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PGD in female carriers of balanced Robertsonian and reciprocal translocations by first polar body analysis

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Preimplantation genetic diagnosis (PGD) using the first polar body (1PB) is a modality of PGD that can be used when the woman is the carrier of a genetic disease or of a balanced chromosomal reorganization. PGD using 1PB biopsy in carriers of balanced chromosome reorganizations has not become generalized. Here, we describe our experience based on the analysis of unfertilized or fresh, non-inseminated control oocytes, by fixing separately the 1PB and the corresponding oocyte, and on the study of six clinical cases of PGD using 1PB biopsy (four Robertsonian translocations and two reciprocal translocations). In fresh oocytes, the chromosome morphology of the 1PB was well preserved, and the results were always concordant for each oocyte–1PB pair. This indicates that the 1PB can be reliably used for the diagnosis of chromosome reorganizations. In these studies the technical problems encountered when performing PGD using 1PB biopsies for chromosome studies are also addressed. Three different strategies of 1PB biopsy (laser beam, partial zona dissection and acid Tyrode's) and two different protocols (intracytoplasmic sperm injection before or after 1PB biopsy) and their effect on the percentage of oocytes diagnosed and the fertilization rate, are discussed. In reciprocal translocation cases, published in the literature or studied by us, in which at least nine oocytes had been diagnosed, a correlation has been found between the frequency of non-disjunction observed and the theoretical recombination rate. To date, PGD by 1PB analysis alone or combined with blastomere biopsies in female carriers of chromosomal rearrangements has been used in 18 cases, with a further six cases reported here. A total of 325 cumulus–oocyte complexes have been obtained, of which 294 were biopsied and 224 were diagnosed. A total of 52 embryos was transferred, 19 of which implanted and 17 produced full-term pregnancies.

Key words: first polar body biopsy/FISH/preimplantation genetic diagnosis/reciprocal translocations/Robertsonian translocations

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Introduction

Preimplantation genetic diagnosis (PGD) is usually based on the analysis of one or two blastomeres obtained from cleavage-stage embryos, but it may also be based on the analysis of the first polar body (1PB). PGD of 1PB biopsies can be used when the woman is the carrier of a genetic disease or of a balanced chromosomal reorganization. Genetic analysis of the 1PB allows the indirect diagnosis of its corresponding oocyte. PGD of 1PB biopsies has the advantage that doubtful or difficult diagnoses can be confirmed by PGD in blastomeres, thus avoiding the need of establishing a pregnancy and its eventual termination.

To date, PGD of 1PB biopsies has been widely used for the diagnosis of monogenic diseases, such as cystic fibrosis (Cieslak *et al*., 2000) in spite of the fact that oocytes with heterozygotic chromosomes (up to 50%) can produce either normal offspring or

affected children. In these cases, confirmation by second polar body (2PB) biopsy or by blastomere biopsy is mandatory. On the other hand, its use in female carriers of balanced chromosome reorganizations has not become generalized, probably because most 1PBs are fixed in interphase or as degenerated chromatin. This may be due to the technical problems encountered in obtaining good chromosome spreads (Verlinsky *et al*., 1996; Munné, 2000), to the difficulty of handling such a small cell as the 1PB, or because the PGD of 1PB biopsies does not allow the analysis of the paternal contribution to the genetic endowment of the embryo.

It must be noted that although over 1000 cycles have been performed for the diagnosis of aneuploidies or genetic diseases in 1PBs (Report of the 9th Annual Meeting of the International Working Group on Preimplantation Genetics, in association with the 11th IVF Congress, 2000) only 28 PGD cycles using 1PB biopsies, and one cycle using 1PB plus blastomere biopsies are included in the data collection II of the ESHRE PGD Consortium Steering Committee (2000), covering the period until May 1, 2000. Of these, only three cycles were performed for the diagnosis of translocations. In contrast, the Report of the 9th Annual Meeting of the International Working Group on Preimplantation Genetics, in association with the 11th IVF Congress (2000) refers to 35 cases of translocation at the St Barnabas centre without providing details on the characteristics of the translocations, or on the results.

The first case of PGD using 1PB biopsy in a female carrier of a balanced translocation was published in 1998 (Munné *et al*.,1998a). Although the procedure is safe, to date only 18 cases of PGD using 1PB biopsy in translocation carriers had been reported. The obstetrical and neonatal outcome of the first 109 live births after PGD for Mendelian disorders and aneuploidies using 1PB biopsies has been reviewed (Strom *et al*., 2000).

Here, we address the technical problems encountered when performing PGD for chromosome studies using 1PB biopsies, describe our experience (six cases) and review all other cases reported so far in the literature (18 cases).

Technical aspects

After several years of experience in the use of hamster and human oocytes for PGD using 1PB biopsies (Durban *et al*., 1998), it has been possible to solve a number of technical problems that can be avoided by following the recommendations given below. The main technical difficulties encountered were: (i) reliability of results; (ii) quality of 1PB chromosome preparations; (iii) fertilization efficiency; and (iv) fluorescence in-situ hybridization (FISH) efficiency and 1PB diagnosis

