere nucleus. Afterward, FISH was performed on the metaphase spread utilizing Metasystems' XCyte mFISH probe, which uses five fluorescent dyes to detect different painting probes at the same time, allowing for identification of all 24 different chromosomes with a single hybridization. Each chromosome is distinguished by a separate or combination of different fluorophores that are separated by appropriate filter sets. Based on the specific fluorochrome combination the chromosomes are given a pseudo-color according to the 24X Cyte labeling scheme. This allows for the analysis of complex numerical aberrations even when there is slight chromosome overlap present as seen in this metaphase spread. (**b**) Karyotype established by utilization of the Isis program revealing complex numerical aberrations consisting of trisomies 17, 20, and 22 and monosomy15

The method has been applied for PGD of paternally derived reciprocal translocations and for confirmation of PGD of chromosomal abnormalities performed by PB1 and PB2 FISH analysis. With the current success rate of blastomere nucleus conversion into metaphase it was applied for clinical cases, which will be described in Sect. 5.

It has recently been reported that the blastomere metaphase can be also obtained without the application of a specific conversion method [93]. To obtain analyzable chromosomes, the embryos were monitored closely the second day after ICSI, to identify the blastomere with nuclear breakdown, which was biopsied and fixed within 1 h. This method was currently modified by 1-h culture of the biopsied blastomere in medium with vinblastine, which resulted in harvest of metaphase chromosomes of good quality (Fig. 2.18).

To get more reproducible results this method was further improved by using chemical agents, which involves morphological selection for biopsy of the largest blastomere with 1–2 large nucleoli within the cell nucleus. Upon embryo biopsy, each blastomere is contained in microdrops of Global culture medium (LifeGlobal, USA) supplemented with Plasmanate (Bayer Biological, USA) 10% vol:vol., containing caffeine (Sigma) (1 mmol/l) and a low dose of colcemid (Sigma) (<0.1 μg/ml) under mineral oil [33, 94]. Blastomeres are incubated at 37°C in an atmosphere of 6% CO_2 and air for approximately 2–3 h or until nuclear membrane breakdown is observed. Afterward, the blastomeres are treated in a hypotonic solution followed by fixation using a cold solution of methanol and glacial acetic acid, 3:1. Careful attention is paid so as not to overspread the chromosomes in order to avoid chromosome loss during fixation. Consequently, not all metaphases are suitable for cytogenetic investigation by G-banding; however, they are suitable for FISH analysis to identify structural rearrangements (Fig. 2.19).

2.2.3 Microarray Analysis

A major recent breakthrough has been in the development and application of microarray technology for PGD of chromosomal disorders, allowing for a highly improved detection of chromosomally abnormal oocytes and embryos [18–29]. Although there are different platforms for 24-chromosome testing, the most adequate for the purpose of PGD at this time is 24sure technology, developed by BlueGnome Ltd, Cambridge, UK, because it can be applied to all the biopsy materials, including PB1, PB2, blastomeres, and blastocyst, allowing completion of the test within 12 h, and providing accurate results in over 90% samples. The protocol consists of at least 6 steps, including amplification (2 h), labeling (2.5 h), hybridization (3.5 h), washing (30 min), scanning (30 min), and data analysis (1 h). The technique tests for all 24 chromosomes for any gain or loss with the bacterial artificial chromosome (BAC) pooling strategy, which, coupled with the uniquely designed software, enables obtaining straightforward results on aneuploidy in a single cell. Currently, two 24sure array formats are used for two applications. BACs spotted on the 24sure array are selected on the basis of having little variations in over 5,000 hybridizations, to deliver the highest level of reproducibility and sensitivity in aneuploidy testing.

To apply the technique for additional analysis for chromosomal rearrangements, 24sure + array format is used, with greater coverage of genome, including subtelomeric and pericentromeric regions, enabling accurate characterization of arm-level aneuploidy and other large-scale structural abnormalities. Both the formats are used with Sure Ref reference DNA, providing a hybridization reference, well matched for quality, to an amplified single cell. Also a sex-mismatched design is used to provide useful reference in interpreting the results, such as the use of male reference in the hybridization, mismatching with X chromosome in the analysis of polar bodies.

