

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

Chas André, Ph.D.
MJ Bioworks, Incorporated



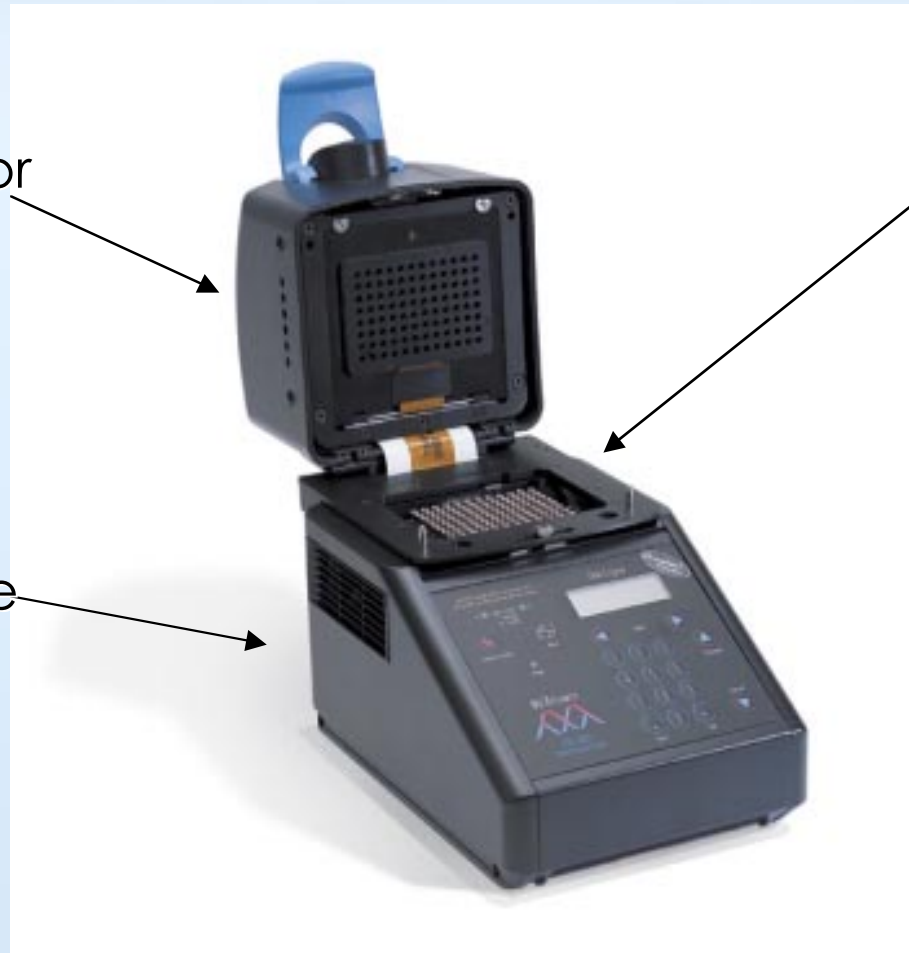
*The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

The Chromo 4™ System

Chromo 4
Real-Time Detector

96-Well Sample
Block

DNA Engine® Base



Chromo 4 System Size



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

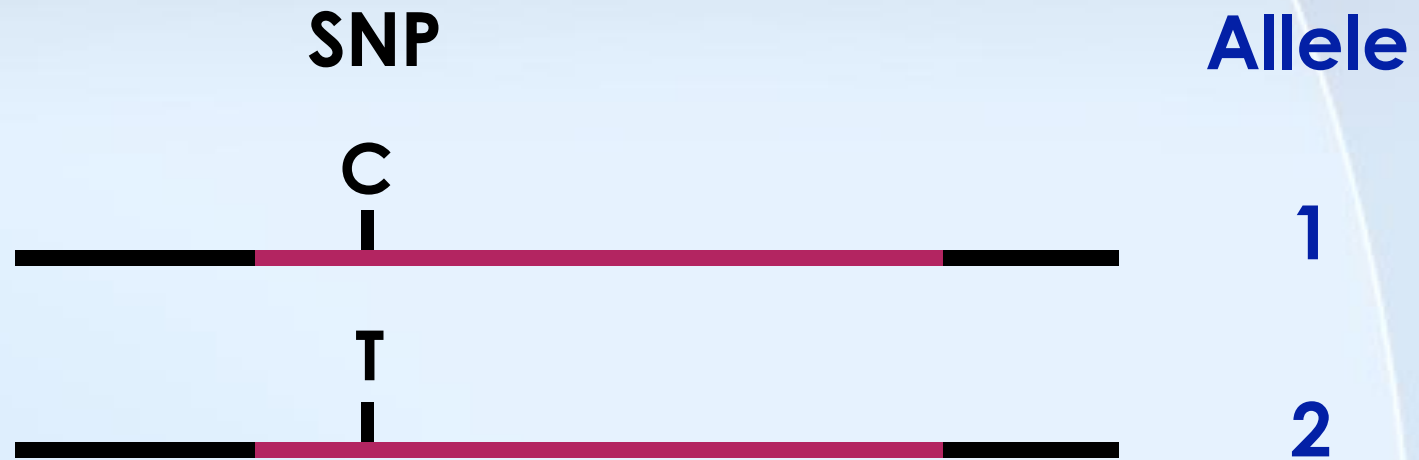
- Introduction to SNPs and haplotypes
- apoE model system
- Haplotyping with allele-specific PCR* and real-time fluorescence detection
- Assay design for haplotyping distant SNPs

*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.

