Preimplantation diagnosis by whole-genome amplification, PCR amplification, and solid-phase minisequencing of blastomere DNA

Tiina Paunio,1,2 Ilkka Reima,3,4 and Ann-Christine Syvänen1

We have developed a new method for preimplantation diagnosis of inherited diseases. Our procedure for the identification of point mutations in single cells combines whole-genome amplification using 15-mer random primers (primer extension preamplification, PEP) with a single locus-specific PCR amplification, followed by detection of the mutation by solid-phase minisequencing. The procedure was evaluated by detecting three disease-causing mutations and seven polymorphic nucleotides located on different human chromosomes from single granulosa and blastomere cells. The correct genotype of the cell was identified at 96% of the nucleotide positions analyzed, showing that a representative part of the genome is amplified during PEP. We estimate that PEP yielded at least 1000 copies of the genome. The quantitative nature of the solid-phase minisequencing method allowed us to notice that preferential amplification of one allele occurs at heterozygous loci during PEP, which is a potential problem in preimplantation diagnosis.

INDEXING TERMS: genotyping • inherited diseases • in vitro fertilization • single-cell analysis • granulosa cells • aspartylglucosaminuria • neuronal ceroid lipofuscinosis • amyloidosis • gelsolin

The ability of the PCR technique to amplify DNA molecules 109-fold permits the analysis of DNA sequence variations in single cells [1]. Consequently, genotyping of individual sperm cells has provided a new approach for genetic mapping (for a review, see ref. 2), and the analysis of blastomeres from in vitro-fertilized (IVF) embryos has been utilized in preimplanta-

tion diagnosis [3]. So far, preimplantation diagnosis has most frequently been used for the sex determination of embryos at risk of X-linked disorders [3, 4], but also for a few autosomally inherited disorders, such as β-thalassemia, cystic fibrosis, and Tay-Sachs disease [5-7].

We undertook this study because of the evident need for preimplantation diagnosis in Finland, where >30 mostly recessively inherited severe disorders, only rarely encountered in other populations, have been enriched [8]. We chose as targets two recessively inherited disorders, aspartylglucosaminuria (AGU) and infantile neuronal ceroid lipofuscinosis (INCL), and one dominantly inherited disorder, familial amyloidosis of the Finnish type [FAF, or gelsolin (GSN)-related amyloidosis], because the disease-causing mutations have been identified in these disorders, and their routine DNA diagnosis is well established [9-11]. No specific cure is available for these disorders, so prevention through genetic counseling and early prenatal diagnosis is so far the only measure available. In addition to the mutations causing AGU, INCL, and FAF, seven polymorphic nucleotides on different human chromosomes were analyzed from granulosa cells and blastomeres to develop a generally applicable procedure for detecting mutations in single cells.

We combined the method for whole-genome amplification, known as primer extension preamplification (PEP) [12], with a single PCR amplification using locus-specific primers, followed by detection of the mutation with a solid-phase minisequencing method [13]. PEP amplification involves multiple rounds of annealing and extension of a mixture of short, random (e.g., 15-mer) oligonucleotide primers and has been shown to be a promising tool for genetic analysis of single cells in several recent studies [7, 14, 15]. The solid-phase minisequencing method detects point mutations in DNA fragments amplified by

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Received February 14, 1996; accepted March 27, 1996.

5 Nonstandard abbreviations: IVF, in vitro fertilization; AGU, aspartylglucosaminuria; INCL, infantile neuronal ceroid lipofuscinosis; FAF, familial amyloidosis of the Finnish type; PEP, primer extension preamplification; AGA, aspartylglucosaminidase; GSN, gelsolin; PPT, palmitoyl protein thioesterase; 3BHSD, 3β-hydroxysteroid dehydrogenase; PROS1, protein S; ADH1, alcohol dehydrogenase; ARSB, aroylsulfatase B; IGF2, insulin-like growth factor II; TBS3, transformation-related protein p53; and FISH, fluorescence in situ hybridization.
PCR. In this method, the variant nucleotide is identified by extension of a primer with a single labeled nucleotide complementary to the nucleotide at the variable position by a DNA polymerase (Fig. 1 illustrates the principle of the method). Not only is the technique highly specific and applicable to the detection of any variable nucleotide, it also allows quantitative determination of the relative amounts of two sequences (e.g., alleles) in a sample [16]. Using the procedure developed in this study, we reproducibly and efficiently amplified a highly representative part of the genome and identified the correct genotype in the majority of the cells analyzed.

