Rapid Cycle PCR, Real Time Analysis, and Hi-Res Melting

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University of Utah

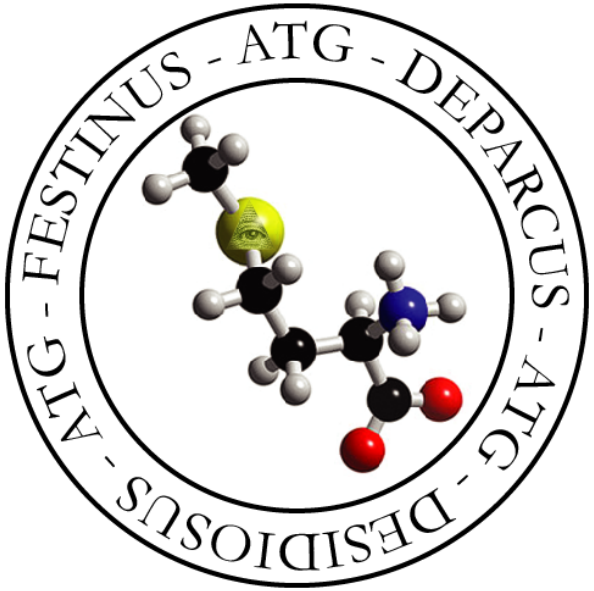
ARUP

Idaho Technology

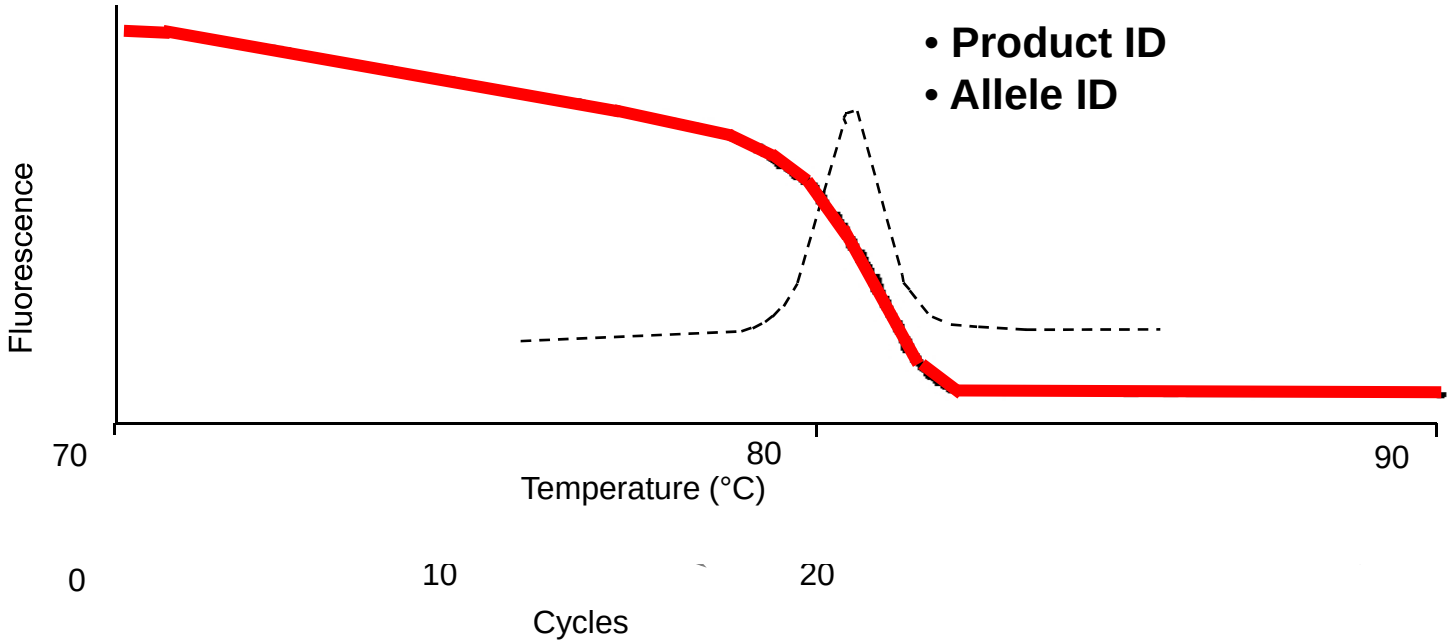
AMP, Oct. 31, 2008

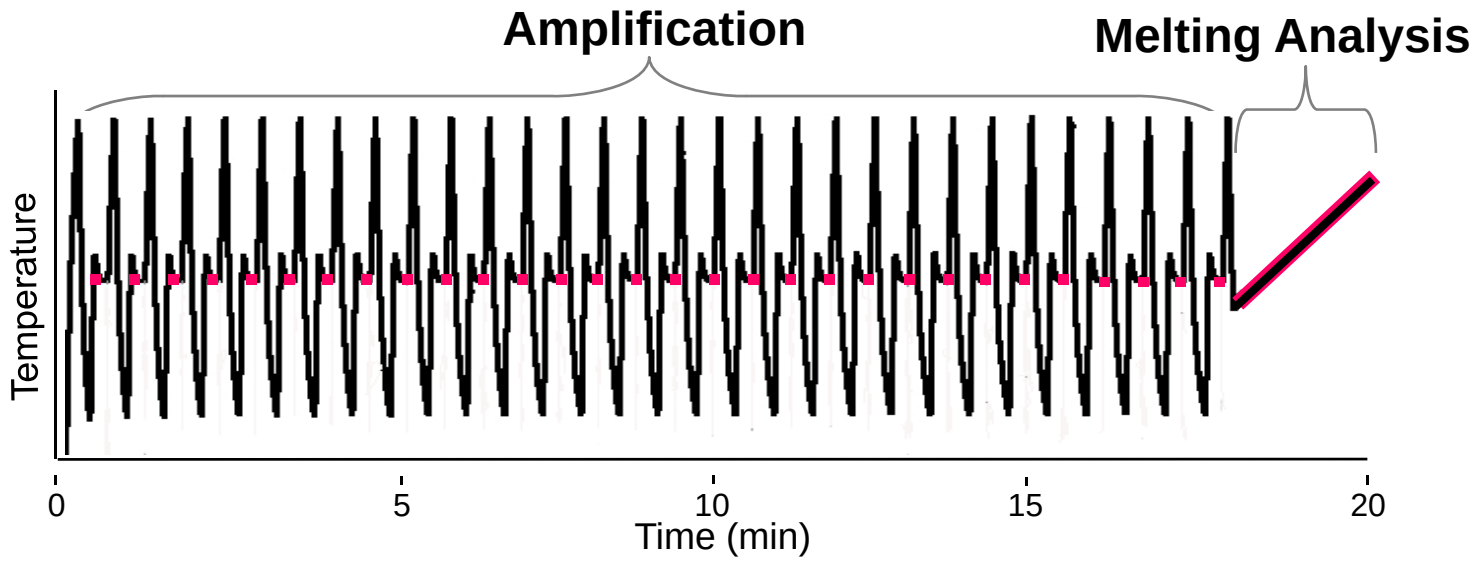
Impatient, Lazy, and Cheap

- Rapid Cycle PCR
  - Fast
- Real-Time Analysis
  - High information content
- High-Resolution Melting
  - Low cost