SNP Introduction

- 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

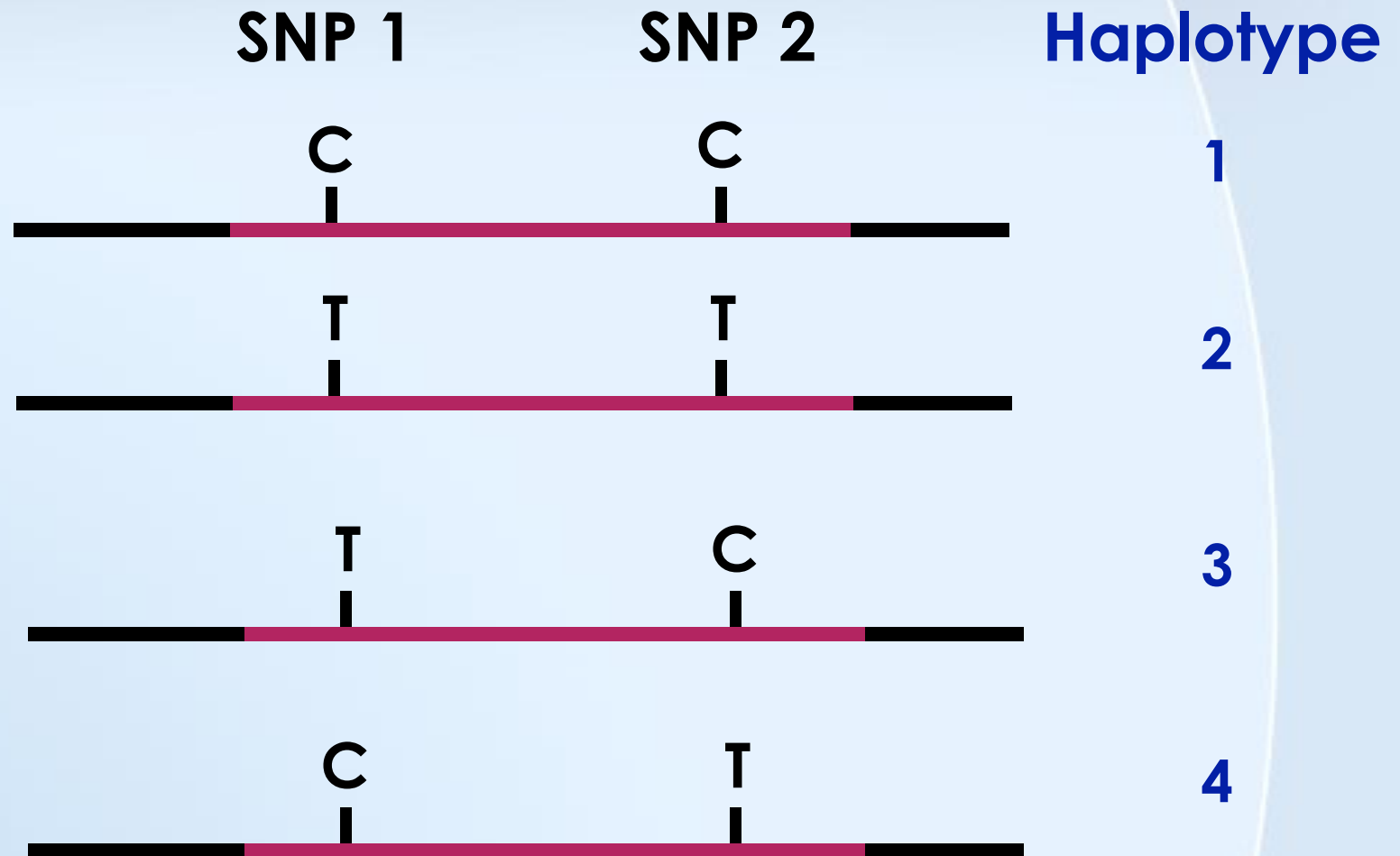
SNP Introduction



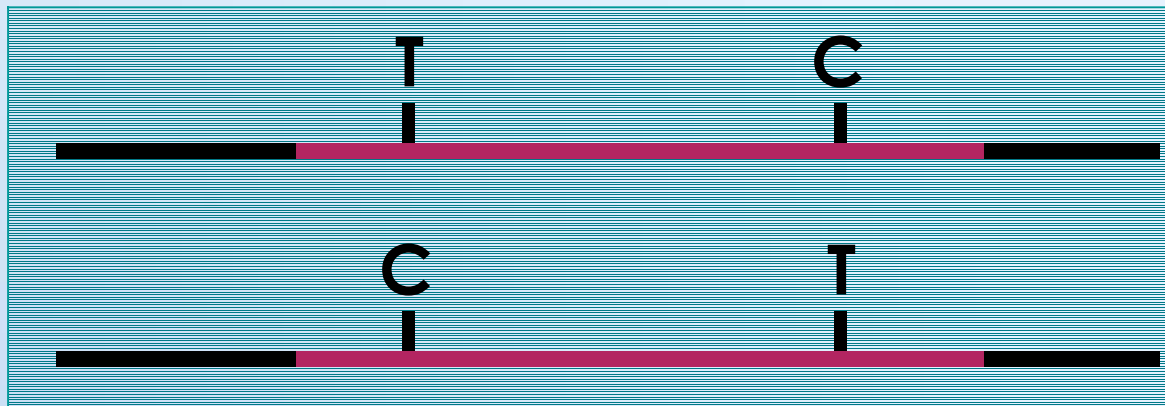
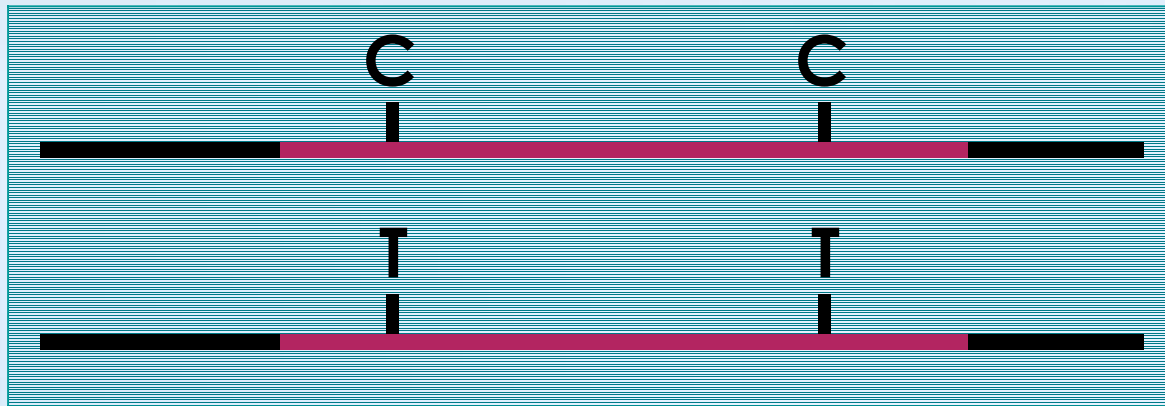
Haplotype Introduction