Materials and Methods

SAMPLES
All samples were collected with permission from the Ethical Committee of the Family Federation of Finland. Human granulosa cells were isolated from the cumulus oophorus surrounding the oocyte. The cells were isolated as a byproduct in the preparation of the oocytes in intracytoplasmic sperm injection, as described in detail previously [17]. Briefly, the oocytes surrounded by the cumulus oophorus were collected and cultured for 1-2 h in Medicult Universal IVF medium, and treated with IVF medium containing 80 U of hyaluronidase enzyme (Type IV-S; Sigma Chemical Co., St. Louis, MO). After separation of the oocytes, the single granulosa cells were rinsed with culture medium without enzyme. The single granulosa cells were transferred to Eppendorf tubes with the use of mouth-controlled sterile micropipettes under a stereomicroscope. Blastomeres of a human four-cell-stage embryo were donated voluntarily by a couple participating in IVF treatment at the Infertility Clinic of the Family Federation of Finland. Blastomeres were collected into the culture medium, the zona pellucida was removed with acid Tyrode's solution, and the blastomeres were isolated as described earlier [18]. The cells were rinsed with culture medium without enzyme before transfer to Eppendorf tubes for lysis.

The individually collected cells were lysed with 5 μL of a solution of 200 mmol/L KOH and 50 mmol/L dithiothreitol and neutralized with 5 μL of a solution of 900 mmol/L Tris-HCl (pH 8.3), 300 mmol/L KCl, and 200 mmol/L HCl, as described previously [19]. The samples were frozen at −80 °C or kept at 0 °C and analyzed immediately. Samples containing large amounts (thousands) of granulosa cells were treated with a rapid lysis procedure [20] before PCR amplification. DNA was extracted from peripheral blood lymphocytes of AGU, INCL, and FAF patients; AGU and INCL carriers; and healthy controls by a standard procedure.

PRIMERS
The polymorphic nucleotides and disease-causing mutations for which the primary PEP products were analyzed are listed in Table 1. The sequences of the PCR and solid-phase minisequencing primers for the 3-β-hydroxy steroid dehydrogenase (3BHSD), protein S (PROST), alcohol dehydrogenase (ADH3), arylsulfatase B (ARSB), insulin-like growth factor II (IGF2), and transformation-related protein p53 (TP53) genes [16], the aspartylglucosaminidase (AGA) gene [9], and the GSN gene [11] have been described previously. The PCR primers for C3 were those designated EX3 (nucleotides 328-345 of the C3 cDNA sequence) and EX4 (complementary to nucleotides 514-531) [27, 30]. The EX4 primer was biotinylated, and the minisequencing primer was identical to nucleotides 344-363 of the C3 cDNA. The sequences of the PCR primers for amplification of the palmitoyl protein thioesterase (PPT) gene were 5'-TGATACATTTCCATCTCTGAGCA (biotinylated) and 5'-GTGAAGGCGATCCTCTGAGCCA, and the minisequencing

Fig. 1. Procedure for detecting point mutations in single cells.
(1) PEP amplification with random 15-mer primers. (2) PCR amplification with locus-specific primers, one of which is biotinylated (>). (3) Affinity capture and denaturation of the PCR products in streptavidin-coated microtiter plate wells. (4) Extension of a detection step primer with a single, labeled nucleotide. (5) Measurement of the incorporated label. (6) Interpretation of the result.
primer was 5'-GCATCTCTGACCCACTGGCC. The primers were synthesized on an Applied Biosystems (Foster City, CA) 392 DNA synthesizer.