Amplification & Analysis



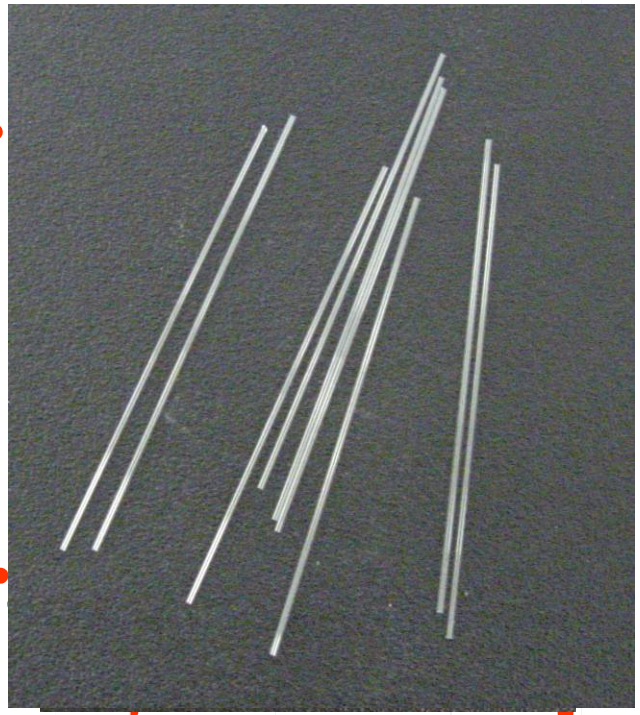


Rapid Cycle PCR

Temperature cycling for PCR

30 cycles

Re-circulating **Hair Dryer**



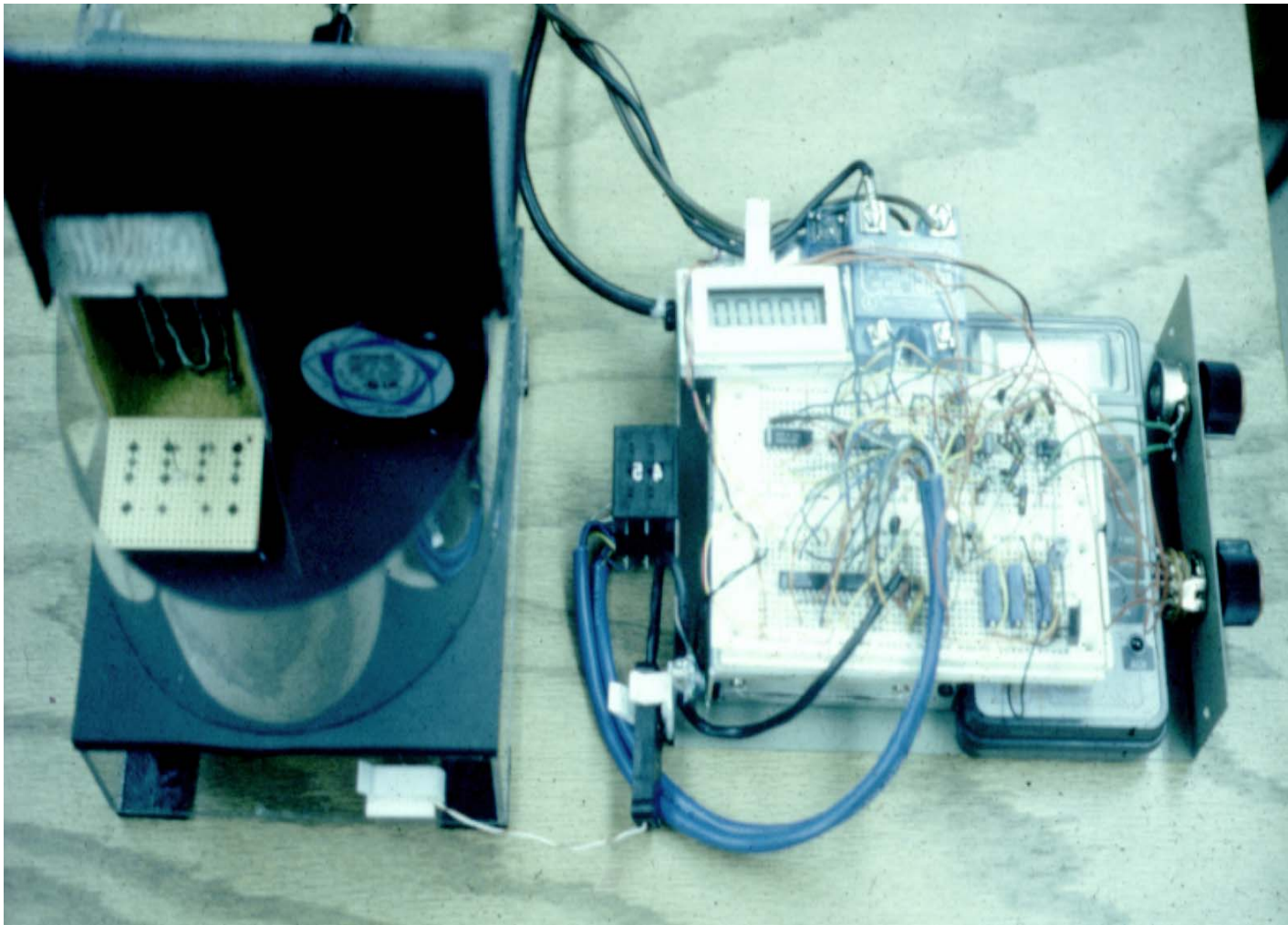
Academic Prototypes

Wood (1988)

Tin (1989)

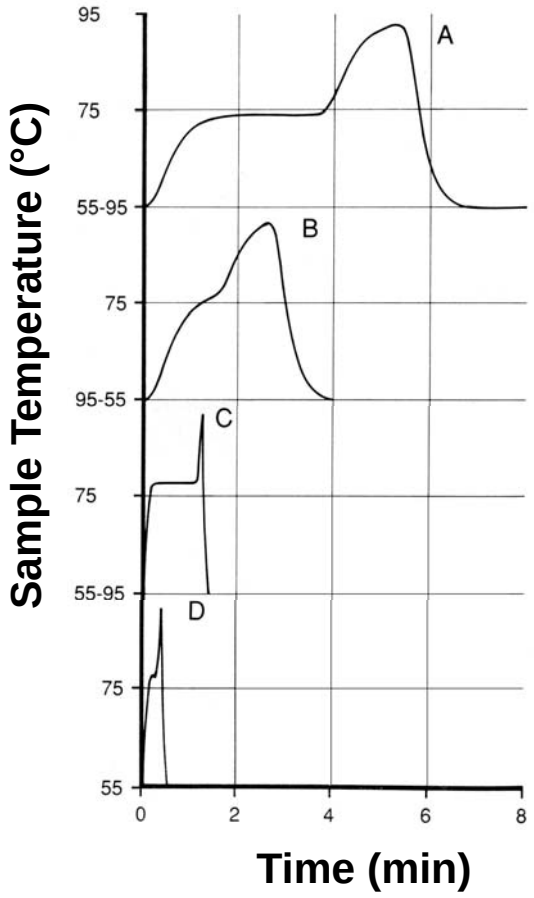
Plastic (1990)

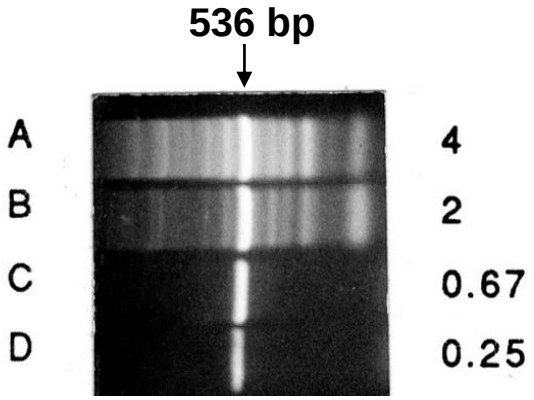
Electronics



Better Temperature Control

Temperature Profiles | **Gel Analysis** | **Time for 30 Cycles (hr)**



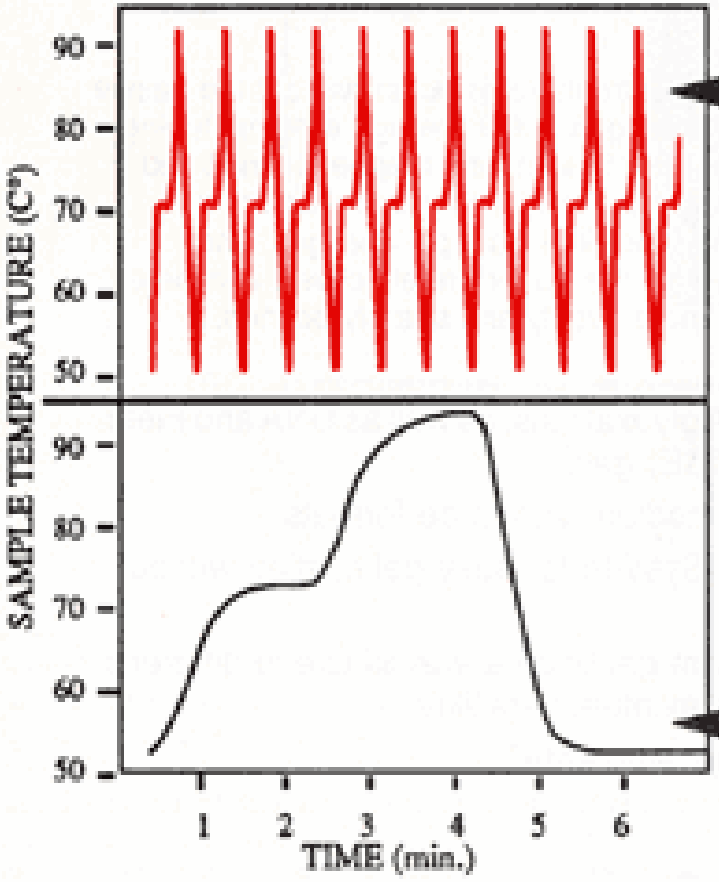


Amplification of a 536 bp β-globin fragment from human genomic DNA

Anal Biochem 1990;***186***:***328-31,*** Biotechniques. 1991;***10***:***76-83***

Late 1990: The Girlfriend Series

Rapid Air Cycling: 30 cycle reactions in 15-30 minutes.

