Haplotype Analysis Using a Novel Amplification Strategy with Real-Time qPCR*

Chas André, Ph.D.
MJ Bioworks, Incorporated

*The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The Chromo 4™ System

Chromo 4 Real-Time Detector

DNA Engine® Base

96-Well Sample Block
Chromo 4 System Size

36cm

37cm

24cm
Chromo 4 Features

- Interchangeable detector
  - Fits on all DNA Engine thermal cycler base units—no tools required
  - Swappable with any of our Alpha™ sample blocks
- User changeable excitation/detection module
- Independent illumination and detection of each well
- Thermal gradient
- Multiplex with up to four colors
- Compact—smallest 96-well real-time system on the market
Overview

• Introduction to SNPs and haplotypes
• apoE model system
• Haplotyping with allele-specific PCR* and real-time fluorescence detection
• Assay design for haplotyping distant SNPs

*The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
• Single Nucleotide Polymorphism (SNP)
  – Most frequent DNA sequence variation in the human genome (1 in ~1000bp)
  – Global effort to identify and catalog SNPs
  – Known to cause inter-individual differences in disease risk and treatment responses
## SNP Introduction

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
</tr>
</tbody>
</table>
Haplotype Introduction

- Combinations of SNPs on a single chromosome
- Associated with a particular version of a gene
- Often the principal determinant of phenotypic consequences for genes with multiple SNPs
- May be associated with important genetic traits by linkage disequilibrium and, therefore, carry more information than individual SNPs
Haplotype Introduction

<table>
<thead>
<tr>
<th>SNP 1</th>
<th>SNP 2</th>
<th>Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>2</td>
</tr>
<tr>
<td>T</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>4</td>
</tr>
</tbody>
</table>
SNP Analysis Cannot Always Determine Haplotype

<table>
<thead>
<tr>
<th>SNP Type</th>
<th>Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-CT</td>
<td>1 2</td>
</tr>
<tr>
<td>CT-CT</td>
<td>3 4</td>
</tr>
</tbody>
</table>
Apolipoprotein E (apoE)

- Key gene in lipid metabolism
- Genetic variations contribute to the risk of developing cardiovascular disease (CVD) and Alzheimer’s disease (AD)
- 3 common haplotypes: E2, E3 and E4
apoE Haplotypes

C  T
EX

T  T
E2

T  C
E3

C  C
E4
apoE Alleles

- Three independent co-dominant alleles:
  
  E2  112 cys (TGC) 158 cys (TGC)
  E3  112 cys (TGC) 158 arg (CGC)
  E4  112 arg (CGC) 158 arg (CGC)

- E3 is the most frequent variant ("wildtype")
- E4 has the highest risk for both CVD and AD
Haplotyping Methods

• Protein structure determination
  – Separation based
  – Affinity based

• DNA structure determination
  – SSCP (single stranded conformation polymorphism)
  – Heteroduplex analysis

• DNA sequencing
  – Genomic DNA (must be cloned)
  – PCR products (must be cloned)

*The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

**Allele-Specific PCR**

<table>
<thead>
<tr>
<th>Reaction 1</th>
<th>Reaction 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'---T</td>
<td>5'---G</td>
</tr>
<tr>
<td>PCR primer with SNP site at 3' end</td>
<td></td>
</tr>
</tbody>
</table>
Mismatches extend at lower efficiency, resulting in delay in product accumulation

SNP is scored based on DIFFERENTIAL amplification of two alleles
Allele-Specific PCR for Haplotyping

Both primers with SNP site at 3’ end

- Mismatches extend at ~1,000 fold lower efficiency, resulting in ~10-cycle delay in product accumulation
- Haplotype is scored based on DIFFERENTIAL amplification of two alleles
- Both matches and mismatches can amplify with similar final quantity of product

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Real-Time Detection with SYBR® Green I

- SYBR Green I fluorescence increases upon binding dsDNA
- Samples illuminated after each PCR cycle
- Graph sample fluorescence vs. cycle

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Differential Amplification
Haplotyping with Real-Time Detection

- Reaction efficiency monitored during exponential phase
- Perfect matches amplify rapidly: early C(t)
- Mismatches amplify with delay: late C(t)
- Comparison of C(t)s for primer combinations gives genotype

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Experimental Outline

• Determine apoE haplotype for 266 patient samples using:
  – Sequencing
  – Differential amplification PCR with SYBR Green I
Primer Sets for apoE Haplotyping

5’———T C G ——— 5’

Forward primer-3’ (112)

GGACATGGAGGACGTG (T)
GGACATGGAGGACGTG (C)
GGGACATGGAGGACGTG

Reverse primer-3’ (158)

GGTACACTGCAGGC (G)
GGTACACTGCAGGC (A)
TGGTACACTGCAGGC

Positive Control
Primer Sets for apoE Haplotyping

5'—T—C—G—5'

Forward primer-3' (112)
- GGACATGGAGGACGTG (T)
- GGACATGGAGGACGTG (C)
- GGGACATGGAGGACGTG

Reverse primer-3' (158)
- GGTACACTGCAGGC (G)
- GGTACACTGCAGGC (A)
- TGGTACACTGCAGGC

C-A combination = Negative Control (EX)
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

