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Genetic characterisation of circulating fetal cells allows non-invasive prenatal diagnosis of cystic fibrosis

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Objectives Cystic fibrosis (CF) is an autosomal recessive disease due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The purpose of this study was to develop a molecular method to characterise both paternal and maternal CFTR alleles in DNA from circulating fetal cells (CFCs) isolated by ISET (isolation by size of epithelial tumour/trophoblastic cells).

Methods The molecular protocol was defined by developing the F508del mutation analysis and addressing it both to single trophoblastic cells, isolated by ISET and identified by short tandem repeats (STR) genotyping, and to pooled trophoblastic genomes, thus avoiding the risk of allele drop out (ADO). This protocol was validated in 100 leucocytes from F508del carriers and subsequently blindly applied to the blood (5 mL) of 12 pregnant women, at 11 to 13 weeks of gestation, whose offspring had a 1/4 risk of CF. Ten couples were carriers of F508del mutation, while two were carriers of unknown CFTR mutations.

Results Results showed that one fetus was affected, seven were heterozygous carriers of a CFTR mutation, and four were healthy homozygotes. These findings were consistent with those obtained by chorionic villus sampling (CVS).

Conclusion Our data show that the ISET-CF approach affords reliable prenatal diagnosis (PND) of cystic fibrosis and is potentially applicable to pregnant women at risk of having an affected child, thus avoiding the risk of iatrogenic miscarriage. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: circulating fetal cells; genetic analyses; non-invasive prenatal diagnosis; cystic fibrosis; allele drop out (ADO)

INTRODUCTION

Cystic fibrosis (CF) is a frequently fatal autosomal recessive inherited disease affecting 30 000 persons (one in 2500 newborns) in the United States (Balinsky and Zhu, 2004), the carrier frequency varying from 1 in 20 to 1 in 40 subjects depending on the geographical area (Cystic Fibrosis Foundation, 2003. http://www.cff.org/about_cystic_fibrosis).

The disease is caused by a chloride channel defect that is attributable to mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR). Approximately, 1000 different mutations have been discovered. Among them, the F508del mutation accounts for 70% of mutated alleles worldwide (Kerem *et al*., 1989). More than ten million Americans carry this mutation and about 80% of babies born with CF are conceived by parents who have no family history of the disease (Fink and Collins, 1997). Not only is the disease relatively frequent, but also has high treatment costs and is usually fatal around 30–35 years of age (Balinsky and Zhu, 2004). These human and health-care costs might justify the widespread implementation of a prenatal genetic screening for carriers of heterozygous mutations of the CFTR gene (Schmidtke, 1998; Balinsky and Zhu, 2004; Watson *et al*., 2004). Actually, the American College of Obstetricians and Gynecologists (ACOG) officially recommends that CF carrier screening be offered to all Caucasian pregnant couples (Gilbert, 2001). However, it has been shown that most interest in screening is among pregnant women (Rowley *et al*., 1998).

Prenatal diagnosis (PND) of inherited monogenic disorders such as CF currently relies on invasive procedures—amniocentesis, chorionic villus sampling (CVS) and fetal blood sampling—which carry a significant risk of miscarriage (0.5, 1 and 3%, respectively) (Ciarleglio *et al*., 2003). Several methods have been proposed to enrich circulating fetal cells (CFCs) from blood and use them in PND (Bianchi, 1999). However, up to now no assay has been shown to be reliable enough for routine application in place of the invasive protocols. In this setting, the availability of a safe and reliable PND of CF would avoid the risk of iatrogenic miscarriage in couples heterozygous for CF mutation and would represent a major step forward in prenatal medicine.

We have previously reported about a powerful approach to enrich rare circulating epithelial tumour/trophoblastic cells on the basis of their size (ISET; isolation by size of epithelial tumour/

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trophoblastic cells) (Vona *et al*., 2000, 2002). When combined with laser microdissection, ISET allows mutation analysis of DNA from single cells demonstrated to be fetal by short tandem repeat (STR) genotyping and uncontaminated with maternal DNA.

This approach accurately detected Spinal Muscular Atrophy in 3 of 12 fetuses at risk for the disease (in agreement with the results of CVS), proving the principle of the method as a potential clinical test (Beroud *et al*., 2003).

Routine application of ISET to non-invasive prenatal diagnosis (NI-PND), however, is subject to a similar technical difficulty as that encountered in preimplantation genetic diagnosis (PGD), that is, the sporadic failure of PCR to amplify one of the two allelic sequences in a single-cell genome—so-called, 'allele drop out' (ADO) (Garvin *et al*., 1998; Hahn *et al*., 1998, 2000; Piyamongkol *et al*., 2003). For instance, ADO implies the risk of a false-positive diagnosis if the fetus is a carrier and the normal allele is lost. This is the case of autosomal recessive disorders secondary to a homozygous mutation. If the mutated allele is lost, on the other hand, the diagnosis is that of a healthy fetus when it is, in fact, a carrier.

In order to overcome this problem, we set out a molecular strategy for NI-PND of CF and assessed its feasibility and reliability. Application of this protocol to 12 couples at risk of having a child affected by CF shows that the new method avoids the risk of ADO, affording a reliable and safe PND of healthy, carrier and affected fetus that can, in principle, be extensively validated and routinely applied in clinical practice.