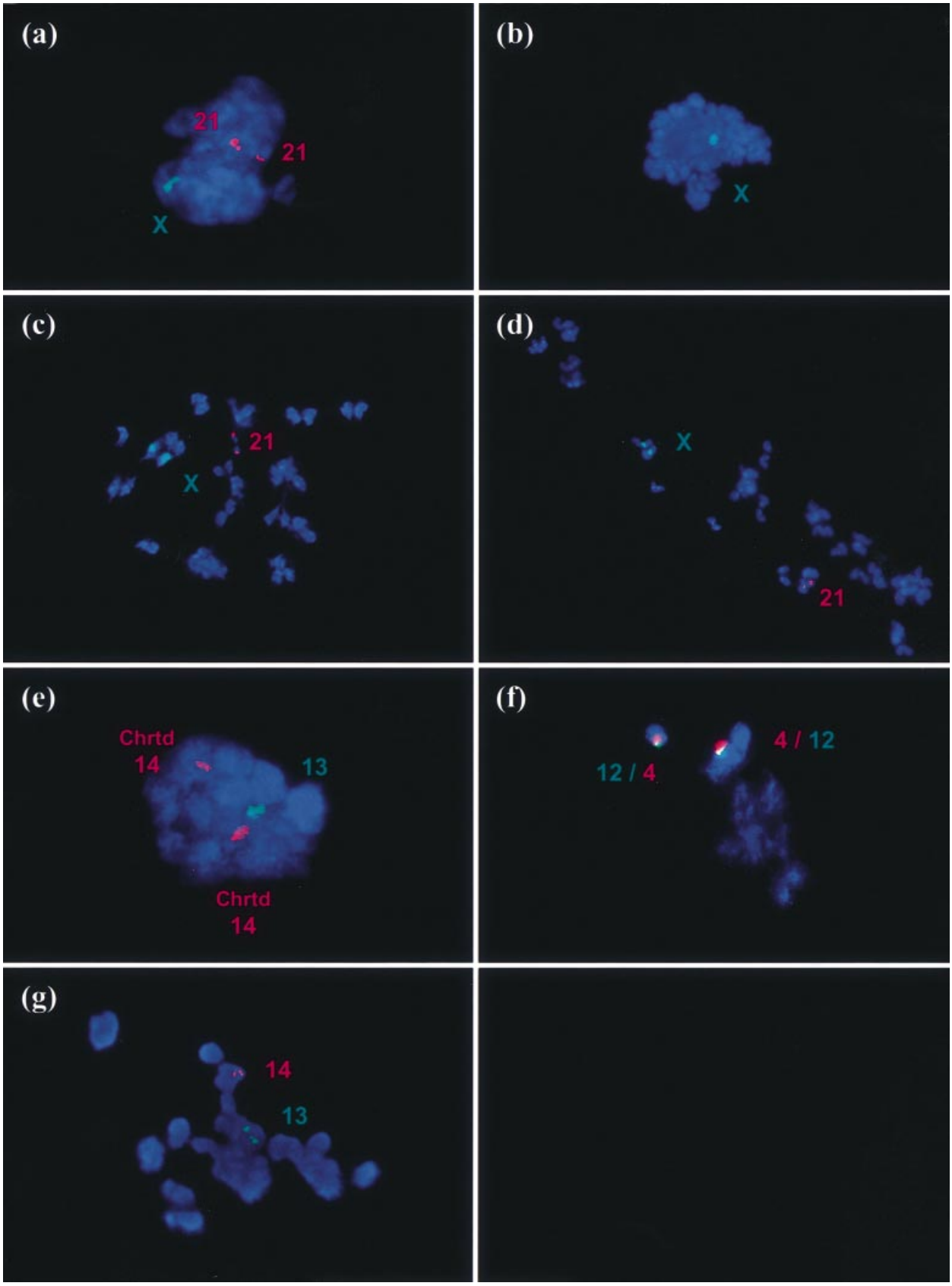
Reliability of results

Analyses of the concordance between the chromosome complements of the 1PB and the metaphase II (MII) oocyte had been previously carried out (Munné *et al*., 1993; Dailey *et al*., 1996; Dyban *et al*., 1996) by jointly fixing the oocyte and the 1PB. All results showed a high degree of concordance in the pair, and demonstrated that a high percentage (>90%) of chromosome complements could be analysed. In order to confirm the reciprocity of the chromosome sets of the 1PB and the oocyte and the possible influence of ageing, a study was carried out in 41 inseminated, unfertilized oocytes and in 18 fresh oocytes donated by patients undergoing IVF cycles or gynaecological operations by fixing the MII oocyte and the 1PB separately. The Ethics Committees of the Universitat Autònoma de Barcelona and of the IVF centres taking part approved the Informed Consent Form and the protocol for the study. The 1PB was separated from the MII oocyte either enzymatically using trypsin (Difco, Detroit, MI, USA) 30 mg/ml in phosphate-buffered saline (PBS) for 2 min, or mechanically by micromanipulation (Narishige, Técnicas Médicas MAB, Spain). Both cells were fixed differently: the 1PB following a published method (Durban *et al*., 1998), and the MII oocyte following a modified Tarkowski's method (Tarkowski, 1966). In order to establish the concordance between the chromosome complement of the 1PB and that of the oocyte, and other than counting, several sets of probes were used, combining centromere probes (CEP), locus-specific probes (LSI), telomere probes (Tel) and whole chromosome painting (WCP) probes. The probes were different depending on the case, but were the same for each oocyte–1PB pair. These directly labelled probes (Vysis, New York, USA and Cambio, UK) were used in single, double and triple FISH sequences in one or two rounds.

In unfertilized oocytes (aged 24–36 h post-puncture), which are the ones normally used to determine the incidence of chromosome abnormalities in female gametes, the chromosome morphology was only preserved in six of 41 1PBs (14.6%), and data were available from only 18 of 41 pairs (43.9%). However, all cases that could be analysed were concordant for the pair, even in two cases of aneuploidy: one pair in which the 1PB was X/21,21 and the oocyte X/0 (Figure 1a and b), and another in which the 1PB was X/1/2 of 21 and the oocyte X/21, 1/2 of 21 as a result of predivision.

In fresh oocytes, the chromosome morphology of the 1PB was preserved in 15 of 18 pairs (83.3%) (Figure 1c and d) and the chromosome complements were concordant in 16 of 16 pairs (100.0%). Two cases could not be analysed due to the presence of membrane and cytoplasmic remnants. All oocytes and 1PBs were chromosomally normal.

Figure 1. (**a**) Abnormal first polar body (1PB) chromosome complement from an unfertilized human oocyte showing two chromosomes 21 and one chromosome X hybridized with LSI 21 spectrum orange and CEP X spectrum green probes. (**b**) The corresponding human metaphase II showing no chromosome 21 and one X chromosome using the same probes. (**c**) Normal, complete 1PB chromosome complement from a fresh (not inseminated) human oocyte showing hybridization with LSI 21 spectrum orange and CEP X spectrum green probes for chromosomes 21 and X respectively. (**d**) The corresponding normal complete human metaphase II (MII) showing hybridization with LSI 21 spectrum orange and CEP X spectrum green probes for chromosomes 21 and X respectively. (**e**) Normal 1PB corresponding to a balanced oocyte from a 45,XX,der(14;21)(q10;q10) patient showing hybridization with WCP 14 spectrum orange and WCP 21 spectrum green probes for chromosomes 14 and 21 respectively. Chromosome 14 shows separated chromatids. Note that although in this spread the chromosome morphology is not well preserved, painting probes show a discrete profile. (**f**) 1PB fixed as chromatin clumps showing two derivative chromosomes corresponding to a normal oocyte from a 46,XX,t(4;12)(q22;q23) patient, hybridized with WCP 4 spectrum orange and WCP 12 spectrum green probes for chromosomes 4 and 12 respectively. (**g**) 1PB with preserved chromosome morphology showing a normal chromosome constitution, corresponding to a balanced oocyte from a 45,XX,der(13;14)(q10;q10) patient, hybridized with WCP 13 spectrum green and WCP 14 spectrum orange probes for chromosomes 13 and 14 respectively.


