

# Scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18 and 21

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**Fluorescence in-situ hybridization (FISH) for application in preimplantation genetic diagnosis (PGD) of aneuploidy has been used successfully, but stringent scoring criteria to score FISH signals have not been developed. In the present study a FISH protocol to simultaneously enumerate chromosomes X, Y, 13, 16, 18, and 21 was used to evaluate two different scoring criteria. The criteria consider hybridization signal size, shape, and vicinity to other signals and nuclear diameter. For this purpose, 74 embryos (412 blastomeres) donated for research had most or all of their cells analysed. The least error-prone criterion (9%) was selected for use in PGD cases. Some probes produced more errors than others, and these criteria may provide clues to improve these probes. The same probe solution was applied to 55 PGD cases and a total of 307 embryos. Of the non-transferred embryos, 67 were fully reanalysed and 1.5% (1/67) of them were falsely diagnosed as normal, while 19% (13/67) were falsely diagnosed as abnormal. Twelve of the patients became pregnant after PGD.**

**Key words:** aneuploidy/blastomere/cytogenetics/embryo/Mosaicism

## Introduction

Fluorescence in-situ hybridization (FISH) allows chromosome enumeration to be performed on interphase cell nuclei, i.e. without the need for culturing cells or preparing metaphase spreads. FISH has been applied to the preimplantation genetic diagnosis (PGD) of X-linked diseases (Griffin *et al.*, 1992; Munné *et al.*, 1993a; Harper *et al.*, 1994), common aneuploidies using either human blastomeres (cells from 2–16-cell stage embryos) or oocyte polar bodies (Munné *et al.*, 1993b, 1995a,b; Verlinsky *et al.*, 1996a,b) and, more recently, translocations (Conn *et al.*, 1998; Munné *et al.*, 1998a,b). However, PGD is still not widely applied due to a lack of standardization. Although FISH is widely used for genetic analyses, its reliability depends on the types of probes, cells and their fixation. Counting criteria and error margins need to be established for each new combination of probes and cell types (Jenkins *et al.*, 1992). Recently we have presented scoring criteria for blastomeres when using X, Y, 13, 18 and 21 probes (Munné and Weier, 1996). The locus-specific 13 and 21 probes used in that study were improved in efficiency over the 13/21  $\alpha$ -satellite. That improvement overcame several problems involving probe 13/21, which are discussed in that study, and also defined criteria to differentiate a split target from two close targets. Errors in FISH applied to blastomeres come mostly from three sources: (i) inaccurate fixation yielding nuclei covered with cytoplasm, or too condensed, in which case the hybridization signals appear too weak, blurred and/or pulverized; (ii) overlaps of signals from the same target can produce false negative results. Overlaps of signals occur more often when the diameter of the fixed nucleus is small (Munné

*et al.*, 1996). Overlaps between signals of different targets may also produce errors, but this likelihood is low because only an exact overlap would produce an error while attention to signal shape can detect most overlaps between two signals of different targets that do not overlap completely; and (iii) single targets can split into two signals and produce false positive results. This can be caused by the fixation procedure (stretched nuclei), or the type of probe, with some probes splitting more than others (i.e.  $\alpha$  satellite probe for chromosome 18, satellite III probe for chromosome Y), or the cellular stage, where double-dotted signals can also represent two sister chromatids after reduplication (Mukherjee *et al.*, 1992, Wyrobek *et al.*, 1994).

In a typical PGD setting, only one or two cells can be analysed from the same embryo, requiring high efficiency and specificity. One of the objectives of our study was to evaluate the efficiency of X, Y, 13, 16, 18, and 21-chromosome specific probes when used simultaneously in single blastomeres. For this purpose, combinational coding of the in-situ hybridization probes with three spectrally distinct fluorescent labels was used to provide visibly distinguishable staining of each of the six chromosome targets (for a review of combinatorial coding, see Fox *et al.*, 1995). The other objective was to analyse the occurrence of false positive and false negative FISH results in relation to the distance between hybridization signals, their shape and size, and the nuclear diameter. For these purposes, embryos donated for research were used to determine the efficiency of the new set of probes, and to develop strict scoring criteria. Once optimal criteria were derived, the same probes were used for PGD.

## Materials and methods

### Source of embryos

Embryos for this study were obtained from two sources. As a first group, embryos donated for research were obtained from the in-vitro fertilization (IVF) programme of The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center, Livingston, USA, and in accordance with guidelines approved by the Internal Review Board of Saint Barnabas Medical Center, including written consent from the patients in each case. Only monospermic embryos developing from bipronucleated zygotes were used for this study. These embryos had most or all of their cells analysed. Embryos donated for research were desegregated on day three of development and their cells were individually fixed following our protocol for blastomeres (Munné *et al.*, 1996).