MATERIALS AND METHODS

Blood collection, ISET and laser capture microdissection

Six millilitres of maternal blood (obtained before CVS) and 1 mL of paternal blood were collected in ethylenediaminetetracetic acid (EDTA) buffer. Paternal and maternal DNA was extracted from 1 mL of blood and 1.5 ng was used for allelotyping with fluoresceinated primers specific for STR markers linked to the CFTR locus (D7S480, D7S486, D7S490 and D7S523) or to other genomic loci (D16S539, D16S3018, D21S1435 and

Table 1—Primer sequences and PCR profiles

Primer name	Primer sequence	PCR profile
D7S480 out	F: 5′-AAAAACCCTGGCTTATGC-3′ R: 5′-AGCTACCATAGGGCTGGAGG-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D7S480 in	F: 5′-**FAM**-CTTGGGGACTGAACCATCTT-3′ R: 5′-TTGCAATGAGCCGAGATCCTG-3′	5 min 94 °C, 40 × (30 s 94 °C, 45 s 55 °C, 30 s 72 °C), 5 min 72 °C.
D7S486 out	F: 5′-GGAATCTGTTCTGGCAATGGAT-3′ R: 5′-TTGCAATGAGCCGAGATCCTG-3′	5 min 94 °C, 40 × (30 s 94 °C, 45 s 55 °C, 30 s 72 °C), 5 min 72 °C.
D7S486 in	F: 5′-**FAM**-AAAGGCCAATGGTATATCCC-3′ R: 5′GCCCAGGTGATTGATAGTGC-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 55 °C, 30 s 72 °C),5 min 72 °C.
D7S490 out	F: 5′-AAGTAATTCTCCTGCCTCAG-3′ R: 5′-AGCTACTTGCAGTGTAACAGCATTT-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D7S490 in	F: 5′-**HEX**-CCTTGGGCCAATAAGGTAAG-3′ R: 5′-AGCTACTTGCAGTGTAACAGCATTT-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 55 °C, 30 s 72 °C), 5 min 72 °C.
D7S523 out	F: 5′-GAATTATAACCGTAACTGATTC-3′ R:5′-GAGATAATGCTTGTCTGACTTC-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D7S523 in	F: 5′-**FAM**-CTGATTCATAGCAGCACTTG-3′ R:5′-AAAACATTTCCATTACCACTG-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D16S539 out	F: 5′-CAGATGCTCGTTGTGCACAA-3′ R: 5′-ATACCATTTACGTTTGTGTGTG-3′	5 min 94 °C, 40 × (30 s 94 °C, 45 s 60 °C, 30 s 72 °C), 5 min 72 °C.
D16S539 in	F: 5′-**HEX**-GATCCCAAGCTCTTCCTCTT-3′ R: 5′-ACGTTTGTGTGTGCATCTGT-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D16S3018 out	F: 5′-GGTCATTGGTCAAGGGCTGCT -3′ R: 5′-TGACAGTGCAGCTCATGGTC-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 61 °C, 30 s 72 °C), 5 min 72 °C.
D16S3018 in	F: 5′-**FAM**-GGATAAACATAGAGCGACAGTTC-3′ R: 5-AGACAGAGTCCCAGGCATT-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D21S1435 out	F: 5′-TTGACATTCTTCTGTAAGGAAGA-3′ R: 5′-AGGCTTGCCAAAGATATTAAAAG-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 45 s 72 °C), 5 min 72 °C.
D21S1435 in	F: 5′-**HEX** -CCCTCTCAATTGTTTGTCTACC-3′ R: 5′-GCAAGAGATTTCAGTGCCAT-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 58 °C, 30 s 72 °C), 5 min 72 °C.
D21S1437 out	F: 5′-TTGTGAATAGTGCTGCAATG-3′ R: 5′-ATGTACACTGACTTGTTTGAG-3′	5 min 94 °C, 40 × (30 s 94 °C, 45 s 60 °C, 30 s 72 °C), 5 min 72 °C.
D21S1437 in	F: 5′-**FAM**- ATGTACATGTGTCTGGGAAGG-3′ R: 5′-TTCTCTACATATTTACTGCCAACA-3′	5 min 94 °C, 40 × (30 s 94 °C, 45 s 58 °C, 30 s 72 °C), 5 min 72 °C.
Delta F508 out	F: 5′-TGGAGCCTTCAGAGGGTAAA-3′ R: 5′-TGCATAATCAAAAAGTTTTCACA-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 55 °C, 30 s 72 °C), 5 min 72 °C.
Delta F508 in	F: 5′-**FAM**-TCT GTT CTCAGT TTT CCTGG-3′ R: 5′-TCT TAC CTC TTC TAG TTG GC-3′	5 min 94 °C, 40 × (30 s 94 °C, 30 s 57 °C, 30 s 72 °C), 5 min 72 °C.

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D21S1437) (see Table 1 for primer sequences and PCR profiles).

The remaining 5 mL of maternal blood was treated by ISET up to 3 h after collection, as previously described, using the ISET device (Metagenex, Paris France (www.metagenex.fr)), and stored at −20 °C (Vona *et al*., 2002; Beroud *et al*., 2003) before analysis. After immunohistochemical analysis with KL1 antibody (Zimmer *et al*., 1993; Vona *et al*., 2002) to identify epithelial cells, single-cell laser capture microdissection (LCM) was performed using the Nikon TE 2000 U (Nikon Paris, France and MMI, Zurich, Switzerland) laser-equipped microscope (for the image of a KL1 positive fetal cell, which is very similar to that of a KL1 positive maternal cell, see Figure 2(C), Vona *et al*., 2002). ISET enriches epithelial cells from blood but also retains approximately 0.02% peripheral blood leukocytes (Vona *et al*., 2000, 2002). Thus, in order to develop the molecular protocol for NI-PND of CF, we treated, by ISET, the blood from five known F508del carriers and laser-microdissected 100 single leukocytes. Cells from F508del carriers were selected as they carry two different alleles (Figure 1(A')), thus the presence of only one allele indicates the occurrence of ADO, whereas identification of the two F508 alleles shows the absence of ADO.