Real-Time qPCR Protocol

- 5 reactions with different primer combinations
- 10µl rxns with 20ng genomic DNA
- 179bp amplicon ~ 75% GC
- Cycling conditions:
  - 95°C, 10min
  - 95°C, 5sec
  - 60°C, 15sec
  - 72°C, 15sec
  - Plate read/40 cycles
  - Melting curve analysis
Haplotype Determination with Allele-Specific PCR and Real-Time Fluorescence Detection

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Haplotype Determination with Allele-Specific PCR and Real-Time Fluorescence Detection

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
## Haplotype Distributions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3/E3</td>
<td>164/266</td>
<td>61.6%</td>
</tr>
<tr>
<td>E3/E4</td>
<td>60/266</td>
<td>22.5%</td>
</tr>
<tr>
<td>E2/E3</td>
<td>29/266</td>
<td>10.9%</td>
</tr>
<tr>
<td>E4/E4</td>
<td>6/266</td>
<td>2.2%</td>
</tr>
<tr>
<td>E2/E2</td>
<td>1/266</td>
<td>0.4%</td>
</tr>
<tr>
<td>E2/E4</td>
<td>4/266</td>
<td>1.6%</td>
</tr>
<tr>
<td>?</td>
<td>2/266</td>
<td>0.8%</td>
</tr>
</tbody>
</table>
## apoE Allele Frequencies

<table>
<thead>
<tr>
<th></th>
<th>apoE2</th>
<th>apoE3</th>
<th>apoE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>de Knijff et al.</td>
<td>0.0 – 14.5%</td>
<td>41-91.1%</td>
<td>6.4-36.8%</td>
</tr>
<tr>
<td>(Hum Mutation 4:195,1994)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MJ samples</td>
<td>6.6%</td>
<td>79.0%</td>
<td>14.4%</td>
</tr>
</tbody>
</table>
Overview

- Introduction to SNPs and haplotypes
- apoE model system
- Haplotyping with allele-specific PCR and real-time fluorescence detection
- Assay design for haplotyping distant SNPs

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Assay Design for Haplotyping

• Smaller amplicons help to maximize differential amplification

SNPs span: 50-200bp

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Assay Design for Haplotyping

- What if the SNPs are not close?

SNPs span: 10,000 bp
Haplotyping Distant SNPs

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
Haplotyping Distant SNPs

SNPs span: 50bp
Phusion™ Polymerase

• New DNA polymerase, Archaeal Family B type
• Same family as Pfu, Tgo, Pfx (Tko/“KOD”), “Vent®” (Tli), “Deep Vent®” (Pab), etc.
• Error-correcting (3’-5’ nuclease)
• Fused to Sso7d - processivity: 28-29 bases
• Produced by Finnzymes Oy (Helsinki)

*Phusion is sold under licensing arrangements with F. Hoffman-LaRoche Ltd., Roche Molecular Systems, Inc., and the Applied Biosystems Group of Applera Corporation. The purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front licensing fee, either by payment to Applied Biosystems, or as purchased, i.e., an authorized thermal cycler. Please see complete licensing information at the end of this presentation. This product includes a limited license under pending patents owned by MJ Bioworks, Inc.
Phusion Polymerase: Direct from Genomic DNA

Human genomic DNA

Template: 10,000 genomes/20µl reaction
30U/ml enzyme, 35 cycles @ 10min/cycle

*Phusion is sold under licensing arrangements with F. Hoffman-LaRoche Ltd., Roche Molecular Systems, Inc., and the Applied Biosystems Group of Applera Corporation. The purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front licensing fee, either by payment to Applied Biosystems, or as purchased, i.e., an authorized thermal cycler. Please see complete licensing information at the end of this presentation. This product includes a limited license under pending patents owned by MJ Bioworks, Inc.
Conclusions

- Allele-specific PCR with real-time fluorescence detection provides a simple, inexpensive and robust method for haplotyping.
- Protocol validated by haplotyping apoE alleles on 266 human samples.
- Long PCR and ligation allows differential amplification haplotyping for SNPs separated by large regions.

The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.
## Acknowledgements

| MJ Research, Incorporated | David Batey, Ph.D.  
|                          | Richard Kurtz, Ph.D. |
| MJ Bioworks, Incorporated  
  Research & Development | Fan Chen, Ph.D.  
|                          | Michael Finney, Ph.D. |
|                          | Vicki Pandey, Ph.D.  
|                          | Jon Menke, Ph.D. |
Opticon, Alpha and Chromo4 are trademarks of MJ Research, Incorporated.
DNA Engine is a trademark registered in the United States belonging to MJ Research, Incorporated.
SYBR is a registered trademark of Molecular Probes, Inc.
Vent & Deep Vent are registered trademarks of New England Biolabs, Inc.
**NOTICE TO PURCHASER: LIMITED LICENSE** The purchase price of Phusion polymerase includes a limited, non-transferable license under issued and pending U.S. and foreign patents owned by MJ Bioworks ("MJB") to use only this amount of the product. No other license under these patents is conveyed expressly or by implication to the purchaser by the purchase of this product. Further information on purchasing licenses to practice methods under MJB's patents may be obtained by contacting the Director of Licensing, MJ Bioworks, 7000 Shoreline Court, South San Francisco CA 94080.