A second group of embryos was obtained from patients undergoing PGD for aneuploidy. These embryos were classified as chromosomally abnormal by PGD or developmentally non-viable prior to full analysis of the embryo for this study. PGD embryos were obtained from couples undergoing preimplantation genetic diagnosis of aneuploidy at the SISMER Reproductive Medical Unit (Bologna, Italy).

Once scoring criteria were developed, based on the results obtained with the above embryos, PGD cases were performed in three centres, Saint Barnabas Medical Center (West Orange, NJ, USA), SISMER (Bologna, Italy), and RBA (Atlanta, GA, USA). Written consent was obtained from all PGD patients in accordance with their protocols. During day three of development, one or two cells per embryo were biopsied, and the embryos returned to culture as described elsewhere (Grifó, 1992). All of the embryos were at the 4–12-cell stage of development at the time of biopsy. Most embryos classified as normal after PGD were transferred to the uterus on the same day of analysis. Only those embryos classified as abnormal or developmentally arrested were fully biopsied on late day 3 or early day 4 and had most or all of their cells analysed. All blastomeres were fixed individually following our protocol for blastomeres (Munné *et al.*, 1996).

### FISH procedure

A FISH scheme described previously for the simultaneous analysis of chromosomes X, Y, 13, 18 and 21 (Munné and Weier, 1996) was modified to include a probe for chromosomes 16. The probe set consisted of a unique sequence locus-specific identifier probes (LSI™) and repetitive sequence centromere enumeration probes (CEP™) obtained from Vysis Inc (Downers Grove, IL, USA). It included LSI 13 (RB-1 locus, 13q14, expanding 440 Kb), LSI 21 (region 21q22.13–q22.2), CEP 16 ( $\alpha$  satellite), CEP 18 ( $\alpha$  satellite, D18Z1), CEP X ( $\alpha$  satellite, DXZ1) and CEP Y (satellite III, DYZ1). The CEP 16  $\alpha$  satellite probe was a Vysis developmental probe that has not been commercialized and has since been superseded by a satellite III probe that does not have the cross-hybridization problem associated with the  $\alpha$  satellite probe. Probes labelled with Spectrum-Orange™, Spectrum-Green™, and Spectrum-Aqua™ were combined in the following quantities to produce visually distinct colours when viewed with the Vysis™ Aqua/Green/Orange triple band-pass filter set (volumes refer to commercial stock solutions except where indicated with specific concentration values): 16  $\mu$ l Spectrum-Aqua CEP Y, 12  $\mu$ l Spectrum-Green CEP Y, 36  $\mu$ l Spectrum-Aqua CEP X, 22  $\mu$ l Spectrum-Aqua CEP 18, 14  $\mu$ l Spectrum-Orange CEP 18, 30  $\mu$ l Spectrum-Orange LSI 13 (200 ng/ $\mu$ l), 24  $\mu$ l Spectrum-Green LSI 13 (200 ng/ $\mu$ l), 30  $\mu$ l Spectrum-Orange LSI 21 (200 ng/ $\mu$ l), and 30  $\mu$ l Spectrum-Green CEP 16 (125 ng/ $\mu$ l). Additional blocker DNA was not added since this was included in the commercial probe solutions. The above probe mixture was concentrated with a Speed-Vac centrifugal evaporator (DyNA VAP; National Labnet company) to a final

volume of 90  $\mu$ l, and added to 210  $\mu$ l of WCP hybridization buffer (Vysis). The resulting hybridization solution (10  $\mu$ l) was applied to the glass slide containing fixed blastomeres and covered with an 18×18 mm coverslip. The slide was then placed for 3 min on a slide warmer preheated to 78°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for 3–4 h. After the hybridization, the slides were washed individually at 72.5°C in 0.4× sodium chloride/sodium citrate (SSC) for 2 min. The slides were then mounted with 10  $\mu$ l of 4',6-diamino-2-phenyl indole (DAPI; Vysis) counterstain in antifade solution and observed with a fluorescent microscope (Olympus BX40 or BX60) at ×900 magnification (×60 immersion lens, ×15 ocular), equipped with the triple-band pass filter set for simultaneous observation of the Spectrum-Orange, Spectrum-Green, and Spectrum-Aqua fluorescence. When viewed through the triple band-pass filter set, the Y-chromosome specific signal appeared white, the X signal as blue, the 18 as magenta, the 13 as orange, the 21 as red, and the 16 as a green domain. A DAPI filter (Olympus) was applied to find the nuclei. Digital computer imaging was not necessary for analysis although it was used for image storage.

