Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide primers

Blair F. Main,* Peter J. H. Jones,[1],* Ross T. A. MacGillivray,† and David K. Banfield†

Division of Human Nutrition and Department of Biochemistry,† University of British Columbia, Vancouver, B. C., Canada V6T 1W5*

Summary A method for apolipoprotein (apo) E genotyping was developed using the polymerase chain reaction (PCR) with allele-specific oligonucleotide primers (ASP). Synthetic oligonucleotides with base-pair mismatches at the 3' terminus were used as primers to amplify the apoE gene in subjects previously phenotyped using isoelectric focusing (IEF). Complementary primer-allele combinations were specifically amplified by PCR, together with a control pair of primers specific to the human prothrombin gene. Identification of genotype by PCR using ASP was consistent with the phenotypes that were determined by IEF for 14 healthy normolipidemic subjects. These results were achieved using DNA isolated from buccal epithelial cells obtained from a mouthwash or DNA extracted from leukocytes. Genotype identification required analysis of the PCR products on an ethidium-stained agarose gel, yielding results 3 h after DNA extraction. In comparison with other current methods, PCR using ASP is suggested as a rapid and simple noninvasive technique for determining population apoE allelic distribution. —**Main, B. F., P. J. H. Jones, R. T. A. MacGillivray, and D. K. Banfield.** Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide primers. *J. Lipid Res.* 1991. **32:** 183–187.

Supplementary key words polymorphism • genotype identification

Apolipoprotein E (apoE), a 34,000 mol wt glycoprotein, is a normal component of plasma chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL) (1). Mature apoE is a 299 amino acid polypeptide that mediates the uptake of apoE-containing lipoproteins by receptor-mediated endocytosis.

The stuctural gene for apoE is polymorphic, coding for three common isoforms of apoE (ϵ2, ϵ3, and ϵ4) with the three alleles producing three homozygous (E2/2, E3/3, E4/4) and three heterozygous (E2/3, E2/4, E3/4) genotypes (2). The common isoforms differ by amino acid substitutions at one or each of two sites, residues 112 and 158. The E2 isoform differs from the most common E3 isoform by a substitution of an Arg for a Cys at amino acid site 158 while E4 differs from E3 due to a substitution of Cys for Arg at site 112 (3). As the E4 isoform has one more positive charge than E3 while E2 has one less positive charge than E3, each isoform can be distinguished by isoelectric focusing (IEF) (4).

Investigators have shown that approximately 50% of the variability in normal serum cholesterol levels is due to genetic differences among individuals (5). It was estimated that as much as 16% of the genetic variance of low density lipoprotein (LDL) cholesterol was due to allelic differences at the apoE gene locus (5). Subjects with the E3/2 phenotype have about 20% lower, and E3/4 subjects have on average 10% higher, levels of LDL cholesterol than subjects possessing the E3/3 phenotype (6, 7). Due to the association between LDL cholesterol levels and atherosclerosis, it has been suggested that apoE polymorphism may play a role in determining the risk of coronary artery disease (8).

In this report, a rapid, simple, noninvasive technique for apoE genotyping is described. This method identifies the six common apoE genotypes with high specificity and would be appropriate for the detection of other, less common apoE mutation alleles. The procedure takes advantage of the polymerase chain reaction (PCR) in conjunction with four allele-specific oligonucleotide primers; each primer is specific for the single base change that results in either Cys or Arg at positions 112 and 158.

MATERIALS AND METHODS

Oligonucleotides

Allele-specific oligonucleotide primers were synthesized on an Applied Biosystems 391A - PCR Mate synthesizer. Primers were designed with the nucleotide change at the 3' end. The primer sequences and their location within the apoE gene are presented in **Fig. 1.**

Subjects

Individuals genotyped using allele-specific primers were previously phenotyped by IEF or immunoblot analysis. This group included 4 E3/2, 7 E3/3, 2 E3/4, and 1 E4/4. After verification of the methodology, 30 additional subjects were genotyped indicating 22 E3/3, 6 E3/4, and 2 E3/2 genotypes.

Genomic DNA preparation

DNA was isolated from buccal epithelial cells obtained from a mouthwash with sterile water or from leukocytes.