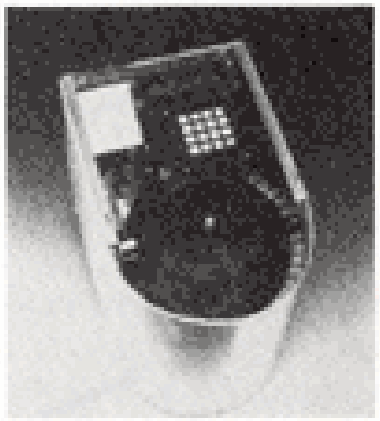


Air Thermo-Cycler

- Improved **specificity.**
- **Lower cost** per sample.
- Ten times **faster** than heat block instruments.
- Closed system ensures **decreased contamination.**

Heat Block Instrument

Introducing the
Air Thermo-Cycler



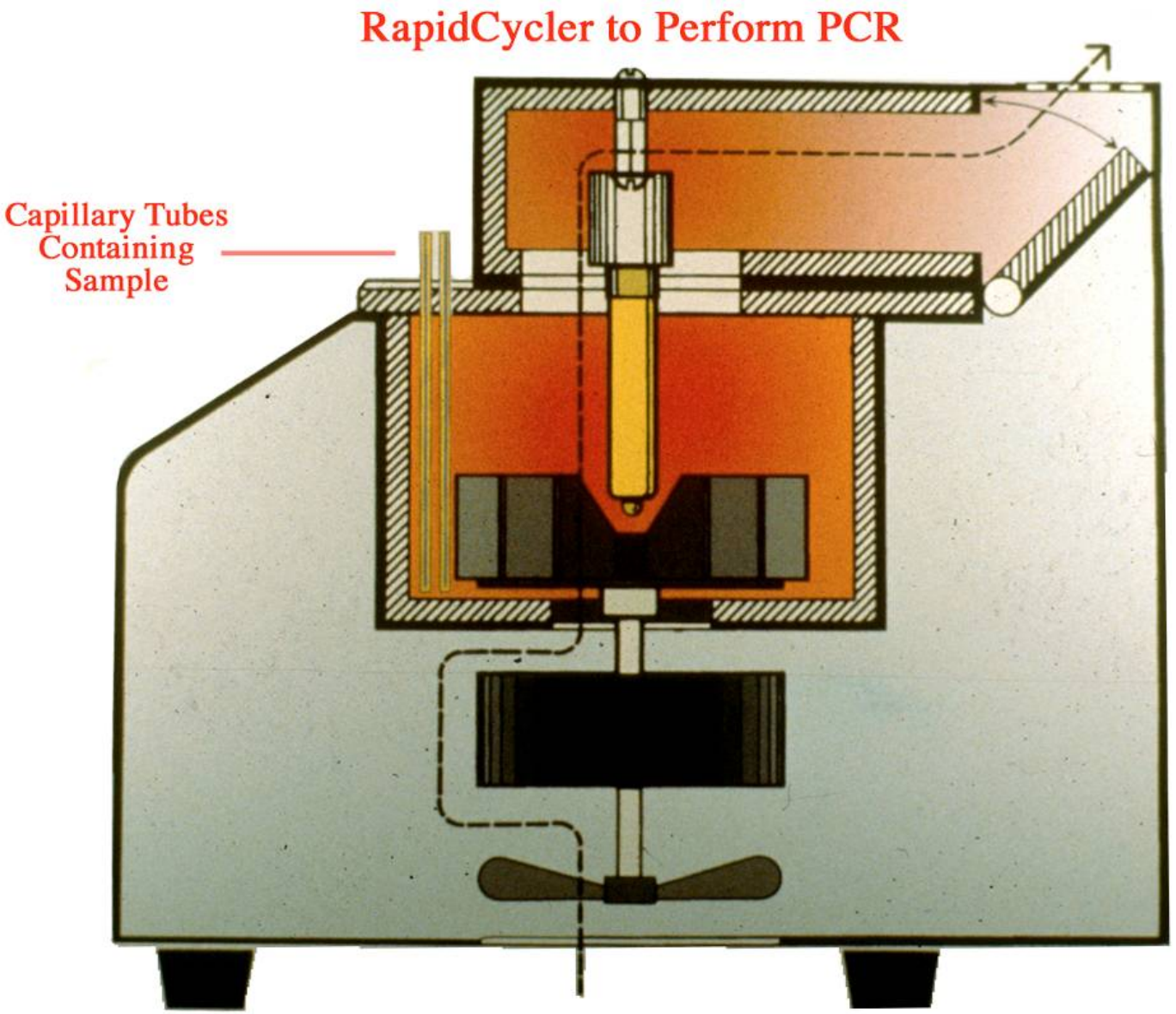
Idaho Technology

1-800-735-6544

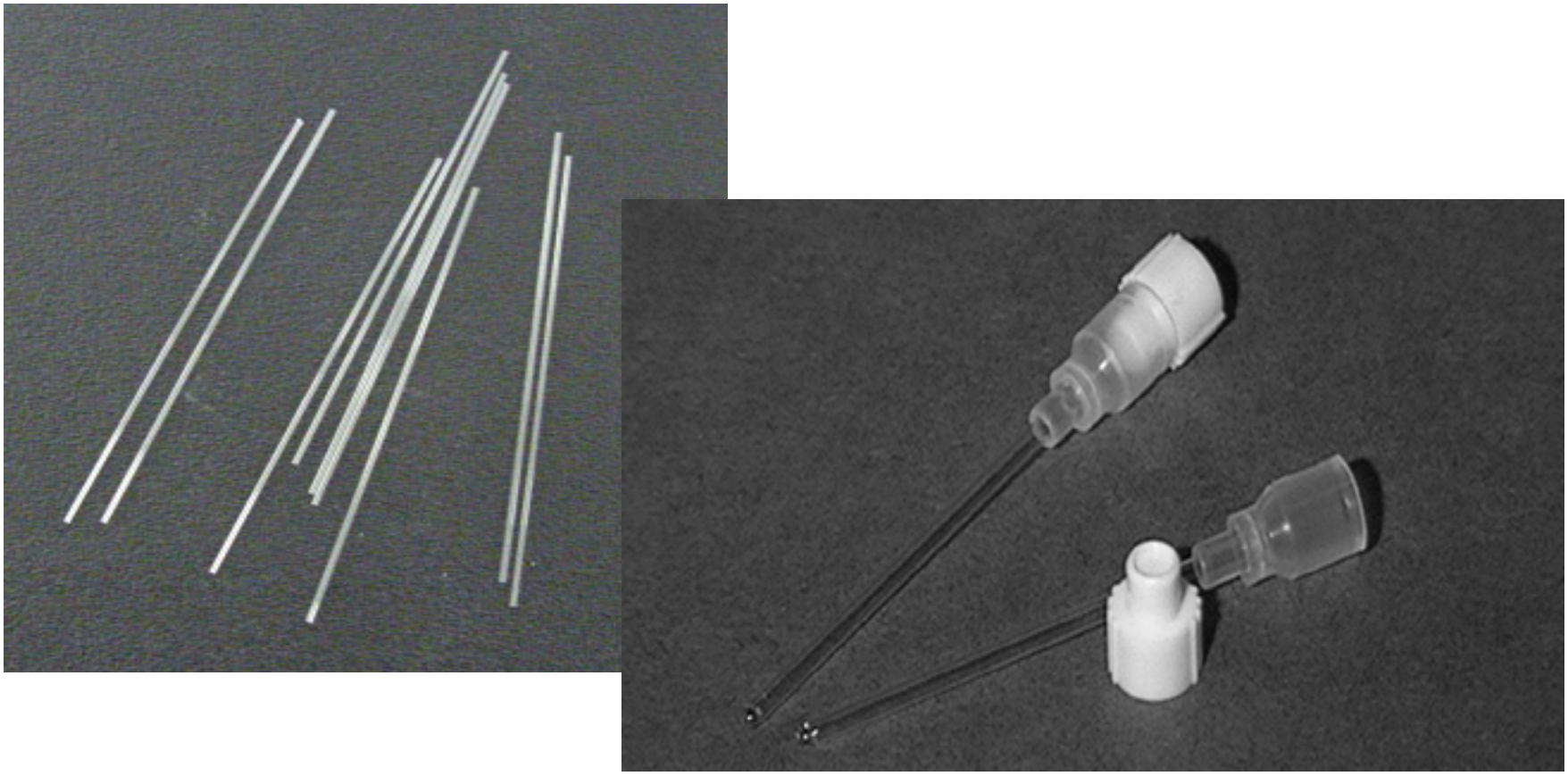
or (208) 524-6354

We are currently beta testing rapid air cycling instruments.