Cell lysis, primer extension preamplification and STR genotyping

Each microdissected cell was lysed in 15 µL of lysis buffer (100 mmol/L Tris-HCl, pH 8; 400 µg/mL proteinase K) for 2 h at 60 °C, followed by proteinase K

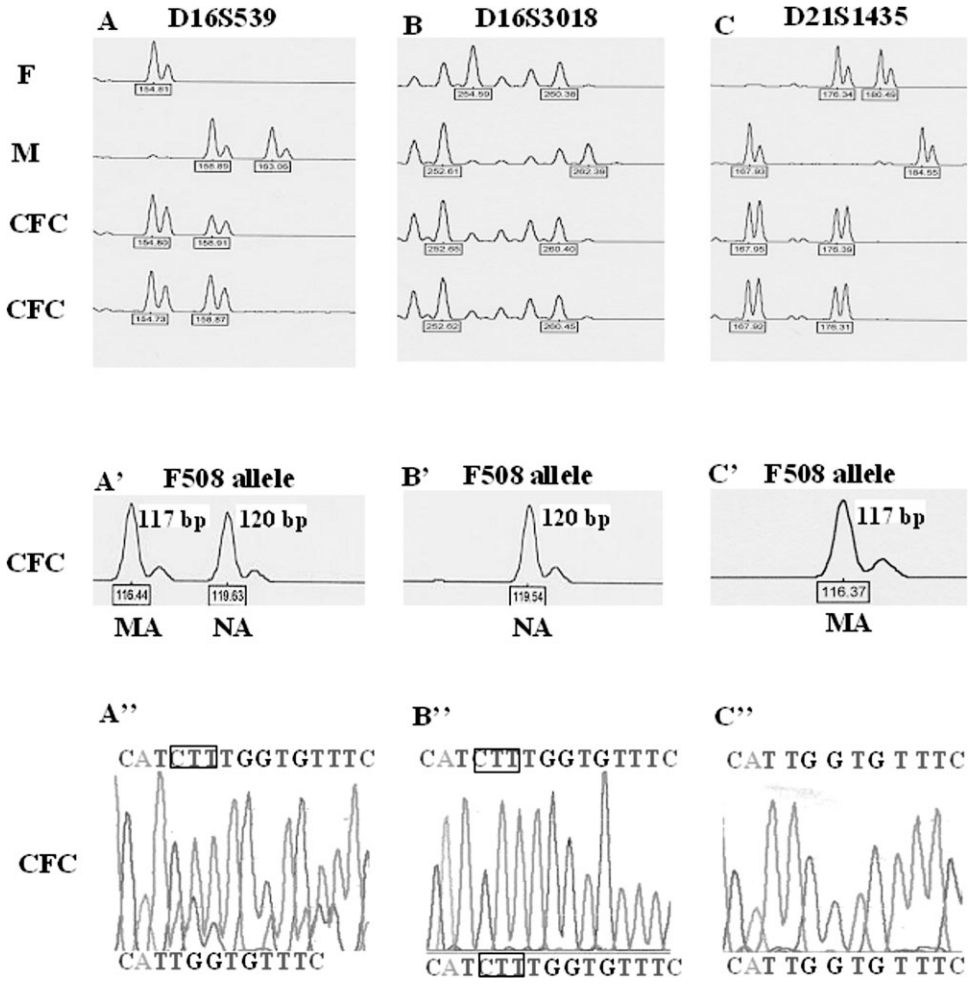

Figure 1—Direct non-invasive prenatal diagnosis (NI-PND) of CF in couples of F508del carriers. A, B, C: STR genotyping analyses were performed on couple 1 with the D16S539 STR marker (A); couple 6 with the D16S3018 STR marker (B) and couple 8 with the D21S1435 STR marker (C). In couple 1 (A), the father (F) is homozygous (one peak) for the target allele and the mother (M) is heterozygous (two peaks). A circulating fetal cell (CFC) from this couple has one paternal allele (left) and one maternal allele (right). In couple 6 (B) and couple 8 (C), both parents are heterozygous for the STR markers and a fetal cell (CFC) has one maternal (left) and one paternal (right) allele. A', B', C': F508 allele genotyping performed on CFCs from couple 1 (A'), couple 6 (B') and couple 8 (C') shows that the fetus of couple 1 is a F508del allele carrier having a mutated allele (MA) and a normal allele (NA), the fetus from couple 6 is completely normal (homozygous presence of the normal allele) and the fetus from couple 8 is affected with CF (homozygous presence of the mutated allele). A", B", C": sequencing of the F508 locus in fetal cells from couple 1 (A"), couple 6 (B") and couple 8 (C") confirms the diagnosis showing both the mutated and normal alleles in the fetus from couple 1 (A"), the homozygous normal allele in the fetus from couple 6 (B") and the homozygous mutated allele in the fetus from couple 8 (C")

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inactivation at 94 °C for 15 min. For primer extension preamplification (PEP) (Zhang *et al*., 1992), to the lysed cell we added 5 μL of a 400 μM solution of random primers (Kit genPEP$_{tm}$ 75 OD, Genetix, Boston, USA), 6 μL of PCR buffer (25 mM MgCl2/gelatin (1 mg/mL), 100 mM Tris-HCl, ph8.3, 500 mM KCl), 3 μL of a mixture of four dNTPs (each at 2 mM) and 1 μL (5 U) of Taq polymerase (Applied Biosystem, Foster City, CA, USA) in a final volume of 60 μL. STR amplifications were performed in 60 μL containing 6 μL of the PEP product, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.5 μM of each STR 'outer' primer and 2 U of Taq Gold (Applied Biosystems, Foster City, CA, USA). Two microliters of a 1 : 10 diluted PCR outer product were re-amplified in 20 μL final volume using 'inner' fluoresceinated STR primers and the same PCR protocol (see Table 1 for primer sequences and PCR profiles). One μL of the 1 : 20 diluted inner PCR product was then mixed with 13.5 μL of deionised Hi-Di formamide and 0.5 μL of Genescan 400 HD (ROX) marker (Applied Biosystems) and loaded into an ABI Prism 3100 automated sequencer (Applied Biosystems). Profiles were analyzed using the Genescan and Genotyper software programs (Applied Biosystems).