### Scoring criteria

A scoring criterion ('mosaicism criterion') for differentiating false-positives and false-negatives from mosaicism has been previously described (Munné *et al.*, 1994), and was used here without modification. The specific FISH signals detected in a given blastomere were considered to reflect a true chromosome constitution in the following instances: (i) blastomeres with two gonosome and two chromosome 13, 16, 18 and 21 specific signals (these were considered to be diploid blastomeres); (ii) embryos in which all the blastomeres had the same abnormality (e.g. aneuploid, haploid or polyploid embryos); (iii) individual blastomeres that have only one signal per chromosome pair (these were considered to be haploid cells); (iv) individual blastomeres that had three or more signals per chromosome pair (these were considered to be polyploid cells); (v) individual blastomeres that had extra or missing signals compensated respectively for the missing or extra signals in sibling blastomeres (these were assumed to belong to an embryo with mosaicism generated by mitotic non-disjunction); (vi) blastomeres showing fewer signals than their sibling blastomeres and belonging to mosaic embryos resulting from the uneven cleavage of a blastomere without previous DNA synthesis (e.g. an embryo with mostly XX 1313 1616 1818 2121 cells, plus XO 130 1616 1818 OO and XO 130 OO OO 2121 cells); (vii) the same criteria (i–vi) applied to multi-nucleated blastomeres; (viii) Blastomeres with less or more than two gonosomes or chromosome 13, 16, 18 or 21 specific signals were considered respectively FISH false-negative or false-positive errors unless one of the prior criteria (ii–vii) applied.

To differentiate between a split target producing two hybridization signals, and two targets close together, our previous criteria ('criteria 1') was compared with a new one ('criteria 2'). Criteria 1 stated that two signals represent two homologue chromosomes when their distance apart was at least two domain diameters (Munné and Weier, 1996). The distance between two hybridization signals, specific for the same chromosome, was measured in domains, with a domain being the diameter of one of these signals, but with domain sizes for each chromosome type having a different area. The design of criteria 2 takes into account five different parameters: total number of signals for the same chromosome, distance between close signals, contacts between close signals, similarity in size between close signals, and similarity in size to a third signal. Criteria 2 is shown in Table I, and it was based on several assumptions: (i) when a signal splits due to stretching it normally splits in unequal domains linked by DNA fibres, and with both domains being smaller than the domain for the homologue chromosome (the fibres, however, can only be differenti-

**Table I.** Criteria 2 for two close signals

Relation to third signal	Domains apart	In contact	Close signals equal to each other	Classified as
No third	>2	No	Yes/no	Two chromosomes
No third	>2	Yes	Yes	Two chromosomes
No third	>2	No	No	One chromosome
No third	0.5–2	<sup>b</sup>	Yes/no	One chromosome
Equal	>2	Yes/no	<sup>a</sup>	Two chromosomes
Equal	0.5–2	<sup>b</sup>	<sup>a</sup>	Two chromosomes
Smaller	>2	No	Yes/no	Two chromosomes
Smaller	>2	Yes	Yes/no	One chromosome
Smaller	0.5–2	<sup>b</sup>	Yes/no	One chromosome

<sup>a</sup>If they are equal to a third signal they have to be equal among them

<sup>b</sup>Connections between signals 0.5–2 domains apart cannot be distinguished from signal blur.

ated from blurred signals when the two pieces of the split signal are far apart, arbitrarily set at less than two domains); (ii) when a homologue has two chromatids in interphase they are assumed to appear as doublets (Arnoldus *et al.*, 1990), the two chromatids are equal in size and both smaller than the domain of the other homologue; (iii) a signal seldom splits into two signals separated more than two domains apart, as shown before (Munné and Weier, 1996); (iv) when a nucleus is very decondensed the centromeric region of chromosome 18 and the satellite-III region of chromosome Y tend to become two domains not linked by DNA fibres (these two domains are invariably unequal in size) (Wyrobek *et al.*, 1994). Chromosomes X and 16, although also studied with centromeric or satellite probes, do split less than chromosomes 18 and Y; (v) when in doubt in a PGD case, it is better to classify a disomic cell as trisomic to avoid any possibility of trisomic offspring.

After comparing the criteria 1 and 2 in donated embryos, the criterion that produced few errors was used for PGD analysis. A  $\chi^2$  test was used to compare error rates.

## Results

### **Validation of scoring criteria: embryos with three or more cells analysed**

A total of 74 embryos were included in this part of the study. These embryos were either chromosomally normal ( $n = 50$ ), or aneuploid embryos ( $n = 24$ ). A total of 429 blastomeres were analysed, of which 412 (96%) produced clear signals after FISH while the rest were damaged ( $n = 15$ ) or the cells were too condensed to produce a clear result ( $n = 2$ ). Examples of normal and trisomic blastomeres are shown in Figure 1. Non-specific hybridization of probes to other than the target chromosomes was commonly observed and was solely due to the probe for chromosome 16. Such cross-hybridization was however far less intense than the specific signals, and did not prevent correct scoring of chromosome 16.