Company Products

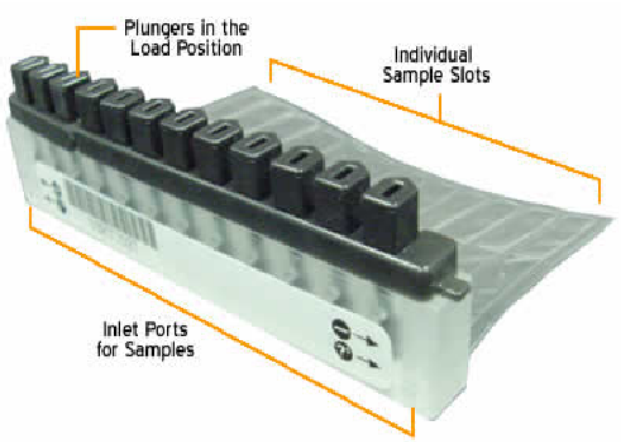


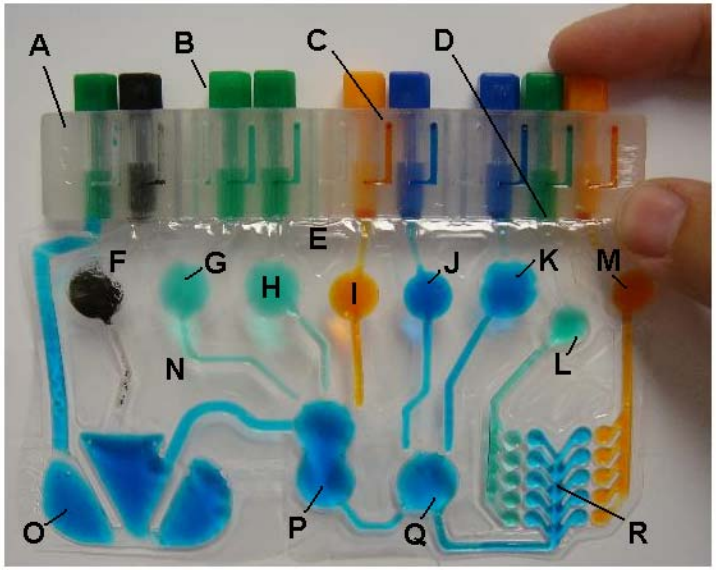
Sample Containers



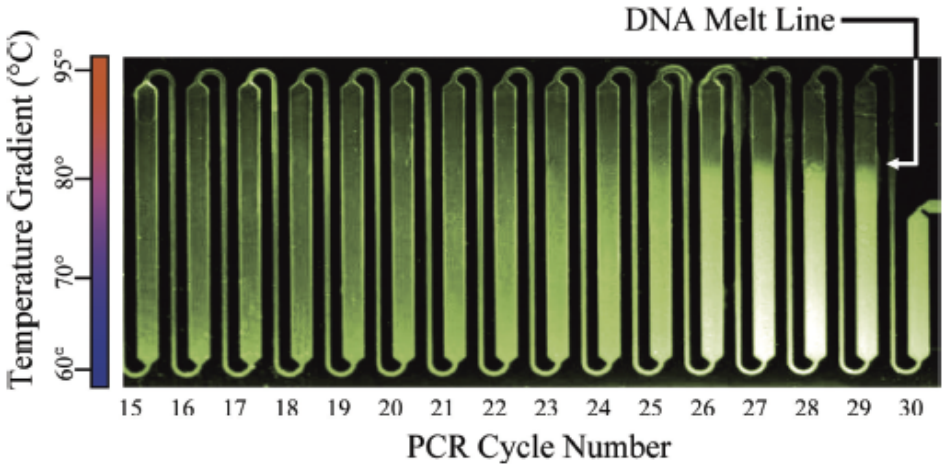
The LightCycler Capillary Cuvette

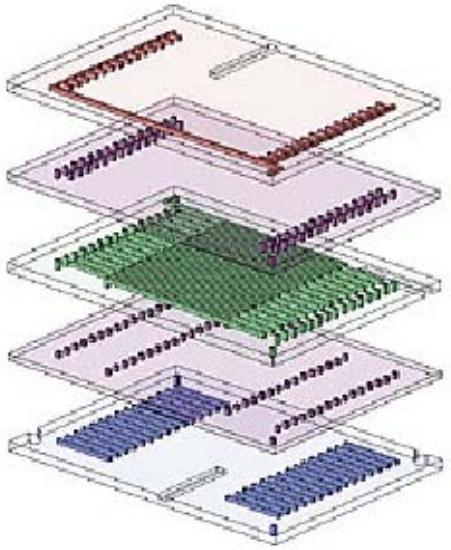
Other Containers for Rapid PCR

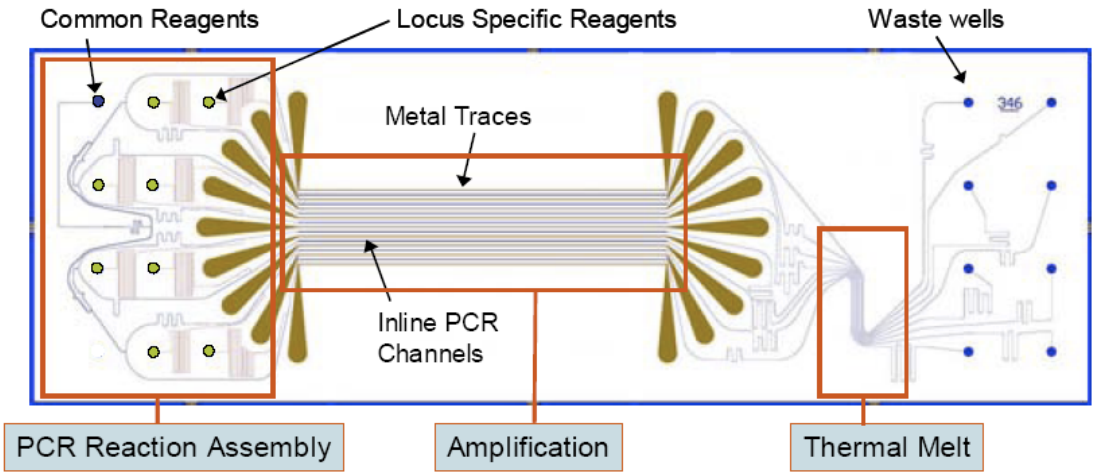