Protocol for single-cell F508 locus amplification

Single-cell genomes were tested individually (6 μL of PEP product in a PCR volume of 60 μL) and in groups of three by pooling 30 μL of the PEP product from each cell and taking 12 μL (from the 90 μL) for the F508 amplification in a PCR volume of 100 μL. Amplifications were performed by using 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.5 μM of each 'outer' primer encompassing the F508 locus and 2 U of Taq Gold (Applied Biosystems, Foster City, CA, USA). Two μL of a 1 : 10 diluted PCR outer product were re-amplified in 20 μL final volume using 'inner' fluoresceinated F508 primers and the same PCR protocol (see Table 1 for primer sequences and PCR profiles). This method avoids the DNA purification steps that carry a high risk of **losing** DNA copies. One μL of the 1 : 20 diluted inner PCR product was then mixed with 13.5 μL of deionised Hi-Di formamide and 0.5 μL of Genescan 400 HD (ROX) marker (Applied Biosystems) and loaded into an ABI Prism 3100 automated sequencer (Applied Biosystems). Profiles were analyzed using the Genescan and Genotyper software programs (Applied Biosystems).

Precautions to avoid carry over of PCR products. Controls of specificity and sensitivity

Precautions to avoid PCR product carryover in our laboratory have been described (Paterlini *et al*., 1995). Briefly, buffer and reaction mix preparation, DNA extraction, and PCR product loading on agarose gel were performed using barrier tips in separate rooms. Protective clothing was used and operators followed specific rules of work. In particular, entry to the 'mix' and to the 'extraction' rooms was forbidden to operators who entered the PCR products loading room the same day. Extensive use of UV light and 0.1 mol/L hydrochloric acid solution after each test enabled efficient cleanup of bench and instruments.

To check for the absence of PCR or sample contamination, tubes with only buffer (negative control) were included at the cell lysis step (one negative control inserted per every cell tested) and run to the end of each PCR run. Thus, an identical number of samples (single-cell DNAs) and negative controls, was analysed. Two controls of sensitivity, the PEP product (6 μL) from a previously tested PCR positive single cell and 1 ng of DNA, were included in each PCR run.

Application of the protocol for NI-PND of CF to 12 pregnant women

We tested the peripheral blood of 12 women at 11–13 weeks of gestation who had requested PND of CF between **J**uly 2003 and **J**uly 2005, including two who had a previous child with compound heterozygosity for CF (a so-called index case; couples 11 and 12, Figure 2, Table 2). All women gave informed consent to this study, which was approved by the local ethics committee.

Circulating epithelial cells shown to be fetal by STR genotyping were tested individually and in pools of three cells for the presence of F508del mutation. Pooling tests were performed, as described above, by mixing 30 μL of the PEP products from three fetal cells and then taking 12 μL (from the 90 μL) for the F508 amplification in a 100 μL final PCR volume. Sequence analyses were performed using the Big Dye terminator sequencing kit (Applied Biosystems) (Vona *et al*., 2002) after purification of the PCR product with MicroSpin S-400RH columns (Amersham Bioscience, Buckinghamshire, UK).

In case of couples where neither or only one of them, was a carrier of the F508del mutation, fetal cell analysis was carried out by an indirect method using informative STR markers linked to the CFTR locus on chromosome 7, thus enabling us to compare the index case and fetus haplotypes.

The non-invasive assay, which takes three days to be performed, was carried out in the Department of Biochemistry of the Necker-Enfants Malades hospital using a completely blind approach by workers who were unaware of results of the invasive analysis carried out in the Department of Medical Genetics of the same hospital.

Statistical analyses

The χ^2 test was used for statistical analyses of between-group frequencies. A P-value of less than 0.05 was considered as significant.

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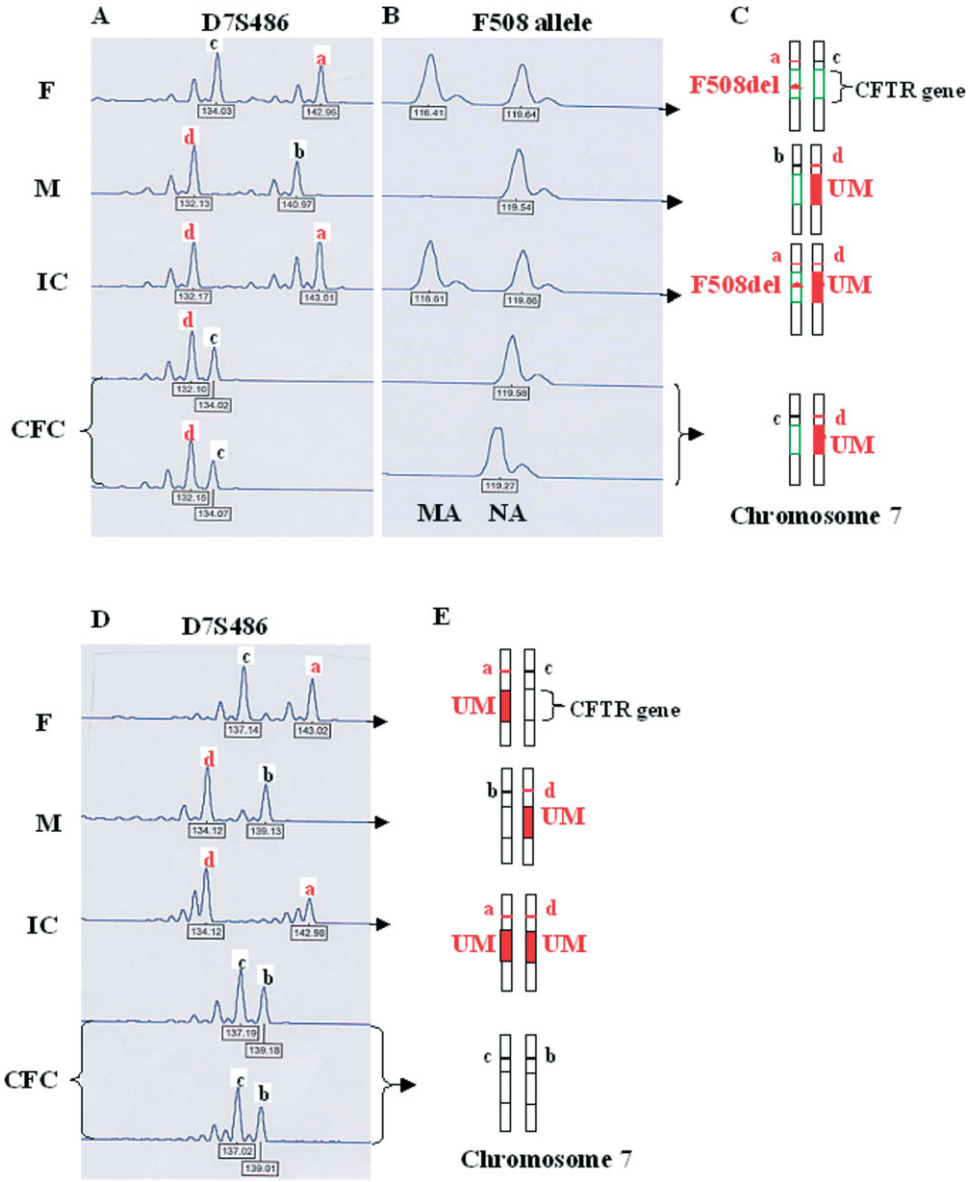

