Improvement in Sensitivity of Allele-specific PCR Facilitates Reliable Noninvasive Prenatal Detection of Cystic Fibrosis

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Background: Cell-free fetal DNA circulating in maternal blood has potential as a safer alternative to invasive methods of prenatal testing for paternally inherited genetic alterations, such as cystic fibrosis (CF) mutations.

Methods: We used allele-specific PCR to detect mutated CF D1152H DNA in the presence of an excess of the corresponding wild-type sequence. Pfx buffer (Invitrogen) containing replication accessory proteins and Taq polymerase with no proofreading activity was combined with TaqMaster PCR Enhancer (Eppendorf) to suppress nonspecific amplification of the wild-type allele. The procedure was tested on DNA isolated from plasma drawn from 11 pregnant women (gestational age, 11–19.2 weeks), with mutation confirmation by chorionic villus sampling.

Results: The method detected 5 copies of the CF D1152H mutant allele in the presence of up to 100,000 copies of wild-type allele without interference from the wild-type sequence. The D1152H mutation was correctly identified in one positive sample; the only false-positive result was seen in a mishandled sample.

Conclusions: This procedure allows for reliable detection of the paternally inherited D1152H mutation and has potential application for detection of other mutations, which may help reduce the need for invasive testing.

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Two common clinical sampling methods used for early prenatal diagnosis are chorionic villus sampling (CVS) and amniocentesis. Both invasive methods carry a small but significant risk to the fetus; procedure-related loss rates are currently 0.23–0.7% (1-4). The potential harm to the fetus involved with CVS and amniocentesis limit their use to women demonstrating certain risk factors (e.g., advanced maternal age and family history of genetic disease). The discovery of circulating fetal DNA in maternal blood has opened up new possibilities for safer (e.g., noninvasive) prenatal diagnosis (5-7).

To date, fetal DNA isolated from maternal plasma and serum has been used to determine fetal gender (8–10), Rh factor (11–13), and genetic disorders such as myotonic dystrophy (14) and achondroplasia (15). Tang et al. (16) used polymorphic markers on the X chromosome to detect DNA from the female fetus in maternal blood. Chen et al. (17) used a similar approach to screen for paternally inherited fetal aneuploidy. Recently, Chiu et al. (18) tested DNA isolated from the maternal circulation for the presence of β-thalassemia major, and González-González et al. (19) used maternal plasma to detect cystic fibrosis (CF) mutation Q890X in the fetus. Additionally, González-González et al. (20) were able to confirm that a fetus had inherited the wild-type allele for the gene that causes Huntington disease from a father who was an asymptomatic carrier of the mutation.

Diagnosis using cell-free fetal DNA is not without complications. The low quantity of fetal DNA in the maternal circulation and interference from an excessive amount of maternal DNA make detection of fetal mutations difficult and increase the chance of false results. Reported diagnostic procedures are not generally appli-
cable to all paternally inherited mutations; they remain limited to specific situations in which the mutation type makes detection of genetic alterations relatively easy. This includes mutations where the nucleotide sequence of the affected allele is changed in such a way that it can be recognized by a restriction enzyme (15,19), cases in which there is a deletion of several nucleotides (18), or cases in which there is an expansion of polymorphic nucleotide repeats (14,20).

Allele-specific PCR (ASPCR) (21) is a PCR-based method with the ability to differentiate among different alleles of the same gene and is frequently used to screen for mutations. The technique was already applied for noninvasive prenatal detection of a single-gene disorder (18); however, that report concerned the specific situation of a microdeletion in the β-thalassemia gene, which makes analysis less difficult. The ability of ASPCR to differentiate between the mutated and wild-type alleles depends on several factors, including the type of mutation (22), the amount of alleles present in the sample, and the ratio between alleles. These factors are responsible for the frequent appearance of false-positive results, leading many researchers to attempt to increase the reliability of ASPCR (23–26). Unfortunately, because of the conditions existing in maternal plasma, these methods are not easily applied to noninvasive prenatal screening.