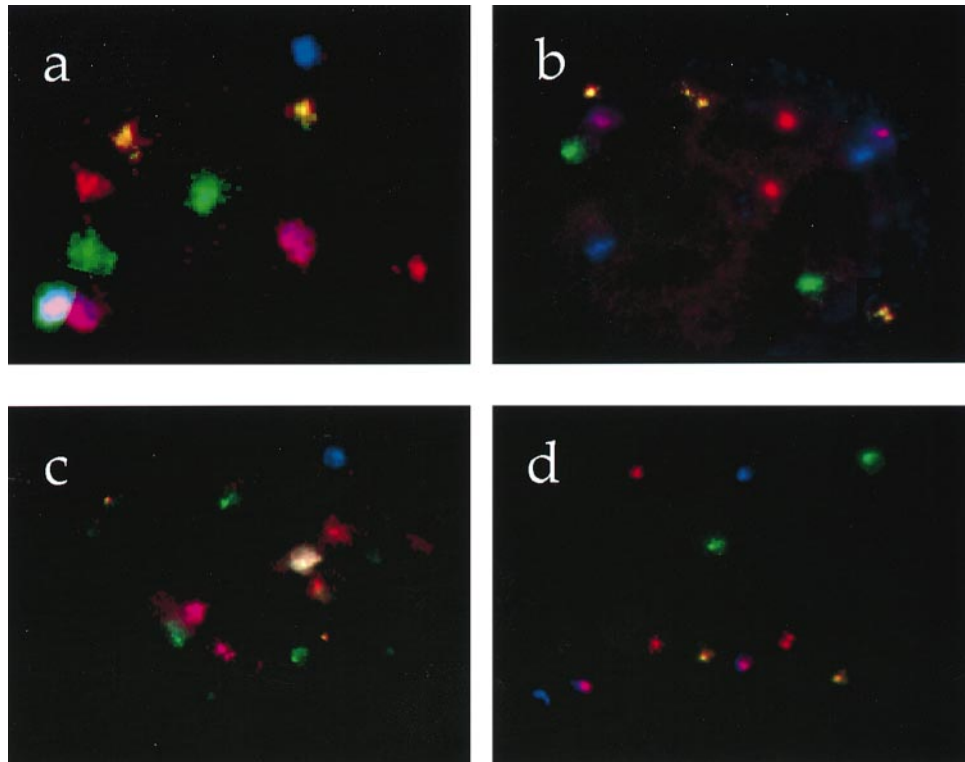
As shown in Table II, criterion 1 produced fewer errors than 2 and therefore criterion 1 was chosen as the best scoring criteria and was used thereafter. According to criteria 1 and the mosaicism criteria to differentiate between mosaicism and false FISH results, the overall error rate was 9% (37/412) for all embryos.

Three types of errors were observed: (i) type A errors ( $n = 21$ ) in which one signal was missing, with no split or close signals for the chromosome affected, while the rest of the

embryo was diploid ( $n = 17$ ), or trisomic ( $n = 4$ ); (ii) type B errors ( $n = 12$ ) produced by the use of the scoring criteria when trying to differentiate a single split signal from two close signals. The most frequent occurrence of type B error was a cell classified as trisomic instead of disomic ( $n = 10$ ), followed by classifying a trisomic cell as disomic ( $n = 2$ ); and (iii) type C errors ( $n = 4$ ) in which an extra signal was observed, with no split or close signals for the chromosome affected, in embryos with the rest of cells being diploid ( $n = 3$ ) or monosomic ( $n = 1$ ). The chromosomes most often involved in misclassification were chromosome 18 (12 errors: six false trisomies, five false monosomies, one false disomy in a monosomy 18), followed by chromosome 21 (10 errors: four false monosomies, four false disomies in trisomies, two false trisomies), chromosome 13 (six false monosomies, two false trisomies), chromosome 16 (three false monosomies, two false trisomies, two false disomies in trisomies), chromosome X (three false monosomies, one false trisomy) and chromosome Y (one false trisomy). Some cells had more than one error, hence the addition of the errors per chromosome is higher than the total error rate.