- 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



SNP Analysis Cannot Always Determine Haplotype



SNP Type Haplotype

CT-CT

1 2

CT-CT

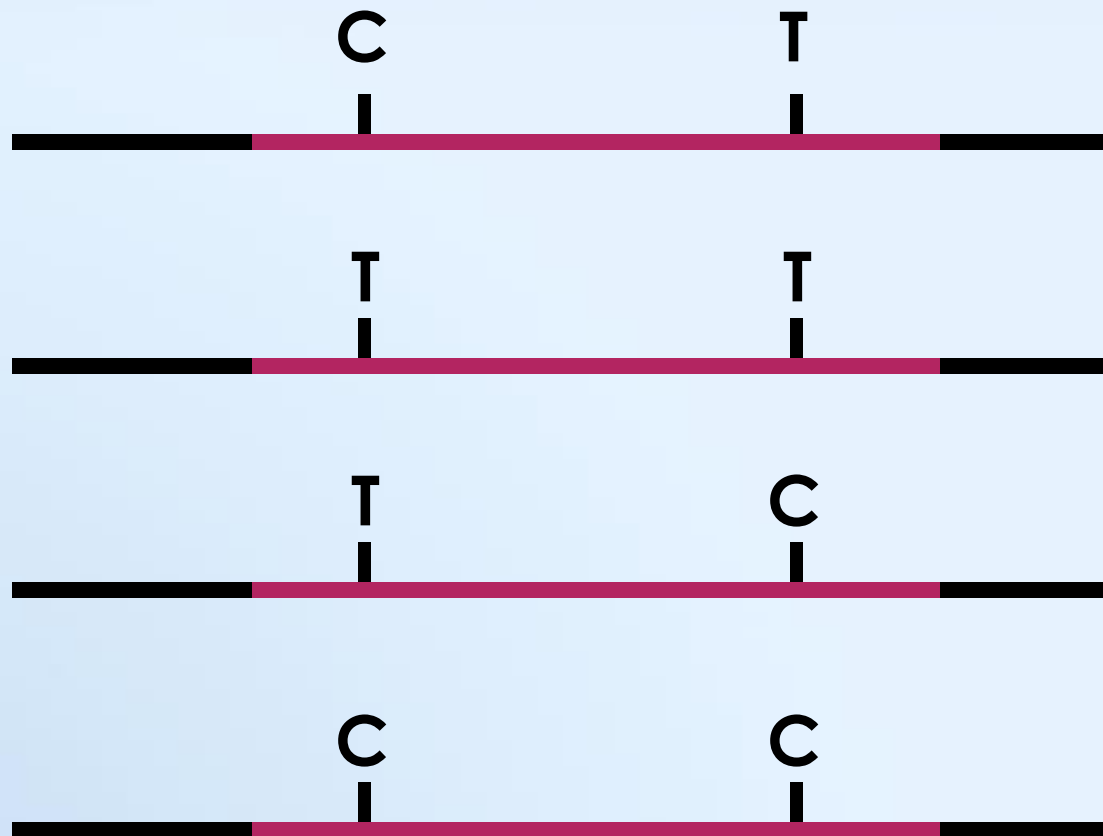
3 4

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

Haplotype



EX

E2

E3

E4

apoE Alleles

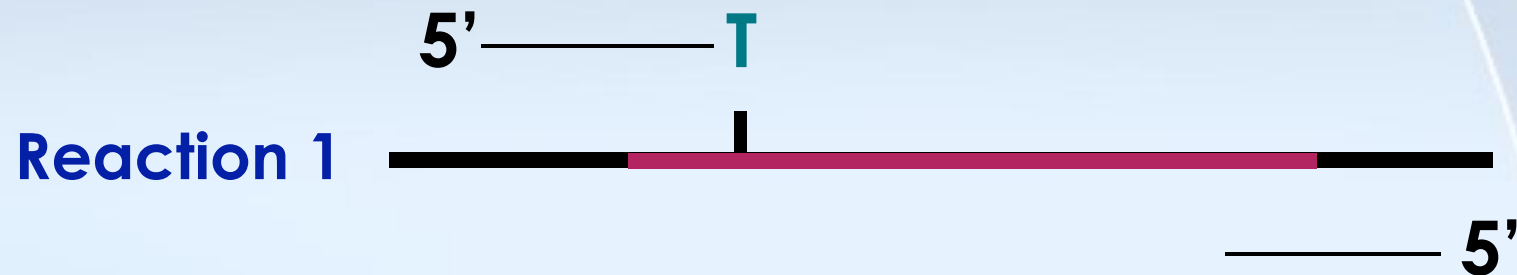
- Three independent co-dominant alleles:
 - E2 112 cys (**T**GC) 158 cys (**T**GC)
 - E3 112 cys (**T**GC) 158 arg (**C**GC)
 - E4 112 arg (**C**GC) 158 arg (**C**GC)
- E3 is the most frequent variant (“wildtype”)
- E4 has the highest risk for both CVD and AD

Haplotyping Methods

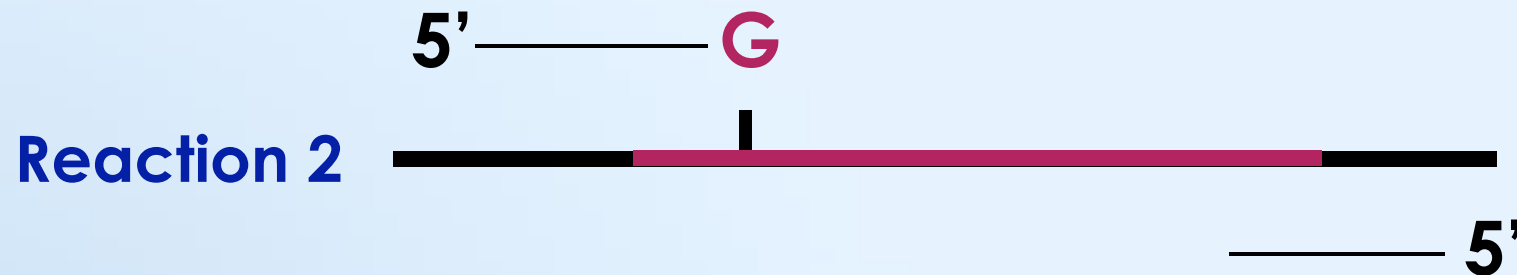
- Protein structure determination
 - Separation based
 - Affinity based
- DNA structure determination
 - SSCP (single stranded conformation polymorphism)
 - Heteroduplex analysis
- DNA sequencing
 - Genomic DNA (must be cloned)
 - PCR products (must be cloned)

*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.

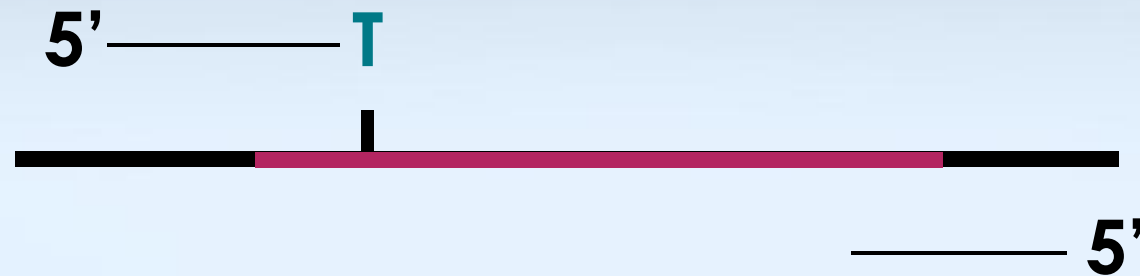
Allele-Specific PCR



PCR primer with SNP site at 3' end

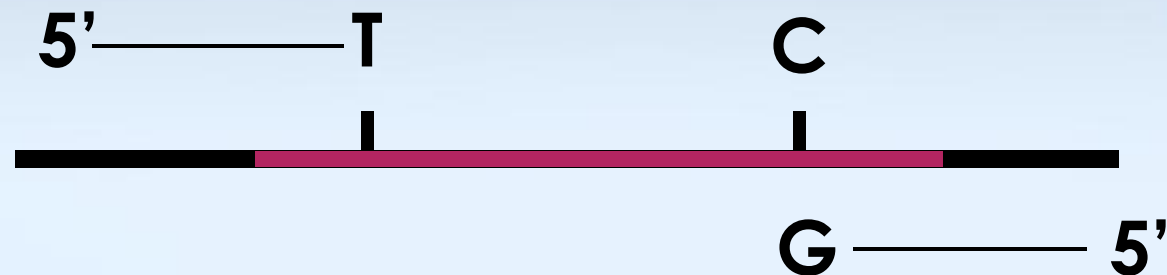


Allele-Specific PCR



- 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

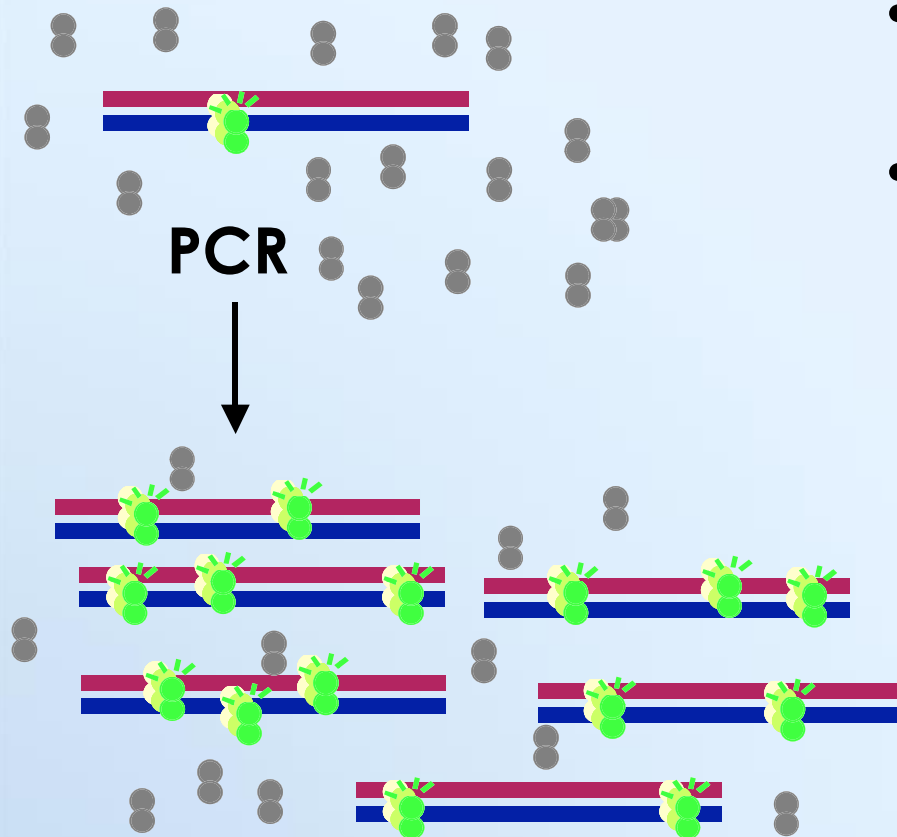


Both primers with SNP site at 3' end

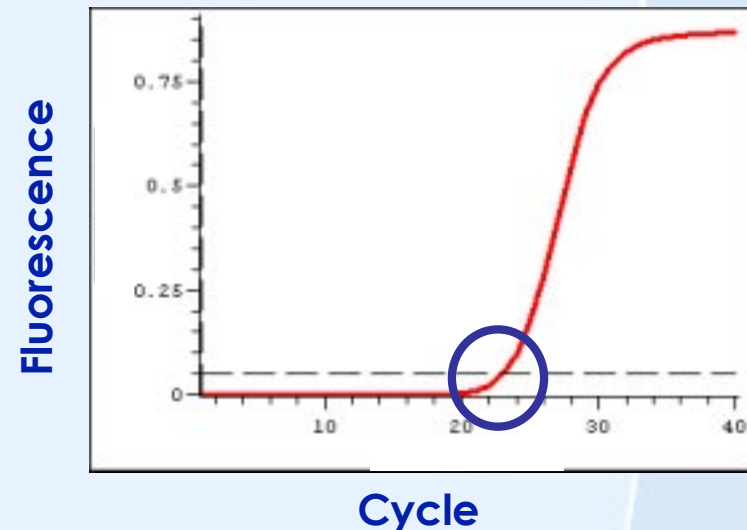
- Mismatches extend at ~1,000 fold lower efficiency, resulting in ~10-cycle delay in product accumulation
- Haplotype is scored based on DIFFERENTIAL amplification of two alleles
- Both matches and mismatches can amplify with similar final quantity of product

