

## Genotyping of Apolipoprotein E: Comparative Evaluation of Different Protocols

Certain forms of naturally occurring genetic variants, commonly referred to as single-nucleotide polymorphisms (SNPs; also see *UNIT 2.9*), have been demonstrated to modulate the risk for various human diseases. One of the most highlighted polymorphic genes is apolipoprotein E (APOE), which has been shown to alter the risk for sporadic late-onset forms of Alzheimer disease (AD; see Rebeck et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993; Corder et al., 1994; Mayeux et al., 1998) as well as cardiovascular disease (Eichner et al., 2002). APOE has three common alleles ( $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ ), differing by the presence of either C or T nucleotides at codons 112 and 158 in the fourth exon of the APOE gene (Fig. 9.14.1). Carriers of one APOE  $\epsilon 4$  allele have 3- to 5-fold increased risk for AD, whereas  $\epsilon 4$  homozygotes are at a 5- to 15-fold elevated disease risk. Moreover, the APOE  $\epsilon 2$  allele may have a protective effect against developing AD (Corder et al., 1994; West et al., 1994). APOE allelic polymorphisms have also been extensively studied in cardiovascular disease; for instance, relative to the APOE  $\epsilon 3$  allele, the  $\epsilon 2$  allele appears to be associated with lower plasma cholesterol levels and  $\epsilon 4$  with higher levels (Hallman et al., 1991; Eichner et al., 2002).

Several different APOE-genotyping techniques have been developed, and the authors of this unit present here four available technologies to assist investigators in selecting the methods appropriate for specific studies. Two traditional and two modern high-throughput genotyping protocols have been modified and validated in order to examine the reliability and cost-benefit aspects of each method. These analyses, based on restriction fragment length polymorphism (RFLP; see Basic Protocol), reverse hybridization (RH; see Alternate Protocol 1), fluorescence polarization (FP; see Alternate Protocol 2), and minisequencing (SNaPshot analysis; see Alternate Protocol 3) were performed on a collection of 42 previously genotyped DNA samples from AD patients who had been evaluated at the Memory Disorders Unit of Massachusetts General Hospital between 1996 and 2001. The results of this study are discussed below (see Commentary).

	<i>APOE</i> <sub>112</sub>		<i>APOE</i> <sub>158</sub>
<i>E2</i>	Cys		Cys
$\epsilon 2$	<b>TGC</b>		<b>TGC</b>
<i>E3</i>	Cys		Arg
$\epsilon 3$	<b>TGC</b>		<b>CGC</b>
<i>E4</i>	Arg		Arg
$\epsilon 4$	<b>CGC</b>		<b>CGC</b>

**Figure 9.14.1** The various APOE alleles and genotypes are defined by the presence of two polymorphic loci at codons 112 and 158 of the APOE gene. The variable positions are shown with bold letters.

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**APOE GENOTYPING BY RFLP ANALYSIS**

This protocol, initially described by Hixson and Vernier (1990) has been in use at the authors' laboratory for routine analysis and serves here as the primary basis of comparison for the other APOE genotyping methods. In principle, RFLP is performed by PCR amplification of the SNP-containing DNA region followed by specific restriction enzyme cleavage of the PCR product to generate allele-discriminating DNA fragments (Fig. 9.14.2). For more extended information on the technique, see *UNIT 2.7*.

**Materials**

3 to 5 ng/μl genomic DNA (isolated from blood, see *UNIT 14.4*) in TE buffer, pH 7.4  
(*APPENDIX 2D*)

APOE oligonucleotide primers:

Forward: 5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3'

Reverse: 5'-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC-3'

100 mM 4dNTP mix (Roche; 25 mM each dNTP; also see *APPENDIX 2D*)

5 U/μl *Taq* DNA polymerase (Roche)

Dimethylsulfoxide (DMSO)

10× PCR amplification buffer containing 15 mM MgCl<sub>2</sub> (Roche)

10 U/μl *Hha*I restriction endonuclease (Promega)

10× buffer C (Promega)

Low-melting agarose (e.g., Fisher)

NuSieve agarose (FMC Bioproducts)

10× TBE buffer (*APPENDIX 2D*)

10 mg/ml ethidium bromide (*APPENDIX 2D*)

6× gel loading buffer (*APPENDIX 2D*)

Additional reagents and equipment for PCR (*UNIT 7.1*) and agarose gel electrophoresis (*UNIT 2.7*)

1. Set up amplification reactions in PCR tubes for a final volume of 25 μl using:
  - 15 to 25 ng genomic DNA (add from 3 to 5 ng/μl genomic DNA preparation)
  - 12.5 pmol APOE forward primer
  - 12.5 pmol APOE reverse primer
  - 6.25 nmol each dNTP (add from 100 mM 4dNTP mix)
  - 1.25 U *Taq* DNA polymerase
  - DMSO to 10% (v/v)
  - 1× PCR amplification buffer/1.5 mM MgCl<sub>2</sub> (add from 10× PCR buffer/15 mM MgCl<sub>2</sub>).
  
2. Perform PCR (*UNIT 7.1*) using the following amplification protocol:
 

Initial step:	10 min	94°C	(denaturation)
32 cycles:	30 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	1 min	72°C	(extension)
Final step:	4 min	72°C	(extension).
  