By relating nuclear diameters and errors, we found that overlaps and close signals decreased with increasing diameter ( $P < 0.01$  and  $P < 0.025$  respectively) from 13% overlaps and 9% close signals ( $n = 104$  nuclei) in nuclei  $< 50 \mu\text{m}$  in diameter to 2% overlaps and 4% close signals ( $n = 92$  nuclei) in nuclei  $> 69 \mu\text{m}$  in diameter. However, the decrease in the number of type A errors with increasing diameter (from 8 to 2%) in nuclei  $< 50 \mu\text{m}$  compared with nuclei  $> 69 \mu\text{m}$  was still not statistically significant.

Different types of possible arrangements when a single target splits or two targets fall close together can occur as shown in Table II. One of the common situations was when two signals were separated by less than two domains and were equal in size to a third signal ( $n = 18$ ). Neither criteria can yet differentiate between split signals, chromatids and close signals, and all three produced errors. In a second situation two signals were separated by less than two domains and were smaller in size than a third signal. These invariably represented a split signal from a single chromosome ( $n = 25$ ). A third common situation was chromosome Y splitting. When chromosome Y was involved it invariably represented a single



**Figure 1.** In-situ hybridization with X, Y, 13, 16, 18 and 21 chromosome-specific DNA probes. The chromosome X-specific probe is shown labelled in blue, chromosome Y in white, chromosome 13 in orange, chromosome 16 in green, chromosome 18 in magenta, and chromosome 21 in red. (a) Normal male blastomere (X, Y, 13, 13, 16, 16, 18, 18, 21, 21) with a slight overlap of the Y signal with an 18 signal; (b) trisomy 13 female blastomere (X, X, 13, 13, 13, 16, 16, 18, 18, 21, 21) with two chromosome 13 split domains; (c) trisomy 16 male blastomere (X, Y, 13, 13, 16, 16, 16, 18, 18, 21, 21); and (d) trisomy 21 female blastomere (X, X, 13, 13, 16, 16, 18, 18, 21, 21, 21) with one chromosome 21 split domain.

**Table II.** Characterization of close signals and evaluation of scoring criteria

Embryo	Relation to third signal	Domains apart	In contact	Close signals equal to each other	X	Y	13	16	18	21	Total no.	No. of chromosomes by criteria		Criteria 1 errors	Criteria 2 errors
												1	2		
Disomic	No third	0.5-2	b	c	1	13				1	15	1	1		
Disomic	Equal	>2*	c	a			1	1	2	2	6	2	2	6	6
Disomic	Equal	0.5-2	b	a	2		3	5	6	2	18	1	2		18
Disomic	Smaller	>2*	No	c			1		2	1	4	2	2	4	4
Disomic	Smaller	0.5-2	b	c	3		6	3	11	2	25	1	1		
<b>Total disomic</b>					<b>6</b>	<b>13</b>	<b>11</b>	<b>9</b>	<b>21</b>	<b>8</b>	<b>68</b>			<b>10</b>	<b>28</b>
Trisomic	Smaller	0.5-2	b	Yes						1	1	1	1	1	1
Trisomic	Equal	>2*	No	a			3		1	1	4	2	2		
Trisomic	Equal	0.5-2	b	a			1				1	1	2	1	
Monosomic	No third	0.5-2	b	Yes			2				2	1	1		
<b>Total aneuploid**</b>					0	0	2	4	0	2	8			2	1

<sup>a</sup>When two signals are equal to a third one, they must be equal among them.

<sup>b</sup>Connections between signals 0.5-2 domains apart cannot be distinguished from signal blur.

<sup>c</sup>Either possibility.

\*Signals more than four domains apart only recorded if they were in contact and/or smaller than third signal.

\*\*Aneuploid for the chromosome splitting or close to an homologous signal.

chromosome. The criteria are planned to account for all chromosomes, but must make an exception for chromosome Y to avoid false XYY trisomies. For chromosomes involved in splitting or associations of chromosomes, probes for chromosomes Y and 18 split the most, while the other chromosomes split less. The differences between chromosomes do not seem

to be related to the type of probe (LSI versus CEP) but to the size of the probe, the bigger, the more splitting.

When these results are compared with our previous protocol in which chromosome 16 was excluded (Munné and Weier, 1996), more errors were found with the present protocol in both spare embryos (9% of 412 cells compared with 5% of

487 cells,  $P < 0.025$ ), false normal PGD results (9% of 11 cells compared with 0% of 21 cells, not significant) and false abnormal PGD results (23% of 56 cells compared with 14% of 43 cells, not significant).

### PGD repeat analysis

The same probe solution was applied to 55 PGD cases and a total of 307 embryos. Of the non-transferred embryos, 67 had their remaining cells analysed. Of the embryos previously classified during PGD as abnormal, 77% (43/56) had their result confirmed. On the other hand, of the embryos previously classified during PGD as normal 91% (10/11) had their result confirmed (Table III). The errors occurred by misdiagnosing a monosomic embryo as normal, 12 normal embryos as monosomic ( $n = 8$ ), trisomic ( $n = 2$ ), or complex abnormal ( $n = 2$ ), and a mosaic embryo with 33% tetraploid cells as tetraploid.