In this study, we focused on improving the accuracy and sensitivity of ASPCR to differentiate alleles to make it suitable for noninvasive prenatal diagnostics. We sought a simple, reliable, single-tube diagnostic assay with potential for general application. CF mutation D1152H was chosen to test our approach. CF is an autosomal disorder caused by many different mutations in the CFTR gene (~1000 described). CF mutation D1152H consists of a single nucleotide substitution (G to C) at nucleotide 3586, changing Asp to His at amino acid position 1152 (27).

### Materials and Methods

**Patients**

This study was approved by the Institutional Review Board of Drexel University College of Medicine, and informed consent was obtained from all patients. The laboratory used ~5–10 mL of blood drawn from the peripheral vein of 11 pregnant women (Table 1) 20–41 years of age during the first and second trimesters of pregnancy. The gestational age of the fetuses ranged from 11 to 19.2 weeks. Blood was collected before the women underwent either amniocentesis or CVS and was processed from less than 1 day to 6 days after collection. Patients 1–5 and 7–11 were used as negative controls. Patient 6 was carrying a fetus known to be heterozygous for the CF D1152H mutation (determined by a commercial laboratory by use of invasive prenatal diagnosis). This patient was a carrier for the CF ΔF508 mutation, and the father of the fetus was a carrier for D1152H. To obtain DNA containing the D1152H mutation for use in the model experiment, we collected ~10 mL of blood from the father.

**Blood processing and extraction of DNA from maternal plasma**

Cell-free plasma samples were obtained by centrifugation of the whole blood at 1600g for 10 min. The supernatant was collected and centrifuged at 16 000g for 10 min to remove all remaining cells (28). Finally, the plasma, free of blood cells, was removed from the microcentrifuge tubes and stored at ~80 °C. DNA was extracted by use of the Qiagen Blood Mini Kit according to the manufacturer’s “Blood and Body Fluid Spin Protocol”, with minor modifications. In our procedure, DNA from 1.6 mL of each patient’s plasma was extracted by use of two Qiagen minicolumns by processing two 400-μl aliquots on each of them. After application of the aliquots

### Table 1. Summary of patient data.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Maternal age, years</th>
<th>Gestational age, weeks</th>
<th>Time between collection and processing of blood, days</th>
<th>Fetal D1152H mutation status</th>
<th>DNA in PCR tubes, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Patient samples used in the first set of experiments.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>19.2</td>
<td>3</td>
<td>−/−</td>
<td>53.3</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>15.5</td>
<td>6</td>
<td>−/−</td>
<td>307.7</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>11.2</td>
<td>1</td>
<td>−/−</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>15.1</td>
<td>3</td>
<td>−/−</td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>18</td>
<td>5</td>
<td>−/−</td>
<td>318.5</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>11</td>
<td>0</td>
<td>+/−</td>
<td>0.3</td>
</tr>
</tbody>
</table>

| B. Blood samples obtained from control patients and processed in 1 day. | | | | | |
| 7 | 32 | 16.5 | 1 | −/− | 1.6 |
| 8 | 20 | 18.1 | 1 | −/− | 1.9 |
| 9 | 41 | 11.4 | 1 | −/− | 2.6 |
| 10 | 34 | 11.5 | 1 | −/− | 3.0 |
| 11 | 20 | 17.4 | 1 | −/− | 13.1 |

* The quantity of DNA in the PCR tubes was determined by the GAPDH assay described in the “Materials and Methods”. |
and according to the manufacturer’s procedure, the DNA immobilized on the columns was eluted with 150 μL of autoclaved water. To obtain efficient recovery of DNA without decreasing its concentration, we eluted the DNA from the column filters according to the following steps. Autoclaved water (150 μL) was applied on the first column, incubated for 3 min at room temperature, and then centrifuged at 6000g for 1 min. The eluate was reloaded on the same column, and the incubation and centrifugation were repeated. Subsequently, the eluate from the final centrifugation step for the first column was applied to the filter of the second column, and the DNA was again recovered by elution, reloading of the eluate, and centrifugation. The final volume of the DNA sample was decreased to ~120 μL because of loss on the centrifugation columns.