Figure 2—Indirect non-invasive prenatal diagnosis (NI-PND) of CF in couples with unknown CFTR mutations. A, B, C: DNA genotyping of couple 11 and their affected child using the informative STR marker D7S486 on chromosome 7 shows that the father (F) and the mother (M) are heterozygous. The father carries alleles **c** and **a,** whereas the mother carries alleles **d** and **b.** Their affected child (IC: index case) carries the **d** and **a** alleles, which must therefore be linked to the mutated CFTR alleles. Two CFCs tested from the ongoing pregnancy carry the **d** and **c** alleles, demonstrating that the fetus is a CF carrier but is not affected. B: DNA genotyping of the F508 locus showing that the father carries a mutated allele (MA) and a normal allele (NA). The index case has compound heterozygosity, carrying the paternal F508del allele and a different maternal CFTR mutation. Two fetal cells are homozygous for the normal F508 allele. Altogether, data from A and B demonstrate that the fetus is a carrier of the maternal CFTR mutation. C: schematic representation of the CFTR alleles and CFTR-linked STR alleles **(a, b, c, d).** Red bar: unknown CFTR mutation (UM). D, E: DNA genotyping of couple 12 with the informative STR marker D7S486 on chromosome 7 shows that the father and the mother are heterozygous and that the index case carries the **a** and **d** D7S486 alleles, which must therefore be linked to the mutated CFTR alleles. Two CFCs from the ongoing pregnancy carry the **c** and **b** D7S486 alleles linked to the non-mutated CFTR alleles, demonstrating that the fetus is completely normal. E. Schematic representation of the CFTR alleles and CFTR-linked STR alleles **(a, b, c, d).** Red bar: unknown CFTR mutation (UM)

RESULTS

F508 locus amplification of CFTR gene

In order to develop a protocol that overcomes the problem of ADO, we used DNA from 100 individual leukocytes from proven F508del carriers. In this case, the normal and the mutated allele are distinguishable as they have a different size (120 bp and 117 bp, respectively) (Figure 1(A')) allowing us to detect the occurrence of ADO. After the F508 single-cell amplification, we checked the PCR efficiency by agarose gel electrophoresis and observed a F508-specific band for 91 single-cell DNAs (91%). Thus, PCR efficiency was 91%. The genescan analysis of the PCR product from the 91 positive cells showed ADO in eight (9%) and accurate

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Table 2—Prenatal diagnosis of cystic fibrosis (CF) by genetic analysis of circulating fetal cells (CFCs) and by chorionic villus sampling (CVS)

Couple	Informative STR markers	No. of microdissected cells	No. of tested single CFCs	CF genotype in single CFCs[a]	No. of tested poolings of 3 CFCs[b]	CF genotype in poolings of 3 CFCs	CF genotype by CVS	Fetal status
1[c]	D7S486/D16S539/D21S1435	10	5	F508del/N	1	F508del/N	F508del/N	CF carrier
2[c]	D16S539/D21S1435/D21S1437	11	5	N/N	1	N/N	N/N	Healthy
3[c]	D7S486/D16S3018/D21S1437	13	7	F508del/N	2[d]	F508del/N	F508del/N	CF carrier
4[c]	D16S539/D16S3018/D21S1435	9	5	N/N	1	N/N	N/N	Healthy
5[c]	D16S3018/D21S1437/D21S1435	9	4	F508del/N	1	F508del/N	F508del/N	CF carrier
6[c]	D7S480/D16S539/D16S3018	8	5	N/N	1	N/N	N/N	Healthy
7[c]	D16S539/D21S1437/D21S1435	12	7	F508del/N	2[d]	F508del/N	F508del/N	CF carrier
8[c]	D7S480/D16S3018/D21S1435	9	4	F508del/F508del	1	F508del/F508del	F508del/F508del	Affected
9[c]	D16S539/D16S3018/D21S1435	16	7	F508del/N	2[d]	F508del/N	F508del/N	CF carrier
10[c]	D7S486/D21S1435/D21S1437	12	5	F508del/N	1	F508del/N	F508del/N	CF carrier
11[e]	D7S486/D7S490/D7S523	13	6	Mut/N		Mut/N	Mut/N	CF carrier
12[f]	D7S480/D7S486/D7S523	11	5	N/N		N/N	N/N	Healthy

STR, short tandem repeat; F508del/N, with heterozygous F508del allele; N/N, with homozygous normal alleles; F508del/F508del, with homozygous F508del alleles; Mut/N, with heterozygous mutated allele.

[a] Accurate amplification and consistent results were obtained in all tested CFCs.