Abbreviations: PCR, polymerase chain reaction; ASP, allele-specific primers; IEF, isoelectric focusing; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; dGTP, deoxyguanosine triphosphate; DNA, deoxyribosyl nucleic acid.

[1]To whom reprint requests should be addressed at: Division of Human Nutrition, 2205 East Mall, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.

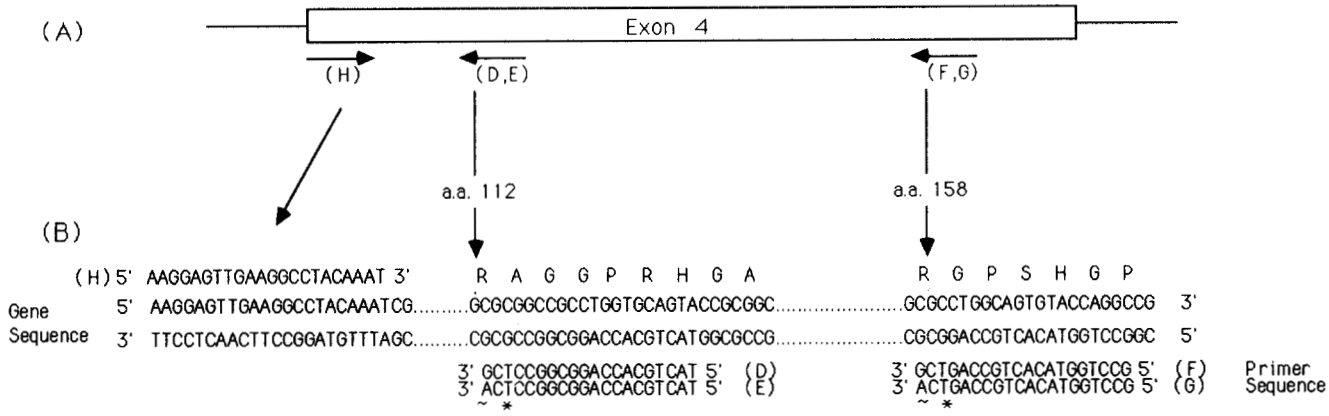

Fig. 1. Allele-specific oligonucleotide primers with 3' base pair mismatch. (A) Orientation of allele-specific oligonucleotide primers on exon 4 coding for the 299 amino acid apoE allele. (B) Specific oligonucleotide primers. The gene sequence is representative of an apoE4 allele; ~ denotes the base that determines the amino acid Cys or Arg; * denotes the G–T deliberate mismatch. Primer D is used in conjunction with the common primer H to give amplification of the ε4 allele. Primer G is used similarly to detect the presence of the ε2 or ε3 allele.

Sufficient DNA for 10 PCR reactions was obtained from a pin-prick method for obtaining blood. The cells were first lysed with 0.5 ml of 0.23 M sucrose, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, and then digested for a minimum of 2 h in 10 mM Tris, pH 8.0, 2 mM EDTA, pH 8.0, 10 mM NaCl, 1% SDS, 0.4 mg/ml proteinase K, and 8 mg/ml dithiothreitol (DTT). The digest was then extracted with phenol-chloroform followed by a 95% ethanol DNA precipitation. The DNA was briefly air dried, then resuspended in 10 μl of water.

In brief, the mouthwash method required the subject to wash his/her mouth with 10 ml sterile water; the cells were then suspended in the above-described sucrose lysis solution. The solution was then centrifuged at 1500 g, the supernatant was poured off, and the lysate was resuspended in 100 μl water. After boiling for 2 min and centrifugation (Eppendorf) for 5 min, the supernatant was removed and used directly in the PCR.

Amplification of genomic DNA

Alleles were determined using the primers described in Fig. 1. PCR amplification was performed in an automated thermocycler (Perkin-Elmer Cetus) as follows. Reactions were carried out in a total volume of 50 μl containing 1 μg DNA, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), and deoxyguanosine triphosphate (dGTP), each at 0.2 mM. The dNTP stock solution was prepared by adjusting the magnesium chloride concentration to 20 mM in 5 mM dNTP. The 10 × buffer consisted of 670 mM Tris-HCl, 15 mM magnesium sulfate, 166 mM ammonium sulfate, and 100 mM β-mercaptoethanol. Also added were 10% dimethyl sulfoxide (DMSO) and 20 pmol of each primer. In addition to apoE primers, all tubes contained prothrombin primers (see Table 1) used as internal controls. Samples were overlaid with mineral oil (Sigma) and the DNA was denatured for 10 min at 96°C. Four units of *Thermus aquaticus* (Taq) polymerase was added to each sample prior to the PCR. Samples underwent 25 cycles with each cycle consisting of a 10-sec denaturation at 96°C, then 30 sec for annealing at 58°C, and an extension of 1 min at 65°C.