DNA USED IN THE MODEL PCR EXPERIMENT
Genomic DNA containing CF mutation D1152H was isolated from 800 μL of whole blood from a heterozygous male carrier (father of the fetus carried by patient 6) by use of the DNA Blood Mini Kit according to the manufacturer’s protocol. DNA samples with various ratios of mutant to wild-type alleles were prepared in the PCR reaction tubes by combining 30 pg of D1152H heterozygous allele with 3, 30, and 300 ng of wild-type DNA obtained from Sigma.

OLIGONUCLEOTIDES
The sequences of oligonucleotide primers 1152F (5'-GATAAGACTTACCAAGCTATCCACATG-3') and 1152R (5'-GAGTTGGTATTATCCGTTAGCCA-3'), used for amplification of DNA carrying the D1152H mutation, were designed with Primer Express 1.5 software (Applied Biosystems) and synthesized by Proligo. Forward primer 1152F is specific for the D1152H mutation and exhibits a perfect match to the mutated sequence. Oligonucleotide 1152R was used as a reverse primer. Primer Des1152F with the sequence 5'-GATAAGACTTACCAAGCTATCACAAG-3' is identical to 1152F except for the second nucleotide from its 3' end. This oligonucleotide was used in the initial experiments and represents a destabilized D1152H mutation-specific primer. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers and fluorescent probe were manufactured by Applied Biosystems according to the sequence listed in Zhong et al. (29). The sequences were as follows: forward primer GAPDH-F, 5'-CCCCACACACATGCACTTACC-3'; reverse primer GAPDH-R, 5'-CTGACCTTGCCGGCTTGTGAT-3'. The fluorescent GAPDH TaqMan probe GAPDH-PRO had the sequence 5'-(FAM)AAAGAGCTAGGAAGGACAG-GCAACTTGGC(TAMRA)-3', where FAM is 6-carboxytetramethylrhodamine.

CONVENTIONAL PCR
All ASPCR amplifications for detection of the D1152H mutation on the CFTR gene were performed on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems).

PCR CONDITIONS USED IN INITIAL ATTEMPTS TO DIFFERENTIATE THE D1152H MUTATED AND WILDTYPE CF ALLELES
PCRs were performed in volumes of 50 μL. The reaction mixture contained the DNA sample, 8 pmol of forward primer 1152F or Des1152F with 8 pmol of reverse primer 1152R, 1× PCR buffer with magnesium (final concentration, 1.5 mM) and deoxynucleotide triphosphates (dNTPs; final concentration of each, 200 μM) from the PCR Core reagent set (cat. no. 1 578 553) supplied by Roche Diagnostics GmbH, and 1 μL of Accuprime Taq DNA polymerase from the Accuprime Taq DNA Polymerase System (cat. no. 12339-016; Invitrogen). Thermal cycling conditions for the PCRs were as follows: 2 min at 94 °C, followed by 49 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

REACTION CONDITIONS PROVIDING HIGH ACCURACY FOR ALLELIC DIFFERENTIATION
PCRs were carried out in volumes of 50 μL containing 30 pmol of forward primer 1152F, 20 pmol of reverse primer 1152R, 7.5 μL of 10× Accuprime Pfx Reaction Mix from the Accuprime Pfx DNA Polymerase Kit (cat. no. 12344-024; Invitrogen), 1 μL of Accuprime Taq DNA polymerase from the Accuprime Taq DNA Polymerase System, 10 μL of 5× TaqMaster PCR Enhancer (Eppendorf), and the DNA sample.