(a)
21
21
X
(b)
X
(c)
21
X
(d)
X
21
(e)
Chrtd
14
13
Chrtd
14
(f)
4 / 12
12 / 4
(g)
14
13

Both the lower frequency of analysable chromosome sets and the increased incidence of chromosome anomalies may be related to ageing, because 1PBs degenerate very quickly, and only chromatin clumps that do not always hybridize correctly can be obtained, and because ageing *in vitro* may induce chromosome alterations.

In 1PBs and MII oocytes, the chromosomes are short and chromatids are widely separated. Furthermore, 1PB chromosomes are fuzzy, probably because 1PB chromosome complements are no longer under the effect of the active maturation promoting factor present in the oocyte (Sagata, 1996). However, the use of FISH allows a high degree of resolution, and several probes can be used on the same chromosomes. Chromosome identification is possible using spectral karyotyping (SKY) (Márquez *et al*., 1998).

Quality of chromosome preparations

Obtaining high-quality chromosome preparations from oocytes or blastomeres is not easy, and in the case of 1PBs it is even more difficult—a difficulty that has been recently acknowledged (Cieslak *et al*., 2000). In fact, the use of fresh oocytes as the most direct approach to obtain chromosome spreads from 1PBs was also first demonstrated only recently (Durban *et al*., 1998). To solve this problem in blastomeres, several authors have used premature chromosome condensation systems, including okadaic acid (Grossmann *et al*., 1995). More recently, high-quality blastomere chromosome complements were obtained by fusing blastomeres with bovine oocytes (Willadsen *et al*., 1999) and mouse zygotes (Evsikov and Verlinsky, 1999), followed by SKY karyotyping. However, the only studies in which percentages are reported (Evisikov and Verlinsky, 1999; Willadsen *et al*., 1999) show that the success rate is not much higher than 50% for any single cell. Furthermore, these approaches are extremely expensive and time-consuming.

In our laboratory, the technique used described to obtain direct chromosome preparations (Durban *et al*., 1998) was used. These preparations were analysed using the most advantageous combination of FISH probes, and as a result of these analyses some factors which appear to be crucial to obtain high percentages of spreads with a preserved chromosome morphology have been identified.

The present protocol allows us not only to obtain 1PB spreads with preserved chromosome morphology, but also a fertilization rate of 73% using laser or partial zona dissection (PZD). The aims of the protocol were to biopsy the 1PB as soon as possible after the oocyte had been aspirated and microinjected. The rationale for each step is discussed below:

Since the purpose is to obtain the 1PB biopsy as soon as possible, it is recommended that the oocyte be denuded from cumulus cells as soon as the cumulus–oocyte complex (COC) is obtained, rather than this being performed ~2 h later, as is usually done in a standard protocol.

Fertilization efficiency

Once the oocyte has been denuded, intracytoplasmic sperm injection (ICSI) should be performed before the 1PB is biopsied. If ICSI is performed 12 h after retrieval and 6 h after 1PB biopsy (as was our case initially), the fertilization rate is only 42.3%. If this period was shortened to 6–7 h after retrieval (Munné, 2000), the fertilization rate increased to 62.5%. Moreover, if ICSI is carried out immediately after retrieval and before 1PB biopsy, the fertilization rate can be increased to 72.2%. The reasons for this are basically two-fold. First, if the 1PB is biopsied before ICSI, the reference of the 1PB is lost, and since the oocyte may rotate inside the zona pellucida (ZP), the ICSI needle can damage the meiotic spindle and prevent the extrusion of the 2PB, producing zygotes with three pronuclei. Alternatively, it may damage the oocyte in such a way that the development of the zygote is arrested after decondensation of the sperm head (zygotes with two pronuclei) or even earlier (zygotes with one pronucleus). Recently, the effects of sperm placement during ICSI relative to the MII spindle on fertility and preimplantation development were evaluated (Blake *et al*., 2000). The results showed that embryos originating from oocytes injected with the polar body positioned at 7 or 11 o'clock implant at a higher rate, although the data are not significant. In the same way, the regions that are safe or dangerous for ICSI in the oocyte, taking into account the position of the 1PB, have also been defined (Hardarson *et al*., 2000). The second reason is that the opening in the ZP produced by the biopsy procedure may result in the artefactual hatching of the oocyte or the zygote. The incidence of these problems is reflected in Tables I and II (see Clinical application and review of results).

The biopsy procedure should be carried out after ICSI has taken place, and using a laser beam or a mechanical procedure to drill the ZP. The laser beam (a 1.48 µm non-contact diode laser; Fertilase, Medical Technologies, Montreux, Switzerland) adapted to an inverted microscope is very convenient, but expensive. This laser system has been never used before for human 1PB biopsy. However, the efficacy of such a system for zona drilling and its usefulness for assisted hatching was initially demonstrated in humans (Germond *et al*., 1995), and subsequently applied to blastocyst biopsy (Veiga *et al*., 1997) and blastomere biopsy (Boada *et al*., 1998). In our hands, biopsy using a laser system after ICSI yielded a high fertilization rate (71.4%). The mechanical procedure is more time-consuming but cheaper, and possible secondary effects on the ZP are avoided. We also obtained a similar fertilization rate (75.0%) using this biopsy system. While in PGD by 1PB analysis only three embryos have been transferred after using the laser system, with the mechanical system 49 embryos have been transferred. Until now, pregnancies have only been obtained using the mechanical system. More cases using the laser system will be needed before statistically valid comparisons can be made.

The use of acid Tyrode's is not recommended (Malter and Cohen, 1989; Santaló *et al*., 1995). Although good quality chromosome preparations may be obtained from 1PBs biopsied with this system, the oocyte may be easily damaged and the fertilization rate is reduced (10.0%).

The 1PB should be removed using a pipette of a diameter slightly larger than that of the 1PB (~20–30 µm). If a smaller micropipette is used, the 1PB may be mechanically damaged and even lysed. In biopsies obtained using a small pipette (~10 µm), only 14.2% of preparations with a preserved chromosome morphology were obtained (Table I, case m; Table II, case H), while using a pipette of a larger diameter this proportion grew to 89.4% (Table I, cases n, p and q; Table II, case I; see also Clinical application and review of results). It is also important to remove

Table I. Clinical case results: preimplantation genetic diagnosis (PGD) of Robertsonian translocations using 1PB biopsy