One of the critical steps of the procedure is whole-genome amplification with the Super Plex Single-Cell Whole-Genome Amplification Kit (BlueGnome Ltd), which is performed according to the manufacturer's instructions. Specific quality control criteria for sample quality and quantity are used to ensure that only specific amplifications are labeled. The fluorescent labeling system

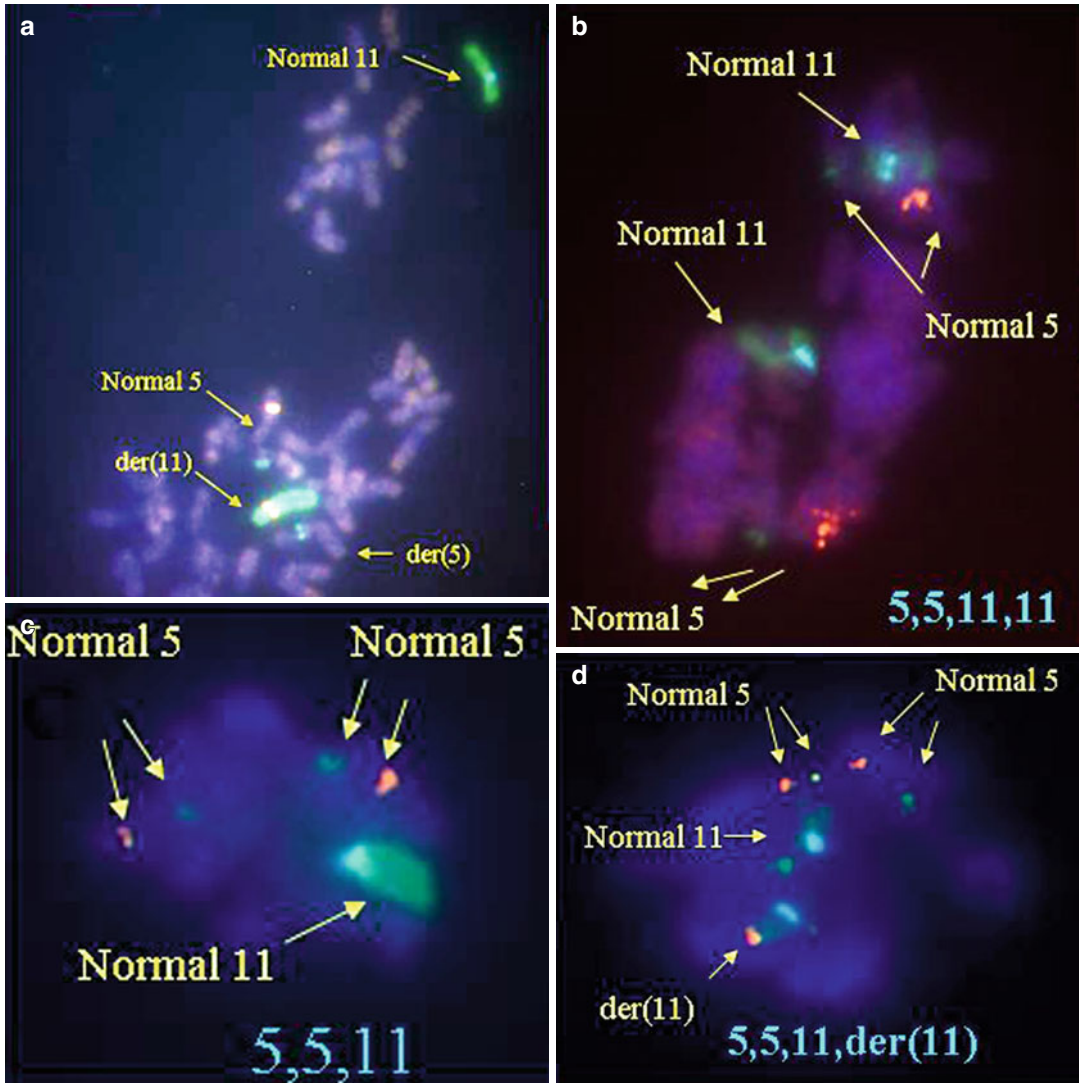

Fig. 2.19 Preimplantation diagnosis for maternally derived rearrangement 46,XX,ins(11;5)(q22.2;q31.1q34) by chemical conversion distinguishing normal from unbalanced embryos. Fluorescent in situ hybridization (FISH) was performed for chromosome 11 using whole chromosome paint 11 (WCP 11) in *green* and the centromeric enumeration probe for chromosome 11 (CEP 11) in *aqua*. Chromosome 5 is identified by a dual probe which targets a region of the p arm LSI D5S23, D5S721 (5p15.2) in *green* and the q arm LSI EGR1 (5q31) in *orange*. The remaining chromosomes are seen in *blue* after the application of DAPI counterstain. (**a**) FISH image of a metaphase from a peripheral blood lymphocyte from the female carrier. Normal chromosome 11 is seen with WCP 11 in *green* and CEP 11 in *aqua*. Derivative 11 is identified by a combination of WCP 11 *green*, CEP 11 *aqua*, and 5q31 in *orange* identifying the inserted segment of chromosome 5 into derivative 11. Normal chromosome 5 is identified by the presence of both 5p15.2 in *green* and 5q31 in *orange*. Derivative 5 is identified by 5p15.2 in *green only* since it lacks the 5q31 region, which has been inserted into the q arm of chromosome 11. (**b**) FISH image of condensed chromosomes isolated from a blastomere from a normal 8-cell embryo showing two normal chromosomes 5 and 11. (**c**) FISH image of condensed chromosomes in close proximity to one another indicating an unbalanced chromosome complement. Two normal chromosomes 5 (*top*) and one normal chromosome 11 (*middle*), and a derivative chromosome 11 (*bottom*) are present. (**d**) FISH image of condensed chromosomes from an unbalanced 8-cell embryo, which shows two normal chromosomes 5 (*top left* and *right*) and one normal chromosome 11 (*lower right*)