RESULTS

Primer design

Initial oligonucleotide primers (**Table 1**, primers A–C) were designed with a single base pair mismatch at the 3' end of the sequence. Results indicated nonspecific amplification unless very stringent annealing temperatures were implemented. At this point various other buffers were tried and the dNTP concentration decreased, yielding inconsistent products. The shifting of the mismatch 1 base pair in from the 3' end yielded the same nonspecific results. These results indicated that a single mismatch was not sufficient to prevent amplification by the PCR. The introduction of an additional mismatch increases the specificity of the primers allowing for specific allele amplification.

Genotypic identification

Each PCR reaction contained the common primer (primer H) and one of the allele-specific primers described in Fig. 1. Therefore, four reaction mixtures are required per subject for genotype diagnosis. Appropriate annealing conditions were determined using an empirical, temperature curve approach. The specific primers were 19 nucleotides in length and the common primer was 22 nucleotides. An attempt was made to combine two different allele-specific primers in one reaction mixture to

TABLE 1. Oligonucleotide primer sequences

Primer	Sequence (5' → 3')
A	TACTGCACCAGGCGGCCGCG
B	TACTGCACCAGGCGGCCGCA
C	ACTGCACCAGGCGGCCGCGC
D	TACTGCACCAGGCGGCCTCG
E	TACTGCACCAGGCGGCCTCA
F	GCCTGGTACACTGCCAGTCG
G	GCCTGGTACACTGCCAGTCA
H	AAGGAGTTGAAGGCCTACAAAT
I	ACAGAATTCCTGGGCTATGAGCTATGCTC
J	ACACTGCAGATAATTCTTTCACGGGCTTG
K	CGGGCCCCGGCCTGGTACACTGCCAGTCA

Primers A and B contain the differentiating nucleotides at the 3' end while primer C has the mismatch one nucleotide in from the 3' end. Primers D and G are designed similarly to A and B but have a second deliberate mismatch (G–T) three nucleotides in from the 3' end. Primer H is the common primer. Primers I and J are the human prothrombin internal controls. Oligonucleotide K is a 29-nucleotide primer similar to primer F.

allow for genotype diagnosis in two reaction mixtures instead of four. The products could be differentiated easily because each set of primers produces a different sized product. Uneven yields of the two product concentrations were observed and may have been due to each primer competing for the same common primer; thus the smaller product always out-competed the larger product. **Fig. 2** depicts subject possessing the apoE 3/3 and E3/2 genotypes.

Initial experiments indicated that the internal control (Exon 13 of the human prothrombin gene) primers would not amplify under the stringent annealing conditions desribed. The annealing time was increased to 3 min and the number of cycles increased from 25 to 35. This gave positive results but increased the occurrence of false products due to nonspecific annealing. To eliminate false annealing the reactions were seeded with a small aliquot, approximately 5 to 10 ng, of cloned human prothrombin and conditions were returned to 25 cycles witha 30-sec annealing time. Results indicated specific amplification of both the human prothrombin segment and the polymorphic sites of apoE.

The 14 subjects used to verify this procedure were previously phenotyped using either the isoelectric focusing method or immunoblot technique. Genotypes identified were E4/4 [1], E3/4 [2], E3/3 [7], E3/2 [4]. The E3/3 genotype yielding a 145 base pair product with primer E, indicating the presence of Cys 112, and a 277 base pair product with primer F indicating the presence of Arg 158. The heterozygote E3/4 produces appropriately sized products with primers E and F identifying the E3 allele, but also produces a 145 base pair product with primer D indicating Arg 112. Homozygous E4/4 subjects react only with primers D and F marking the presence of Arg 112 and Arg 158, while E2/2 homozygotes react only with primers E and G indicating Cys 112 and Cys 158. In all reactions a 500 base pair fragment was observed indicating a successful amplification of the internal control.