Oocytes analysed	1PB	Preserved 1PB chromosome morphology	ICSI versus biopsy	1PB diagnosis	Blastomere diagnosis	Oocyte or embryo diagnosis	ICSI result and transfer
Case m: 45,XX,der(14;21)(q10;q10)							
1PB-1	Fragmented	–	Post	–	–	–	Artefactual hatched 2 PN
1PB-2	Fixed	No	Post	Undiagnosed	–	No result	Fertilized
1PB-3	Fixed	No	Post	21	–	14 der14;21	3 PN
1PB-4	Lost during biopsy	–	Post	–	–	–	Degenerated
1PB-5	Fixed	No	Post	21 der14;21	–	14	Fertilized
1PB-6	Fragmented	–	Post	–	–	–	3 PN
1PB-7	Fixed	No	Post	14 21 der14;21	–	0	3 PN
1PB-8	Fixed	No	Post	14 21	–	der14;21	3 PN
1PB-9	Fixed	No	Post	Undiagnosed	–	No result	Lysed
1PB-10	Fixed	No	Post	21 der14;21	–	14	Artefactual hatched 2 PN
1PB-11	Fixed	No	Post	21 14	–	der14;21	1 PN
1PB-12	Lost during biopsy	–	Post	–	–	–	Fertilized
1PB-13	Fixed	No	Post	Undiagnosed	–	No result	Fertilized
1PB-14	Fixed	No	Post	21 14sc	–	der14;21	Fertilized and transferred
1PB-15	Fixed	No	Post	Undiagnosed	–	No result	Fertilized
Case n: 45,XX,der(13;14)(q10;q10)							
1PB-1	Fixed	No	–	der13;14	–	13 14	Damaged during biopsy
1PB-2	Lost during fixation	–	Post	–	–	No result	Fertilized[a]
1PB-3	Fixed	Yes	Post	13 der13;14	–	Unbalanced	Fertilized
1PB-4	Lost during fixation	–	Post	–	–	No result	Unfertilized
Case p: 45,XX,der(13;14)(q10;q10)							
1PB-1	Fixed	Yes	Pre	13	–	14 der13;14	Fertilized
1PB-2, Blt-2	Lost during fixation	–	Pre	–	14 der13;14	14 der13;14	Fertilized
1PB-3, Blt-3	Lost during biopsy	–	Pre	–	14 der13;14	14 der13;14	Fertilized
1PB-4	Fixed	Yes	Pre	13 der13;14	–	14	Fertilized
1PB-5	Fixed	Yes	Pre	14 der13;14	–	13	Degenerated
1PB-6, Blt-6	Fragmented	–	Pre	–	Alternate	Alternate	Fertilized and transferred
1PB-7, Blt-7	Lost during fixation	–	Pre	–	Undiagnosed	No result	Fertilized
1PB-8	Fixed	Yes	Pre	der13;14	–	13 14	Fertilized
1PB-9	Fixed	Yes	Pre	der13;14	–	13 14	Degenerated
1PB-10	Fixed	Yes	Pre	14	–	13 der13;14	3 PN
1PB-11, Blt-11	Fragmented	–	Pre	–	Unbalanced	Unbalanced	Fertilized
1PB-12	Fixed	Yes	Pre	13 der13;14	–	14	Degenerated
1PB-13, Blt-13	Lost during biopsy	–	Pre	–	Unbalanced	14 der13;14	Fertilized
1PB-14	Fixed	Yes	Pre	13 14	–	der13;14	Fertilized and transferred
Case q cycle 1: 45,XX,der(13;14)(q10;q10)							
1PB-1, Blt-1	Fixed	Yes	Pre	Undiagnosed	13 der13;14	13 der13;14	Fertilized
1PB-2	Lost during fixation	–	Pre	–	–	13 der13;14	Unfertilized[b]
Case q cycle 2: 45,XX,der(13;14)(q10;q10)							
1PB-1	Fixed	Yes	Pre	der13;14	–	13 14	Fertilized and transferred
1PB-2, Blt-2	Fixed	Yes	Pre	0	–	13 14 der13;14	Fertilized

[a]Transferred by decision of the couple on day 3
[b]Fixed and analysed by FISH.
Blt = blastomere; ICSI = intracytoplasmic sperm injection; 1PB = first polar body; PN = pronucleus; sc = separated chromatids.

the 1PB when it has become completely detached from the oocyte (otherwise, the membrane may be ruptured), but as soon as possible after ICSI (<30 min), because the 1PB must be biopsied before the 2PB is extruded.

Once biopsied, the 1PB and its corresponding oocyte are placed in numbered, but separate drops of medium. The reason for using separate drops is that, in a single drop, one cell may be damaged while handling the other. The drops are prepared with freshly filtered medium, to avoid the presence of debris that could be confused with the 1PB.

The hypotonic shock needed to obtain chromosome spreads should also be performed as quickly as possible. To that end, treatment with 1% trisodium citrate for 10 min (Durban *et al.*, 1998) was changed to a treatment with 0.5% trisodium citrate for 5 min. In clinical cases, use of the long hypotonic treatment led to only 17.3% of spreads having a preserved chromosome

Table II. Clinical case results: preimplantation genetic diagnosis (PGD) of reciprocal translocations using 1PB biopsy

Oocytes analysed	1PB	Preserved 1PB chromosome morphology	ICSI versus biopsy	1PB diagnosis	Blastomere diagnosis	Oocyte or embryo diagnosis	ICSI result and transfer
Case H: 46,XX,t(4;12)(q22;q23)							
1PB-1	Lost during biopsy	–	–	–	–	Lost during fixation	Not inseminated[a]
1PB-2	Fixed	No	Post	der12	–	4 der4 12	Degenerated
1PB-3	Lost during biopsy	–	–	–	–	Lost during fixation	Not inseminated[a]
1PB-4	Fixed	Yes	Post	der4 der12	–	4 12	Artefactual hatched 2 PN
1PB-5	Fragmented	–	–	–	–	12 der12	Not inseminated[a]
1PB-6	Fixed	No	Post	0	–	4 der4 12 der12	Unfertilized
1PB-7	Fixed	No	Post	0	–	4 der4 12 der12	Fertilized
1PB-8	Fixed	No	Post	Undiagnosed	–	No result	Artefactual hatched and unfertilized
1PB-9	Fixed	Yes	Post	4 der4 der12	–	12	Fertilized
1PB-10	Lost during fixation	–	–	–	–	12 der12	Not inseminated[a]
1PB-11	Lost during fixation	–	–	–	–	12 der12	Not inseminated[a]
1PB-12	Fixed	No	–	4 12 der4	–	der12	Not inseminated[a]
1PB-13	Fixed	No	–	4 der4	–	12 der12	Not inseminated[a]
1PB-14	Fixed	No	Post	12 der4 der12	–	4	Degenerated
1PB-15	Lost during fixation	–	–	–	–	Not analysable	Not inseminated[a]
1PB-16	Fixed	Yes	Post	Undiagnosed	–	No result	Fertilized
Case I: 46,XX,t(1;18)(p34.3;q12.3)							
1PB-1	Fixed	Yes	Pre	1 18 der18	–	der1	Unfertilized[a]
1PB-2	Fixed	Yes	Pre	der1 der18	–	1 18	Unfertilized[a]
1PB-3	Lost during fixation	–	Pre	–	–	der1 18	1 PN[a]
1PB-4, Blt-4	Fixed	Yes	Pre	Undiagnosed	Undiagnosed	Undiagnosed	Fertilized
1PB-5	Fixed	Yes	Pre	1 der18	–	der1 18	Unfertilized[a]
1PB-6, Blt-6	Lost during fixation	–	Pre	–	Undiagnosed	Undiagnosed	Unfertilized[a]
1PB-7	Fragmented	–	–	–	–	Undiagnosed	1 PN[a]
1PB-8	Fixed[b]	No	Pre	Undiagnosed	–	1 der18	Unfertilized[a]
1PB-9	Fixed	Yes	Pre	18 der18	–	1 der 1	3 PN[a]
1PB-10	Lost during biopsy	–	Pre	–	–	Undiagnosed	Degenerated[a]