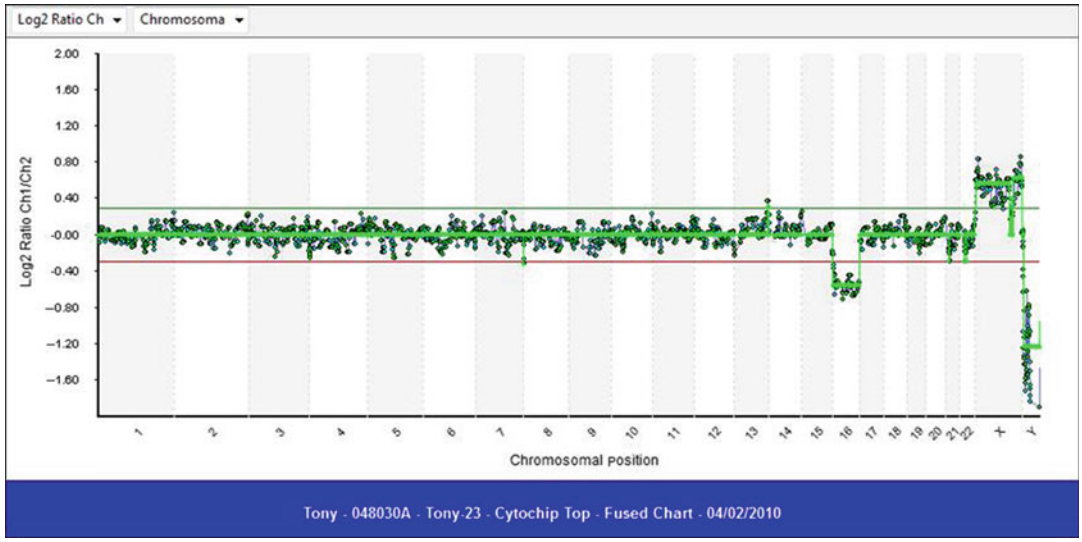

Fig. 2.20 Results from a PB1 carrying a loss of chromatid 16, obtained by array-CGH analysis

(BlueGnome Ltd) is used for the labeling of the amplified samples of biopsy materials, as well as labeling of a commercially available reference DNA. Test and reference DNA co-precipitation, their denaturation, array hybridization, and the post-hybridization washes are done according to protocol provided by the manufacturer.

A laser scanner is used to excite the hybridized fluorophores, and to read and store the resulting images of hybridization, using the special software provided by Bluegnome. Chromosome profiles are examined for gain or loss, or for structural rearrangement. Figure 2.20 shows the results of PB1 analysis, demonstrating trisomy 16.

Preliminary data on array-CGH (24sure by BlueGnome) testing of the first and second polar bodies, and the resulting oocytes, were consistent with FISH results, suggesting that array-CGH may be the method of choice for PGD of aneuploidies [24–27]. An ESHRE Task Force pilot study demonstrated an accurate identification of chromosomal status in 89% of oocytes tested, based on microarray analysis of PB1 and PB2 [26, 27]. When combined with embryo biopsy to detect paternally derived aneuploidies and those originating from mitotic errors, this could provide an improved detection and avoidance of aneuploid embryos from transfer. However, as evidenced from inconsistencies between the predicted and observed types of errors in cleaving embryos [24] and by the high prevalence of mosaicism at this stage, blastocyst biopsy may appear a better choice, taking also into consideration the recent reports on a higher accuracy and improved clinical outcome following the application of 24-chromosome testing coupled with blastocyst biopsy [23, 28, 29].