Microfluidics and Rapid Cycling

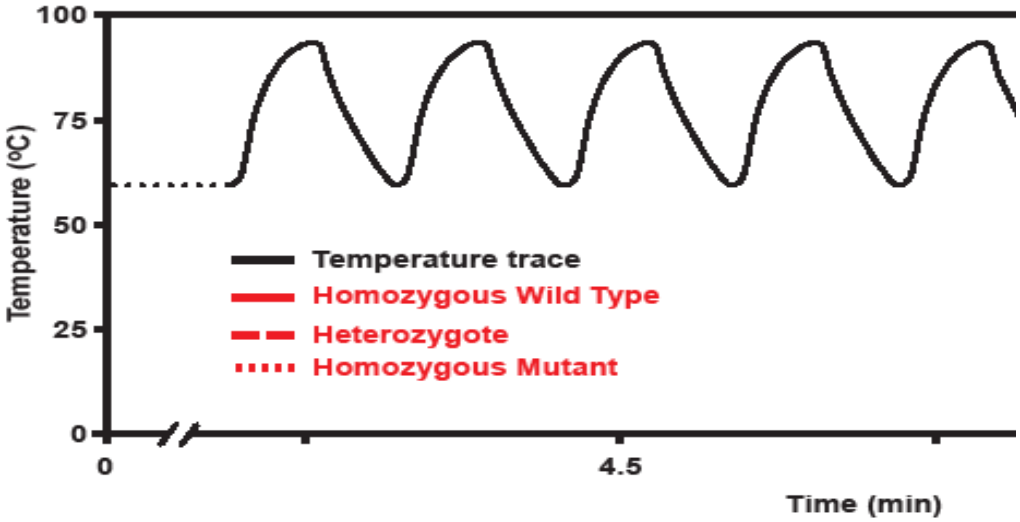




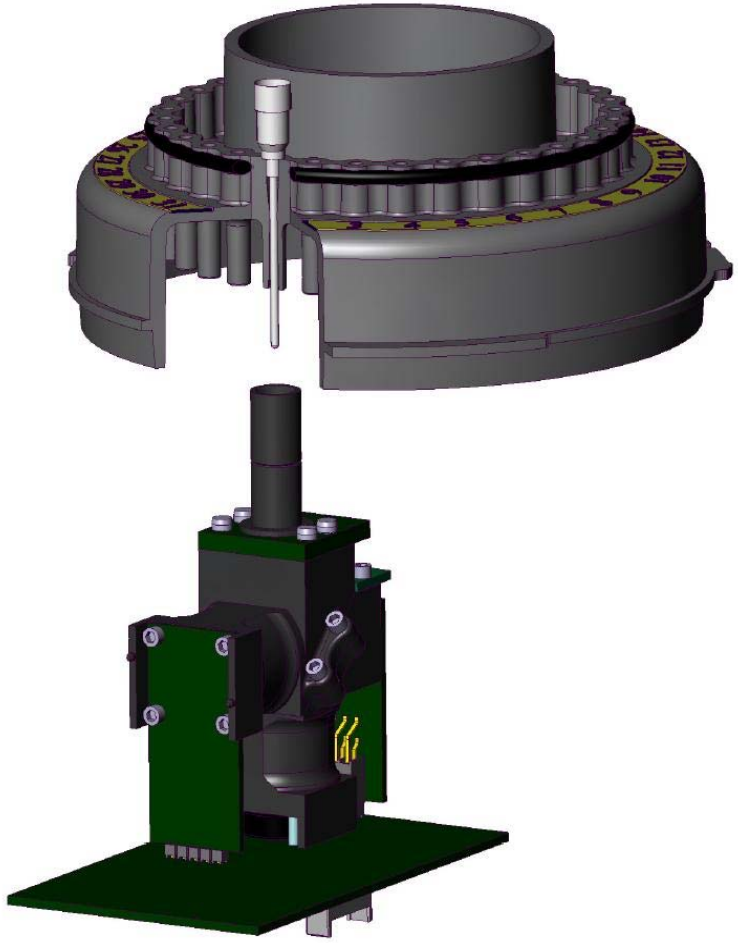


35 Cycles in <5 min

(modifed LightScanner 32)

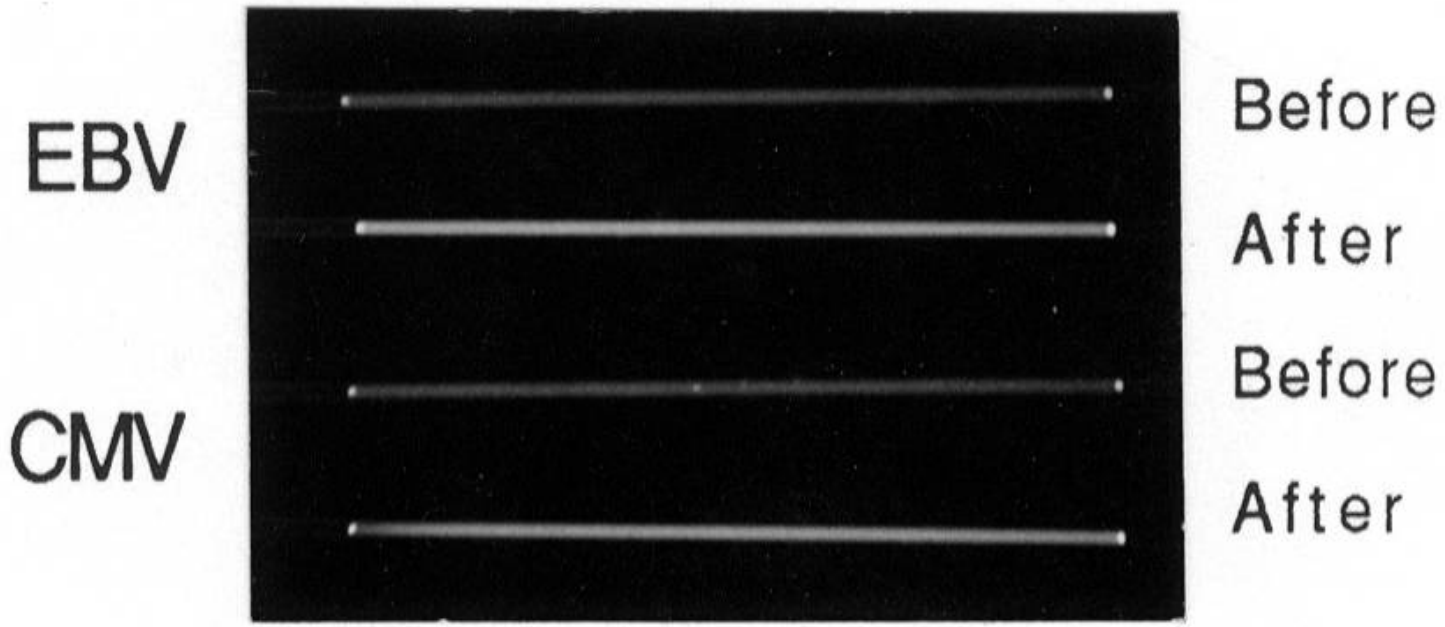


CODIS Region 83bp (C/T SNP)
rs876724
Turbo Cycle protocol: 60-93
35 Cycles (4 min 48 s)
High resolution melt time- 27s