For prenatal DNA analysis, 10 μL of DNA isolated from maternal plasma was used in the PCR. This amount is equivalent to the DNA content of ~110 μL of plasma before processing. The amount of DNA used for PCR analysis in our model experiments was variable and is discussed in the section concerning preparation of DNA for the model PCR experiment. Thermal cycling conditions of PCR reactions were as follows: 2 min at 94 °C, followed by 50 cycles at 94 °C for 30 s, 58 °C for 1 min, and 68 °C for 45 s, with a final hold at 68 °C for 5 min.

To obtain results from amplifications performed on conventional PCR instruments, we analyzed 10 μL from each 50-μL reaction on 3% agarose gels containing ethidium bromide in 1× Tris-borate-EDTA buffer (30). The fluorescence of the DNA fragments was visualized and recorded with a FluorChem 8800 Imaging System (Alpha Innotech).

REAL-TIME PCR
A TaqMan assay for the GAPDH gene was used to determine the total amount of circulating DNA present in the maternal plasma samples (29). The forward primer was GAPDH-F, and the reverse primer was GAPDH-R.
Fluorescent TaqMan probe GAPDH-PRO was used to obtain the DNA amplification profile of the PCR.

TaqMan real-time PCR analysis was performed on the ABI Prism 7700 Sequence Detector (Applied Biosystems). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, and 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The PCR amplifications were carried out in a 50-μL reaction volume and contained 10 μL of DNA, 300 nM each primer, 100 nM GAPDH TaqMan probe, and the following components from the TaqMan PCR Core Reaction Kit: 5 μL of 10× buffer A, 3.5 mM magnesium chloride, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 0.025 U/μL AmpliTaq Gold, and 0.01 U/μL AmpErase. The AmpErase enzyme contains a uracil N-glycosylase activity that, when used with dUTP (instead of dTTP), prevents PCR product carryover.

To determine the amount of DNA in samples isolated from maternal plasma, we ran PCRs containing 3, 30, and 300 ng of female DNA (Sigma) in parallel and used them as standards. DNA samples were analyzed in triplicate.

Results

ASSAY DEVELOPMENT AND ANALYSIS OF ARTIFICIAL MODEL SAMPLES

On the basis of data concerning the amount of fetal DNA in maternal blood (31) and considering the gestational ages of the analyzed pregnancies, the DNA isolation procedure, and the size of the DNA aliquots used for the PCR, we expected approximately two or more copies of the fetal allele to be present in each of the PCRs performed on plasma DNA isolates. However, because of frequent inconsistencies in PCR amplification when working with quantities of target lower than five copies—probably caused by an uneven distribution of intact DNA target molecules among the samples—we decided to include five copies of the mutated allele in all aliquots of D1152H-positive artificial specimens used in PCRs. These artificial specimens were used to develop the D1152H mutation detection assay. Considering that the diploid DNA content of one cell is 6.6 pg (28), five copies of the mutated allele should be present in ~30 pg of DNA isolated from a heterozygous D1152H carrier.

According to Lo et al. (32), fetal DNA represents 3.4–6.2% of the total cell-free DNA circulating in the maternal blood. Thus, to make model D1152H-positive samples that resemble conditions in maternal plasma—and to allow for biological variation and additionally to test the limits of our procedure—we prepared several PCR samples by mixing 30 pg of D1152H heterozygous carrier DNA (isolated from the father of the fetus carried by patient 6) with 3, 30, and 300 ng of wild-type female DNA (Sigma).

We decided to use ASPCR in the development of an assay for noninvasive prenatal screening. However, our initial experiments, using mutation-specific primer 1152F, reverse primer 1152R, Accuprime Taq polymerase without 3’-exonuclease activity (Invitrogen), dNTPs and reaction buffer providing a final magnesium chloride concentration of 1.5 mM (Roche), failed to differentiate between the D1152H mutated and wild-type CF alleles (Fig. 1A).

Thus, to make the ASPCR technique applicable to our specific needs, we had to develop conditions that would suppress the nonspecific amplification caused by the interfering presence of excessive maternal DNA.

