

# Haplotype Analysis using a Novel Real-Time Amplification Strategy on the MJ Research® Opticon™ Continuous Fluorescence Detection System

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## Abstract:

Single nucleotide polymorphisms (SNPs) are well recognized as markers for inter-individual differences in disease risk and treatment response in humans. For genes containing multiple SNPs, however, the haplotype (combination of SNPs on one chromosome) is often the principal determinant of phenotypic consequences. The human apolipoprotein E (APOE) gene encodes a protein that plays a key role in the transport and metabolism of plasma cholesterol and triglycerides. An individual's APOE haplotype influences the risk for cardiovascular disease and late-onset Alzheimer disease.

We have developed a protocol to rapidly genotype human APOE haplotype alleles using a novel real-time qPCR strategy with SYBR® Green I. The three common isoforms of apoE are encoded by haplotypes involving two diallelic SNPs of thymine/cytosine at nucleotide positions 3937 and 4075. We determined the APOE haplotypes based on the differential amplification of alleles using primer sets that contain specific terminal bases for SNP interrogation. DNA samples from 264 individuals were analyzed using the MJ Research DNA Engine Opticon® Continuous Fluorescence Detection System. The frequencies of each apoE allele were: apoE2 – 6.6%, apoE3 – 79%, and apoE4 – 14.4%. Our results are consistent with previous reports. Validation of the real-time qPCR results by DNA sequencing analysis establishes the DNA Engine Opticon system as an efficient and reliable platform for genotyping.

## Objective

To demonstrate a new haplotyping technique: differential amplification of alleles with different combinations of SNP-interrogating primers. The human ApoE gene served as a model system and SYBR® Green I was the detection chemistry.

## Introduction

### SNPs = Single-Nucleotide Polymorphisms

- are the most frequently occurring DNA sequence variation in the human genome
- are markers for inter-individual differences in disease risk and treatment response in humans

### Haplotype = the combination of SNPs on one chromosome

- often is the principal determinant of phenotypic consequences
- cannot be determined by simple SNP-typing techniques

### APOE = human apolipoprotein E

- plays a key role in the transport and metabolism of plasma cholesterol and triglycerides
- polymorphisms influence the risk of cardiovascular disease and late-onset Alzheimer's Disease
- has three common isoforms encoded by haplotypes involving two diallelic SNPs of thymine/cytosine at nucleotide positions 3937 and 4075

### Possible combinations of SNPs in APOE gene

nucleotide 3937	nucleotide 4075	Haplotype
T	T	E2
T	C	E3
C	C	E4
C	T	Ex

Has not been detected in any human population; Serves as negative control.

## Methods

### Materials

- Human DNA from anonymous clinical blood samples was isolated by MaximBio, using their genomic DNA isolation kit (EXT-0001).
- 20ng DNA from each sample was used as template for PCR with the Qiagen QuantiTect™ SYBR Green PCR kit (204143).
- Reaction components were assembled in low-profile microplates (MJ Research, MLL-9651) and sealed with ultra-clear strip caps (MJ Research, TCS-0803).

### Primers:

#### The 3'-terminal base of each primer corresponds to a specific SNP allele

Forward primers correspond to the SNP at position 3937:

F(T): 5'-GGA-CAT-GGA-GGA-CGT-G(T)-3'

F(C): 5'-GGA-CAT-GGA-GGA-CGT-G(C)-3'

Reverse primers correspond to the SNP at position 4075:

R(G): 5'-GGT-ACA-CTG-CAG-GC(G)-3'

R(A): 5'-GGT-ACA-CTG-CAG-GC(A)-3'

The 3'-terminal bases that hybridize to the SNP nucleotide are enclosed in parentheses. The primers amplified a 169bp amplicon from total genomic DNA.

#### Reactions using primers that lack the SNP-interrogating nucleotide were performed as positive controls (E+)

F(pos): 5'-GGG-ACA-TGG-AGG-ACG-TG-3'

R(pos): 5'-TGG-TAC-ACT-GCA-GGC-3'

## Real-time Quantitative PCR

Amplification was performed on a DNA Engine Opticon® continuous fluorescence detection system (MJ Research) using the following conditions:

### Quantitative PCR reaction components

Component	Stock Concentration	Volume	Final Concentration
Genomic DNA	10ng/μl	2.0μl	2.0ng/μl
Forward Primer	10μM	0.2μl	0.2μM
Reverse Primer	10μM	0.2μl	0.2μM
QuantiTect SGI PCR mix	2X	5.0μl	1X
ddH <sub>2</sub> O		2.6μl	
Total Volume		10.0μl	

\*Primer annealing temperature was experimentally determined for allelic discrimination using the temperature gradient feature of the DNA Engine Opticon system. The post-amplification melting curve showed no non-specific amplification products.