3. Incubate the 25-μl PCR product 2 to 3 hr with 5 U of *Hha*I restriction endonuclease in 1× buffer C at 37°C.

4. Prepare a 4% agarose gel solution by combining 1.5 g low-melting agarose and 4.5 g NuSieve agarose with 150 ml of 1× TBE buffer. Mix thoroughly and heat in a microwave oven until the agarose has completely resolved. Add 150 μl of 10 mg/ml ethidium bromide (0.15 mg), mix evenly with the liquid gel, and cast immediately in a gel tray of an appropriate size. Insert combs for 15 to 20 μl wells.

**CAUTION:** *Ethidium bromide is toxic; wear gloves at all times.*

*See UNIT 2.7 for additional details of agarose gel electrophoresis.*

5. When the gel has polymerized, cover it completely with 1× TBE buffer and remove the combs carefully. Load each well with a mixture of 10 μl cleaved PCR product (from step 3) and 2 μl of 6× gel loading buffer.
6. Run the gel at 125 V for ~45 min and analyze immediately in a gel reader.

*The different APOE genotypes are recognized by the DNA fragment lengths (Fig. 9.14.2).*

### **APOE GENOTYPING BY REVERSE HYBRIDIZATION**

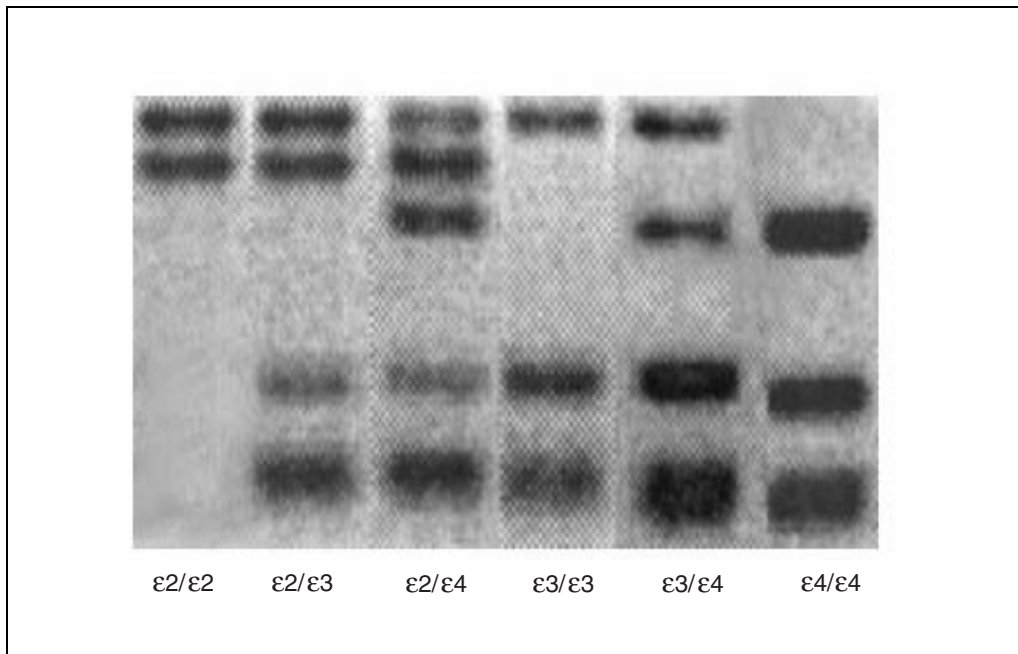
The following method is based on hybridization of amplified biotinylated DNA with APOE allele-specific oligonucleotide probes immobilized as parallel lines on membrane strips (Fig. 9.14.3). The protocol was established by Innogenetics, and all reagents required are provided in a kit (INNO-LiPA ApoE; Nishimura et al., 1998). The respective compositions and concentrations of the reagents are therefore not known to the user.

**ALTERNATE  
PROTOCOL 1**

#### **Materials**

3 to 5 ng/μl genomic DNA (isolated from blood, see *UNIT 14.4*) in TE buffer, pH 7.4  
(*APPENDIX 2D*)

INNO-LiPA ApoE kit (Innogenetics) including:  
ApoE amplification buffer



**Figure 9.14.2** APOE genotyping by restriction fragment length polymorphism analysis. The band patterns generated after electrophoresis on an ethidium bromide–agarose gel can be translated into the different APOE genotypes. All naturally occurring APOE genotypes are shown, also the rare  $\epsilon 2/\epsilon 2$  form, not discussed in the unit.

**Clinical  
Molecular  
Genetics**

**9.14.3**

ApoE primer mix  
MgCl<sub>2</sub> solution  
Glycerol solution  
Membrane strips  
Hybridization solution  
Stringent wash solution  
Denaturation solution  
Conjugate, substrate  
Conjugate diluent  
Substrate solution  
Substrate buffer  
Rinse solution  
Test troughs  
Incubation trays (all provided in the kit)  
*Taq* DNA polymerase (Roche)  
45°C water bath with shaking platform  
Calibrated thermometer  
Additional reagents and equipment for PCR (*UNIT 7.1*)

### **Perform PCR**

1. Set up amplification reactions in PCR tubes for a final volume of 50 µl using:

15 to 25 ng genomic DNA (add from 3 to 5 ng/µl genomic DNA preparation)  
10 µl amplification buffer (from INNO-LiPA ApoE kit)  
10 µl ApoE primer mix (from INNO-LiPA ApoE kit)  
10 µl MgCl<sub>2</sub> solution (from INNO-LiPA ApoE kit)  
10 µl glycerol (from INNO-LiPA ApoE kit)  
1 U *Taq* polymerase.