[a]Fixed and analysed by FISH.
[b]Matured *in vitro* on day 0.
Blt = blastomere; ICSI = intracytoplasmic sperm injection; 1PB = first polar body; PN = pronucleus.

morphology. By comparison, using the short hypotonic treatment led to 94.1% of the preparations having a preserved chromosome morphology.

Fixation is also a crucial step in obtaining good quality chromosome preparations. The fixative is placed on the slide and allowed to spread while evaporating; in this way, artefactual chromosome losses are reduced to a minimum. However, higher quality preparations of fixed 1PBs were also obtained when a change was made from using a solution of ethanol:acetic acid (1:1) at room temperature to using a methanol:acetic acid (1:1) solution prepared at least one month in advance and kept in the freezer at –20°C. The use of cold fixative is a classic approach. The reason why a fixative prepared in advance spreads better is unknown, but it is recommended that the fixative is tested on a clean, empty slide before being used to fix fresh 1PBs; moreover, it should only be used if spreading is satisfactory. It is also recommended that the fixative be added as soon as the drop of hypotonic solution begins to dry. In this way, the 1PB is fixed *in situ*, and the risk of losing the 1PB or some chromosomes is almost completely eliminated.

In order to increase the efficiency of FISH, it is important to eliminate membrane and cytoplasmic remnants (Cieslak *et al.*, 2000). This is done by treating the slide with pepsin (Sigma, Spain) at 50 µg/ml in HCl 10 mmol/l at 37°C for 0.5–5 min, rinsing at room temperature with purified water, and allowing the slide to air-dry before adding a drop of freshly prepared Carnoy's fixative (methanol:acetic acid, 3:1). After cleansing, the slide is checked under phase contrast microscopy, and the protocol repeated if membrane remnants are still present. Before performing the first and second rounds of FISH, the preparations are treated with 50 mmol/l $MgCl_2$/PBS for 5 min, post-fixed with 1% formaldehyde in 50 mmol/l $MgCl_2$/PBS for 10 min, cleansed in PBS for 5 min, and then dehydrated in 70, 80 and 95% ethanol (5 min each) to better preserve the morphology of the chromosomes during the following, successive rounds of FISH.

Images are captured under phase contrast and also after 4,6-diamidino-2-phenylindole (DAPI) staining, because in these images the morphology of the chromosomes is well preserved. To that end, slides are stained with a 25% DAPI II (Vysis, New York, USA)/75% Antifade (Vectashield, CA, USA) solution for

Table III. Robertsonian translocations: patient data and 1PB biopsy

Pt. no.	Reference	Female karyotype	Age (years)	SA (*n*)	COC (*n*)	Biopsy technique	1PBs biopsied (*n*)[a]
a	Munné *et al*. (2000)	45,XX,der(14;21)(q10;q10)	–	–		PZD	32
b	Munné *et al*. (2000)	45,XX,der(14;21)(q10;q10)	–	–		PZD	21
c	Munné *et al*. (2000)	45,XX,der(14;21)(q10;q10)	–	–	101	PZD	32
d	Munné *et al*. (2000)	45,XX,der(14;21)(q10;q10)	–	–		PZD	16
e	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	11
f	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	13
g	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	15
h	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–	77	PZD	13
i	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	11
j	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	6
k	Munné *et al*. (2000)	45,XX,der(13;14)(q10;q10)	–	–		PZD	8
m	Present study	45,XX,der(14;21)(q10;q10)	32	3	15	Laser	13
n	Present study	45,XX,der(13;14)(q10;q10)	37	5	5	Laser	4
p	Present study	45,XX,der(13;14)(q10;q10)	37	4	18	Laser	12
q	Present study	45,XX,der(13;14)(q10;q10)[b]	36	0	4	PZD	4

[a]Fragmented 1PBs are not included.
[b]Referred to the centre because her sister experienced three spontaneous abortions and had two children who were carriers of the translocation.
COC = cumulus–oocyte complex; 1PB = first polar body; PZD = partial zona dissection; SA = spontaneous abortion.

Table IV. Reciprocal translocations: patient data and 1PB biopsy

Pt. no.	Reference	Female karyotype	Age (years)	SA (*n*)	COC (*n*)	Biopsy technique	1PBs biopsied (*n*)[a]
A	Munné *et al*. (1998a)	46,XX,t(4;14)(p15;q24)	37	2	8	PZD	6
B	Munné *et al*. (1998b)	46,XX,t(7;20)(q22;q11.2)	28	4	8	PZD	8
C	Munné *et al*. (1998b)	46,XX,t(14;18)(q22;q11)	36	5	18	PZD	8
D	Munné *et al*. (1998c)	46,XX,t(12;20)(p13.1;q13.3)	43	1	9	PZD	2
E	Munné *et al*. (1998d)	46,XX,t(11;16)(q21;q22)	32	5	19	PZD	19
F	Willadsen *et al*. (1999)	46,XX,t(9;11)(p24;q12)	37	>2	6	PZD	5
G	Willadsen *et al*. (1999)	46,XX,t(11;16)(q21;q22)	31	>2	11	PZD	11
H	Present study	46,XX,t(4;12)(q22;q23)	35	2	16	Laser	15
I	Present study	46,XX,t(1;18)(p34.3;q12.3)	35	2	10	Tyrode	9

[a]Fragmented 1PBs are not included.
COC = cumulus–oocyte complex; 1PB = first polar body; PZD = partial zona dissection; SA = spontaneous abortion.