**PROCEDURES**

**Prevention of contamination.** To avoid contamination, we carried out the PEP and PCR amplifications in laboratories located in separate buildings. DNA-free reagents and equipment were used, and the amplification reactions were set up by using positive-displacement pipettes in hoods equipped with an ultraviolet light. Samples of the cell medium, lysis and neutralization solutions, and water were included in each amplification experiment to confirm the absence of contaminating DNA. The PCR products were analyzed in a laboratory physically separated from the PCR laboratory.

**PEP.** The genome of the lysed single cells was amplified for 50 cycles of 1 min at 92 °C, 2 min at 37 °C, a ramping step of 10 s/°C to 54 °C, and 4 min at 55 °C. Random degenerate 15-mer primers at 33 μmol/L were used in accordance with the PEP procedure described by Zhang et al. [12], with the following two modifications: We increased the pH of the PEP buffer (2.5 mmol/L MgCl2, 0.1 g/L gelatin, 10 mmol/L Tris- HCl) to 8.3 from 8.0, and, instead of Taq DNA polymerase, we used 5 U of a cloned thermostable DNA polymerase derived from *Thermus brocianus* F500 (Finzymes, Helsinki, Finland). When genomic DNA samples were amplified, KCl was added to the PEP buffer to give a final concentration of 50 mmol/L. After PEP, the 60-μL samples were divided into 5-μL aliquots and stored at −20 °C until further use or kept at 0 °C and analyzed immediately.

**PCR.** Each of the 10 loci were amplified in individual 100-μL PCR reactions from 5-μL aliquots of the PEP products. The PCR mixtures contained 20 pmol of biotinylated primer, 100 pmol of unbiotinylated primer, the four dNTPs at 0.2 mmol/L, and 1.7 U of Dynazyme II DNA polymerase in buffer supplied with the enzyme (Finzymes). The amplification reactions were carried out for 40 cycles of 1 min at 94 °C, 1 min at a temperature optimal for the primer pair (58 °C for loci 3BHSD, PROS1, IGFL, TP53, and GSN; 54 °C for ADH3 and ARSB; 62 °C for C3; 55 °C for AGA; and 52 °C for PPT), and 1 min at 72 °C in a programmable heat block. The correct size of the PCR products, ranging from 75 to 226 bp, and the quality of the PCR products were initially assessed by electrophoresis in 2% agarose gels.

**Solid-phase minisequencing.** To allow duplicate assays for both nucleotides, we captured four 10-μL aliquots of the PCR products in streptavidin-coated microtiter wells (Combiplate 8; Labsystems, Helsinki, Finland). The wells were washed, and the captured DNA was denatured with 50 mmol/L NaOH as described previously [16]. The minisequencing reaction mixture contained 10 pmol of one detection step primer, 3.7 kBq of one tritiated dNTP—[3H]dATP, 274–311 TBq/mmol TRK 633; [3H]dCTP, 237 TBq/mmol TRK 625; [3H]dTTP, 100–118 TBq/mmol TRK 627; or [3H]dGTP, 422 TBq/mmol TRK 576 (Amersham, Little Chalfont, UK)—and 0.05 U of Dynazyme II DNA polymerase in 50 μL of its buffer. The reaction was allowed to proceed for 10 min at 50 °C. The wells were washed, and the extended primer was eluted by treating the wells with 60 μL of 50 mmol/L NaOH. The incorporated radioactivity was measured with a liquid scintillation counter. Table 3 (discussed later) illustrates representative count per minute (cpm) values obtained in the assay.