2. Perform PCR (*UNIT 7.1*) using the following amplification protocol:

Initial step:	5 min	95°C	(denaturation)
30 cycles:	30 sec	95°C	(denaturation)
	20 sec	60°C	(annealing)
	20 sec	72°C	(extension)
Final step:	10 min	72°C	(extension).

### **Perform reverse hybridization using INNO-LiPA ApoE kit**

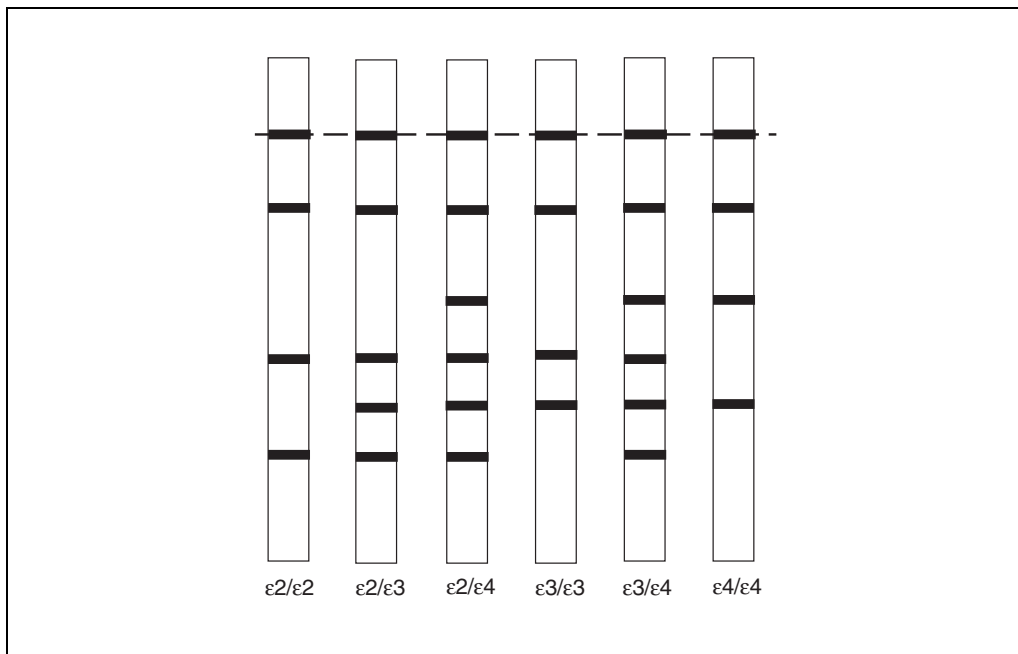
3. In a test trough mix 10 µl of the PCR product in with 10 µl denaturation solution and incubate 5 min at room temperature.

*Each of the following incubation, washing, and rinsing steps are carried out in a volume of 1 ml in a water bath on a shaking platform. The same test trough is used through the procedure and previous reagents must be removed before new ones are added.*

4. Incubate the membrane strips in a mix of denatured DNA and the ready-to-use hybridization solution, for 30 min at 45°C.

*A high-quality calibrated thermometer should be used to control the temperature in these hybridization steps (see Critical Parameters and Troubleshooting).*

5. Wash the strips in wash solution at 45°C once for 10 to 20 sec and then again for 10 min.



**Figure 9.14.3** With the reverse hybridization technique, nitrocellulose strips display different patterns of parallel bands, from which the APOE genotypes can be interpreted. All naturally occurring APOE genotypes are shown, including the rare  $\epsilon 2/\epsilon 2$  form, not discussed in the unit. The uppermost two lines are a marker and a control line, respectively.

6. Rinse the strips twice in 1:5 (i.e., 20% rinse solution/80% water) rinse solution at room temperature, each time for 1 min.
7. Add 1 ml of 1:100 conjugate solution to each test trough and incubate the strips in this solution at room temperature for 30 min.
8. Rinse the strips twice in 1:5 rinse solution at room temperature, each time for 1 min.
9. Incubate the strips in the ready-to-use substrate buffer at room temperature for 1 min.
10. Add 1 ml of 1:100 substrate solution to each test trough and incubate the strips in this solution at room temperature for 30 min.
11. Rinse the strips twice in water at room temperature, each time for 3 min.
12. Read out the respective APOE genotypes from the membrane strip band pattern (Fig. 9.14.3).