5 min. To remove DAPI, slides are rinsed in 0.1%Tween 20/2×SSC at room temperature under constant agitation for 5 min, dehydrated in 70, 80 and 95% ethanol (5 min each), and then air-dried.

FISH is performed in different rounds and using different probes depending on the characteristics of the chromosome reorganization. Note that even in cases of poorly spread 1PB chromosome complements it is possible to distinguish separated chromatids using painting probes (Figure 1e). In most cases, the first round of FISH is carried out using the appropriate combination of centromere, telomere and/or locus-specific probes, and the second round using WCP probes. In this way, it is usually possible to diagnose all successfully fixed 1PBs. If needed, the diagnosis can be confirmed by PGD using blastomere biopsies. However, in some cases the translocation can be so terminal that it will not be detected with WCP probes, or even telomeric probes. In these cases, probes specifically produced for the region(s) involved may be used (Munné *et al*., 1998c).

Clinical application and review of results

Until now, and including our own experience, only 24 cases of female translocation carriers using 1PB analysis have been performed. Eighteen cases had been reported in the literature: 11 Robertsonian translocations (Munné *et al*., 2000) (Table III, cases a–k) and seven reciprocal translocations (Munné *et al*., 1998a–d; Willadsen *et al*., 1999) (Table IV, cases A–G). So far, we have had the opportunity to analyse using PGD of 1PBs in four female carriers of Robertsonian translocations (Table III, cases m, n, p and q) and in two female carriers of a reciprocal translocation (Table IV, cases H and I). Full details of these cases are given in Tables I and II. The protocol was progressively changed to include the modifications detailed above, which were used in full only in cases p and q (Table III), and in case I (Table IV). In a recent report (Cieslak *et al*., 2000), the authors referred to several cases studied, but gave no details on the results from any of them.

Table V. Robertsonian translocations: results of 1PB analysis

Pt no.	1PBs biopsied[a]	Probes	No. of oocytes			
			Normal (%)	Balanced (%)	UnB (%)	ND (%)[b]
a	32	WCP14, WCP21[c]	9 (28.1)	8 (25.0)	11 (34.3)	4 (12.5)
b	21	WCP14, WCP21	7 (33.3)	1 (4.7)	11 (52.3)	2 (9.5)
c	32	WCP14, WCP21	10 (31.2)	4 (12.5)	10 (31.2)	8 (25.0)
d	16	WCP14, WCP21	6 (37.5)	5 (31.2)	4 (25.0)	1 (6.2)
e	11	WCP13, WCP14[c]	6 (54.5)	1 (9.0)	4 (36.3)	0
f	13	WCP13, WCP14	9 (69.2)	3 (23.0)	1 (7.6)	0
g	15	WCP13, WCP14	3 (20.0	2 (13.3)	6 (40.0)	4 (26.6)
h	13	WCP13, WCP14	4 (30.7)	4 (30.7)	5 (38.4)	0
i	11	WCP13, WCP14	7 (63.6)	0	2 (18.1)	2 (18.1)
j	6	WCP13, WCP14	2 (33.3)	0	3 (50.0)	1 (16.6)
k	8	WCP13, WCP14	4 (50.0)	2 (25.0)	1 (12.5)	1 (12.5)
m	13	WCP14, WCP21	0	3 (23.0)	4 (30.7)	6 (46.1)
n	4	R1:LSI13, Tel14q/R2: W13, W14	0	1 (25.0)	1 (25.0)	2 (50.0)
p	12	R1:LSI13, Tel14q/R2: W13, W14	1 (8.3)	2 (16.6)	5 (41.6)	4 (33.3)
q	4	R1:LSI13, Tel14q/R2: W13, W14	1 (25.0)	0	1 (25.0)	2 (50.0)

[a]Fragmented 1PBs are not included.
[b]1PBs lost during biopsy or fixation, or FISH not informative.
[c]An LSI13 or LSI21 probe was also added in some cases when it became available.
1PB = first polar body; ND = no diagnosis; UnB = unbalanced.
Probes: WCP, LSI, CEP, Tel (Vysis); W (Cambio); cep, 4ptel, wcp (Oncor).

Table VI. Reciprocal translocations: results of 1PB analysis

Pt no.	1PBs biopsied[a]	Probes	No. of oocytes			
			Normal (%)	Balanced (%)	UnB (%)	ND (%)[b]
A	6	WCP4, 4ptel, wcp14	0	2 (33.3)	3 (50.0)	1 (16.6)
B	8	WCP7, CEP7, WCP20, cep20	0	3 (37.5)	3 (37.5)	2 (25.0)
C	8	WCP14, WCP18, CEP 18	1 (12.5)	1 (12.5)	3 (37.5)	3 (37.5)
D	2	CEP12, YACs 858D9, 922C8	1 (50.0)	0	1 (50.0)	0
E	19	WCP11, CEP11, CEP16	5[c] (26.3)	1 (5.2)	5 (26.3)	8 (42.1)
F	5	WCP9, CEP9, WCP11, 11qtel	0	2 (40.0)	0	3 (60.0)
G	11	WCP11, CEP11, WCP16	3[d] (27.2)	1 (9.0)	3[e] (27.2)	4 (36.3)
H	15	R1: CEP4, CEP12/R2: W4, W12	0	1 (6.6)	7 (46.6)	7 (46.6)
I	9	R1: Tel18q, CEP18, W18/ R2: W1, W18	1 (11.1)	0	3 (33.3)	5 (55.5)

[a]Fragmented 1PBs are not included.
[b]1PBs lost during biopsy or fixation, or FISH not informative.
[c]Two as a result of chromatid recombination.
[d]One as a result of recombination between chromatids and not whole chromosomes.
[e]One haploid.1PB = first polar body; ND = no diagnosis; UnB = unbalanced.

In our cases, a total of 80 oocytes was recovered from six patients. Of these oocytes, 63 (78.7%) were mature, and 57 of them could be processed for 1PB analysis, while six were fragmented 1PBs (Tables I and II). The remaining oocytes were either lysed during removal of the cumulus cells (8.7%) or immature oocytes (12.5%). In four cases 1PB biopsy was carried out drilling the ZP with a laser beam, in one case it was carried out by PZD, and in another case using acid Tyrode's.

In summary, PGD by 1PB analysis has been used in 24 female translocation carriers; 325 COCs have been obtained (220 from Robertsonian translocation carriers and 105 from reciprocal translocation carriers), 294 (90.5%) of the oocytes were biopsied (211 from Robertsonian translocation carriers and 83 from reciprocal translocation carriers), and 224 (68.9%) of the oocytes were diagnosed by FISH (174 from Robertsonian translocation and 50 from reciprocal translocation carriers).