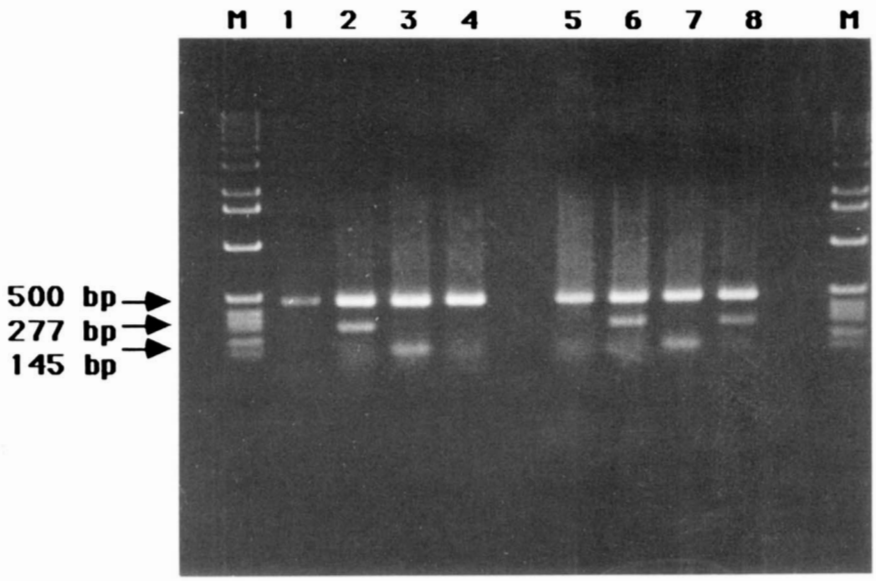

Fig. 2. Apolipoprotein E genotyping using the polymerase chain reaction and allele specific oligonucleotide primers. Ethidium bromide-stained agarose gel showing the products from the PCR reactions, as described in Methods, of apoE 3/3 and apoE 3/2 genotypes. Lane 1 is negative (primer D), lane 2 is positive (primer F), lane 3 is positive (primer E), and lane 4 is negative (primer G) indicating the homozygous E 3/3 genotype. Lanes 5 through 8 are the reactions for subject 2. Lane 5 (primer D) is negative indicating the absence of the $\epsilon 4$ allele; lanes 6 through 8 are positive with primers E, F, and G, indicating the presence of E3 and E2 yielding a genotype of apoE 3/2.

DISCUSSION

Despite the identification of apoE as a genetic factor contributing significantly to within-population variation in plasma cholesterol level, methods for apoE phenotyping remain time-consuming and labor-intensive. Isoelectric focusing (IEF) on a single polyacrylamide cylindrical gel has been the method of choice in apoE phenotyping in the past (9). This method requires a prolonged ultracentrifugation step and is often unreliable due to variation in isoform staining intensity and distance of migration during the electrophoresis procedure. A more recent alternative approach to diagnosing apoE phenotypes is use of a combination of isoelectric focusing of delipidated serum and immunoblotting (10, 11). This method identifies apoE phenotypes immunologically using a double antibody reaction and does not require the long ultracentrifugation step used in IEF (11). However, a highly specific antibody preparation is required.

More recently, a DNA amplification technique has been developed using the polymerase chain reaction with allele-specific oligonucleotide probes (12–16). These probes are used in conjunction with the amplified DNA product from the PCR to detect cysteine–arginine interchanges at residues 112 and 158 of the apoE allele that distinguish the various isoforms. Specific amplification of the apoE allele using the PCR is followed by hybridization with radioactively labeled allele-specific oligonucleotide probes, which is multi-step and requires radioisotope. Restriction isotyping of apoE is another method that uses oligonucleotides to amplify the apoE gene (17). The PCR products are digested with a restriction enzyme and then separated by electrophoresis on polyacrylamide gels. The restriction isotyping method requires an incubation step which extends this technique's diagnosis time.

To develop a simple, specific method for identification of apoE genotypes, we have modified the previously reported PCR technique. The modifications eliminate the need for radiolabeled probes as well as all incubation, hybridization, and washing procedures. This new technique requires only an agarose gel for an assay and can diagnose an apoE genotype within 3 h after DNA isolation.