**INTERPRETATION OF THE RESULTS**

At homozygous nucleotide positions, a positive signal (>1000 cpm) is generated in one of the minisequencing reactions; at heterozygous positions, signals are generated in the reactions corresponding to both nucleotides at the variable site. The result of the assay is expressed as the ratio R between the [3H]dNTP incorporated in the reaction with one of the variable nucleotides and the [3H]dNTP incorporated with the other variable nucleotide. Calculation of R eliminates sample-to-sample variations from differences in PCR efficiency. R values fall into three distinct categories, which define the genotype of the sample. In homozygous samples, R is >10 or <0.1. At heterozygous nucleotide positions, the R value is affected by the specific activities and the number of [3H]dNTPs that will be incorporated into the alleles. If the nucleotide to be detected at the site of the mutation is followed by one or more identical nucleotides, two or more [3H]dNTPs will be incorporated. Thus, R for a heterozygous sample usually is # 1. Given that both the specific activities and the nucleotide sequence are known in advance, and that R always differs by at least 10-fold from that obtained in homozygous samples at the same locus, the interpretation of the results is unequivocal. The R value is proportional to the ratio between two amplified alleles, even when they are present in ratios other than homozygous (2:0) and heterozygous (1:1).
primers specific for the GSN gene was followed by detection of the nucleotide (G) at position 654 by the solid-phase minissequencing method [11]. We found that PEP with a random mixture of 15-mer primers [12], followed by a single PCR with specific primers for 40 cycles, reproducibly yields a sufficient amount of product to generate a positive signal of >1000 cpn in the minissequencing reaction from a single copy of the diploid genome. Less efficient was a modified PEP procedure in which random 6-mer primers and two-phase amplification [31] were used for a total of 50 cycles; this procedure required 50 copies of the genome for a positive signal after a single locus-specific PCR. A similar result to that obtained with random 15-mer primers in the PEP was obtained with GSN-specific primers in two consecutive PCRs, each for 40 cycles and each with a heminested primer in the second amplification. An obvious advantage of whole-genome amplification as compared to two specific PCRs with nested primers is that the former yields material for several independent PCR amplifications with locus-specific primers. Therefore, the procedure used in all further experiments consisted of amplification of the genome of the single cells by PEP with 15-mer primers, followed by a single PCR amplification with locus-specific primers and detection of the variable nucleotide by the solid-phase minissequencing method. The steps of the optimal procedure are illustrated in Fig. 1.

GENOTYPING OF 10 LOCI FROM SINGLE GRANULOSA CELLS
Granulosa cells were chosen as the model cells for evaluating the method. These cells surround the ovum and are easily isolated (after collection of ova) by a method similar to that used in the separation and analysis of single blastomeres. The genome of 11 granulosa cells isolated from one individual were analyzed for seven common single-nucleotide polymorphisms located on different chromosomes and for the AGU, INCL, and FAF mutations (Table 1).

The genotype of the individual from whom the granulosa cells had been obtained was determined at each locus by analyzing samples of several thousand cells by a single specific PCR amplification without PEP, followed by solid-phase minissequencing. As indicated by the R values <0.1 in this sample (control 2 in Table 2), the individual is homozygous for one of the polymorphic nucleotides at three loci (PROS1, ADH3, and TP53). As expected, the individual carries the normal nucleotide in both alleles at the site of the disease-causing mutations in the PPT, AGA, and GSN genes. The individual is heterozygous for the polymorphism at the 3BHSD, ARSB, IGF2, and C3 loci.

A positive minissequencing result reflecting a detectable amount of PCR product in the second amplification was obtained from the single granulosa cells at 100 of the 102 loci analyzed, indicating that a representative part of the genome had been amplified by the PEP procedure. By reamplifying with specific primers dilutions of the PCR products corresponding to the two negative samples, we found that the reason for the negative result was failure of the PEP. At the six loci for which the individual had been shown to be homozygous, the genotype of the single cells was unequivocally homozygous at all loci at which a PCR product was obtained (Table 2).

Interestingly, we observed differences in the relative amounts of the two alleles amplified at each of the four heterozygous loci. To clarify the reason for these different allele ratios (Table 2), we repeated the minissequencing analysis of the PCR-amplified ARSB locus for four samples (cells 1, 3, 5, and 7) that had shown particularly large differences in R values (Table 3, experiment 2). PCR amplification with the primers specific for the ARSB locus was also repeated from the initial PEP amplification product of these cells; this was followed by the minissequencing assay (Table 3, experiment 3). The differences in R values between the cell samples obtained in the first analysis (Table 3, experiment 1) remained unaltered in these two experiments (F = 0.055; P = 0.947) [32], which shows that the reason for the different allele ratios is random variation in the efficiency of the PEP amplification between the two alleles. Possible introduction of mutations during PEP by the degenerate primers was excluded by carrying out a minissequencing assay for the two other nucleotides (C and T); neither was detected at the site of the variable nucleotide in the ARSB gene. In cell 1 at the ARSB locus and in cells 4 and 6 at the IGF2 locus (Table 2), preferential amplification of one of the alleles during the PEP procedure would have resulted in mistyping of the heterozygous sample as homozygous, according to the stringent criterion of 10-fold differences in R values between the genotypes.