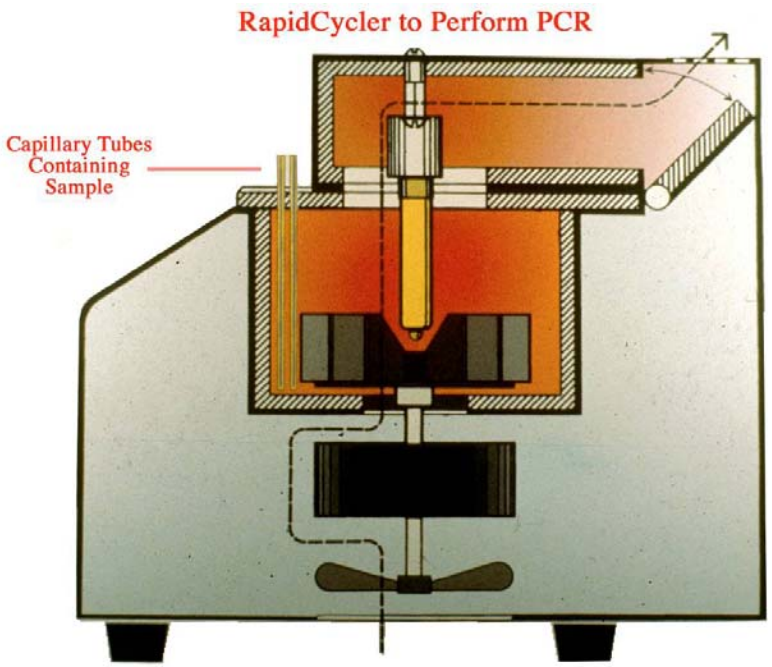
Real Time Analysis

Monitoring PCR with Fluorescence



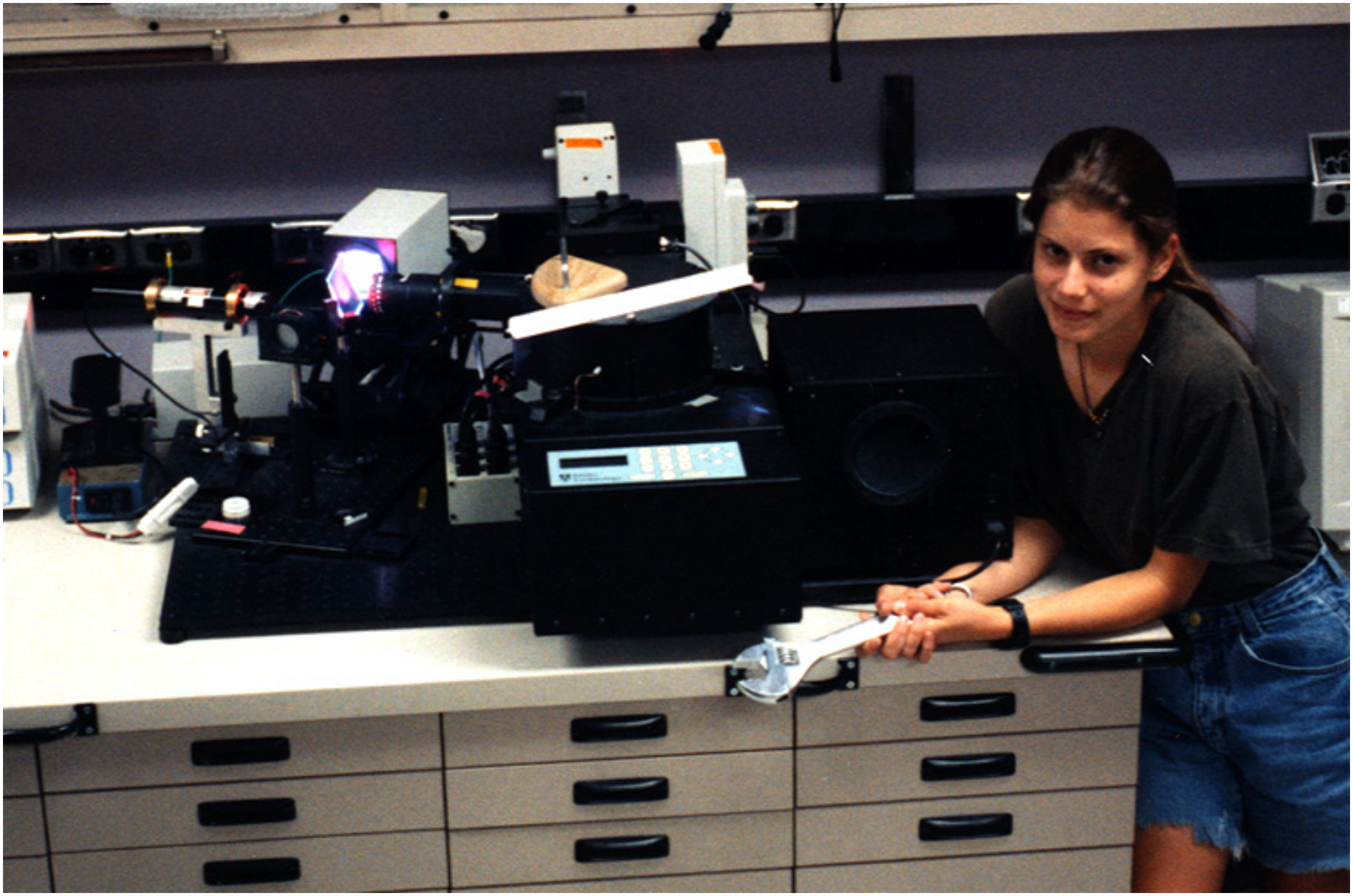
Ethidium Bromide / Transilluminator

1993: Monitoring Fluorescence during Amplification

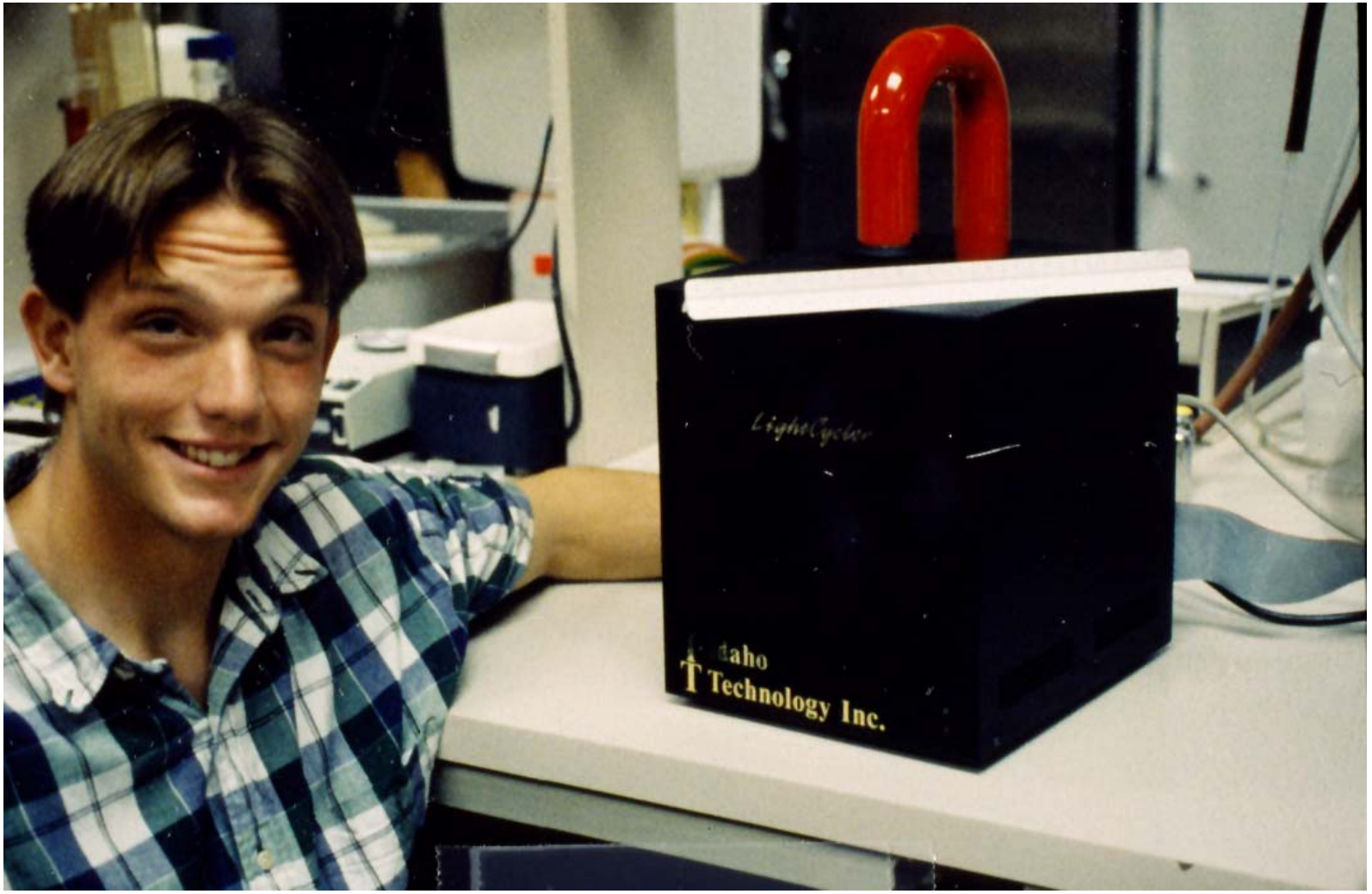


1994: RapidCycler + Fluorimeter