This approach has opened the possibility of combining aneuploidy testing for 24 chromosomes, with PGD for single-gene disorders, which was first performed for G_{M1} gangliosidosis, which is an autosomal-recessive lysosomal storage disorder [95]. Of 10 embryos tested by SNP Microarray on blastocyst biopsy, 7 were euploid and 3 aneuploid, while 2 embryos were determined as affected with G_{M1} gangliosidosis, 5 embryos were mutation carriers, and 3 were normal. By combining the results of these tests, 5 embryos were found to be suitable for transfer. This novel approach was then extended to a variety of genetic conditions, and also applied together with preimplantation HLA typing, to investigate feasibility of a combined test for 24-chromosome aneuploidy, with PGD for single-gene disorders and preimplantation HLA typing. The list included gangliosidosis GM1,

isolated hypertrophic cardiomyopathy, cystic fibrosis, Fragile –X, neurofibromatosis type 1, Nieman Pick disease, and congenital gangliosidosis. A total of 77 embryos were tested, including 43 by blastocyst biopsy in 6 and 34 by blastomere biopsy in 4 cycles. Of 77 embryos tested, 30 (39%) were predicted to be aneuploidy-free and also unaffected. Overall, such embryos were available for transfer in all but one cycle, and transferred without freezing in the majority of cycles performed by blastomere biopsy (unpublished data).

At the present time, this combined approach is applied in approximately 100 cycles, including aneuploidy testing together with Mendelian disorders and HLA typing, as described above, and also together with PGD for translocations [96], demonstrating the possibility of testing for multiple indications in a single comprehensive test, avoiding additional biopsy procedures. This also improves the efficiency of PGD, as the transfer of aneuploid embryos is excluded in cycles from patients of advanced reproductive age.

So, presented data show that the PB approach, based on PB1 and PB2 analysis, as well as genetic analysis of single blastomeres or blastocyst cells, may presently be applied for PGD of single-gene disorders, aneuploidies, and translocations. Based on this analysis, the embryos resulting from unaffected oocytes are transferred back to patients within the implantation window, while those predicted as affected are followed up by PCR or FISH analysis to confirm the PB or blastomere diagnosis. The results of genetic analysis using both sources of biopsy material will be presented below, showing the accuracy and reliability of PGD for single-gene and chromosomal disorders.

References

1. Preimplantation Genetic Diagnosis International Society (PGDIS). Guidelines for good practice in PGD: program requirements and laboratory quality assurance. Reprod Biomed Online. 2008;16:134–47.
2. Preimplantation Genetic Diagnosis International Society (PGDIS). 10th International congress on preimplantation genetic diagnosis. Reprod Biomed Online. 2010;20:S1–42.
3. Kuliev AM. Expanding indications for preimplantation genetic diagnosis. Expert Rev Obstet Gynecol. 2011;6:599–607.
4. Handyside AH, Kontogiani EH, Hardy K, Winston RML. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature. 1990;344:768–70.
5. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM. Analysis of the first polar body: preconception genetic diagnosis. Hum Reprod. 1990;5: 826–9.
6. International Working Group on Preimplantation Genetics. Current progress in preimplantation genetic diagnosis. J Assist Reprod Genet. 1993;10:353–60.
7. International Working Group on Preimplantation Genetics. Preimplantation diagnosis of genetic and chromosomal disorders. J Assist Reprod Genet. 1994; 11:236–43.
8. Griffin DK, Handyside AH, Penketh RJA, Winston RML, Delhanty JDA. Fluorescent in-situ hybridization to interphase nuclei of human preimplantation embryos with X and Y chromosome specific probes. Hum Reprod. 1991;6:101–5.
9. Griffin DK, Wilton LJ, Handyside AH, Winston RML, Delhanty JDA. Dual fluorescent in-situ hybridization for the simultaneous detection of X and Y chromosome specific probes for the sexing of human preimplantation embryonic nuclei. Hum Genet. 1992;89: 18–22.
10. Munne S, Lee A, Rozenwaks Z, Grifo J, Cohen J. Diagnosis of major chromosome aneuploidies in human preimplantation embryos. Hum Reprod. 1993;8:2185–91.
11. Munne S, Weier HUG, Stein J, Grifo J, Cohen J. A fast and efficient method for simultaneous X and Y in-situ hybridization of human blastomeres. J Assist Reprod Genet. 1993;10:82–90.
12. Delhanty JDA, Griffin DK, Handyside AH. Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridisation (FISH). Hum Mol Genet. 1993;2:1183–5.
13. Munné S, Weier HUG, Grifo J, Cohen J. Chromosome mosaicism in human embryos. Biol Reprod. 1994; 51:373–9.
14. Verlinsky Y. Preimplantation genetic diagnosis. J Assist Reprod Genet. 1996;13:87–9.
15. International Working Group on Preimplantation Genetics. Current status of preimplantation diagnosis. J Assist Reprod Genet. 1997;14:72–5.
16. Munne S, Morrison L, Fung J, et al. Spontaneous abortions are reduced after preconception diagnosis of translocations. J Assist Reprod Genet. 1998;15:290–6.
17. Verlinsky Y, Kuliev A. Preimplantation genetics. J Assist Reprod Genet. 1998;15:215–8.
18. Munne S, Wells D, Cohen J. Technology requirements for preimplantation genetic diagnosis to improve assisted reproduction outcomes. Fertil Steril. 2010;92:408–30.