*The banding on the strips is produced by the conversion of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) into a purple/brown precipitate.*

### APOE GENOTYPING BY FLUORESCENCE POLARIZATION (FP)

FP is performed in microtiter plates and is based on an initial PCR of the SNP-containing DNA region combined with an elongation reaction step in which the incorporation of differently fluorophore-labeled nucleotides enables discrimination between the various alleles (Fig. 9.14.4) (Chen et al., 1999). The described procedure is a combination of a modified standard PCR protocol and an adjustment of a protocol recommended by Perkin-Elmer Life Sciences for SNP genotyping by FP. The primer elongation reaction is mainly based on reagents provided in the AcycloPrime-FP SNP Detection Kit (Perkin-Elmer Life Sciences).

**ALTERNATE  
PROTOCOL 2**

**Clinical  
Molecular  
Genetics**

**9.14.5**

## Materials

3 to 5 ng/μl genomic DNA (isolated from blood, see *UNIT 14.4*) in TE buffer, pH 7.4

(*APPENDIX 2D*)

APOE oligonucleotide primers for PCR:

Forward: 5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3'

Reverse: 5'-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC-3'

40 mM 4dNTP mix (Roche; 10 mM each dNTP; also see *APPENDIX 2D*)

5 U/μl *Taq* DNA polymerase (Roche)

Dimethylsulfoxide (DMSO)

10× PCR amplification buffer containing 15 mM MgCl<sub>2</sub> (Roche)

Exo-SAP-IT (USB)

Oligonucleotide primers for SNPs (elongation reaction):

5'-GGC GCG GAC ATG GAG GAC GTG-3' (APOE<sub>112</sub>)

5'-CGG CCT GGT ACA CTG CCA GGC-3' (APOE<sub>158</sub>)

AcycloPrime-FP SNP Detection Kit (Perkin-Elmer Life Sciences) including:

10× reaction buffer

AcycloTerminator mix

AcycloPol DNA polymerase

96-well PCR plates (MJ Research)

Thermal cycler accommodating 96-well PCR plates

80°C water bath

96-well plate reader capable of fluorescence polarization measurements (e.g.,

Victor series from Perkin-Elmer Life Sciences)

Additional reagents and equipment for PCR (*UNIT 7.1*)

1. Set up amplification reactions in wells of a 96-well PCR plate for a final volume of 30 μl using:

30 to 50 ng genomic DNA (add from 3 to 5 ng/μl genomic DNA preparation)

6 pmol APOE forward primer

6 pmol APOE reverse primer

0.5 nmol each dNTP (add from 40 mM 4dNTP mix)

1.25 U *Taq* DNA polymerase

DMSO to 10% (v/v)

1× PCR amplification buffer/1.5 mM MgCl<sub>2</sub> (add from 10× PCR buffer/15 mM MgCl<sub>2</sub>).

2. Perform PCR using the following amplification protocol:

Initial step: 10 min 94°C (denaturation)

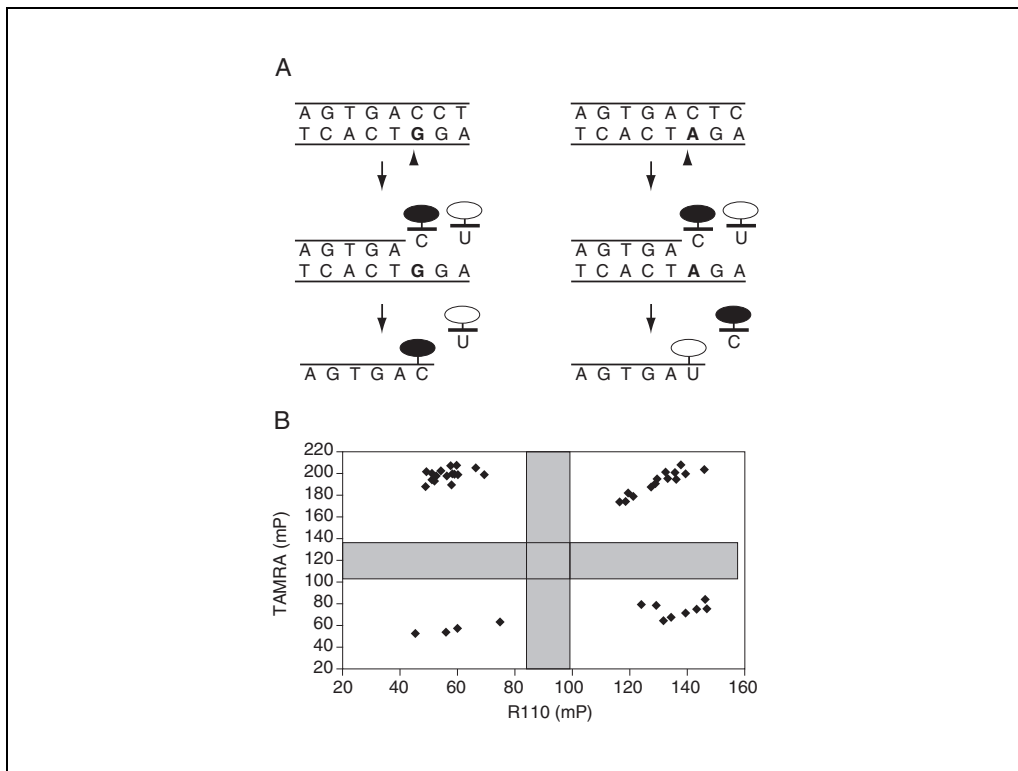
32 cycles: 30 sec 94°C (denaturation)

30 sec 56°C (annealing)

1 min 72°C (extension)

Final step: 7 min 72°C (extension).