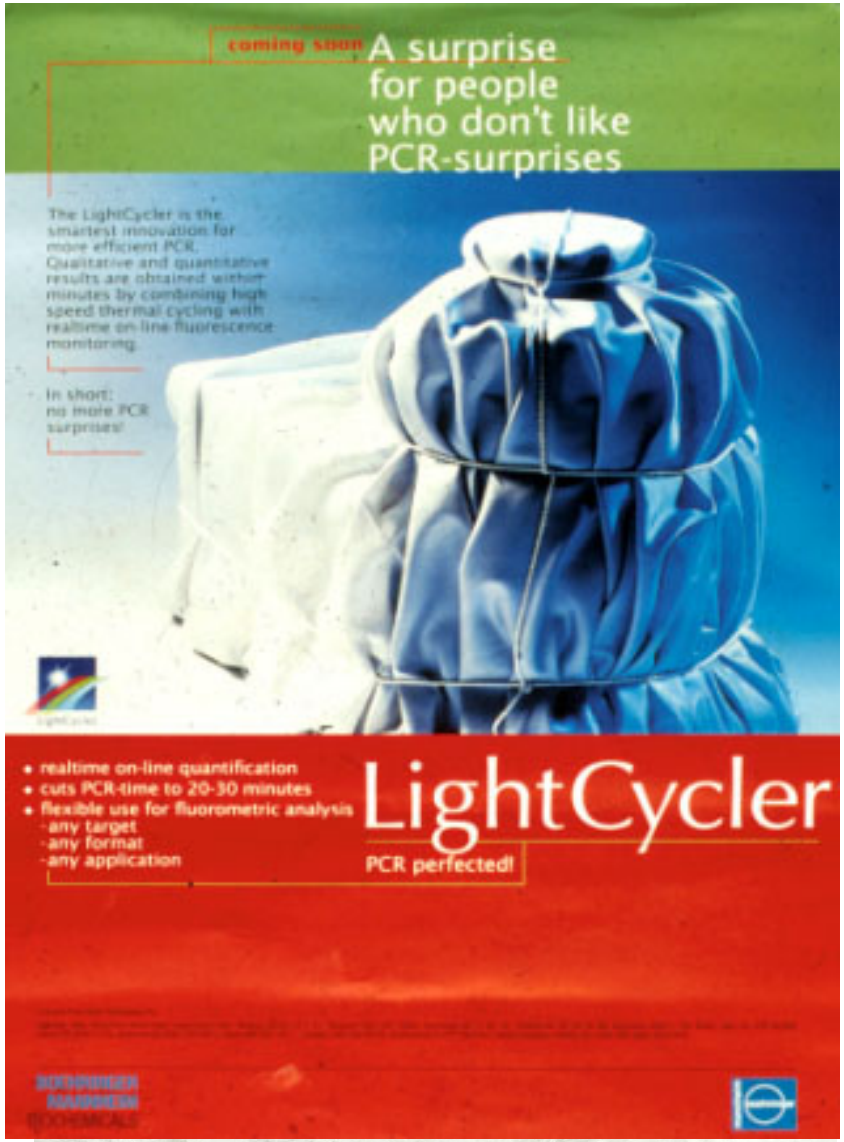
1995: LightCycler Prototype

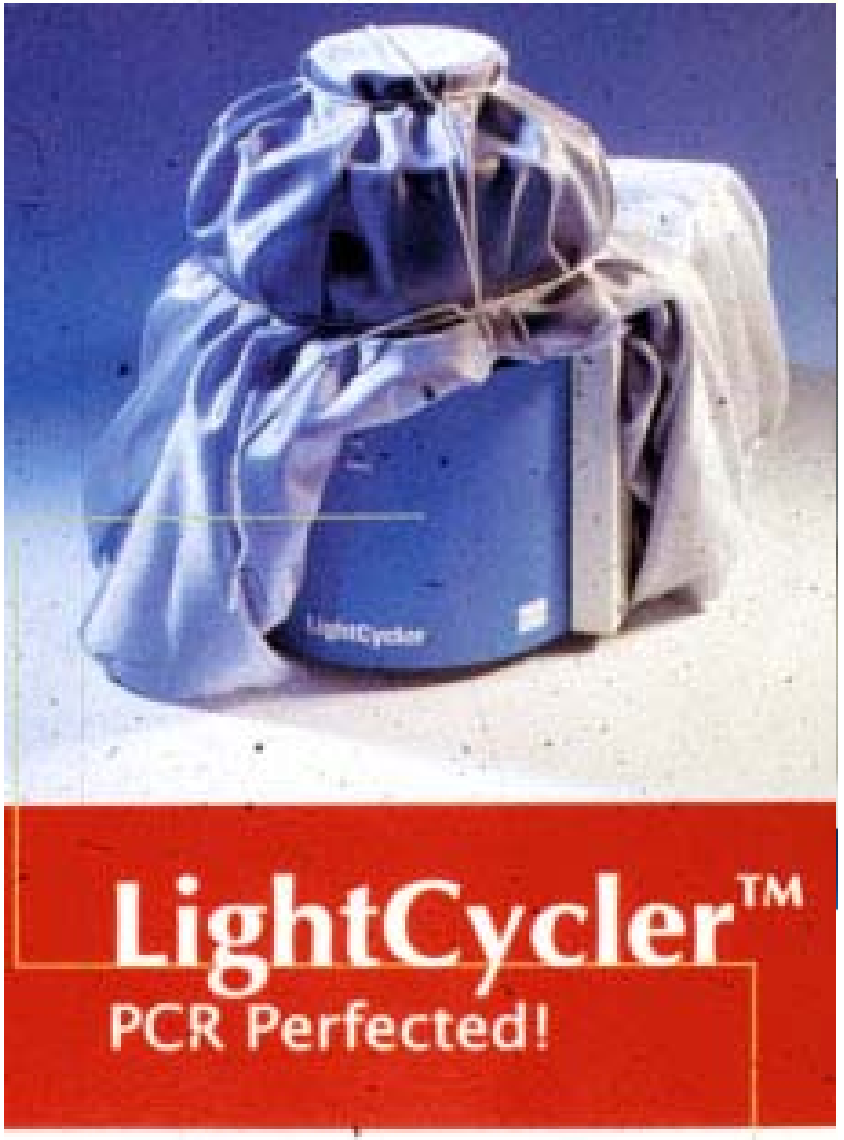


1996: LightCycler 24



Licensing to Boehringer Mannheim (1997)





1998: Roche LightCycler

1998 – Instrument Comparision

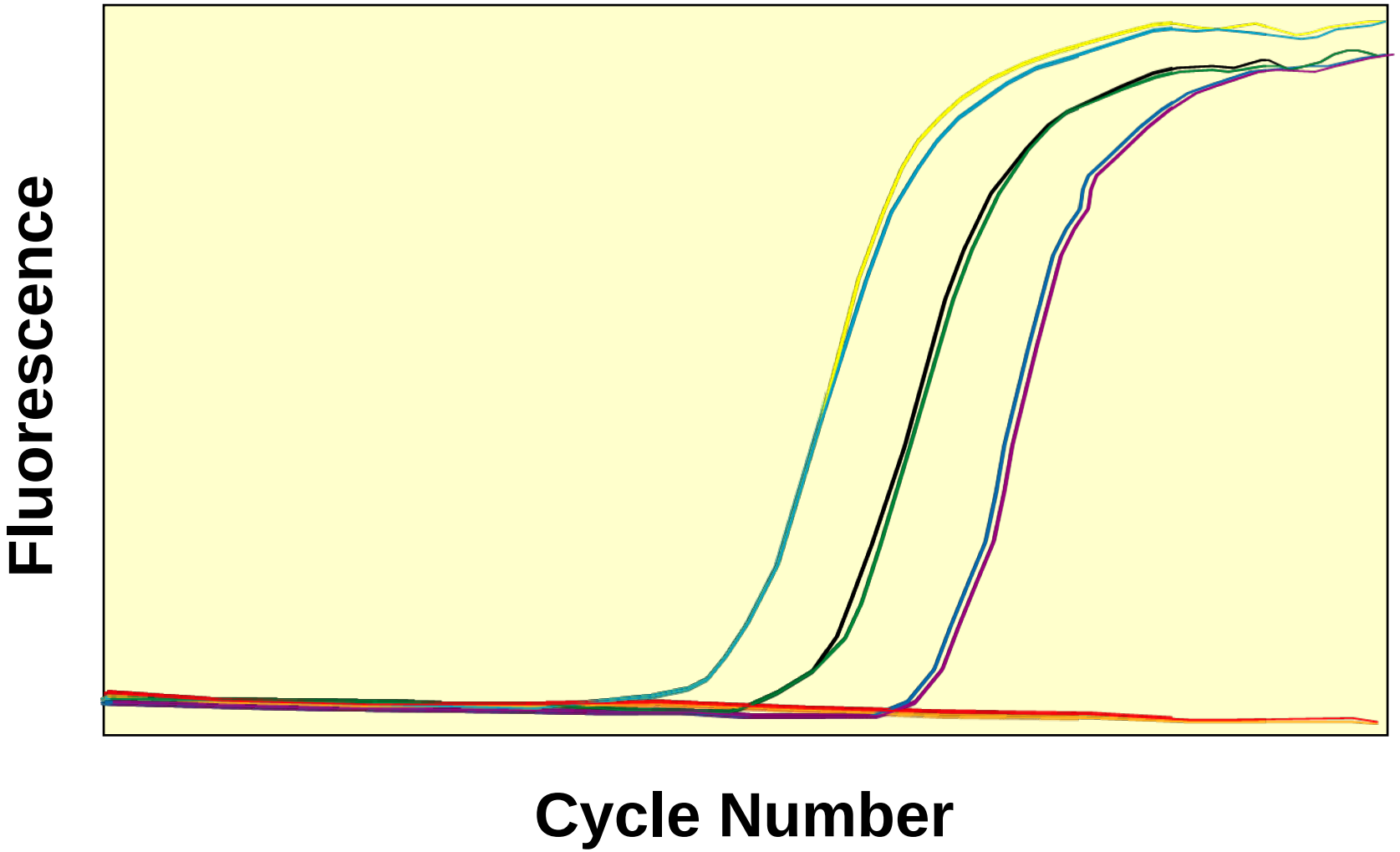
1998-2008: LightCycler 10-year Anniversary