### Other results

By combining all the cells from spare embryos and PGD cases, a total of 871 cells was analysed. From those cells an interesting observation was made, in that the type of fixation seems to be related to type A errors. During fixation, one to two drops of fixative are dropped on top of the blastomere, then the blastomere lysed and, after that, another drop was added to eliminate the remnants of the cytoplasm. We found that if only one drop of fixative was added at the beginning of fixation (pre-lysis), fewer errors were observed than with two drops (one drop: 9/569 (1.6%) missing signals versus two drops: 19/302 (6.3%) missing signals,  $P < 0.001$ ). Neither the day of fixation (day 3 or day 4) nor characteristics of embryo development correlated with type A errors, nor was any other correlation found with other types of errors.

### Pregnancy outcome after PGD

A total of 55 PGD cases, involving 307 analysed embryos, were performed in three different centers using the probe combination evaluated here. A total of 113 embryos were transferred in 45 cases (all biopsied and analysed). Pregnancy results indicated that 12 patients became pregnant, 14 fetal heartbeats being detected.

### Discussion

FISH analysis for PGD of aneuploidy has been attempted at the oocyte, zygote or early cleavage-stage embryo (Munné *et al.*, 1993b, 1995a,b, Harper *et al.*, 1995a,b; Munné and Weier, 1996; Verlinsky *et al.*, 1996a,b). None of these stages permit analysis of more than one or two cells, therefore, very efficient multi-probe solutions and scoring criteria should be developed. More cells can be available through blastocyst biopsy (Benkhalifa *et al.*, 1993, Muggleton-Harris *et al.*, 1995) but PGD of aneuploidy at that stage has not yet been attempted.

The present protocol allows simultaneous screening of the aneuploidies at risk of developing to term, plus the screening for aneuploidy 16, the most common abnormality found in spontaneous abortions. In addition, two scoring criteria have been assessed to minimize errors caused by split or close

signals (type B error). Strict scoring criteria for FISH analysis have previously been developed for spermatozoa and were based solely on distance between same-chromosome signals (Wyrobek *et al.*, 1994; Martin and Rademaker, 1995; Robbins *et al.*, 1995). However, compared with our previous protocol (Munné and Weier, 1996) in which the chromosome 16 probe was not used, the scoring reliability decreases from 95 to 91% in spare embryos ( $P < 0.025$ ), from 0% false normal PGD results to 9% and from 14% false abnormal PGD results to 23%. This decrease was expected because each additional probe added to a probe solution increases the potential for error, due to the error produced by the hybridization of the probe, and by the complexity of scoring multiple signals. Still, a 9% PGD misdiagnosis of abnormal embryos as normal could be considered acceptable because many more embryos are aneuploid for chromosome 16 (12% in women aged  $\geq 40$  years; Benadiva *et al.*, 1996). In addition, most misdiagnoses of normal embryos as abnormal are due to false monosomies. Since autosomal monosomies are lethal and rarely implant, in the event that not enough morphologically and genetically good embryos are available for transfer, potential monosomies could also be transferred with minimal risk. This policy is being used in our labs under such circumstances.

Both scoring criteria were designed to minimize false negatives in trisomic embryos at the expense of classifying some normal embryos as abnormal. This bias is understandable in an IVF setting, where excess embryos are routinely produced, but only two to four embryos can be transferred at a time. In this respect, criteria 1 (Munné and Weier, 1996) produced far fewer misdiagnoses.

The present set of probes provides an acceptably low error rate, although this can still be improved in several ways. The probes for chromosomes 18 and Y should be substituted by locus-specific probes, which presumably will not split as often nor be polymorphic. The reassuring observation that the new probe for chromosome 13 (expanding 440 Kb) splits far less than the old one (expanding 280 Kb), indicates that signal splitting is also a function of location in addition to length. The probe for chromosome 16 should be made more specific because slight changes in denaturation temperature, as low as 0.5°C less than optimal, can render the slide unreadable due to an excess of non-specific chromosome 16 cross-hybridization. Use of a unique sequence (LSI) probe for chromosome 16 should eliminate this problem in the future.