DETECTION OF THREE DISEASE-CAUSING MUTATIONS IN GENOMIC DNA
As shown in Table 2, the normal nucleotide at the position of the mutation in the PPT, AGU, and GSN genes was reproducibly identified from single granulosa cells. To ensure that the actual disease-causing mutation was also detected, we analyzed genomic DNA isolated from INCL and AGU patients homozygous for the respective mutations, from INCL and AGU carriers, and from a patient heterozygous for the FAF mutation. Samples containing 7 and 70 pg of genomic DNA, corresponding to ~1- and 10-diploid genome equivalents, respectively, were amplified by PEP. The mutant and normal nucleotides were correctly identified in all homozygous DNA samples (Table 4). Also in this experiment, we observed variations in the ratios between the amplified alleles in the heterozygous samples. Preferential amplification of the mutant allele in one of the samples from an INCL carrier would have resulted in mistyping of this sample as homozygous mutant. The variation in R values in this experiment could also result from stochastic pipetting errors that may have occurred when diluted DNA samples containing low copy numbers of the genome were added to the PEP amplification reaction mixtures.

ANALYSIS OF BLASTOMERES FROM A HUMAN EMBRYO
Finally, after successful detection of the GSN and AGA genes in single murine blastomeres with primers for the mouse GSN and AGA genes, respectively (data not shown), four single blastomeres isolated from a four-cell-stage human embryo were analyzed. Primers specific for the human PPT, AGA, and GSN genes and for the seven polymorphic loci were used. A signal in
Table 2. Genotyping* of seven polymorphic loci and three disease-causing genes on different chromosomes from single granulosa cells by PEP, PCR, and solid-phase minisequencing.

<table>
<thead>
<tr>
<th></th>
<th>3BHSO</th>
<th>PRO51</th>
<th>ADH3</th>
<th>ARSB</th>
<th>IGF2</th>
<th>TP53</th>
<th>C3</th>
<th>PPT</th>
<th>AGA</th>
<th>GSN</th>
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<td>AG</td>
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<td>0.006</td>
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<td>AG</td>
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Control*:

- Control 1 = 10 granulosa cells subjected to the PEP amplification.
- Control 2 = lysate of several thousands of granulosa cells subjected to specific PCRs and solid-phase minisequencing without PEP amplification.

* Genotypes are designated according to the [(3H)]dNTP incorporated in the minisequencing reaction. The nucleotide of the noncoding strand of the PPT and AGA genes is identified. The results are mean values of duplicate cell samples. Triplicate samples of the culture medium, a sample containing the cell lysis and neutralization solutions, and a sample containing water were included as negative controls in the PEP amplification. Additional negative controls were included in each specific PCR amplification and minisequencing assay. The cpm values from the negative controls were <100 cpm in all samples. Background values were not subtracted from the positive signals (>1000 cpm) before calculation of the R values. The R values are mean values of duplicate minisequencing assays from the same specific PCR product. n.d., not determined; —, no detectable signal obtained.

* Control 1 = 10 granulosa cells subjected to the PEP amplification. Control 2 = lysate of several thousands of granulosa cells subjected to specific PCRs and solid-phase minisequencing without PEP amplification.
the minisequencing assay was generated at all amplified loci (Table 5). According to the R values obtained, the cells of the embryo appeared, as expected, to be homozygous for the normal nucleotide in the PPT, AGA, and GSN genes, and also at the ADH3, TP53, and C3 loci. The R values also showed that the embryo was heterozygous for the polymorphism at the 3BHSD, PROS1, ARSB, and IGF2 loci, but again variations in the relative amount of the amplified alleles were observed. In blastomere 2, the embryo would have been typed as homozygous for nucleotide G at the ARSB locus, whereas the embryo was clearly heterozygous at the ARSB locus in the three other blastomeres.