19. Treff NT, Levy B, Su J, Northrop LE, Tao X, Scott RT. SNP microarray-based 24 chromosome aneuploidy screening is significantly more consistent that FISH. Mol Hum Reprod. 2010;16:583–9.
20. Johnson DS, Gemelos G, Ryan A, et al. Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol. Hum Reprod. 2010;25:1066–75.
21. Treff NT, Sue G, Tao X, Levy B, Scott RT. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. Fertil Steril. 2010; 94:2017–21.
22. Gutierrez-Mateo C, Colls P, Sanchez-Garcia J, Escudero T, Prates R, Wells D, Munne S. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. Fertil Steril. 2011;95:953–8.
23. Fragouli E, Alfarawati S, Daphnis DD, et al. Cytogenetic analysis of human blastocyst with the use of FISH, CGH, and aCGH: scientific data and technical evaluation. Hum Reprod. 2011;26:480–90.
24. Kuliev A, Zlatopolsky Z, Kirillova I, Spivakova J, Cieslak-Janzen G. Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing. Reprod Biomed Online. 2011;22:2–8.
25. Gabriel AS, Thornhill AR, Ottolini CS, et al. Array comparative genomic hybridization on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans. J Med Genet. 2011;48:433–7.
26. Geraedts J, Collins J, Gianaroli L, et al. What next for preimplantation genetic screening? A polar body approach. Hum Reprod. 2010;25:575–7.
27. European Society of Human Reproduction and Embryology (ESHRE). Abstracts of the 26th annual meeting of European Society of Human Reproduction and Embryology. Hum Reprod. 2010;25 Suppl 1.
28. Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening in the blastocyst stage. Fertil Steril. 2010;94:1700–6.
29. Scott RT, Tao X, Ferry KM, Treff NR. A prospective randomized controlled trial demonstrating significantly increased clinical pregnancy rates following 24 chromosome aneuploidy screening: biopsy on day 5 with fresh transfer. Fertil Steril. 2010;94 Suppl S2:O–05.
30. Munné S, Sandalinas M, Escudero T. Outcome of premplantation genetic diagnosis of translocations. Fertil Steril. 2000;73:1209–18.
31. ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium. Best practice guidelines for preimplantation genetic diagnosis/screening (PGD/PGS). Hum Reprod. 2011;26:14–46.
32. Verlinsky Y, Tur-Kaspa I, Cieslak J, Bernal A, Morris R, Taranissi M, Kaplan B, Kuliev A. Preimplantation testing for chromosomal disorders improves reproductive outcome of poor prognosis patients. Reprod BioMed Online. 2005;11:219–25.
33. Kuliev A, Kuliev A, Cieslak Jansen J, Zlatopolski Z, et al. Conversion and Non-conversion approach to preimplantation diagnosis for chromosomal rearrangements in 475 cycles. Reprod BioMed Online. 2010;21:93–9.
34. The Practice Committee of the Society for Assisted Reproductive Technology and Practice Committee of the American Society for Reproductive Medecine. Preimplantation genetic testing: a practice committee opinion. Fertil Steril. 2007;88:1497–504.
35. Munne S. Preimplantation genetic diagnosis of numerical and structural chromosome abnormalities. Reprod Biomed Online. 2002;4:183–96.
36. Verlinsky Y, Cieslak J, Evsikov S, Galat V, Kuliev A. Nuclear transfer for full karyotyping and preimplantation diagnosis of translocations. Reprod Biomed Online. 2002;5:302–7.
37. Kuliev A, Verlinsky Y. Thirteen years' experience of preimplantation diagnosis: report of the fifth international symposium on preimplantation genetics. Reprod Biomed Online. 2004;8:229–35.
38. International Working Group on Preimplantation Genetics. Preimplantation diagnosis: an integral part of assisted reproduction. Report of the 9th Annual Meeting International Working Group on Preimlantation Genetics, in association with the 11th IVF Congress. Sydney, May 10, 1999. J Assist Reprod Genet. 2000;16:161–4.
39. International Working Group on Preimplantation Genetics. 10th anniversary of preimplantation genetic diagnosis. Report of the 10th Annual Meeting of International Working Group on Preimlantation Genetics, in conjunction with 3rd International Symposium on Preimplantation Genetics. Bologna, June 23, 2000. J Assist Reprod Genet. 2001;18:66–72.
40. International Working Group on Preimplantation Genetics. Preimplantation genetic diagnosis – experience of three thousand clinical cycles. Report of the 11th Annual Meeting International Working Group on Preimlantation Genetics, in conjunction with 10th International Congress of Human Genetics. Vienna, May 15, 2001. Reprod BioMed Online. 2001;3:49–53.
41. Verlinsky Y, Munne S, Cohen J, et al. Over a decade of preimplantation genetic diagnosis experience – a multi-center report. Fertil Steril. 2004;82:292–4.
42. Verlinsky Y, Rechitsky S, Verlinsky O, et al. Preimplantation diagnosis for p53 tumor suppressor gene mutations. Reprod Biomed Online. 2001;2:102–5.
43. Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Preimplantation diagnosis for fanconi anemia combined with HLA matching. JAMA. 2001;285:3130–3.
44. Verlinsky Y, Rechitsky S, Sharapova T, Morris R, Tharanissi M, Kuliev A. Preimplantation HLA typing. JAMA. 2004;291:2079–208.
45. Kuliev A, Verlinsky Y. Current feature of preimplantation genetic disgnosis. Reprod Biomed Online. 2002;5:296–301.

46. ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium. Data collecttion II. Hum Reprod. 2002;15:2673–83.
47. Liebaers I, Desmyttere S, Verpoest W, De Rycke M, Staessen C, Sermon K, Devroey P, Haentjens P, Bonduelle M. Report on a consecutive series of 581 children born after blastomere biopsy for preimplantation genetic diagnosis. Hum Reprod. 2010;25:275–82.
48. Verlinsky Y, Kuliev A. Atlas of preimplantation genetic diagnosis. New York, London: Parthenon; 2000.
49. Verlinsky Y, Milayeva S, Evsikov S, et al. Preconception and preimplantation diagnosis for cystic fibrosis. Prenat Diagn. 1992;12:103–10.
50. Verlinsky Y, Rechitsky S, Cieslak J, et al. Preimplantation diagnosis of single gene disorders by two-step oocyte analysis using first and second polar pody. Biochem Mol Med. 1997;62:182–7.
51. Kaplan B, Wolf G, Kovalinskaya L, Verlinsky Y, et al. Viability of embryos following second polar body removal in a mouse model. J Assist Reprod Genet. 1995;12:747–9.
52. Kuliev A, Rechitsky S, Laziuk K, Verlinsky O, Tur-Kaspa I, Verlinsky Y. Pre-embryonic diagnosis for Sandhoff disease. Reprod Biomed Online. 2006;12:328–33.
53. Kuliev A, Rechitsky S, Verlinsky O, Strom S, Verlinsky Y. Preembryonic diagnosis for sickle cell disease. Mol Cell Endocrinol. 2001;183:S19–22.
54. Larsen WJ. Human embryology. New York: Churchill Livingstone; 1994.
55. Willadsen S, Munne S, Schmmel T, Cohen J. Applications of nuclear sperm duplication. Fifth Internationa Symposium on Preimplantation Genetics. Antalya, 5–7 June, 2003. p. 35.
56. Munne S, Willadsen S, Schmmel T, Cohen J. Nuclear sperm duplication as a tool to study mosaicism. Fifth Internationa Symposium on Preimplantation Genetics. Antalya, 5–7 June, 2003. p. 55–6.
57. Lacham-Kaplan O, Daniels R, Trounson A. Fertilization of mouse oocytes using somatic cells as male germ cells. Reprod Biomed Online. 2001;3:205–11.
58. Tesarik J, Nagy ZP, Sousa M, et al. Fertilized oocytes reconstructed from patient's somatic cell nuclei and donor ooplast. Reprod Biomed Online. 2001;2: 160–4.
59. Galat V, Verlinsky Y. Haploidization of somatic cell nuclei by cytoplasm of human oocytes. Reprod Biomed Online. 2002;4 Suppl 2:48–9.
60. Galat V, Ozen S, Rechitsky L, Verlinsky Y. Is haploidization by human mature oocytes real? Fifth Internationa Symposium on Preimplantation Genetics. Antalya, 5–7 June, 2003. p. 36–7.
61. McArthur S, Marshall J, Wright D, de Boer K. Successful pregnancies following blastocyst (day 5) biopsy and analysis for reciprocal and Robertsonial translocations. Fifth Internationa Symposium on Preimplantation Genetics. Antalya, 5–7 June, 2003. p. 33.