3. Transfer 5 μl of the PCR product from each reaction to a well of a new PCR plate. Incubate with 2 μl of Exo-SAP-IT for 1 hr at 37°C.
4. Inactivate Exo-SAP-IT by incubating 15 min at 80°C.



**Figure 9.14.4** (A) In FP, after a traditional PCR, an elongation reaction is performed in which the amplicons are mixed with a primer ending one base pair immediately upstream of the SNP, combined with nucleotides conjugated with two fluorescent dyes of different excitation-emission profiles (TAMRA or R110); black and white ovals). The labeled nucleotides terminate the transcription, and the dye emits its light in different planes depending on whether it is bound (match) or unbound (mismatch) to the DNA-polymer. (B) After analysis in a plate reader for fluorescence polarization, the different APOE alleles can be defined by the quadrants in which the cases cluster. CC homozygotes are positioned in the lower right, TT homozygotes in the upper left, and CT heterozygotes in the upper right quadrant. Apart from one blank, the four lower left samples have not incorporated any fluorophore and are thus considered failures. The graph illustrates the outcome for the APOE<sub>112</sub> locus. The APOE genotypes are given by combining this data with the corresponding graph for the APOE<sub>158</sub> locus. Abbreviation: mP, millipolarization units.

5. Set up elongation reactions for each of the two APOE SNP sites in wells of a 96-well PCR plate for a final volume of 20  $\mu$ l using:

- 7  $\mu$ l of the 244-bp PCR-product/inactivated enzyme (step 4)
- 50 pmol SNP primer (APOE<sub>112</sub> or APOE<sub>158</sub>)
- 2  $\mu$ l 10 $\times$  reaction buffer
- 1  $\mu$ l AcycloTerminator mix
- 0.05  $\mu$ l AcycloPol DNA polymerase

6. Perform a primer elongation reaction using the following protocol:

- |               |        |      |                |
|---------------|--------|------|----------------|
| Initial step: | 2 min  | 95°C | (denaturation) |
| $x$ cycles:   | 15 sec | 95°C | (denaturation) |
|               | 30 sec | 55°C | (annealing)    |
| Final step:   | 2 min  | 15°C | (cooling)      |

where  $x$  is 30 cycles for APOE<sub>112</sub> or 60 cycles for APOE<sub>158</sub>.

7. Centrifuge the samples at 150  $\times$   $g$  (1000 rpm in a Sorvall H1000B rotor, 4°C).

8. Analyze the samples on an FP plate reader.

*Example results are illustrated in Figure 9.14.4.*

## APOE GENOTYPING BY SNaPshot ANALYSIS

SNaPshot analysis is a technique based on minisequencing (Makridakis and Reichardt, 2001). In this protocol, the DNA is amplified by PCR resulting in a 217-bp fragment containing both APOE SNP sites. In a second step, the PCR product is used as template for a minisequencing reaction with allele-specific primers of different lengths ending one base pair upstream of the respective polymorphic site. The presence of dideoxynucleotides, labeled with different fluorophores, will terminate the elongation at the polymorphic site, and the read-out is given by the different migration distances of the fluorescent fragments (Fig. 9.14.5). The reagents for the minisequencing step are provided in a kit (SNaPshot multiplex system, Applied Biosystems).

### Materials

3 to 5 ng/μl genomic DNA (isolated from blood, see *UNIT 14.4*) in TE buffer, pH 7.4  
(*APPENDIX 2D*)

APOE oligonucleotide primers:

Forward: 5'-CCA AGG AGC TGC AGG CGG CGC A-3'

Reverse: 5'-GCC CCG GCC TGG TAG ACT GCC A-3'

100 mM 4dNTP mix (Roche; 25 mM each dNTP; also see *APPENDIX 2D*)

*Taq* DNA polymerase (Roche)

Dimethylsulfoxide (DMSO)

10× PCR amplification buffer containing 15 mM MgCl<sub>2</sub> (Roche)

QiaQuick DNA purification kit (Qiagen)

Oligonucleotide primers for minisequencing:

5'-CGG ACA TGG AGG ACG TG-3' (APOE<sub>112</sub>)

5'-TTT TTT TTT TCC GAT GAC CTG CAG AAG-3' (APOE<sub>158</sub>)

SNaPshot mix (containing fluorescent [F]ddNTPs; Applied Biosystems)

Calf intestinal phosphatase (CIP; New England Biolabs)

DNA size standard ladder (GeneScan-120 LIZ, Applied Biosystems)

Hi-Di formamide (Applied Biosystems)

96- or 384-well PCR plates (MJ Research)

75°C water bath

ABI PRISM 3100 Genetic Analyzer (Applied Biosystems)

GeneMapper 2.0 or GeneScan software (Applied Biosystems)

Additional reagents and equipment for PCR (*UNIT 7.1*)

**NOTE:** The amplification primers used in the SNaPshot procedure are different from those used in the Basic Protocol, since the protocols were developed in different laboratories.