[b] Pooling of primer extension preamplification (PEP) aliquots from three CFCs.

[c] F508del carriers.

[d] Consistent results were obtained from the two poolings of three CFCs.

[e] One parent is F508del carrier and one is carrier of unknown mutation.

[f] Carriers of unknown mutations.

amplification of both alleles in the remaining 83, giving a PCR accuracy of 91%.

Because ADO is stochastic and affects one or the other allele at a frequency of around 10% (one in 10 analyses, thus 0.1), it will affect the same allele at a frequency of one in 20 analyses (0.05). Thus, we reasoned that if we pooled the DNA from three or more fetal cells, the frequency of ADO affecting the same allele would drop to 0.0001 ($0.05 \times 0.05 \times 0.05 = 0.000125$). We thus blindly pooled (by an operator unaware of previous results) the PEP products from the 91 leukocyte DNA samples into 30 random batches of three and carried out the F508 amplification and fragment analysis as before. In this case, we observed a F508-specific band in all the PCR analyses (100%) and a significantly higher PCR efficiency compared to single-cell tests ($p < 0.001$). We also consistently observed the absence of ADO in all the 30 pooling tests (100%), giving à significantly higher PCR accuracy ($p < 0.001$ vs single-cell tests).

We explain this observation by the fact that, in the pooling analyses, the probability to include DNA sequences coming from only one (and the same) of the two alleles in the whole mix is, as calculated before, very low (1/10 000). We concluded that by pooling 30 μL of the PEP products from three fetal cells and taking 12 μL for the PCR, we can markedly increase the probability of having bi-allelic sequences.

Application of the protocol to 12 couples at risk of having a child with CF

We then applied this improved method to blood cells obtained from mothers at risk of having a fetus affected by CF. STR genotyping analyses allowed us to identify four to seven fetal cells from 5 mL of maternal blood (Figure 1(A), (B) and (C)). Overall, approximately 50% of microdissected and genotyped cells were proven to be fetal by the genetic analysis (Table 2). These fetal cells were tested individually and also as a pool of three cells. In couples 1–10 (Table 2, Figure 1), where both parents were F508del carriers, we studied the DNA extracted from fetal cells with primers encompassing the F508 locus (Figure 1). This assay identified the F508del mutation, characterised by three base pair (CTT) deletions giving a PCR product of 117 bp instead of 120 bp, (Figure 1(A') and (C')). We thus identified heterozygous fetuses carrying the F508del mutation (couples 1, 3, 5, 7, 9 and 10) (Figure 1(A')), normal homozygous fetuses (couples 2, 4 and 6) (Figure 1(B')) and one CF homozygous mutant fetus (couple 8) (Figure 1(C')) (Table 2).

In six individual fetal cells, the results we obtained by genotyping were confirmed by sequencing the F508-locus-specific PCR product (Figure 1(A"), (B") and (C")). (Sequence analysis was not systematically performed as the data obtained by genotyping were very clear.) We obtained the same results by testing each CFC individually and by pooling CFCs in groups of three (Table 2). The frequency of ADO was lower in samples from single CFCs (0/35 F508del heterozygous fetal cells) than from single leukocytes (8/91). We did not observe ADO in pools of PEP products from three fetal cells.

NI-PND of CF was also performed in couples carrying unknown CFTR mutations. In these cases, the DNA from an affected sibling (index case) was available and allowed us to identify STR alleles linked to the mutated CFTR alleles. In couple 11 (Table 2), the father was a carrier of the F508del mutation and the mother was a carrier of an unknown mutation (Figure 2(A), (B) and (C)). Individual CFCs analysis with informative STR primers linked to the CFTR locus on chromosome 7 (indirect diagnosis) (Figure 2(A)), and by primers specific to the F508 locus (Figure 2(B)), showed the absence of the F508del mutation and the presence, in heterozygous form, of the maternal allele carrying the unknown mutation that was identified by its presence in the index case genome. Thus, the fetus was a carrier of the unknown maternal mutation. In couple 12 (Table 2), the father and the mother were carriers of unknown mutations (Figure 2(D) and (E)). Fetal cells analysis with informative STR primers on chromosome 7 (indirect diagnosis) showed the homozygous absence of the mutated alleles previously identified in the index case genome. Thus, the fetus was completely normal.

This work was performed with a completely blind protocol, and consistent results were obtained by the non-invasive method (ISET-CF) carried out in the Department of Biochemistry of the Necker-Enfants Malades hospital, and by the invasive method, carried out on chorionic villus samples (CVS) by an independent team in the Department of Medical Genetics of the same hospital (Table 2).

Protocol for NI-PND of CF

On the basis of the results obtained, we have defined the following protocol for NI-PND of CF. DNAs extracted from maternal (1 mL) and paternal blood (1 mL) are compared by using STR primers to identify informative STR markers (Figure 3). ISET is used to enrich circulating fetal trophoblasts from the maternal blood (5 mL). Trophoblastic cells, identified by KL1 immunostaining and cell morphology, are then individually removed from the ISET filter by LCM, and their DNA is subsequently analyzed after cell lysis. The whole single-cell genome is first amplified by random primer extension PEP (final volume: 60 μL). Aliquots of the PEP product (6 μL) are used for single-cell genotyping with informative STR markers to determine whether the DNA is of fetal (having both maternal and paternal alleles) or maternal origin. For F508del analysis (direct diagnosis of CF), aliquots (30 μL) of the PEP product from three fetal cells are pooled and 12 μL used for amplification with F508-specific primers (in a final PCR volume of 100 μL) allowing us to avoid the risk of ADO. Informative STR markers on chromosome 7 allow indirect diagnosis of CF (if an index case is available). In this case, there is no need to pool aliquots from three cells since both maternal and paternal alleles have to be visualised for the diagnosis. In other words, if ADO occurs in