**Discussion**

Preimplantation diagnosis of IVF embryos offers a relevant alternative to conventional prenatal diagnosis of couples at risk of conceiving a child with a difficult hereditary disorder that has a well-characterized molecular genetic background. We report here a procedure generally applicable for the analysis of single blastomeres potentially carrying disease-causing mutations. The technique comprises whole-genome amplification by PEP with random 15-mer primers [12], a single PCR amplification with locus-specific primers, and identification of the mutation by a solid-phase minisequencing method [13]. By successful analysis of human single granulosa cells and blastomeres at 10 loci in coding regions on different chromosomes, we showed that a highly representative part of the genome was amplified by PEP. PEP failed to yield a product that was amplifiable by the locus-specific PCR in only 2 cases out of 146.

Surprisingly, our procedure, which is based on a single specific PCR amplification of the PEP product, was equally or even more specific and sensitive than most previously described procedures for PEP-based single-cell analyses, which utilize nested or heminested primers in a second specific PCR amplification [7, 14, 15]. In our experience, ~100-1000 copies of the genome are required to detect a variable nucleotide from genomic DNA by a single PCR for 40 cycles and solid-phase minisequencing. Assuming equal efficiency of the PCR for PEP products and genomic DNA, and taking into account that 1/12 of the PEP product was amplified by the specific PCRs, we estimate that PEP amplification generated at least 1000 copies of the genome. This figure is much larger than the frequently cited estimate by Zhang et al. [12], according to which PEP amplification yields 78% probability at least 30 copies of any sequence of the genome from a haploid cell. The notion that substantially more than 30-60 copies of the diploid genome were generated by PEP amplification is supported by a calculation based on the following: (a) We observed a positive signal in the minisequencing assay by analyzing 1/10 of the PCR products generated after 40 amplification cycles of 1/12 of the total PEP product; (b) 40 PCR cycles at 100% efficiency can theoretically yield a 10^{12}-fold amplification; (c) according to the specific

### Table 3. Demonstration of uneven amplification of alleles at the ARSB locus during PEP with granulosa cells.

<table>
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<td>1540b</td>
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<td>135a</td>
<td>1300a</td>
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<td>148a</td>
<td>2650a</td>
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*The data from Experiment 1 are those presented in Table 2. Experiment 2 was a separate minisequencing assay of the same PCR product as in Experiment 1. Experiment 3 was a separate PCR and minisequencing assay performed with the initial PEP product.

b Cpm incorporated; means of duplicate minisequencing assays.
c Control was a lysate of several thousand granulosa cells subjected directly to PCR without PEP.

### Table 4. Detection of INCL, AGU, and FAF mutations in single copies of genomic DNA by PEP, PCR, and solid-phase minisequencing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of DNA (pg)*</th>
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<th>AGU</th>
<th>Genotype</th>
<th>FAF</th>
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<td>GG</td>
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<td>70</td>
<td>200</td>
<td>AA</td>
<td>20</td>
<td>GG</td>
<td>1; 4</td>
<td>AG</td>
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<td>Carrier</td>
<td>7</td>
<td>50c; 2; 0.3</td>
<td>AT</td>
<td>0.5; 1</td>
<td>GC</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>70</td>
<td>3</td>
<td>AT</td>
<td>0.3</td>
<td>GC</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>7</td>
<td>0.009; 0.006; 0.01</td>
<td>TT</td>
<td>0.01; 0.01; 0.02</td>
<td>CC</td>
<td>0.03; 0.01</td>
<td>GG</td>
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<tr>
<td></td>
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<td>0.007</td>
<td>TT</td>
<td>0.07</td>
<td>CC</td>
<td>0.02; 0.01</td>
<td>GG</td>
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</table>

*Triplicate samples of DNA from the same individual were analyzed.

The INCL and AGU patient and carrier samples and the FAF patient sample were analyzed for mutations in PPT, AGA, and GSN genes, respectively.