Other types of errors were not the result of splitting or close signals but were caused by the complete absence of a signal (type A errors). One source of type A errors could be the overlap of chromosome signals. As indicated in a previous study and also shown in this one, the smaller the diameter of the nucleus, the more overlaps and missing signals (Munné *et al.*, 1996). However, since we saw only 7% overlap between five types of chromosomes, that means that an overlap between two signals for the same chromosome (which would produce a missing signal) would occur only 1.4% of the time, which is far less than the 5% of type A errors observed. Another source of type A errors could be the loss of DNA during fixation. This could occur through the loss of micronuclei, and/or extra-nuclear chromosomes and DNA fibres, because,

**Table III.** Preimplantation genetic diagnosis (PGD) repeat analysis

Embryo	No. of cells analysed	PGD diagnosis	Re-analysis diagnosis	Misdiagnosed cell	Embryo
01	6	Normal	Normal	No	No
02	8	Normal	Normal	No	No
03	3	Normal	Normal	No	No
04	3	Normal	Monosomy 16	Yes	Yes
05	6	Normal	Normal	No	No
06	4	Normal	Normal	No	No
07	6	Normal	Normal	No	No
08	5	Normal	Normal	No	No
09	7	Normal	Normal	No	No
10	4	Normal	Normal	No	No
11	8	Normal	Normal	No	No
12	3	ABN complex	Mosaic chaotic, 100%	No	No
13	8	ABN complex	Mosaic chaotic, 50%	No	No
14	2	ABN complex	Mosaic chaotic, 100%	No	No
15	5	ABN complex	Trisomy 21, mosaic chaotic 50%	No	No
16	2	ABN complex	Mosaic chaotic, 100%	No	No
17	6	ABN complex	Mosaic chaotic, 100%	No	No
18	3	ABN complex	Mosaic chaotic, 66%	No	No
19	8	ABN complex	Normal	Yes	Yes
20	3	ABN complex	Polyploid mosaic 100%	No	No
21	9	ABN complex	Mosaic chaotic, 78%	No	No
22	3	ABN complex	Mosaic chaotic, 100%	No	No
23	6	ABN complex	Haploid mosaic	No	No
24	3	ABN complex	Mosaic chaotic, 100%	No	No
25	9	ABN complex	Normal	Yes	Yes
26	5	Haploid	Haploid mosaic	No	No
27	2	Haploid	Mosaic chaotic, 100%	No	No
28	3	Haploid	Haploid	No	No
29	6	Haploid	Mosaic aneuploid (16), 100%	No	No
30	6	Monosomy 13	Monosomy 13	No	No
31	9	Monosomy 13	Normal	Yes	Yes
32	8	Monosomy 13	Normal	Yes	Yes
33	3	Monosomy 13	Mosaic chaotic, 66%	No	No
34	3	Monosomy 13, 16	Normal	Yes	Yes
35	6	Monosomy 13, Trisomy 16	Monosomy 13, trisomy 16, and mosaic 33%	No	No
36	4	Monosomy 16	Monosomy 16	No	No
37	13	Monosomy 16	Mosaic 2N/4N, 33%*	Yes	Yes
38	5	Monosomy 16	Normal	Yes	Yes
39	7	Monosomy 16	Monosomy 16	No	No
40	9	Monosomy 16, 21	Monosomy 16, 21 and mosaic 25%	No	No
41	6	Monosomy 18	Monosomy 18	No	No
42	9	Monosomy 18, 16	Mosaic anaphase lag (16, 18, 21), 43%	No	No
43	2	Monosomy 21	Normal	Yes	Yes
44	3	Monosomy 21	Mosaic 2N/4N, 33%*	Yes	Yes
45	8	Monosomy X	Mosaic chaotic, 100%	No	No
46	4	Monosomy X	Mosaic chaotic, 100%	No	No
47	2	Monosomy X, 21, Trisomy 16	Monosomy X, 21, Trisomy 16	No	No
48	3	Monosomy Y, 18	Normal	Yes	Yes
49	3	Tetraploid	Mosaic 2N/4N, 33%*	No	Yes
50	8	Tetrasomy 21	Tetrasomy 21	No	No
51	4	Triploid	Mosaic chaotic, 75%	No	No
52	3	Triploid	Triploid	No	No
53	5	Trisomy 13	Normal	Yes	Yes
54	6	Trisomy 16	Trisomy 16	No	No
55	5	Trisomy 16	Mosaic aneuploid (16, X), 50%	No	No
56	8	Trisomy 16	Trisomy 16	No	No
57	2	Trisomy 18	Normal	Yes	Yes
58	4	Trisomy 18	Mosaic chaotic, 100%	No	No
59	7	Trisomy 21	Trisomy 21, 16	No	No
60	4	Trisomy 21	Mosaic aneuploid (21), 100%	No	No
61	7	Trisomy 21	Trisomy 21	No	No
62	5	Trisomy 21	Trisomy 21	No	No
63	3	Trisomy 21	Trisomy 21	No	No
64	6	Trisomy X	Trisomy X	No	No
65	4	Trisomy X	Trisomy X	No	No
66	11	Trisomy XXY	Monosomy 18	Yes	No
67	4	Trisomy XXY	Mosaic aneuploid (X), 50%	No	No