Real-Time Detection with SYBR[®] Green I

● Unbound SYBR Green I
● Bound SYBR Green I



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



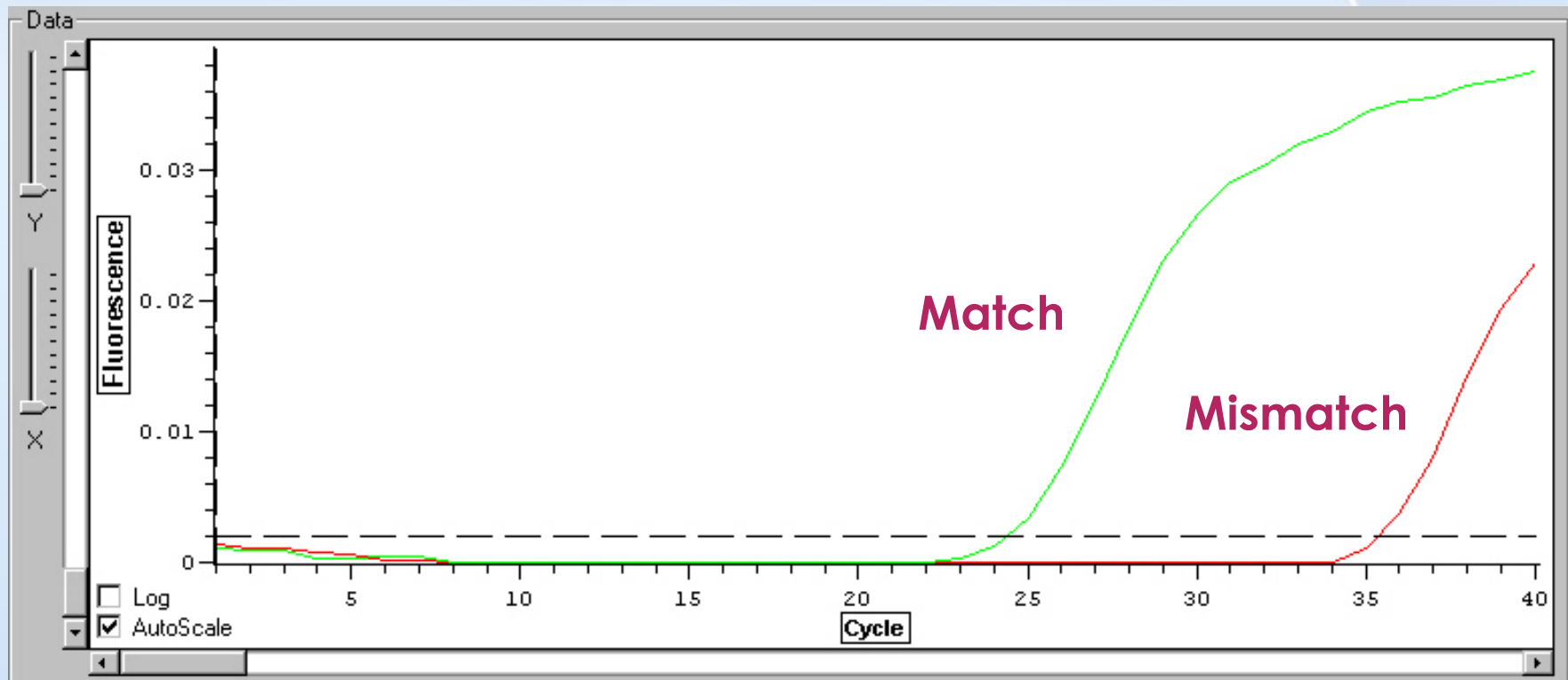
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

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

Early and Late C(t) with Real-Time Detection on the Opticon™ System

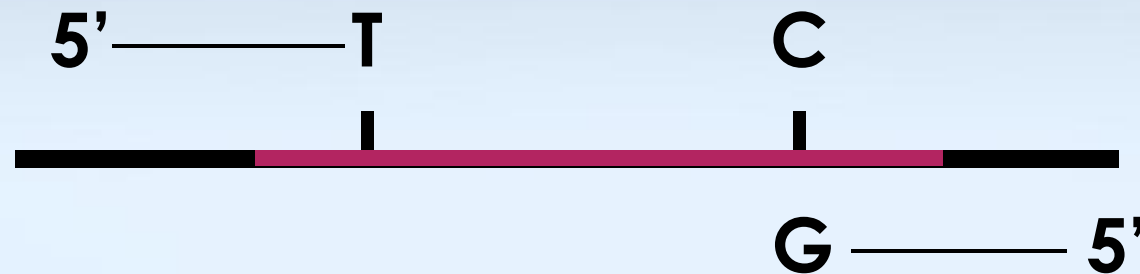


The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

Experimental Outline

- Determine apoE haplotype for 266 patient samples using:
 - Sequencing
 - Differential amplification PCR with SYBR Green I

Primer Sets for apoE Haplotyping



Forward primer-3' (112)

GGACATGGAGGACGTG (T)
 GGACATGGAGGACGTG (C)
 GGGACATGGAGGACGTG

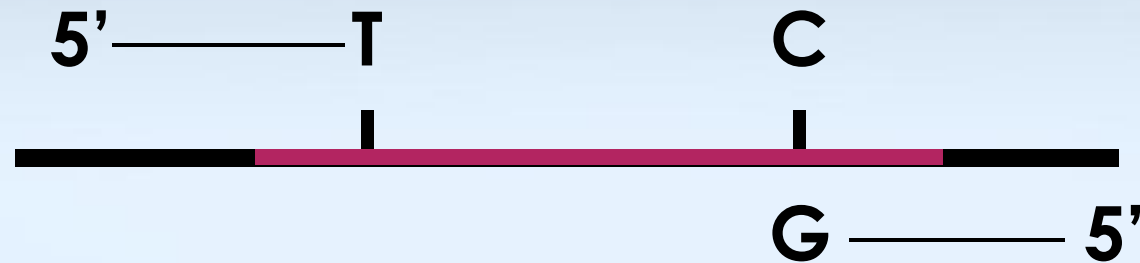
Positive Control

Reverse primer-3' (158)

GGTACACTGCAGGC (G)
 GGTACACTGCAGGC (A)
 TGGTACACTGCAGGC

Positive Control

Primer Sets for apoE Haplotyping



Forward primer-3' (112)

GGACATGGAGGACGTG (T)
 GGACATGGAGGACGTG (C)
 GGGACATGGAGGACGTG

Reverse primer-3' (158)