This sample shows only a 4- or 6-fold difference in R value compared with that of the homozygous mutant (AA) samples and would have been falsely genotyped as homozygous mutant (AA) according to the criterion of 10-fold differences of R values between the genotypes.
activity of the [³H]dNTPs used, incorporation of \(2 \times 10^{10}\) molecules of [³H]dNTP is required for a signal of >1000 cpm in the minisequencing assay. If only 60 copies of the diploid genome had been present in the PEP product, a positive signal in the minisequencing reaction would be obtained only if the PCR and minisequencing reactions occur with 100% efficiency, which is hardly the case. Our estimate of the efficacy of PEP is in accordance with a recent study in which theoretical modeling of the PEP amplification procedure suggested that PEP generates substantially higher yields than has been previously estimated [33].

In the solid-phase minisequencing method, sequence variants are distinguished by using a DNA polymerase to specifically extend the 3' end of a primer that has been annealed immediately upstream of a variable nucleotide position. The reaction is highly specific, allowing unequivocal genotyping at any variable position. Because the assay is carried out in a solid-phase format, the results are directly obtained as objective numeric R values, which define the genotype of the samples. At all analyzed homozygous nucleotide positions, the correct genotype was identified, indicating that mutations are not introduced by the random primers used for the PEP amplification. The absence of mutations introduced by the PEP primer was also demonstrated experimentally by analyzing all four nucleotides by the solid-phase minisequencing method at two loci. Furthermore, the solid-phase minisequencing method is a particularly favorable tool for determination by PCR of the ratio between two sequences (e.g., alleles) that differ from each other by a single nucleotide and are present as a mixture in a sample. Because the two sequences are essentially identical, they are amplified with equal efficiency regardless of whether the PCR amplification is in its exponential phase or not [34]. Therefore, our method avoids the preferential amplification of one of the alleles in a heterozygous sample, which has been shown to occur during PCR because of differences in size or sequence of the allele [35]. In the present study, the solid-phase minisequencing method revealed uneven distribution between the alleles in the final PCR product at all heterozygous loci analyzed. As could be expected, and as was shown experimentally, this variation in ratios between the alleles originates from the PEP, presumably by uneven amplification of the alleles during the initial PEP cycles, where only a few copies of the genome are present, and not from the PCR with locus-specific primers. At 4 of the 52 heterozygous nucleotide positions analyzed from human single cells at which a detectable PCR product was obtained, preferential amplification of one of the alleles would have resulted in the genotyping of a heterozygous sample as homozygous, according to the stringent criterion of 10-fold differences in R values between the genotypes. However, at each of the four mistyped positions, incorporation of the [³H]dNTP corresponding to the missing nucleotide was higher than the background values of the true homozygote samples. In previous studies, we have shown that the solid-phase minisequencing method allows quantitative detection of a mutant sequence present as <1% of a sample [9, 36]. The rate of correct genotyping of single cells by the present procedure can thus be further improved by applying less stringent criteria for defining the genotypes, which is feasible in practice after the method has been carefully standardized for the particular nucleotide position to be analyzed. In a study in which fluorescent PCR products amplified from single cells were detected with high sensitivity by a DNA-sequencing instrument, Findley et al. clearly showed that the problem of preferential amplification of one allele was substantially less than in conventional, less-sensitive detection methods [37]. A drawback of detecting PCR products with a DNA sequencer is that the method can only be used for sex determination or for detecting mutant alleles that differ in size from the normal allele (e.g., the alleles carrying the cystic fibrosis Δ-F508 deletion mutation) and not for detecting point mutations.

The risk of mistyping a heterozygous sample as homozygous because of preferential amplification of the normal allele during PEP is a serious problem in the preimplantation diagnosis of dominantly inherited disorders because failure to detect the disease-causing allele would result in a false prediction of the health status of the embryo. The same problem can be encountered in recessive disorders caused by compound heterozygous mutations. However, the problem can be overcome by analyzing two individual cells from the same embryo, which greatly increases the probability of correct genotyping.