We first experimented with changing the sequence of mutation-specific primer 1152F by altering nucleotides located close to the 3’ end of the primer. Primers with destabilizing nucleotide substitutions were previously reported to improve ASPCR-based allelic differentiation (32). The results of one such PCR experiment—using destabilized mutation-specific forward primer Des1152F, reverse primer 1152R, Accuprime Taq polymerase without 3’-exonuclease activity (Invitrogen), and dNTPs together with reaction buffer providing a final magnesium chloride concentration of 1.5 mM (Roche), are shown in Fig. 1B. Detailed reaction conditions are described in the “Material and Methods”.

As can be seen from comparison of panels A and B in Fig. 1, the use of destabilized primer Des1152F improved reaction specificity by increasing suppression of the wild-type CF allele amplification. Unfortunately, wild-type genomic DNA at a concentration of 3 ng in a 50-μL PCR reaction still produced the amplification band. The band
was extremely weak and difficult to see. Gel analysis of PCR performed with 6 ng of wild-type DNA already showed strong amplification. Thus, based on these data, the system is not acceptable for diagnostic applications in which higher suppression of the wild-type CF allele is required to avoid false-positive results.

By contrast, the combination of a primer pair containing perfectly matched D1152 mutation-specific forward primer 1152F and reverse primer 1152R, Pfx PCR buffer from the Accuprime Pfx DNA Polymerase Kit (Invitrogen), Accuprime Taq polymerase (without proofreading activity; from the Accuprime Taq DNA Polymerase System), and TaqMaster PCR Enhancer (Eppendorf) allowed consistent detection of 5 copies of the CF D1152H mutant allele in the presence of up to \( \sim 100 \, 000 \) copies of the wild-type allele without interference from the wild-type sequence. The amplification of up to 300 ng of genomic DNA in a PCR (equivalent to \( \sim 100 \, 000 \) copies of the wild-type allele) was completely suppressed.

The results of the model PCR experiment are shown in Fig. 2. Approximately 5 copies of the D1152H mutated allele contained in 30 pg of D1152H heterozygous genomic DNA was amplified in the presence of 0–300 ng of wild-type female DNA (i.e., up to \( \sim 100 \, 000 \) copies of the wild-type allele). No amplification was observed with 0–300 ng of female DNA in the absence of the D1152H mutated allele.

**Analysis of DNA Samples Isolated from Maternal Plasma**

*Testing for the presence of the D1152H mutation.* We studied DNA from the plasma of a woman known to be carrying a fetus with the CF D1152H mutant gene inherited from the father (Table 1, patient 6) and five controls. The sample from patient 6 (Fig. 3A, lanes 2–4) showed amplification of the mutant D1152H allele in all three reactions. The reduced intensity of the PCR product in lane 2 is most likely caused by competing formation of a primer-dimer that produced a band of lower molecular weight than the CF gene fragment. Samples from four of five control patients (Fig. 3, B and C) showed no amplification of the mutant D1152H allele, but patient 2 (Fig. 3C, lanes 5–7) showed a band in two of three amplifications. By contrast, the results from the CVS of patient 2 and a blood test performed on the father of the pregnancy were both negative for the D1152H mutation.

*Comparison of DNA content among DNA samples isolated from maternal plasma of patients.* The model experiment worked reliably and reproducibly in all PCRs containing up to 300 ng of wild-type DNA. This amount corresponds to \( \sim 2.8 \mu g/mL \) (\( \sim 424 \, 000 \) genome equivalents/mL) of cell-free DNA in plasma and, based on previously reported results (10, 29), is substantially higher than would be expected in a wild-type maternal sample during pregnancy. However, to determine whether any of the samples contained DNA concentrations greater than this, we performed real-time PCR using primers and probe specific to the GAPDH gene on all patient samples to determine the total amount of DNA. This was accomplished by use of a method similar to the one described by Zhong et al. (29), with 3, 30, and 300 ng of female DNA as the standards. The amounts of DNA present in DNA isolates obtained from maternal plasma of patients 1–6 were calculated from real-time PCR threshold cycle values and are shown in Table 1A.