62. Mastenbroek S, Twisk M, Van Echten-Arends J, et al. In vitro fertilization with preimplantation genetic screening. NEJM. 2007;357:9–17.
63. Rechitsky S, Verlinsky O, Strom C, et al. Experience with single-cell PCR in preimplantation genetic diagnosis: how to avoid pitfalls. In: Hahn S, Holzgreve W, editors. Fetal cells in maternal blood. New developments for a new millennium. 11th fetal cell workshop. Basel: Karger; 2000. p. 8–15.
64. Rechitsky S, Verlinsky O, Amet T, et al. Reliability of preimplantation diagnosis for single gene disorders. Mol Cell Endocrinol. 2001;183:S65–8.
65. Verlinsky Y, Rechitsky S, Verlinsky O, Strom C, Kuliev A. Polar body based preimplantation diagnosis for X-linked genetic disorders. Reprod Biomed Online. 2002;4:38–42.
66. Rechitsky S, Strom C, Verlinsky O, et al. Allele drop out in polar bodies and blastemeres. J Assist Reprod Genet. 1998;15:253–7.
67. Rechitsky S, Strom C, Verlinsky O, et al. Accuracy of preimplantation diagnosis of single-gene disorders by polar body analysis of oocytes. J Assist Reprod Genet. 1999;16:192–8.
68. Findlay I, Quirke P. Fluorescent polymerase chain reaction: part I. A new method allowing genetic diagnosis and DNA fingerprinting of single cells. Hum Reprod Update. 1996;2:137–52.
69. Kuliev A, Rechitsky S, Verlinsky O, et al. Birth of healthy children following preimplantation diagnosis for thalassemias. J Assist Reprod Genet. 1999; 16:219–25.
70. Rechitsky S, Verlinsky O, Kuliev A, Ozen S, Masciangelo C, Lifchez A, Verlinsky Y. Preimplantation genetic diagnsis for familial dysautonomia. Reprod Biomed Online. 2003;6:488–93.
71. Sermon K, Seneca S, De Rycke M, et al. PGD in the lab for triplet diseases-myotonic dystrophy, Huntington's disease and Fragile-X syndrome. Mol Cell Endocrinol. 2001;183:S77–85.
72. Katz MG, Mansfield J, Gras L, Trounson A, Cram DS. Diagnosis of trisomy 21 in preimplantation embryos by single-cell DNA fingerprinting. Reprod Biomed Online. 2002;4:43–50.
73. Harper J, Wells D, Piyamongkol W, et al. Preimplantation genetic diagnosis for single gene disorders: experience with five single gene disorders. Prenat Diagn. 2002;22:525–33.
74. Ray PF, Gigarel N, Bonnefont JP, Attie T, Hamamah S, Frydman N, Vekemans M, Frydman R, Munnich A. First specific preimplantation genetic diagnosis for ornithine transcarbamilase deficiency. Prenat Diagn. 2000;20:1048–54.
75. Verlinsky Y, Rechitsky S, Verlinsky O, Strom C, Kuliev A. Preimplantation diagnosis for long-chain 3-hydroxiacyl-CoA dehydrogenase deficiency. Reprod Biomed Online. 2001;2:17–9.
76. Renwick P, Trussler J, Braude P, Ogilvie CM. Preimplantation genetic haplotyping: 127 diagnostic