Diagnosis of individuals with alpha 1-antitrypsin deficiency or cystic fibrosis has used similar approaches to identify point mutations (18–20). In each application, the identical principal is used; oligonucleotides with a 3' mismatched base pair will not function as primers in the PCR mixture. In some cases a single mismatch is not sufficient to prevent nonspecific amplification and a second deliberate mismatch must be incorporated into the oligonucleotide (18). Investigators have used this technique to detect different mutations in an allele selectively. Neubauer, Newbauer, and Edison (21) used this differential PCR method to detect allelic loss of the β-interferon gene. Human immunodeficiency virus studies have investigated the effects of different primer-template mismatches on DNA amplification efficiency (22). Kwok and co-workers (22) found that some mismatches (A:G, G:A, and C:C) reduced PCR product yield drastically while mismatches involving a T amplified efficiently.

Specificity of a primer for the template may be modulated by at least three means: incorporation of a deliberate base-pair mismatch, altering annealing temperature, or by decreasing dNTP concentration. We originally tried to differentiate the apoE alleles using primers with only a single mismatch at the 3' end and results from the agarose gel indicated a large amount of nonspecific amplification. New oligonucleotides were synthesized with the mismatch 1 base pair from the 3' end; however, a significant amount of nonspecific amplification was observed. The addition of a further deliberate mismatch enhanced the specificity greatly. We have obtained optimal results using a more stringent annealing temperature of 58°C because lower temperatures also resulted in nonspecific amplification. To allow for amplification of the larger human prothrombin segment, the reactions are seeded with a cloned DNA sequence of the human prothrombin gene, allowing amplification of the internal control indicating each PCR mixture contained the appropriate reagents. The restriction sites incorporated into the internal control primers were used for other purposes than apoE genotyping.

Initially, amplification of the various apoE alleles yielded inconsistent amounts of product. Primer K was synthesized in an attempt to alleviate this problem by increasing the length to 29 nucleotides. Aspecific amplification was observed at a variety of different annealing temperatures and buffer conditions. Winship (23) observed that G–C-rich sequences were difficult to amplify and added DMSO to the PCR mixture. This is thought to reduce the secondary structure of DNA resulting in amplification of G–C-rich sequences. The addition of 10% DMSO to our PCR mixtures increased the yield of certain apoE segments specifically even though DMSO is thought to reduce *Taq* polymerase activity by approximately 50% (24).

Presently, this novel PCR–allele-specific primer method of apoE genotyping offers advantages of simplicity and cost compared with other methods. Obtaining DNA from a mouthwash also obviates the need for venipuncture (25). In addition, the method is based on nucleotide sequence variation; therefore, by using other specific primers, uncommon structural mutants of the apoE allele could be analyzed. Using past methods such as IEF and immunoblotting, these alleles cannot be detected. This new procedure may enable large populations

to be screened rapidly and accurately, thus contributing to our understanding of apoE's allelic distribution and its regulation of plasma cholesterol levels.

The authors appreciate the assistance and insight of B. Chow, W. Funk, and B. Ritchie. Also greatly appreciated are the efforts of J. Frohlich, J. Hill, H. Pritchard, and R. Roe for phenotyping subjects by isoelectric focusing and immunoblotting. This research was supported by a grant from the British Columbia Health Care Research Foundation.

Manuscript received 22 June 1990 and in revised form 10 September 1990.