The amount of DNA in PCRs of two samples (patients 2 and 5) exceeded 300 ng. As mentioned before, the sample from patient 2 provided a false-positive result. This higher amount of DNA may explain the failure of the method because the reliability of the assay was only tested
up to 300 ng of DNA. It appears that 300 ng is close to the limit of the assay, and analysis of samples containing amounts of DNA exceeding this limit is not reliable. The quantity of DNA in the other samples was <300 ng.

Because of the large differences in the amount of DNA in the samples, we reviewed all records of patients 1–6. The relevant information is summarized in Table 1A. From that data, it can be seen that there was considerable variation in the length of time between initial blood draw and the start of blood processing (ranging from 0 to 6 days). Patients 2 and 5, with DNA amounts exceeding 300 ng, both showed abnormal delays in the start of blood processing (6 and 5 days, respectively). Samples processed more quickly had lower amounts of DNA. This finding is logical. The blood was collected in tubes containing EDTA, which stabilizes DNA and prevents its degradation by nucleases. However, any delay in processing can lead to lysis of the intact cells with an increase in the proportion of maternal cell-free DNA in the samples, which can subsequently interfere with ASPCR.

**Testing of an ASPCR-Based D1152H Mutation Assay on Appropriately Handled Maternal Blood Samples Obtained from D1152H-Negative Control Pregnancies**

To demonstrate the reliability of the assay in terms of suppression of nonspecific amplification interference from the wild-type CFTR allele, we isolated new negative DNA control samples from appropriately processed maternal blood specimens corresponding to pregnancies with no D1152H mutation (Table 1B, patients 7–11). Maternal plasma used for DNA extraction was separated from blood cellular components no more than 1 day after blood collection, which dramatically decreased in the quantity of total DNA present in DNA aliquots used in PCRs (Table 1).

PCRs with primers 1152F and 1152R, PfX reaction buffer, Accuprime Taq Polymerase without 3′-exonuclease activity, and PCR additive (Eppendorf) were set up and performed as described in the “Material and Methods”. The results of the experiments are shown in Fig. 4. Fig. 4A corresponds to analysis of samples from patients 7–9, and Fig. 4B represents results of PCR performed on samples from patients 10 and 11 and negative and positive controls. The negative amplification results of PCRs performed on samples 7–11 demonstrate that the assay does not produce false-negative signals in cases where blood samples are processed no more than 1 day after blood drawing.

**Discussion**

After searching for a suitable method that would allow detection of fetal mutations in maternal blood samples, we decided to use ASPCR, a PCR-based technique often used for genotyping. However, a frequent cause of failure of ASPCR is uneven distribution of different alleles of the same gene in a sample, which leads to false-positive results produced by the abundant form of the gene. Additionally, the low quantities of the target allele and reduced amplification efficiency resulting from the stringent conditions essential for allelic differentiation requires a high number of PCR cycles to detect amplification products. The high number of cycles is usually responsible for the accumulation of nonspecific amplification products and thus higher interference by the excessive allele.

On the basis of our experience with ASPCR, it is possible to detect certain types of genetic alterations present at concentrations <5 copies in a PCR tube and simultaneously suppress up to ~1000 copies of the wild-type allele (3 ng of genomic DNA). Considering the ratio of fetal to maternal DNA present in plasma (11) and the expected amount of total DNA in PCRs using the experimental setup described in the “Material and Methods”, this amount of allelic differentiation should be sufficient for noninvasive prenatal diagnosis of paternally inherited disorders. However, this applies only to a limited number of mutation types, and moreover, the amount of maternal DNA in the PCR sample can, in certain situations, exceed 3 ng and interfere with the assay. A significant increase in maternal DNA in the blood can occur as a result of several situations, including (a) certain diseases, such as pre-eclampsia (29); (b) viral infections, which can cause increased blood cell count and/or lysis of the cells; and (c) delayed processing or mishandling of the blood. It is possible to measure the amount of DNA in a sample by real-time PCR or fluorometry and exclude all samples with increased amounts of DNA from analysis. This, however, would reduce the usefulness of the procedure, limiting it to only “ideal” specimens.