cycles demonstrating a robust, efficient alternative to direct mutation testing on single cells. Reprod Biomed Online. 2010;20:470–6.
77. Schrurs BM, Winston RML, Handyside AH. Preimplantation diagnosis of aneuploidy using fluorescent in-situ hybridization: evaluation using a chromosome 18-specific probe. Hum Reprod. 1993;8:296–301.
78. Munne S, Magli MC, Bahce M, Fung J, Legator M, Morison L. Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births: XY, 13, 14, 15, 16, 18, 21, 22. Prenat Diagn. 1998;18:459–1466.
79. Bahce M, Cohen J, Munné S. Preimplantation genetic diagnosis of aneuploidy: were we looking at the wrong chromosomes? J Assist Reprod Genet. 1999;16: 154–9.
80. Gianaroli L, Magli MC, Ferraretti AP, Munne S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with poor prognosis: identification of the categories for which it should be proposed. Fertil Steril. 1999;72:837–44.
81. Munne S, Magli C, Cohen J, et al. Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. Hum Reprod. 1999;14:2191–9.
82. Munne S, Sandalinas M, Escudero T, et al. Improved implantation after preimplantation genetic diagnosis of aneuploidy. Reprod Biomed Online. 2003;7: 91–7.
83. Munné S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates and maternal age are correlated with chromosome abnormalities. Fertil Steril. 1995;64:382–91.
84. Harper JC, Coonen E, Handyside AH, et al. Mosaicism of autosomes and sex chromosomes in morphologically normal monospermic preimplantation human embryos. Prenat Diagn. 1994;15:41–9.
85. Evsikov S, Cieslak J, Verlinsky Y. Survival of unbalanced translocations to blastocyst stage. Fertil Steril. 2000;74:672–6.
86. Dyban A, Fredine M, Severova E, Cieslac J, Wolf G, Kuliev A, Verlinsky Y. Detection of aneuploidy in human oocytes and corresponding first polar bodies using FISH. 7th International Conference on Early Prenatal Diagnosis. Jerusalem, May 22–27, 1994; (Abstract #97).
87. Dyban A, Fredine M, Severova E, et al. Detection of aneuploidy in human oocytes and corresponding first polar bodies by FISH. J Assist Reprod Genet. 1996;13:72–7.
88. Munné S, Daily T, Sultan KM, Grifo J, Cohen J. The use of first polar bodies for preimplantation diagnosis of aneuploidy. Hum Reprod. 1995;10:1014–120.
89. Verlinsky Y, Cieslak J, Freidine M, Ivakhnenko V, Wolf G, Kovalinskaya L, White M, Lifchez A, Kaplan B, Moise J, Ginsberg N, Strom C, Kuliev A. Pregnancies following pre-conception diagnosis of commom aneuploidies by fluorescent in-situ hybridization. Hum Reprod. 1995;10:1923–7.
90. Verlinsky Y, Evsikov S. Karyotyping of human oocytes by chromosomal analysis of the second polar body. Mol Hum Reprod. 1999;5:89–95.
91. Verlinsky Y, Evsikov S. A simplified and efficient method for obtaining metaphase chromosomes from individual human blastomeres. Fertil Steril. 1999;72:1–6.
92. Willadsen S, Levron J, Munne S, et al. Rapid visualization of metaphase chromosomes in single human blastomeres after fusion with in-vitro matured bovine eggs. Hum Reprod. 1999;14:470–4.
93. Tanaka A, Nagayoshi M, Awata S, Mawatari Y, Tanaka I, Kusunoki H. Preimplantation diagnosis of repeated miscarriage due to chromosomal translocations using metaphase chromosomes of a balstomere biopsied from 4–6 cell stage embryo. Fertil Steril. 2004;81:30–4.
94. Shkumatov A, Kuznyetsov V, Cieslak J, Ilkevitch Y, Verlinsky Y. Obtaining metaphase spreads from single blastomeres for PGD of choromosomal rearrangements. Reprod Biomed Online. 2007;14:498–503.
95. Brezina PR, Benner A, Rechitsky S, Kuliev A, Pomerantseva E, Pauling D, Kearns WG. Single-gene testing combined with single nucleotide polymorphism microarray preimplantation genetic diagnosis for aneuploidy: a novel approach in optimizing pregnancy outcome. Fertil Steril. 2011;95:1786.
96. Treff NR, Northrop LE, Kasabwala K, Su J, Levy B, Scott RT. Single nucleotide polymorphism microarray-based concurrent screening of 24-chromosome aneuploidy and unbalanced translocations in preimplantation human embryos. Fertil Steril. 2011;95:1606–1612.e2.