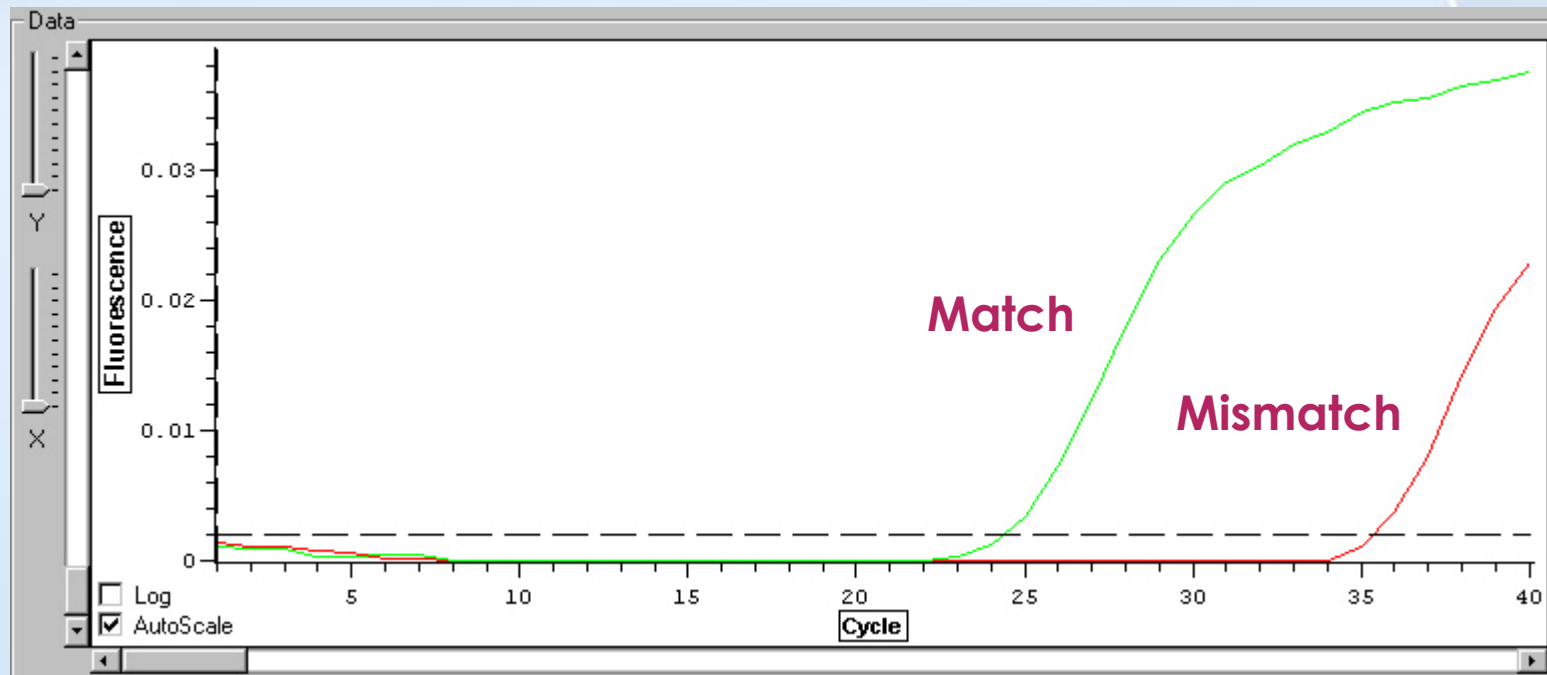
GGTACACTGCAGGC (G)
 GGTACACTGCAGGC (A)
 TGGTACACTGCAGGC

C-A combination = Negative Control
 (EX)

Real-Time qPCR Protocol

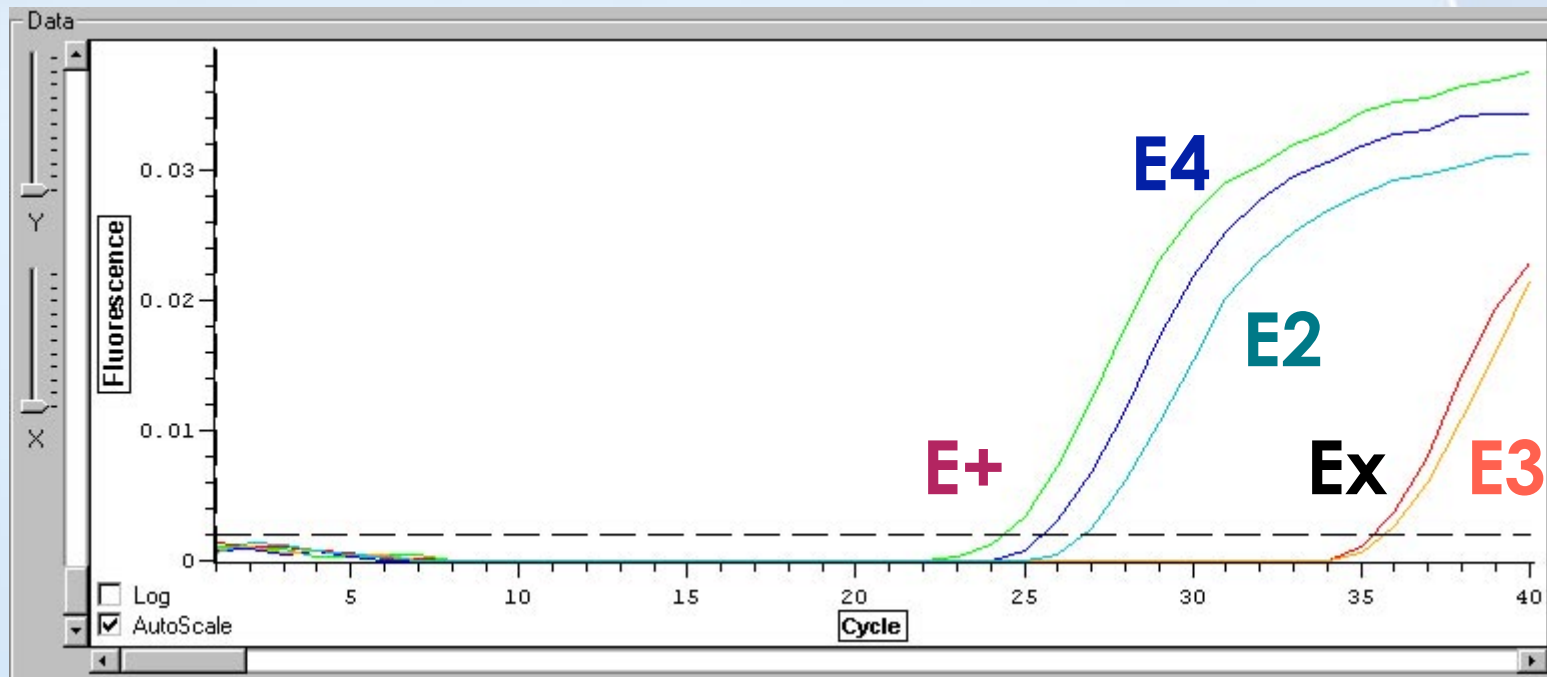
- 5 reactions with different primer combinations
- 10 μ l rxns with 20ng genomic DNA
- 179bp amplicon ~ 75% GC
- Cycling conditions:
 - 95°C, 10min
 - 95°C, 5sec
 - 60°C, 15sec
 - 72°C, 15sec
 - Plate read/40 cycles
 - Melting curve analysis

Haplotype Determination with Allele-Specific PCR and Real-Time Fluorescence Detection