### Real-time PCR cycling program

1. 95°C 10 min
2. 95°C 5sec
3. 60°C 15sec\*
4. 72°C 15sec
5. Plate read
6. Go to step 2, 39 more times
7. 72°C 10min
8. Melting curve analysis: 65°C to 98°C, 0.2°C/read, 1sec hold\*
9. 72°C 10min
10. 10°C Forever
11. END

## Haplotype determination:

1. Each DNA sample was run in five PCR reactions: four reactions with different combinations of SNP-interrogating forward and reverse primers and a positive control reaction.

2. The amplification efficiency of each reaction is characterized by the corresponding C(t) value, an indication of the cycle number at which a sample's fluorescence signal exceeds background fluorescence.

3. The C(t) values obtained with each combination of primers were compared for each DNA sample to determine haplotype.

- Lower C(t) values => SNPs of haplotype match the 3'-ultimate nucleotides of both forward and reverse primers. Such values will always be obtained in reactions containing the positive control primers (F(pos)-R(pos)).
- Higher C(t) values => SNPs of haplotype don't match primers. Such values will always be obtained in reactions containing the negative control primers (F(C)-R(A)).

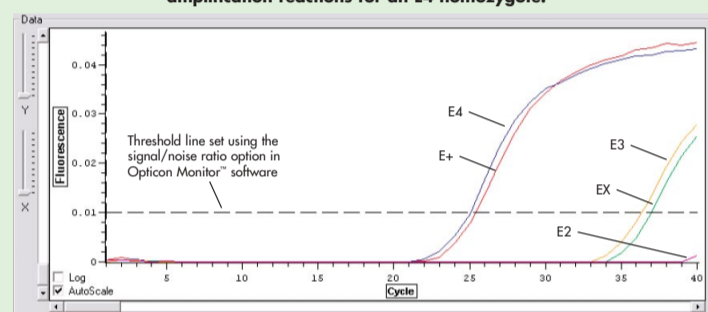
### Each haplotype is amplified most efficiently by specific primer pairs

Haplotype	nucleotide 3937	nucleotide 4075	Primer pairs that allow efficient amplification
E2	T	T	F(T) - R(A) F(pos) - R(pos)
E3	T	C	F(T) - R(G) F(pos) - R(pos)
E4	C	C	F(C) - R(G) F(pos) - R(pos)
EX	C	T	F(C) - R(A)

Because the following haplotype has not been detected in any human populations, the primer pair F(C) - R(A) is not expected to amplify any samples efficiently. It therefore serves as a negative control.

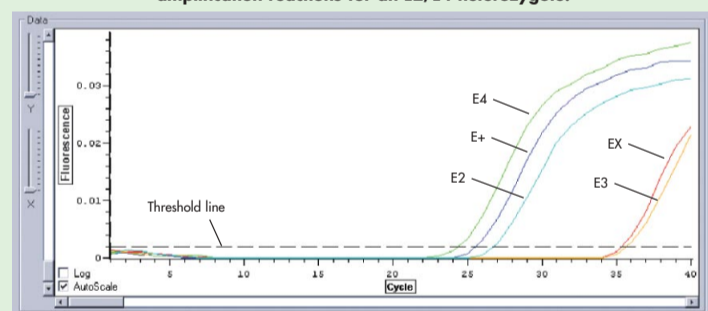
## Results

### Representative fluorescence traces from all five amplification reactions for an E4 homozygote.



Primers corresponding to the E4 [F(C)-R(G)] haplotype yielded a C(t) value similar to positive control [E+: F(pos)-R(pos)]. Primers corresponding to the E2 [F(T)-R(A)] and E3 [F(T)-R(G)] haplotypes yielded C(t) values similar to negative control [EX: F(C)-R(A)]. Therefore this patient was classified as an E4 homozygote.

### Representative fluorescence traces from all five amplification reactions for an E2/E4 heterozygote.



Primers corresponding to the E4 [F(C)-R(G)] and E2 [F(T)-R(A)] haplotypes yielded C(t) values similar to the positive control [E+: F(pos)-R(pos)]. Primers corresponding to the E3 [F(T)-R(G)] haplotype produced a C(t) value similar to negative control [EX: F(C)-R(A)]. Therefore this patient was classified as an E2/E4 heterozygote.

### Frequency of individual genotypes in 264 patient DNA samples\*

Genotype	Number of Individuals	Genotype Frequency
E3/E3	164	62.10%
E3/E4	60	22.70%
E2/E3	29	11.00%
E4/E4	6	2.30%
E2/E4	4	1.50%
E2/E2	1	0.40%

\*Sequence analysis of all 264 patient samples showed complete concordance with real-time qPCR results.

## Summary

We present a rapid, economical method for accurately scoring the combination of SNPs in a human haplotype.

Monitoring product accumulation during the exponential phase of amplification, in contrast to traditional end-point analysis, is important for this protocol, since reactions with primer matches or mismatches may eventually accumulate equivalent amounts of products.

Haplotype analysis using real-time qPCR requires the discrimination between priming events from perfectly matched and single (3' base) mis-matched primers. Critical parameters include, 1) careful design and validation of primer sequences, 2) optimization of annealing temperature, and 3) precision thermal cycling, particularly in the thermal excursion from denaturation to annealing temperatures.

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