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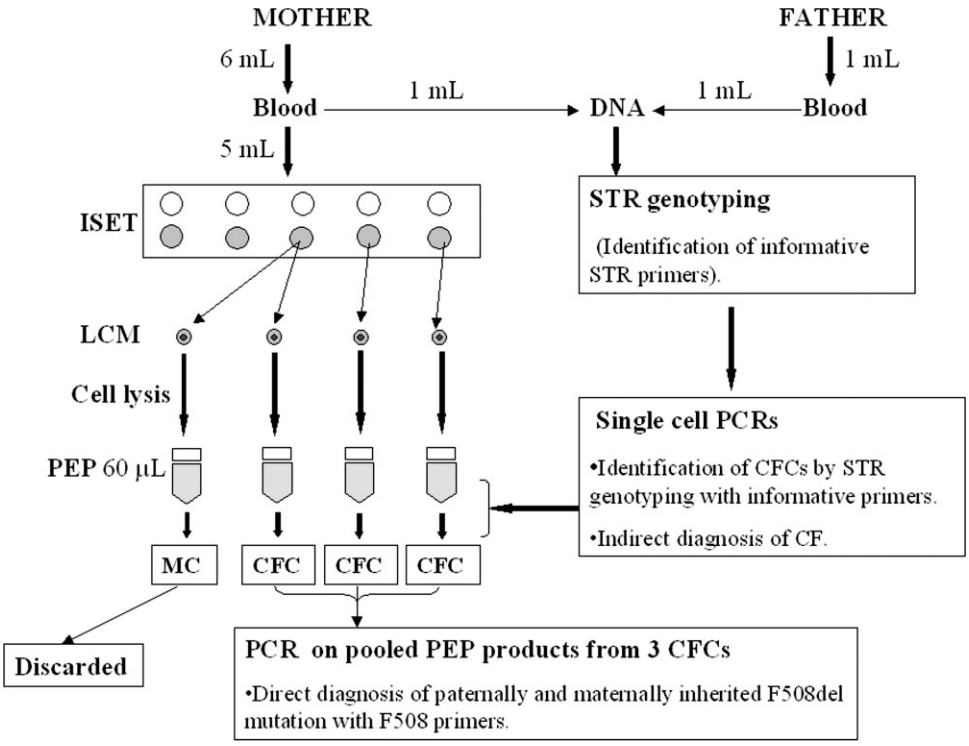

Figure 3—Protocol for non-invasive prenatal diagnosis (NI-PND) of cystic fibrosis. See the main text for details. STR, short tandem repeat; ISET, isolation by size of epithelial tumour/trophoblastic cells; LCM, laser capture microdissection; PEP, primer extension preamplification; MC, maternal cells; CFC, circulating fetal cell

this setting, it can be identified and the cell is discarded or re-tested.

DISCUSSION

Our study shows that reliable NI-PND of CF is feasible and may be applicable in a routine clinical setting. In fact, clinical application of our method to fetal cells isolated from the blood of 12 mothers at 1/4 risk of having a child affected by CF, in a blind protocol, has shown that reliable diagnosis was obtained in all cases, with the precise identification of mothers carrying healthy fetuses, carriers or affected fetuses.

The method involves isolating CFCs by ISET and laser microdissection, single-cell STR genotyping to determine the presence of the paternal marker (i.e. to confirm the fetal origin of the cells), and mutation analysis of a pool of three genetically proven fetal cells, which significantly brings the probability of ADO close to zero. These characteristics make the test reliable and potentially applicable in the clinic as a safe alternative to invasive PND procedures.

The development of a non-invasive, safe and reliable PND of genetic diseases remains an ambitious goal (Bianchi, 1999; Bianchi *et al*., 2002). In fact, it has been hampered by the difficulty in enriching rare fetal cells, with the minimum contamination by maternal cells. This problem is mainly due to the fact that it is still not possible to define the fetal origin on the basis of immunolabelling of a cell surface antigen. Among CFCs, only nucleated erythrocytes and trophoblasts are thought to be eliminated after delivery, allowing the diagnosis of the ongoing pregnancy (Bianchi *et al*., 1996). Enrichment of CFCs has been obtained in the past by using different protocols of density gradient separation (van Wijk *et al*., 1996; von Eggeling *et al*., 1997; Al-Mufti *et al*., 1999; de Graaf *et al*., 1999; Bianchi *et al*., 2002; Yang *et al*., 2003; Sitar *et al*., 2005), differential cell lysis (Krabchi *et al*., 2001; Voullaire *et al*., 2001), combined or not combined with positive and/or negative immuno–based isolation (van Wijk *et al*., 1996; Koumantaki *et al*., 2001; Martel-Petit *et al*., 2001; Bianchi *et al*., 2002; Yang *et al*., 2003). In order to identify and count CFCs, the enriched cells have been analysed by FISH (Oosterwijk *et al*., 1998; Krabchi *et al*., 2001; Bianchi *et al*., 2002; Al-Mufti *et al*., 2004), PRINS (Orsetti *et al*., 1998; Krabchi *et al*., 2001, 2004) or PCR analysis (Bianchi *et al*., 1997; Bohmer *et al*., 2002; Chen *et al*., 2004), mostly targeted to Y sequences. However, FISH is not highly sensitive (Krabchi *et al*., 2004), as labelling of 100% cells is uncommon, so decreasing the overall rate of identified fetal cells and, like the more sensitive method PRINS (Krabchi *et al*., 2004), does not identify fetal cells from female fetuses. However, a recent work has shown the possibility to identify CFCs by FISH and PRINS without previous enrichment (Krabchi *et al*., 2006). While this method is appealing, it implies the analysis of 20 to 30 slides in order to look for CFCs in only one mL of blood and has been addressed to mothers carrying a male fetus, thus leaving unsolved the problem of NI-PND in mothers carrying a female fetus.