The FISH strategies used so far include WCP (Table V; cases a–k and m) (Figure 1f and g) combined with telomere, centromere or locus-specific probes in a single hybridization round (Table VI; cases A–G) or in two successive hybridization rounds (Table V; cases n–q; cases H and I).

In Robertsonian translocations, the percentages of 1PB diagnosis have been ~80%, with three cases reaching 100% (Table VII). In reciprocal translocations (Table VIII) the

Table VII. Robertsonian translocations: preimplantation genetic diagnosis (PGD) using 1PB biopsy

Pt no.	1PB diagnosis[a]	Embryos transferred	Pregnancy	Outcome
a	87.5			
b	90.4			
c	75.0	Authors summarized 16 cycles of PGD using 1PB biopsy from 11 patients. Seven achieved pregnancy after 41embryo transfers with 13 implantation sacs. One pregnancy (two fetuses) was ongoing, and of 11 fetuses, nine were chromosomally normal and two were balanced.		
d	93.7			
e	100.0			
f	100.0			
g	73.3			
h	100.0			
i	81.8			
j	83.3			
k	87.5			
m	53.9	1	0	–
n	50.0	0[b]	–	–
p	75.0	2[c]	1	SA, 7th week
q	50.0	0+1[d]	–	–

Table VIII. Reciprocal translocations: preimplantation genetic diagnosis (PGD) using 1PB biopsy

Pt no.	1PB Diagnosis[a]	Embryos transferred	Pregnancy	Outcome
A	83.4	0+2[b]	1	SA, 7th week
B	75.0	2	2	2 babies
C	62.5	0	–	–
D	100.0	1	0	–
E	57.9	0+0[b]	0	–
F	40.0	1	1	1 baby
G	63.6	1	1	–
H	53.4	0	–	–
I	44.4	0	–	–

[a]Fragmented 1PBs are not included.
[b]Two cycles of PGD and two embryo transfers.
SA = spontaneous abortion.

Table IX. Comparison between the observed segregation in oocytes or embryos from female reciprocal translocation carriers and the frequency of one or more interstitial chiasma in each arm involved in the reorganization

Female karyotype	Type of cell	Diagnosed cells	Type of segregation								Interstitial chiasmata (%)[a]		Non-disjoined homologues (%)[b]		Reference
46,XX,t(A;B)			Nor	Bal	Ad1	Ad2	3:1A	3:1B	4:00	ND or mosaic	A	B	A	B	
46,XX,t(11;16)(q21;q22)	1PB, Blt	17	2	6	2	1	3	1	1	1	24.0	2.0	17.6	23.5	Munné *et al.* (1998d)
46,XX,t(5;8)(p13;p23)	Blt	9	2	0	3	0	0	0	0	4	0	0	0	0	Pierce *et al.* (1998)
46,XX,t(6;21)(q13;q22.3)	Blt	9	1	0	2	1	2	3	0	0	55.0	5.0	44.4	22.2	Conn *et al.* (1999)
46,XX,t(11;16)(q21;q22)	1PB, Blt	10	4	2	2	0	1	1	0	0	24.0	2.0	10.0	10.0	Willadsen *et al.* (1999)
46,XX,t(4;12)(q22;q23)	1PB	11	1	0	0	4	3	1	2	0	81.0	52.0	81.8	63.8	Present study

[a]Frequencies of cells with one or more chiasma in the interstitial region on the arm of either chromosome A or B involved in the reorganization (Laurie and Hultén 1985a,b).
[b]Frequencies of cells showing a segregation with non-disjunction for either chromosomes A or B.
Ad1 = adjacent 1; Ad2 = adjacent 2; Bal = balanced; Diag. = diagnosed; hom. = homologues; ND = no diagnosis; Nor = normal,

percentages of 1PB diagnosis have varied considerably, from 40 to 80%, with a single case in which only two oocytes were biopsied and diagnosis was possible in both (100%).

Tables V and VI show the well-known tendency of many female carriers of balanced translocations to produce high percentages of unbalanced oocytes, which cannot be used for fertilization or, if fertilized, result in abnormal embryos which also cannot be transferred.

Summarising data of PGD by 1PB analysis in reciprocal translocation female carriers, 60.3% (105/174) of the oocytes diagnosed were either normal ($n = 69$) or balanced ($n = 36$), while 39.7% (69/174) were unbalanced.

With regard to Robertsonian translocations (Table V), 10 cases of Rob (13;14) and five cases of Rob (14;21) have been studied (Munné *et al.*, 2000). As can be seen in Table V, the percentage of unbalanced oocytes varied widely, from 7.6 to 52.3%, although in most cases the frequency of unbalanced oocytes ranged from 25 to 40%. Summarising data of PGD by 1PB analysis in Robertsonian translocations, 44% (22/50) of the oocytes diagnosed were either normal ($n = 11$) or balanced ($n = 11$), while 56% (28/50) were unbalanced.

It should be noted that in Robertsonian translocations, if the number of oocytes available for biopsy is high (i.e. >10), the percentage of diagnosed 1PBs tends to be higher than the mean (80%) (Table VII). On the other hand, in reciprocal translocations the percentage of diagnosed oocytes does not increase with the number of oocytes available.

PGD using 1PB biopsy, as a consequence, provides information from a large number of the oocytes including those that ultimately will not be fertilized, thus allowing for more reliable genetic

counselling. Summarising all the cases in which PGD of 1PB biopsies was used, 76.2% (224/294) of the oocytes biopsied were diagnosed.

As indicated above, after PGD using 1PB biopsy it is possible to carry out a PGD using blastomere biopsies in those embryos not diagnosed due to different causes (fragmented 1PB, 1PB lost during biopsy, 1PB lost during fixation, 1PB showing chromosomes with two different chromatids after a meiotic recombination process, or 1PB with non informative FISH). To date, PGD in 1PB biopsies has been used in combination with blastomere biopsies in six cases (Table IV, case E from Munné *et al*., 1998c; Table IV, cases F and G from Willadsen *et al*., 1999; and Table IV, case I and Table III, cases p and q of the present report). In five of these (all except case I) the 1PB biopsy was performed using the laser system or PZD; the frequency of undiagnosed oocytes after 1PB analysis was 43.3% (23/53), a percentage that was reduced to16.9% (9/53) after PGD blastomere analysis. Only three embryos were diagnosed as normal or balanced after the double biopsy procedure, and were finally transferred. Two of them implanted, with one producing a normal, full-term pregnancy, indicating that embryo development was not affected. However, many more cases will have to be analysed to draw reliable conclusions.