1. Set up amplification reactions in 96- or 384-well PCR plates for a final volume of 25 μl using:

30 to 40 ng genomic DNA (add from 3 to 5 ng/μl genomic DNA preparation)

4 pmol forward APOE primer

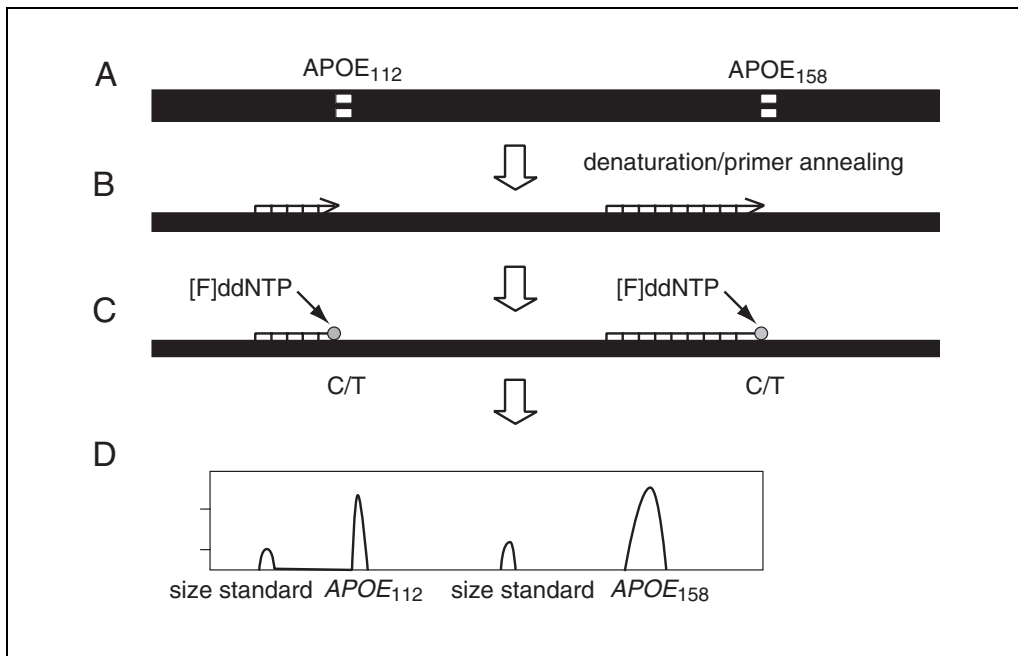
1.4 pmol reverse APOE primer

5 nmol each dNTP (add from 100 mM 4dNTP mix)

0.5 U *Taq* DNA polymerase

DMSO to 10% (v/v)

1× PCR amplification buffer/1.5 mM MgCl<sub>2</sub> (add from 10× PCR buffer/-15 mM MgCl<sub>2</sub>).



**Figure 9.14.5** APOE genotyping with the SNaPshot method (ABI PRISM SNaPshot Multiplex kit). (A) A 217-bp fragment, encompassing the APOE<sub>112</sub> and APOE<sub>158</sub> locus, is amplified in an initial PCR. (B) In a minisequencing step, primers ending one base pair 5' of the APOE<sub>112</sub> and APOE<sub>158</sub> polymorphic sites are mixed in one reaction. (C) Incorporation of fluorescently labeled ddNTP is followed by (D) separation of the labeled primers on the ABI 3100 Genetic Analyzer capillary system.

2. Perform PCR using the following amplification protocol:

Initial step:	10 min	94°C	(denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	30 sec	72°C	(extension)
Final step:	10 min	72°C	(extension).

3. Remove excess dNTPs and primers with the QIAquick purification kit according to the kit instructions.

4. Set up minisequencing reactions in wells of 96- or 384-well PCR plates for a final volume of 10 µl using:

- 1 µl of the 217 bp PCR product (step 3)
- 2 pmol of each of the two minisequencing primers (APOE<sub>112</sub> and APOE<sub>158</sub>)
- 5 µl SNaPshot mix

*In the minisequencing step both polymorphisms are identified in the same reaction.*

5. Perform PCR in 96- or 384-well plates using the following amplification protocol:

25 cycles:	10 sec	96°C	(denaturation)
	5 sec	50°C	(annealing)
	30 sec	60°C	(extension).

6. To minimize interference from the unincorporated [F]ddNTPs, incubate samples 1 hr at 37°C with 2.5 U calf intestinal phosphatase (CIP).

7. Inactivate CIP by incubating 15 min at 75°C.

- Mix the SNaPshot product with a DNA size standard ladder and Hi-Di formamide and run on an ABI PRISM 3100 Genetic Analyzer.
- Analyze the data with GeneMapper 2.0 or GeneScan software to identify APOE genotypes (Fig. 9.14.5).

## COMMENTARY

### Background Information

Several different methods have been developed for APOE genotyping, of which RFLP analysis (UNIT 2.7) has been the most widely used (Hixson and Vernier, 1990). In this method, DNA is subjected to PCR with primers flanking the region containing the two polymorphic loci, after which the amplicon is cleaved by a restriction enzyme. After electrophoretic separation on an agarose/ethidium bromide gel, the PCR products generate a characteristic pattern, which reflects the APOE genotypes. Other general genotyping methods include strategies to incorporate labeled nucleotides into immobilized single-stranded DNA, such as the reverse hybridization technique (Saiki et al., 1989).