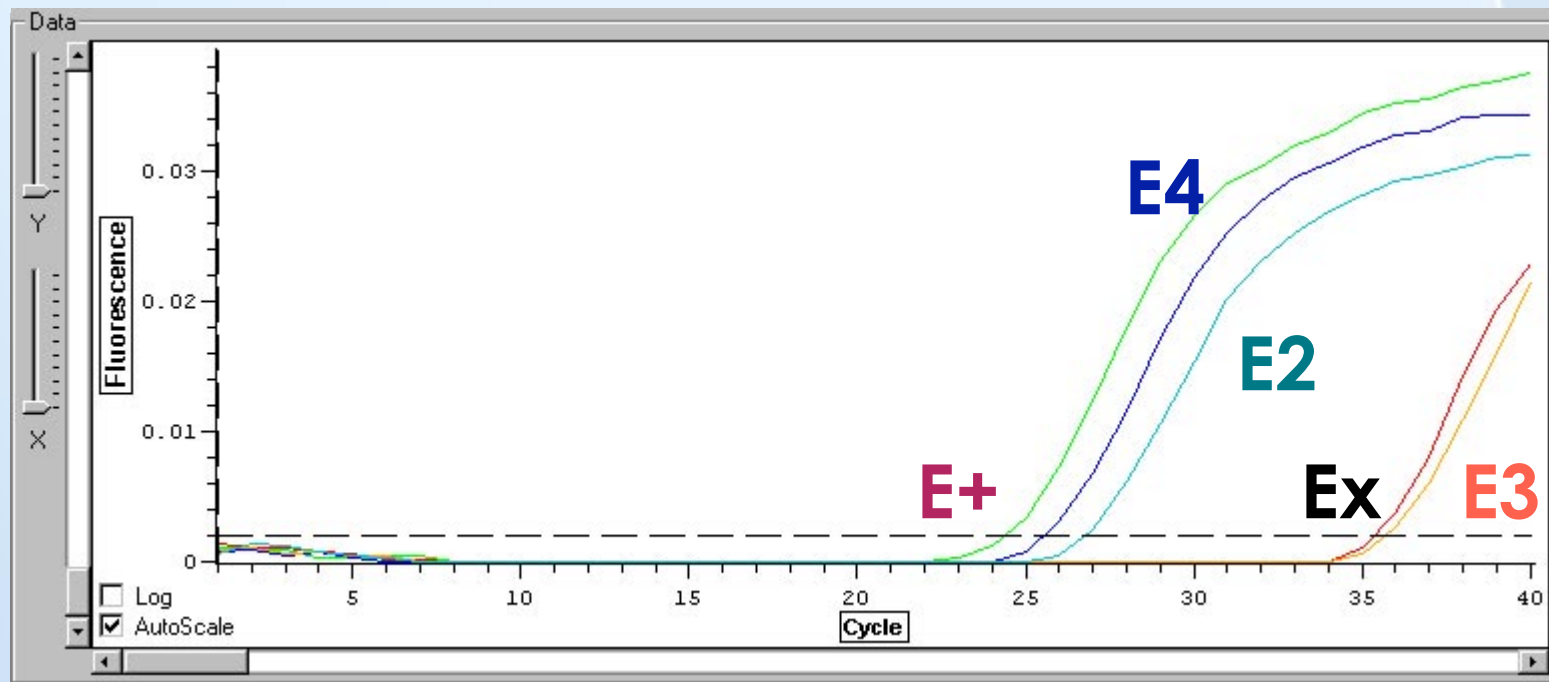
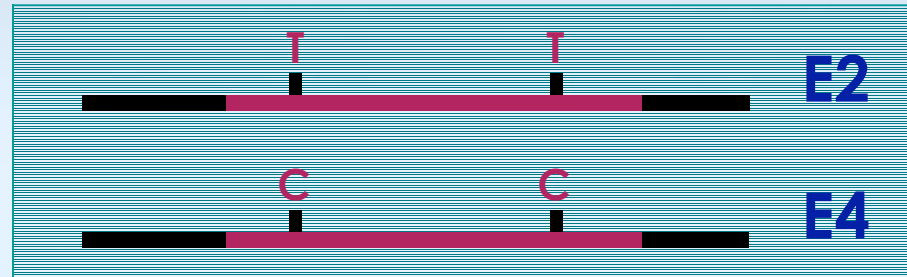
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

Haplotype Determination with Allele-Specific PCR and Real-Time Fluorescence Detection



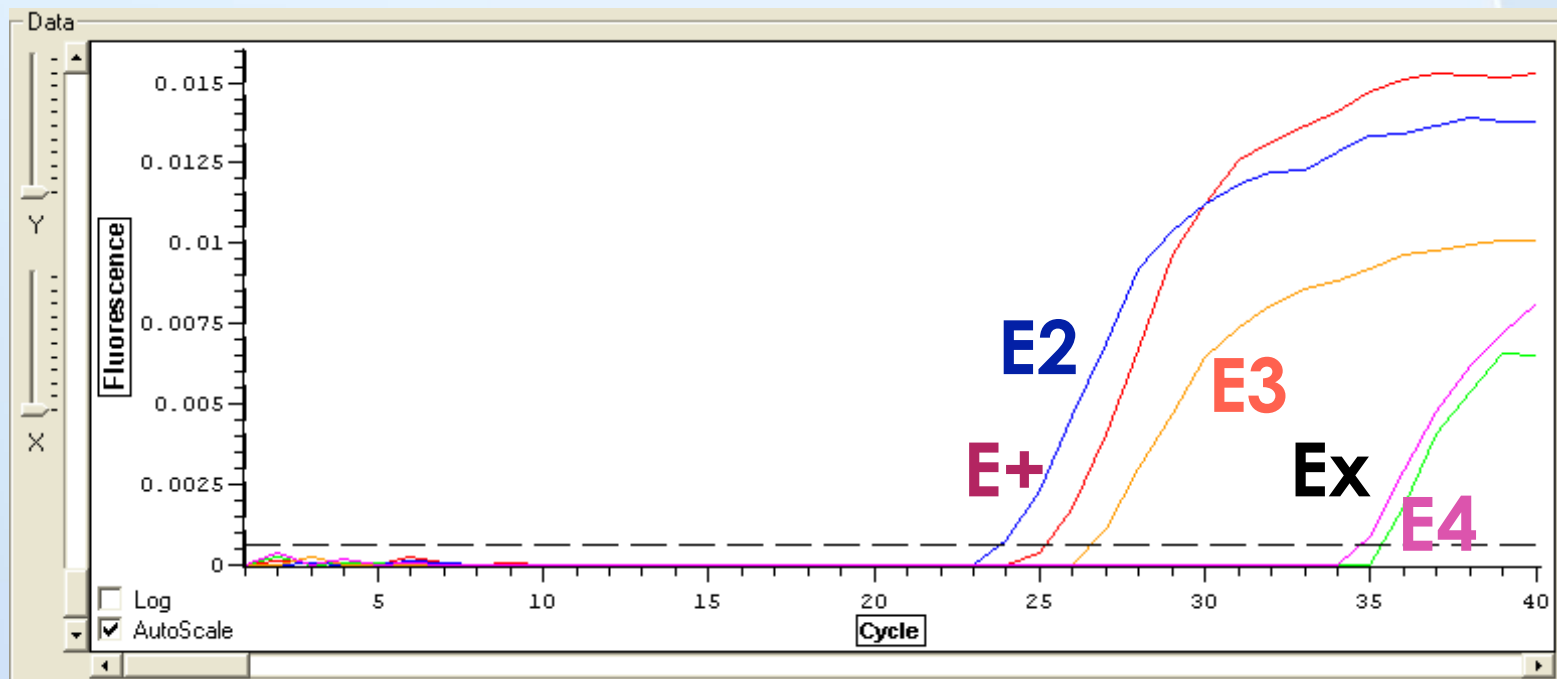
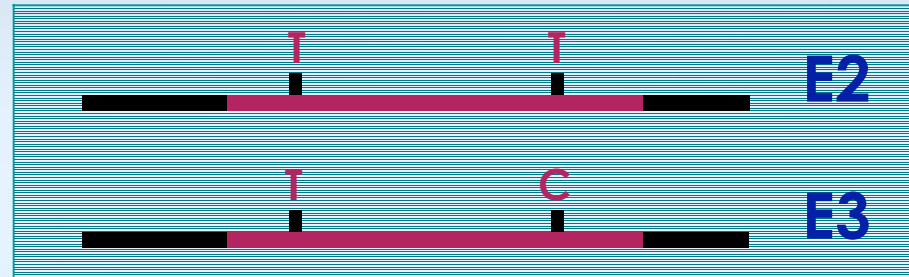
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