To date, and using PGD by 1PB analysis, 52 embryos have been transferred, 19 implanted, and 17 gave a positive heart beat test (pregnancy rate 32.7%). The ESHRE PGD Consortium (2000) considered 175 cases carriers of a chromosome disorder performed by PGD using blastomere analysis; 308 embryos were transferred, 40 implanted and 30 gave a positive heart beat test (pregnancy rate 9.7%).

Theoretical considerations on chromosome segregation

In Robertsonian translocations there is no correlation between the number of chiasmata and the type of segregation, because the number of chiasmata in the trivalent does not depend on the presence of recombination hot spots. The resolution of chiasmata is not interfered with, by the presence of centromere regions, and segregation depends on the orientation of the centromeres.

Otherwise, in reciprocal translocations the presence of a high frequency of recombination between homologues could favour the adjacent II type of segregation following the mechanism proposed (Lamb *et al*., 1996) to explain one of the origins of non-disjunction, while the presence of open configurations would favour the 3:1 and 4:0 types of segregation. Thus, the segregation resulting from the resolution of the quadrivalent could be influenced by the presence of an increased number of chiasmata in one or both interstitial regions, and by the presence or absence of chiasmata in the pairing regions of the quadrivalent.

We have tried to determine if a correlation exists between the presence of recombination hot spots in the interstitial segments of these reciprocal translocations in which at least nine oocytes had been diagnosed, and the type of segregation observed (Table IX). Since no data on recombination frequencies for the female karyotype exist, we have used published data (Laurie and Hultén, 1985a,b) in the male karyotype at metaphase I. The data should be valid, taking into account that the only regions in which the human male has a higher frequency of recombination than the human female are the terminal telomeric regions (Ledbetter, 2000). By applying Pearson's correlation test to the frequency of non-disjunction of homologues in each translocation and the frequency of chiasmata in the interstitial segments (Laurie and Hultén, 1985a,b), a correlation coefficient of 0.8698 ($P < 0.01$) was found, which was highly significant. Thus, it is suggested that resolution of the quadrivalent and segregation of the chromosomes involved in the translocation is highly related to the presence or absence of interstitial chiasmata. This is supported by the absence of adjacent II segregation in one reported case of a translocation 46,XX,t(5;8)(p13;q23) in which the frequency of interstitial chiasmata in the chromosomes involved was zero (Pierce *et al*., 1998).

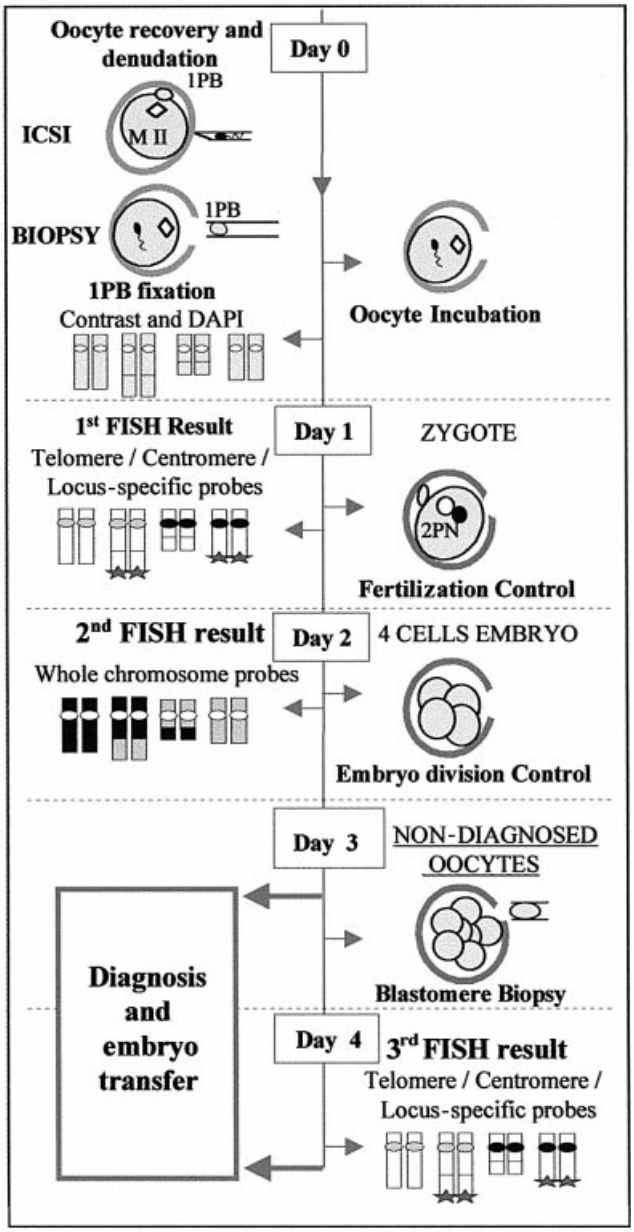


Figure 2. Proposed preimplantation genetic diagnosis (PGD) protocol. If FISH of the 1PB is not informative, or one of the chromatids shows recombination, a blastomere biopsy is performed.

Conclusions

These conclusions are based on our personal experience with PGD using 1PB biopsy and on previously published data (Munné *et al*., 1998a–c, 2000; Willadsen *et al*., 1999). The following considerations (see also Figure 2) can be useful to obtain a higher fertilization and diagnostic efficiency, as well as better 1PB chromosome preparations. Other equally valid diagnostic strategies can also be found (Munné *et al*., 1998a–c, 2000; Willadsen *et al*., 1999).

First, in our hands it is useful to perform ICSI in all oocytes with an apparently normal 1PB previous to PZD or laser biopsy. Second, it seems adequate to remove the 1PB once detached from the oocyte, using a smooth-opening pipette with a diameter slightly larger than the 1PB. Third, as hypotonic treatment it is most effective to use 0.5% trisodium citrate for 5 min, whilst methanol:acetic acid (1:1) which has been kept in the freezer is most effective as a fixative. Fourth, it is a good strategy to obtain phase contrast microscopy and DAPI images before FISH is applied. Subsequently, centromere, locus-specific, yeast artificial chromosomes, telomere or WCP probes for the chromosomes involved in the translocation can be used. Fifth, in case of undiagnosed oocytes due to fragmented 1PBs, 1PBs lost during biopsy or fixation, or even in cases in which the 1PB seems to have recombinant chromatids, it is possible to perform blastomere biopsy.

As a final conclusion, in female carriers of chromosome reorganizations, PGD using 1PB seems to be at least as good an approach as PGD of blastomeres, to select the oocytes to be fertilized. To date, the results obtained using PGD of 1PBs are far better than those obtained using blastomere biopsies, although this might be related to the characteristics of the translocations analysed, to a different behaviour of the translocation depending on the sex of the carrier, or even to chance, taking into account the low number of embryos transferred using these two procedures

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