In recent years, high-throughput genotyping strategies in a 96- or a 384-well format have been designed, such as microarrays (UNIT 2.9), the TaqMan assay (UNIT 2.10), FP (Gibson et al., 1997; Hsu et al., 2001), and SNaPshot analysis (Makridakis and Reichardt, 2001), a technique based on minisequencing. In FP and SNaPshot analysis a DNA region containing the SNP of interest is first amplified in a conventional PCR, after which the amplicons are incubated with elongation primers (ending one base pair upstream of the polymorphic locus) and fluorescently labeled nucleotides. The read-out of FP is based on the polarization properties between bound and unbound fluorophores. In SNaPshot analysis, the alleles are scored based on the incorporated fluorophore and the distance migrated in the electrophoresis.

As shown in Table 9.14.1, the four methods all successfully genotyped ~90% of the samples analyzed and yielded no false positives. Details of the outcome including troubleshooting and technical modifications are described below.

### Critical Parameters and Troubleshooting

In a pilot test, all four methods for APOE genotyping worked accurately with either no optimization efforts or only minor ones (Table 9.14.1). However, for each method, ~10% of samples could not be genotyped, even after a second attempt. Two of the samples failed with

three of the four applied methods; these failed to be appropriately amplified in the initial PCR preceding the RFLP analysis and RH, indicating that the DNA quality of these samples was poor. All DNA samples had been extracted according to the same protocol and were originally of a sufficiently good quality (since APOE genotyping had been successfully performed with the RFLP protocol).

The DNA concentrations, ~3 to 5 ng/μl, were found to be lower in some but not all of the samples that failed with the respective techniques. Moreover, there was no correlation between storage time and the likelihood for a successful outcome of the re-genotyping. After the initial analysis, the samples had been stored in TE buffer at 4° for a period of 1 to 6 years, and it is possible that failed samples were compromised due to breakdown during this storage period.

Apart from stringent precautions in the extraction, handling, and storage of DNA, another recommendation for all PCR-based genotyping techniques is to optimize each protocol internally. For example, slightly different annealing temperatures may be required in different laboratories due, e.g., to apparatus-related calibration differences. Such optimizations are best carried out by using PCR instrumentation in which a temperature-gradient based reaction in the 96-well format can be performed.

In addition to the general troubleshooting aspects, common to all PCR-based techniques, each method analyzed was found to have strengths and weaknesses. In RFLP analysis, the read-out depends on gel electrophoresis, which sometimes fails. The authors of this unit have compared several gel compositions and found that the agarose mixture described in the Basic Protocol produced the most reliable outcome. RH (Alternate Protocol 1) is very temperature sensitive with respect to its hybridization step, where an appropriate and constant temperature is critical. By using a high-quality calibrated thermometer, this potential pitfall is easily avoided.

In the PCR used for the FP protocol (Alternate Protocol 2), higher initial DNA concentrations (30 to 50 ng/μl) gave a better separation of the sample clusters at the end. A critical procedural issue is keeping the concentrations

**Table 9.14.1** Comparative Notes on the Different APOE Genotyping Protocols Evaluated in this Study

Method	Success rate	Start-up time	Total run time/run time per sample	Start-up cost	Run cost per sample	Equipment <sup>a</sup>	General comments
RFLP analysis (see Basic Protocol)	90%	Varies	6 hr/10 min (if 36 samples)	\$5,000	\$0.50	Basic PCR engine	Widely applied and inexpensive; interpretation sometimes difficult
RH (see Alternate Protocol 1)	88%	None. Worked directly	4 hr/6-7 min (if 36 samples)	\$10,000	\$1.00	Basic PCR engine, shaking water bath	Convenient for small-scale investigators
FP (see Alternate Protocol 2)	90%	1 week (4 runs of modifications and primer testing)	5-6 hrs/3-4 min (if full 96-well plate)	\$50,000	\$1.50	Microtiter plate PCR engine, FP reader, computer, microtiter plate centrifuge	Rapid and convenient genotyping of larger number of samples; expensive equipment needed
SNaPshot analysis (see Alternate Protocol 3)	93%	None. Worked directly	9 hr/3 or 6 min (if full 96- or 384-well plate)	\$200,000 (\$75,000-400,000 depending on instrument)	\$1.00	Microtiter plate PCR engine, automated sequencer	Rapid and convenient genotyping of larger number of samples; expensive equipment needed

<sup>a</sup>In addition to standard laboratory equipment such as gel trays, power supply, centrifuge, pipettors

of oligonucleotide primers and dNTPs lower than in the standard PCR reaction (since an excess of either of these could interfere with the subsequent primer elongation). For the elongation step, the authors have also established that a 10-fold increased primer concentration (compared to the manufacturer's recommendation) improved the separation of clusters. The elongation step should theoretically work with either one of the primer options. However, it is the authors' experience that one of the two primers is always preferable, so test runs of both the forward and reverse versions should be performed. For the FP protocol described, it was found that the forward primer worked best for APOE<sub>112</sub>, whereas the reverse variant was the most suitable for APOE<sub>158</sub>. Moreover, the elongation reaction for the APOE<sub>158</sub> had to be extended to a higher number of cycles to yield an outcome that was comparable to APOE<sub>112</sub>. This difference in efficiency might be due to the fact that the APOE<sub>158</sub> site is close to the 3'-end of the APOE amplicon generated after the first PCR reaction. Another

oligonucleotide primer design for the DNA amplification, where a slightly larger amplicon is generated, may improve the efficiency. However, the PCR product from the authors' amplification protocol is 244 bp, which already is somewhat larger than the recommended size of 200 bp for FP amplicons. A further optimization of the FP protocol may then have to be based on the use of two different primer sets for the amplification of APOE<sub>112</sub> and APOE<sub>158</sub>, respectively.