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Cell-free fetal DNA in maternal plasma, accounting for 3.4–6.2% of the total plasma DNA (Lo *et al*., 1998), affords determination of fetal sex and the fetal Rhesus D status (Costa *et al*., 2001, 2002), but does not seem to be a reliable approach to NI-PND of recessive disorders. Detection of the mutated paternal allele in the maternal plasma is possible (Li *et al*., 2004, 2005), but only when it is different from the mutated maternal allele. In this case, it allows us to identify an increased risk (50%) for the fetus to be affected, but not to perform a non-invasive PND. However, the low quantity of fetal DNA in the maternal circulation and interference with maternal DNA increase the chance of false results, suggesting the use of allele-specific PCR approaches (Nasis *et al*., 2004) and limiting the analyses to specific situations in which the detection of mutation is relatively easy (Chiu *et al*., 2002; Gonzalez-Gonzalez *et al*., 2002, 2003). If the mutated paternal allele (different from the maternal allele) is not found in maternal plasma, it could be possible, in theory, to make a diagnosis of 'non affected' fetus (without the possibility to distinguish between a healthy and a heterozygous carrier). Practically, though, since the proportion of fetal and maternal DNA in maternal plasma may be variable, it seems very risky to make a diagnosis of 'non affected' fetus by maternal plasma analysis. Furthermore, in the case of couples where both parents carry the same mutation (ex.: F508del), it is not possible to distinguish the paternal from the maternal mutated allele by maternal plasma analysis, and even the detection of 'increased risk' is not possible.

Our new approach follows a previous report (Griffin and Ferguson-Smith, 1999) that has identified genotyping of isolated fetal cells as a promising route to develop a NI-PND. The rare circulating trophoblasts are enriched by ISET because they are larger than peripheral blood leukocytes. Immuno-cytological identification allows us to rule out cells with apoptotic morphology (Kolialexi *et al*., 2004). This enrichment is so efficient that fetal cells (4 to 7) have been found in a single sample of 5 mL of blood taken from all 51 mothers tested up to now in our laboratory. Single-cell genotyping after laser microdissection allows us to identify fetal cells unequivocally according to the bi-parental contribution to their genome and to address further molecular analyses to a 'pure' fetal genome. At the step of single-cell genotyping, even if ADO occurs, the consequence is that the single-cell DNA is incompletely characterised. This leads us to test the DNA with other sets of informative primers and, if ADO persists, to discard the cell. There is no risk of making an incorrect diagnosis. We actually observed that genotyping single cells by using three STR informative markers increases the probability of having an accurate single-cell genotyping (visualisation of the two alleles) to 95% of tested cells (data not sown) and that approximately half the microdissected cells have a fetal genome (Table 2). Finally, pooling half of the PEP product (30 μL of 60 μL) from groups of three proven fetal cells and taking 12 μL for F508 amplification allows performing the mutation analysis on a higher amount of 'pure' fetal DNA, preventing false-positive diagnosis due to the loss of a normal allele by ADO. It has been previously shown that mixing the DNA from two or more single cells almost completely prevents the risk of ADO (Piyamongkol *et al*., 2003), but these studies were performed on whole-cell DNA from fresh cells that had not been amplified by PEP. Amplification of DNA from fixed single cells (rare cells can be enriched by ISET only after fixation by the ISET dilution buffer) is less sensitive than that of DNA from fresh cells, as fixation is known to induce DNA breaks. However, the PCR protocol we have developed affords the amplification of DNA from fixed cells. It also avoids the risk of ADO, when performing direct diagnosis of CF (detection of F508del mutation), by pooling the DNA from groups of three fetal cells. In this protocol, the use of aliquots of the PEP product is relevant as it makes it possible to carry out PCR analyses both on a single-cell genome (for STR genotyping) and on pools of genomes from several fetal cells (for direct CF diagnosis).

The optimised PEP protocol includes optimal DNA conservation (Nagy *et al*., 2005) (we store ISET filters for NI-PND at −20 °C), proteinase K treatment to lysate cell's proteins (we comparatively tested several protocols), and assessment of the quality of the degenerate primers used for PEP (we check every primers lot before its use in NI-PND protocols). We confirm that PEP applied to DNA from single cells does not prevent the occurrence of ADO (Hahn *et al*., 1998), however, our results show that ADO is prevented by mixing large aliquots of the PEP products obtained from at least three cells.

Results obtained by analysing 100 cells tested individually and in pools of three cells showed that the probability that ADO, which occurs randomly, fail to amplify the same allele in all three cells is extremely low (1/10 000). Since in routine protocols the NI-PND can be repeated in three to five pools of three fetal cells obtained from a blood sample of 10 mL, and the test could also be repeated in two subsequent blood samples taken from the same mother, the risk of misdiagnosis should be really brought to zero.

In single-cell testings, we observed a higher incidence of ADO among the 91 lymphocytes (9%) than among the 35 fetal cells (0%), all heterozygous for the F508del allele. We think that this difference is due to the fact that, at variance with lymphocytes DNAs, the fetal cells DNAs tested with F508 primers had been previously selected as DNA showing two STR alleles at the genotyping step. We could also speculate that the compact nature of the DNA in lymphocytes might explain their higher rate of ADO as the DNA in these cells is expected to be less accessible to PCR primers.

We demonstrate here that NI-PND for CF is possible even when both parents carry unknown CFTR mutations (indirect diagnosis), provided that the DNA from an index case (an affected child from the same couple) is available and that informative STR primers on the chromosome 7, where the CFTR gene is located, are identified. In practice, the DNA from an index case is usually available as the affected child is very often the only indication that the parents are carriers of CFTR mutations other than F508del. In this indirect diagnosis, there is no need to pool fetal cells to avoid mistakes

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because both maternal and paternal STR alleles, required for the diagnosis, have to be visualised. Thus, the presence of the two alleles ensures absence of ADO and reliable diagnosis (cells giving ADO are discarded).

In conclusion, we developed an ISET-based protocol affording reliable and safe PND of healthy, carrier and CF affected status.

This optimised strategy—which can be speeded up by automated laser microdissection and development of dedicated kits for PCR analyses—should bring NI-PND of CF closer to clinical application.

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