E2/E4 Individual



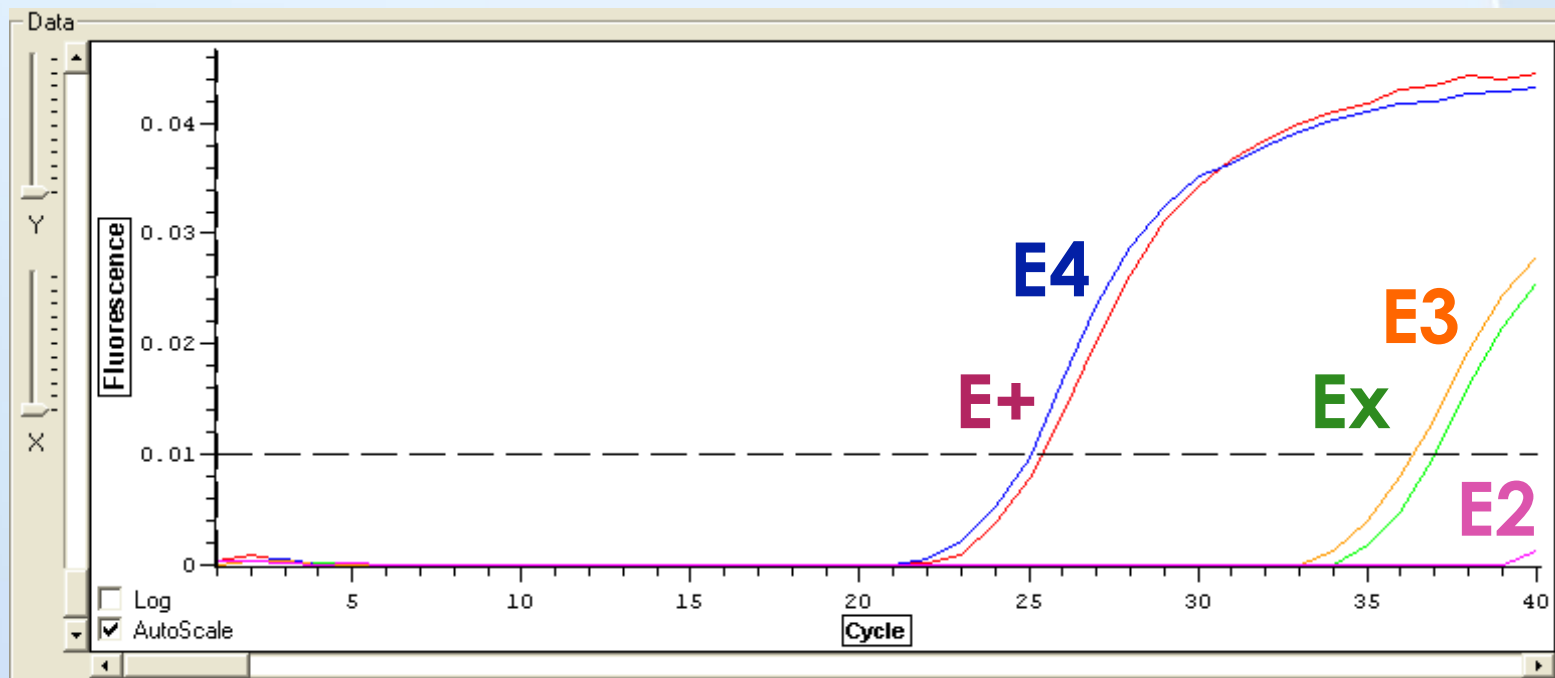
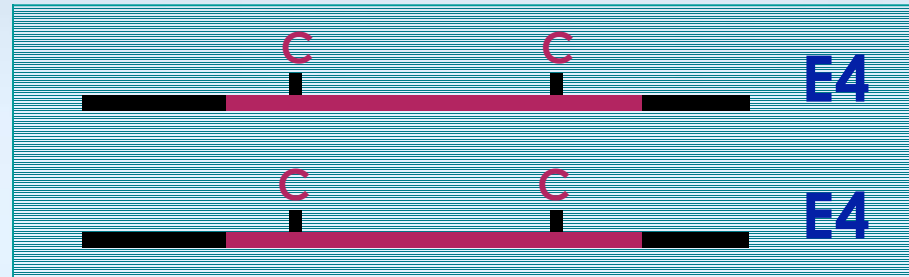
The polymerase chain reaction (PCR) is a process covered by patents owned by Hoffman-LaRoche, Inc. & F. Hoffmann-LaRoche Ltd. Users should obtain proper license to perform the reaction. Additional licensing information is presented at the end of this presentation.

E2/E3 Individual



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.

E4/E4 Individual



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

Genotype	Number	Percent
E3/E3	164/266	61.6%
E3/E4	60/266	22.5%
E2/E3	29/266	10.9%
E4/E4	6/266	2.2%
E2/E2	1/266	0.4%
E2/E4	4/266	1.6%
?	2/266	0.8%

apoE Allele Frequencies

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

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

Assay Design for Haplotyping

- Smaller amplicons help to maximize differential amplification



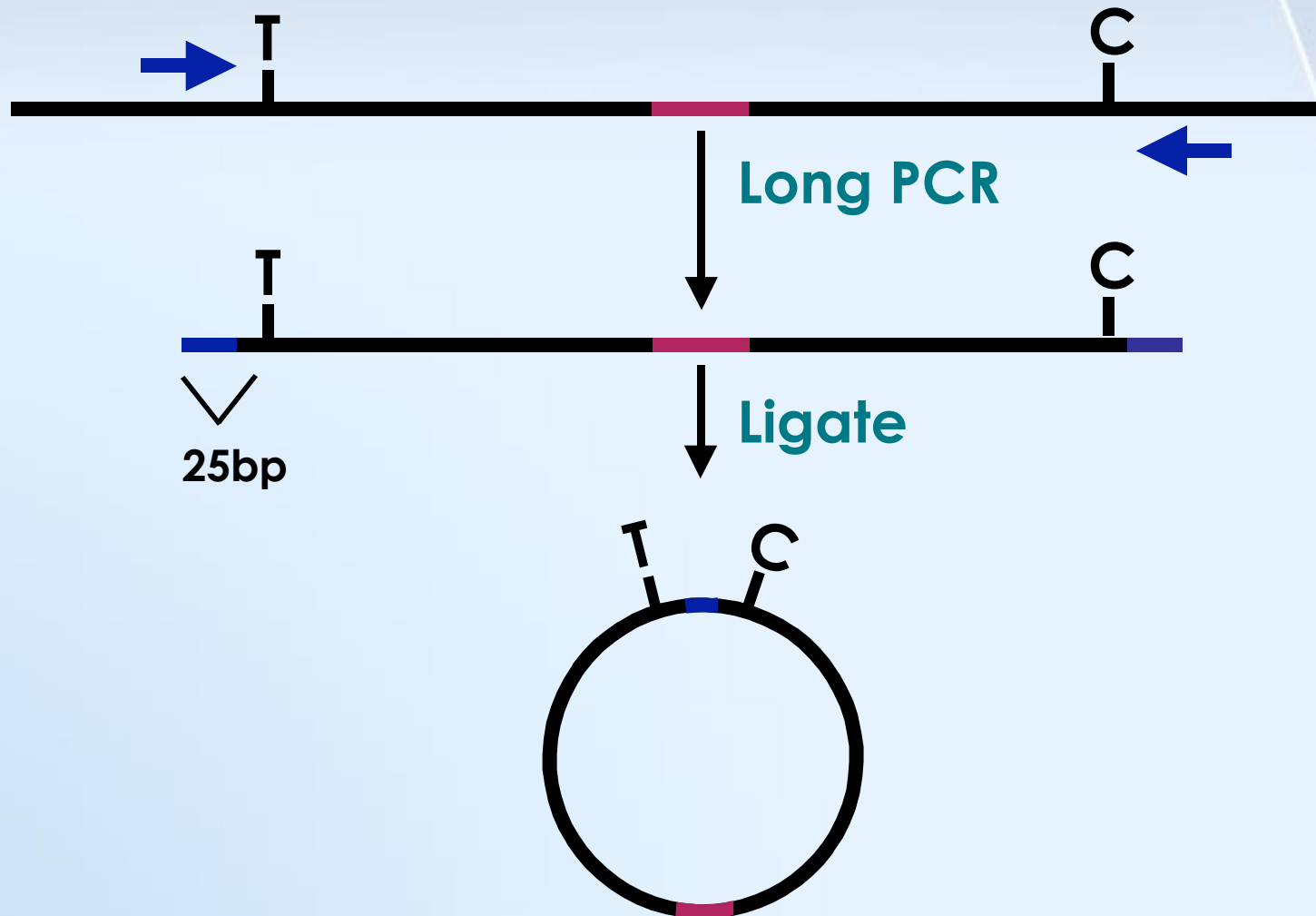
Assay Design for Haplotyping

- What if the SNPs are not close?