Also for SNaPshot analysis, the authors wish to stress the importance of optimizing the primer design, especially if several primers are to be used in the minisequencing reaction. First, the annealing temperature has to exceed the melting temperature of the respective primers in the minisequencing reaction. Second, the primers have to differ by 4 to 6 nucleotides in length (e.g., by the addition of a nonhomologous poly(dT) tail to one of the primers) in order to read the gel.

## Anticipated Results

The 42 cases included in the analysis had originally been APOE-genotyped (by RFLP) and represented the following genotypes:  $\epsilon 2/\epsilon 3$  ( $n=11$ ),  $\epsilon 2/\epsilon 4$  ( $n=6$ ),  $\epsilon 3/\epsilon 3$  ( $n=9$ ),  $\epsilon 3/\epsilon 4$  ( $n=8$ ),  $\epsilon 4/\epsilon 4$  ( $n=8$ ). When re-genotyping the cases 1 to 6 years later with the traditional genotyping strategies, the authors of this unit were able to successfully determine the genotypes on 38 (RFLP analysis) and 37 (RH) subjects, respectively. The high-throughput approaches yielded correct genotypes in 38 (FP) and 39 (SnaPshot analysis) of the 42 cases (Table 9.14.1). The four different methods tested in the authors' laboratory thus seem to have about the same specificity and did not lead to any false-positive results. The failure rate of 7% to 12% corresponds fairly well to the 5% to 10% of failed cases seen in the authors' laboratory when routinely performing APOE genotyping by RFLP analysis on freshly prepared DNA.

## Cost

There are significant differences in the start-up costs involved with the different methods (Table 9.14.1). Apart from standard laboratory equipment, e.g., gel trays, power supplies, and pipettors, more expensive apparatus is needed for FP and SNaPshot analysis, whereas RH can be performed with a moderate initial investment. Apart from standard equipment, RFLP analysis only requires a basic PCR engine. After the initial investments have been made, the run cost also varies, with approximately \$0.50/sample for RFLP analysis, \$1/sample for RH, \$1.50/sample for FP, and \$1/sample for SNaPshot analysis (Table 9.14.1). All estimates are based on current standard prices, e.g., for primers, enzymes, and reagents that are needed for the different protocols.

## Selecting a technique

Taken together with time considerations (see below), the number of samples to be run can influence the selection of technique. RH can adequately handle volumes of 25 to 50 samples per month. The cost per sample for this technique is higher than for RFLP analysis, but the protocol is easier to follow, with the reagents conveniently provided in a kit. Moreover, the reproducibility is likely to be better than that of a technique where the read-out relies on interpretation of bands on a gel. Apart from a PCR engine (needed for all forms of genotyping), the only more exclusive piece of equipment needed for RH is a shaking water bath.

For those laboratories that genotype more than 50 samples per month, or for those that want to standardize their procedures, FP or SNaPshot analysis may be appropriate. The FP system (provided by Perkin-Elmer Life Sciences) has the lower investment costs of the two and seems to work very reliably once the PCR conditions have been optimized. SNaPshot analysis initially requires a more costly investment, but the price for each sample analyzed is lower than for FP. In addition, pooling of the samples further reduces cost and labor, and also facilitates scaling up to the 384-well format. SNaPshot analysis can thus be adapted for laboratories requiring a very high throughput.

Apart from large capacity, the automated plate-based genotyping strategy benefits from flexibility; as soon as the investigator wants to analyze a new SNP believed to be important in human diseases, it is just a matter of designing new primers for the reactions involved and optimize the conditions anew. The drawback is the high initial cost. For the small-scale investigator, investments of ~\$50,000 for FP to ~\$200,000 for SNaPshot analysis might be hard to justify if molecular genetics is not the primary focus of the laboratory.

## Time Considerations

RH turned out to be the method with the shortest start-up time, whereas FP required some initial modifications of the standard protocol provided by the manufacturer (Perkin-Elmer Life Sciences) in addition to the recommended comparison of efficiency between the forward and the reverse primer version in the elongation reaction (Table 9.14.1). Moreover, the run time differed for each procedure, with one run of RFLP analysis taking ~6 hr, one run of RH taking ~4 hr, one run of FP between 5 and 6 hr, and the SnaPshot analysis ~9 hr (Table 9.14.1). In addition, the authors took into account the different number of samples that, for practical reasons, can be handled with the various techniques. Both FP and SNaPshot, for which 96 (or even 384) samples could be analyzed simultaneously, were time-saving strategies compared to RFLP analysis and RH, where a maximum of 36 samples could be handled conveniently.

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