Thus, to make ASPCR reliable and useful for clinical noninvasive prenatal testing, we focused on improving the accuracy of allelic differentiation to the extent that
interference by the wild-type allele would not be a limiting factor. Several published improvements in ASPCR already exist, but to our knowledge, all of those studies reported improved accuracy only in cases in which a sufficient quantity of the underrepresented allele allowed the use of a relatively low number of PCR cycles.

Recently, Invitrogen introduced several new PCR-related products. Two of them, the Accuprime Taq DNA Polymerase System and the Accuprime Pfx DNA Polymerase Kit, differ dramatically from PCR reagent sets supplied by other companies. According to the manufacturer, the reaction buffers included in these reagent sets contain thermostable proteins belonging to the replication accessory protein group (33, 34). Details regarding the proteins included in the proprietary buffers were unavailable. Replication accessory proteins are included in the PCR buffers to reduce the appearance of nonspecific amplification products resulting from annealing of primers to incorrect but similar sequences.

Considering the previously mentioned problems with the accuracy of ASPCR for allelic differentiation, we hypothesized that adding replication accessory proteins to our reactions would suppress potential false-positive results caused by high amounts of the wild-type allele. Because the mutation-specific primer is designed to perfectly match its mutated target, annealing of this primer to the wild-type allele would cause a mismatch at the 3' end of the primer. Thus, it is likely that replication accessory proteins would help destabilize mismatched primers and inhibit undesired amplification of the wild-type allele.

The results presented here demonstrate that inclusion of the buffer from the Pfx System (Invitrogen) containing an advanced mixture of replication accessory proteins substantially improved the ability of ASPCR to differentiate alleles. However, the Accuprime Pfx polymerase (with proofreading activity) that is supplied with the Accuprime Pfx DNA Polymerase Kit had to be replaced by the Taq polymerase (without proofreading activity) from the Accuprime Taq DNA Polymerase System. The Pfx polymerase would have repaired mismatches formed between mutation-specific primers and the wild-type allele during PCR, which would have led to amplification of the wild-type DNA.

To reduce possible inconsistencies in PCR amplifications, we included TaqMaster PCR Enhancer (Eppendorf) in the reactions. During our PCR experiments, we found that including this additive also helped decrease the formation of primer-dimer artifacts. These dimers can compete with the desired PCR product and reduce its yield.

We discovered that our ASPCR-based method loses its reliability and provides occasional false-positive signals when the amount of wild-type genomic DNA in the PCR exceeds 300 ng. This was the case with the mishandled blood sample of patient 2, which contained an extremely high amount of DNA. False-positive results, however, can be prevented by processing of maternal blood samples within 24 h after blood draw, as is demonstrated in Fig. 4. It should also be noted that (a) it is beneficial to separate plasma from the cellular components of blood before shipping samples from remote locations and (b) maternal blood serum should never be used as a source of fetal DNA when ASPCR technology-based assays are used to diagnose patients because serum contains a substantially higher amount of maternal DNA than blood plasma (5).

In conclusion, this report provides evidence that our improved ASPCR method could be useful for noninvasive prenatal diagnosis. On the basis of our current experience with DNA extracted from blood plasma and on the data shown in Table 1, the amount of DNA present in 10-μL aliquots of DNA isolates obtained from patient samples processed no more than 1 day after blood collection does not even exceed 15 ng. This amount is 20 times lower than the 300 ng of wild-type genomic DNA that can be added to the 50-μL PCR and still not produce a false-positive result. However, as with all newly developed diagnostic assays, the procedure should be repeated on a larger number of samples to fully explore its reliability and sensitivity. In addition, the earliest week of pregnancy during which the mutation is detectable has yet to be determined. On the basis of our results, we believe that this assay can be applied to the noninvasive prenatal diagnosis of other paternally inherited mutations.

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References