SNPs span:
10,000 bp

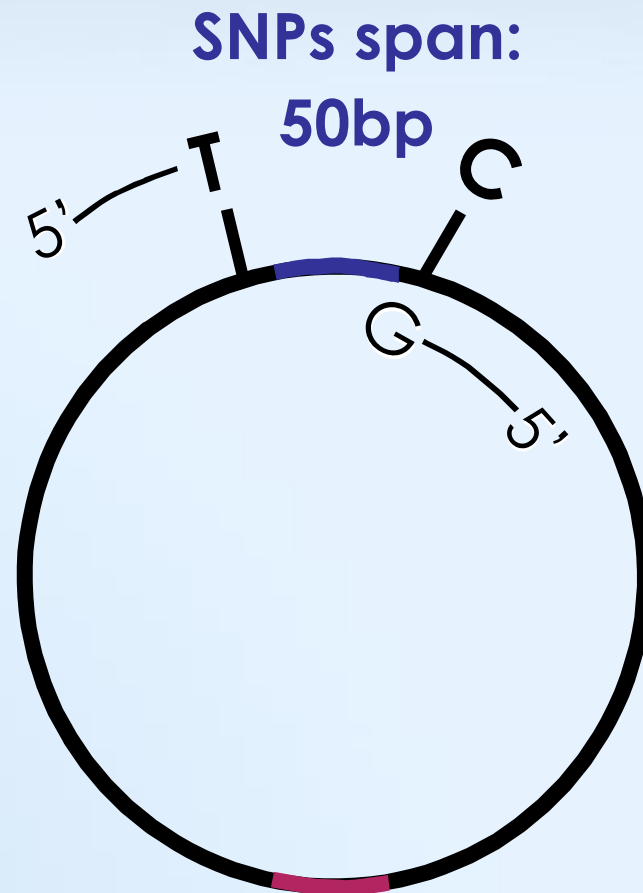


Haplotyping Distant SNPs



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





Phusion™ Polymerase

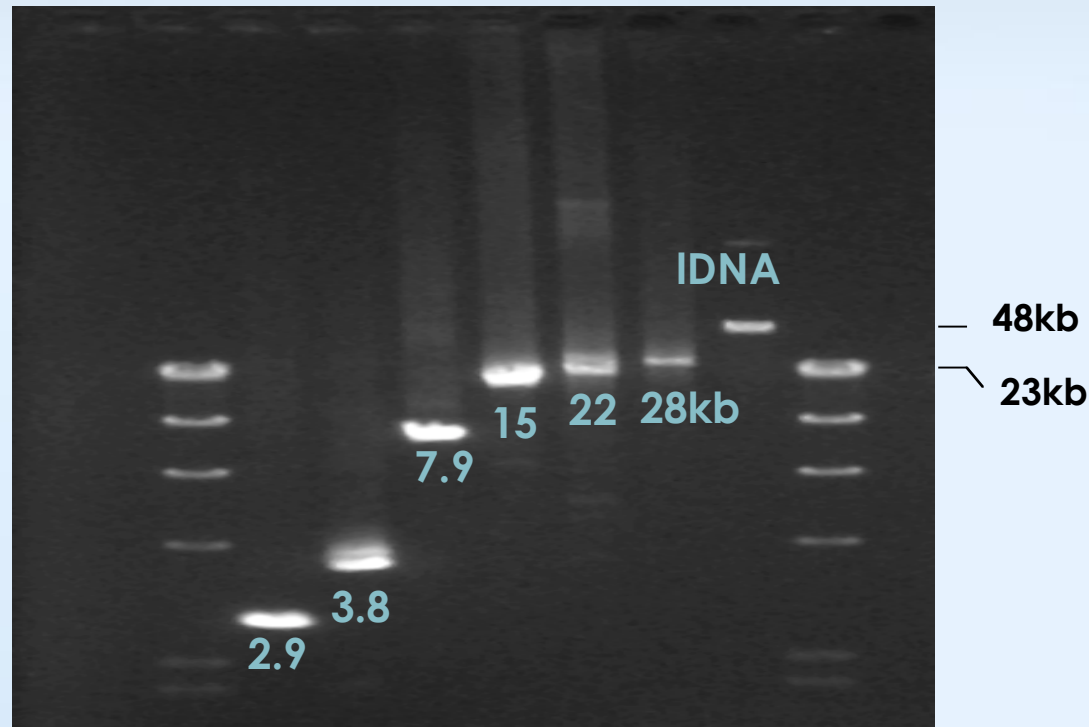
- New DNA polymerase, Archaeal Family B type
- Same family as *Pfu*, *Tgo*, *Pfx* (*Tko*/"KOD"), "Vent®" (*Tli*), "Deep Vent®" (*Pab*), etc.
- Error-correcting (3'-5' nuclease)
- Fused to *Sso7d* - processivity: 28-29 bases
- Produced by Finnzymes Oy (Helsinki)

*Phusion is sold under licensing arrangements with F. Hoffman-LaRoche Ltd., Roche Molecular Systems, Inc., and the Applied Biosystems Group of Applied Biosystems 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/20 μ l 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 Applied Biosystems, Inc. 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



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.

PCR* Licensing Information

The following table reflects MJ Research's understanding of the requirements set by Roche and its licensees for particular classes of users to purchase PCR licenses in the United States. This table is not endorsed by Roche, and all thermal cycler users are encouraged to contact Roche for further information.

"Authorization" of thermal cyclers: The Applied Biosystems division of Applied Biosystems Corporation holds the exclusive license from Roche to grant sub-licenses to perform PCR in specific fields, such as research and forensics. Since 1992, Applied Biosystems has generally issued licenses in the form of a running royalty with sale of specific licensed enzymes, which carry a label stating that reactions must be run in an "authorized" thermal cycler.

MJ Research does not sell "authorized" thermal cyclers and believes that ABI's right to charge a thermal cycler "authorization" fee is probably legally unenforceable in the U.S. (this matter is under review by the U.S. Federal District Court). However, Applied Biosystems will sell such an "authorization" for any individual thermal cycler. It may be prudent in some circumstances to "authorize" an MJ cycler after purchase.

Addresses for contacting Hoffmann-LaRoche:

In the United States:

Licensing Manager
Roche Molecular Systems, Inc.
1145 Atlantic Avenue
Alameda CA 94501 USA
(510) 814-2970 • Fax: (510) 814-2977

In other nations:

PCR Licensing Manager
F. Hoffmann-La Roche Ltd.
Building 222/350
CH-4002 Basel, Switzerland
41 61 687 3031 • Fax: 41 61 687 2113

Type of Use	PCR* for Human & Animal Diagnostics		Other PCR ¹	Non-PCR ¹
	with Diagnostic Service License ²	with Licensed Test Kits ²	Research, Forensics, etc.	Cycle Sequencing, Prins, etc. ³
Thermal Cycler License*	Usually none (see actual terms of license)	Usually none (see actual terms of license)	Thermal cycler "authorized" for PCR	None
Enzyme License*	Usually none (see actual terms of license)	Usually none (see actual terms of license)	Enzyme with PCR label license	None

* This table refers to PCR licenses only; other processes, and particular types of enzyme or thermal cycler, may require separate licenses.

¹ For definitive information on where your application fits, please contact Roche.

² For information on PCR Diagnostic Product Licenses or PCR Diagnostic Service Licenses, please contact Roche.

Note: Please do not construe the information on this page as legal advice—for more information, please contact your institution's legal department.

N.B. For each column, both the indicated thermal cycler license and the indicated enzyme license are required.

***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 MJBs patents may be obtained by contacting the Director of Licensing, MJ Bioworks, 7000 Shoreline Court, South San Francisco CA 94080.