REFERENCES

1. Havel, R. J., N. Yamada, and D. M. Shames. 1987. Role of apolipoprotein E in lipoprotein metabolism. *Am. Heart J.* **2:** 470–474.
2. Zannis, V. I., P. W. Just, and J. L. Breslow. 1981. Human apoE isoprotein subclasses are genetically determined. *Am. J. Hum. Genet.* **33:** 1033–1041.
3. Rall, S. C., K. H. Weisgraber, and R. W. Mahley. 1982. Human apoE: the complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
4. Wardell, M. R., P. A. Suckling, and E. D. Janus. 1982. Genetic variation in human apoE. *J. Lipid Res.* **23:** 1174–1182.
5. Sing, C. F., and J. Davignon. 1985. Role of the apoE polymorphism in determining normal plasma lipid and lipoprotein variation. *Am. J. Hum. Genet.* **37:** 268–285.
6. Utermann, G. 1986. The apoE system: genetic control of plasma lipoprotein concentration. *In* Advances in Experimental Medicine. A. Angel and J. Frohlich, editors. Plenum Press, New York. 261–273.
7. Schaefer, E. J., R. E. Gregg, and E. Ghiselli. 1986. Familial apoE deficiency. *J. Clin. Invest.* **78:** 1206–1219.
8. Kuusi, T., M. S. Nieminem, C. Ehnholm, H. Y. Jarvinen, M. Valle, E. A. Nikkila, and M. R. Taskinen. 1989. ApoE polymorphism and coronary artery disease. *Arteriosclerosis.* **9:** 237–241.
9. Warnick, G. R., C. Mayfield, J. J. Albers, and W. R. Hazzard. 1979. Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type III. *Clin. Chem.* **25:** 279–284.
10. Havekes, L. M., P. de Knijff, U. Beisiegel, J. Havinga, M. Smit, and E. Klasen. 1987. A rapid micromethod for apolipoprotein E phenotyping directly in serum. *J. Lipid Res.* **28:** 455–463.
11. Kamboh, M. I., R. E. Ferrell, and B. Kottke. 1988. Genetic studies of human apolipoproteins. V. A novel rapid procedure to screen apoE polymorphism. *J. Lipid Res.* **29:** 1535–1543.
12. Weisgraber, K. H., Y. M. Newhouse, and R. W. Mahley. 1988. ApoE genotyping using the polymerase chain reaction and allele-specific oligonucleotide probes. *Biochem. Biophys. Res. Commun.* **57:** 1212–1217.
13. Houlston, R. S., C. Snowden, F. Green, K. G. M. M. Alberti, and S. E. Humphries. 1989. ApoE genotypes by polymerase chain reaction and allele-specific oligonucleotide probes: no detectable linkage disequilibrium between apoE and apoC-II. *Hum. Genet.* **83:** 364–368.
14. Emi, M., L. L. Wu, M. A. Robertson, R. L. Myers, R. A. Hegele, R. R. Williams, R. White, and J. M. Lalouel. 1988. Genotyping and sequence analysis of apolipoprotein E isoforms. *Genomics.* **3:** 373–379.
15. Smeets, H. J. M., J. Poddighe, P. M. J. Stuyt, A. F. H. Stalenhoef, H. H. Ropers, and B. Wieringa. 1988. Identification of apoE polymorphism by using synthetic oligonucleotides. *J. Lipid Res.* **29:** 1231–1237.
16. Funke, R., S. Rust, and G. Assman. 1986. Detection of apoE variants by an oligonucleotide "melting" procedure. *Clin. Chem.* **32:** 1285–1289.
17. Hixson, J. E., and D. T. Vernier. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *HhaI J. Lipid Res.* **31:** 545–548.
18. Ballabio, A., R. A. Gibbs, and C. T. Caskey. 1990. PCR test for cystic fibrosis deletion. *Nature.* **343:** 220.
19. Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith, and A. F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17:** 2503–2516.
20. Okayama H., D. T. Curiel, M. L. Brantly, M. D. Holmes, and R. G. Crystal. 1989. Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J. Lab. Clin. Med.* **114:** 105–113.
21. Neubauer, A., B. Neubauer, and L. Edison. 1990. Polymerase chain reaction-based assay to detect allelic loss in human DNA: loss of beta-interferon gene in chronic myelogenous leukemia. *Nucleic Acids Res.* **18:** 993–998.
22. Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res.* **18:** 999–1005.
23. Winship, P. R. 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Res.* **17:** 1266.
24. Lawyer, F. C., S. Stoffel, R. K. Saiki, K. Myambo, R. Drummond, and D. H. Gelfand. 1989. Isolation, characterization, and expression in *E. coli* of the DNA polymerase gene from *Thermus aquaticus. J. Biol. Chem.* **264:** 6427–6437.
25. Lench, N., P. Stanier, and R. Williamson. 1988. Simple noninvasive method to obtain DNA for gene analysis. *Lancet.* **1:** 1356–1358.