The procedure developed in this study has several advantages in clinical practice. The use of a single, specific PCR amplification after PEP instead of two consecutive PCR amplifications, as used in previous PEP-based methods developed for single-cell

| Table 5. Analysis of single blastomeres from a human embryo by PEP, PCR, and solid-phase minisequencing at 10 loci. |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Blasto-mere | 38H5D | PRO51 | ADH3 | ARS8 | IGF2 | TPS3 | C3 | PPT | AGA | GSN |
| R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type | R | Geno-type |
| 1 | 0.3 | AC | 0.2 | AG | 0.008 | GG | 0.7 | AG | 0.01 | GG | 14 | CC | 0.02 | TT | 0.01 | GG | 0.02 | GG |
| 2 | 0.3 | AC | 1 | AG | 0.02 | GG | 0.008 | GG | 6 | AG | 0.005 | GG | 16 | CC | 0.05 | TT | 0.02 | GG | 0.04 | GG |
| 3 | 0.6 | AC | 0.8 | AG | 0.007 | GG | 1 | AG | 0.004 | GG | 11 | CC | 0.02 | TT | 0.02 | GG | 0.02 | GG |
| 4 | 0.6 | AC | 0.8 | AG | 0.01 | GG | 0.8 | AG | 4 | AG | 0.008 | GG | 13 | CC | 0.02 | TT | 0.01 | GG | 0.03 | GG |

The genotypes are designated according to the [³H]dNTP incorporated in the minisequencing reaction. The nucleotide of the noncoding strand of the PPT and AGA genes is identified. Triplicate samples of the culture medium and the cell lysis and neutralization solutions, and a sample containing water were included as negative controls in the PEP amplification. An additional negative control was included in each specific PCR amplification and minisequencing assay. The cpm values of the negative controls were <100 cpm in all samples. Background values were not subtracted from the positive signals (>1000 cpm) before calculation of R values. R values are mean values of duplicate minisequencing assays from the same specific PCR product.
analysis \[14, 15\], reduces the time required for the analysis. Also, a single PCR amplification reduces the risk of DNA contaminations by minimizing the number of manipulations of the samples. Prevention of contamination is of crucial importance, especially in preimplantation diagnosis, where single cells are analyzed and the analysis cannot be repeated. In the future, the risk of contamination associated with methods based on DNA amplification may be avoided by using methods that detect the mutations directly from blastomeres by fluorescence in situ hybridization (FISH). So far, diagnosis of single cells by FISH has been applied to X-linked disorders, where determination of the sex of the embryo and transfer female embryos can be used only as a preventive tool \[38\]. Detection of single-nucleotide variations by FISH will require the development of probes with more-sensitive labels and higher specificity than those available today; e.g., the recently devised "padlock" probes are promising \[39\]. Finally, the solid-phase minisequencing method can be applied for the detection of all disease-causing mutations under the same reaction conditions. In this study, we detected single-nucleotide changes, but the method can be designed for the identification of both small and large deletions.

We conclude that whole-genome amplification by PEP followed by identification of the disease-causing mutation by the solid-phase minisequencing method is feasible for preimplantation diagnosis; however, the risk of preferential amplification of one of the alleles has to be considered in the diagnosis of dominantly inherited disorders and of recessive disorders caused by compound heterozygous mutations. The possibility of false genotyping can be decreased by analyzing two individual cells from the same embryo at the eight-cell stage and by standardization of the solid-phase minisequencing method to allow reliable detection of a small minority of the mutant sequence in the presence of a vast excess of the normal sequence. Consequently, the procedure developed here can be used not only for preimplantation diagnosis of recessive disorders in an isolated population but also for a wide range of hereditary diseases that have been characterized at the molecular level. Obviously, our procedure will also be highly useful for genotyping multiple loci from samples containing a limited number of cells in applications other than preimplantation diagnosis.

We thank Katri Virtanen, Hannele Pihlaja, Mari Sipilä, and Anne Nyberg for their invaluable technical assistance; Jouni Vesa and Elina Hellsten for providing unpublished sequence information on the PPT gene; Markus Perola for advice on statistics; and Outi Hovatta, Eero Lehtonen, Jorma Palo, and Leena Peltonen for encouraging us throughout the study. Grants from the Maud Kuistila Foundation, the University of Helsinki, and the Academy of Finland are acknowledged.

References