\*Mosaic embryos with low percentage (<38%) of tetraploid cells were considered to be mostly normal. If they were classified as abnormal during PGD, the embryo was considered to be misdiagnosed.

while the nucleus of the cell can be observed throughout the fixation process, micronuclei can get lost because they are too small to be seen. For instance, we found a strong correlation between one type of fixation (two drops added before cell breakage) and higher loss of chromosomes. However, later observations in our laboratory (unpublished) seem to indicate that the loss of DNA actually occurs when a drop of fixative is applied after cytoplasm lysis. In the present study, we compared only one versus two drops of fixative before lysis followed in both cases by another drop post-lysis. The drops pre-lysis allow the cytoplasm to expand, the more drops the more expansion, while the drop post-lysis removes cytoplasm debris, and probably some anuclear DNA. It is therefore possible that the loss of DNA is higher after adding a drop post-lysis when the cell is more expanded (2 drops pre-lysis instead of one) as was observed in this study. Currently we recommend two drops pre-lysis followed by no drops post-lysis, which with appropriate humidity conditions (40%) allow a good spreading, little cytoplasm debris, and minimal loss of DNA.

Type B errors, those produced by the inability of the scoring criteria to differentiate between close and split signals, were not reduced when criteria 2 were applied. Therefore, criteria based on the shape of split and/or close signals were not enough to avoid errors. Intensity of the signals would be a better or complementary measure. In addition polymorphic sites (alpha-satellite probes X, Y, 16 and 18) cannot be reliably assessed based only on size.

Less common are type C errors. These could be caused by the presence of auto-fluorescent debris or by scoring errors in which signals with similar colours (white and pink, yellow and white, etc.) are confused and counted as belonging to an incorrect chromosome type. Confusion in distinguishing different targets from one another could be reduced or eliminated by using a separate spectrally distinct fluorophore to stain each target. This would also eliminate confusion caused by the overlap of different chromosome targets. Two new different fluorochromes are currently being tested (Spectrum Red and Spectrum Gold; Vysis).

The fact that we found more errors in PGD than in spare embryos could be due to two reasons. Firstly, the fixation could have a harsher effect at the earlier time of embryo biopsy (07:00 to 11:00, early third-cell division) than at the later time spare embryos were fully desegregated and fixed (15:00 to 17:00, late third-cell to early four-cell division). The second reason could be the fact that scoring is still very dependent upon the individual. Although the criteria can be objectively applied when only one cell is present, when one or more cells from the same embryo have already been scored and recorded, it is possible to be unconsciously biased towards results that are already expected from the results on the first cells. Although we tried to be as impartial as possible, this situation cannot be completely avoided. For this reason, the true error rate for this probe solution should be closer to the one obtained after repeated PGD analysis compared with the one obtained from spare embryos.

Several groups have applied multiple-colour multiple-probe FISH analysis in tissues other than blastomeres or gametes

using mixtures of two or three fluorophores to detect three or more chromosomes simultaneously (Nederlof *et al.*, 1990; Dauwerse *et al.*, 1992; Ried *et al.*, 1992a; Morrison, 1993; Ward *et al.*, 1993; Divane *et al.*, 1994; Fox *et al.*, 1995; Morrison and Legator, 1997). In this respect, the present study probably achieves the maximum number of chromosomes detectable by eye using three fluorophores. Other combinations of these three labels are too close in colour to be scored reliably. Currently, five or more fluorescent probe labels are available, permitting the detection of all 24 chromosome types simultaneously with the aid of image analysis (Schröck *et al.*, 1996; Speicher *et al.*, 1996). However, the resolution of small signals instead of whole chromosomes and the occurrence of overlapping signals sharing one or more colours in their ratios may also cause misdiagnosis. For this reason, the ideal assay would use 24 different fluorophores, each with excitation and emission peaks well separated from the other fluorophores.

In conclusion, the present FISH protocol for PGD of aneuploidy increases the number of chromosomes being detected with only a small increase in error rate. Further improvements in scoring criteria, such as taking into account signal intensity, may reduce the number of errors observed. In the meantime criteria 1, based on between-signal separation, is still better than criteria 2, based on between-signal separation and shape. Other improvements could be the use of chromosome specific fluorochromes, which improve efficiency and the number of chromosomes analysable in a single cell. Similarly, differences in fixation methods may reduce the number of missing signals. Even taking into account these limitations, the test has been applied successfully for PGD analysis of aneuploidy.

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