- Adoption by Clinical Laboratories
- First FDA Approved Genetic Tests (2003)
- Open Platform

LightCycler 2.0

LightCycler 480

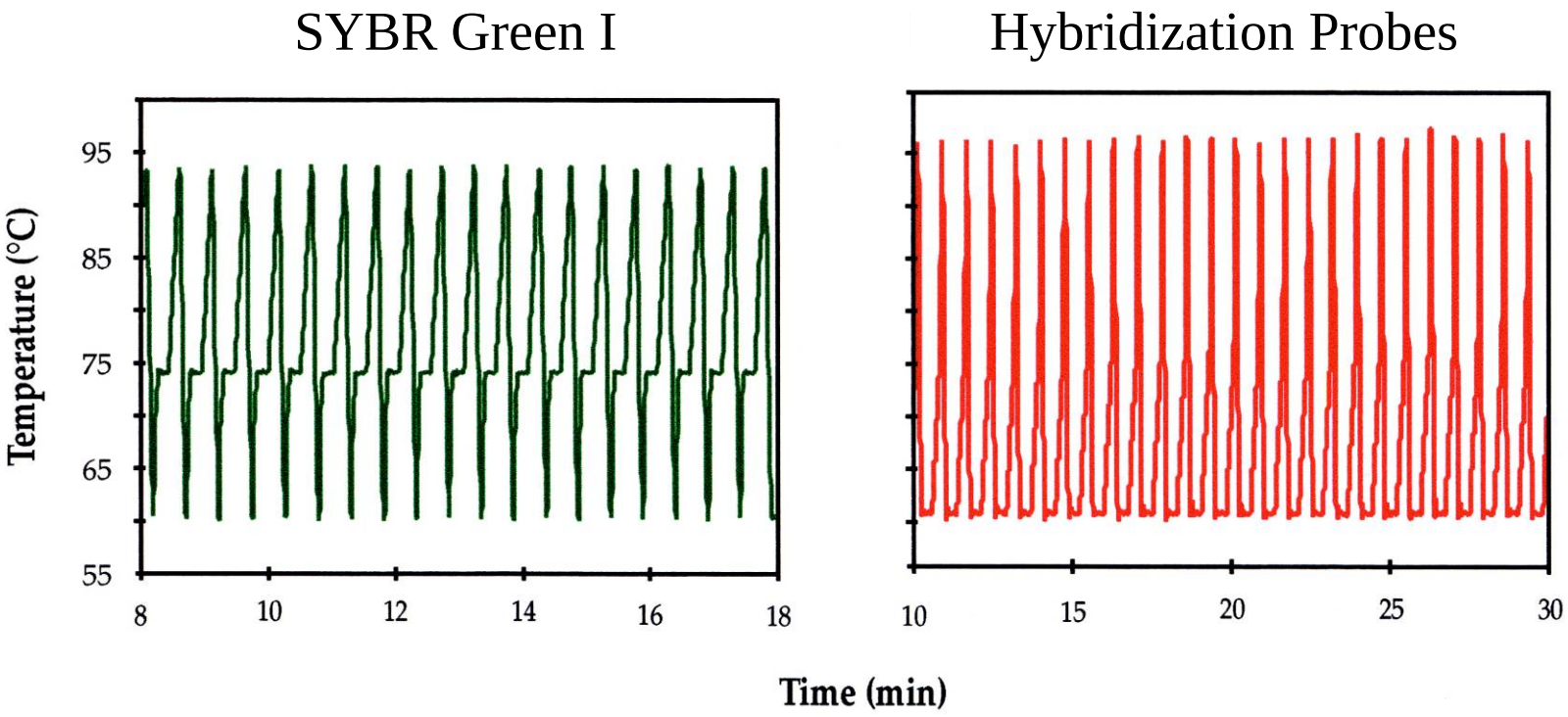
Real-Time Monitoring of PCR



Real-Time Parameters

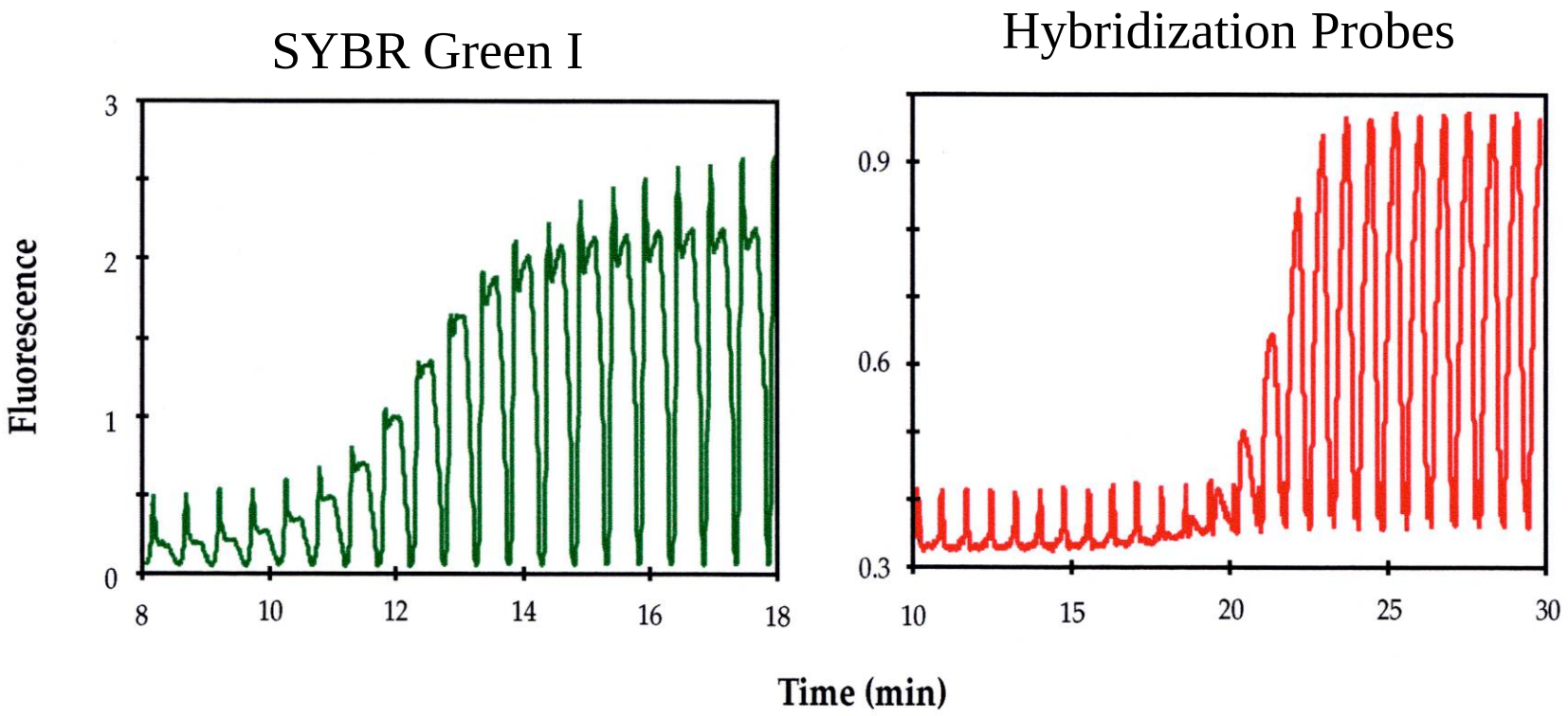
1. **Time**
2. **Temperature**
3. **Fluorescence**
    - **Once Each Cycle (Quantification)**
    - **With Changing Temperature**
        - **Product melting**
        - **Probe melting**

Temperature vs. Time
(Rapid Cycle PCR)



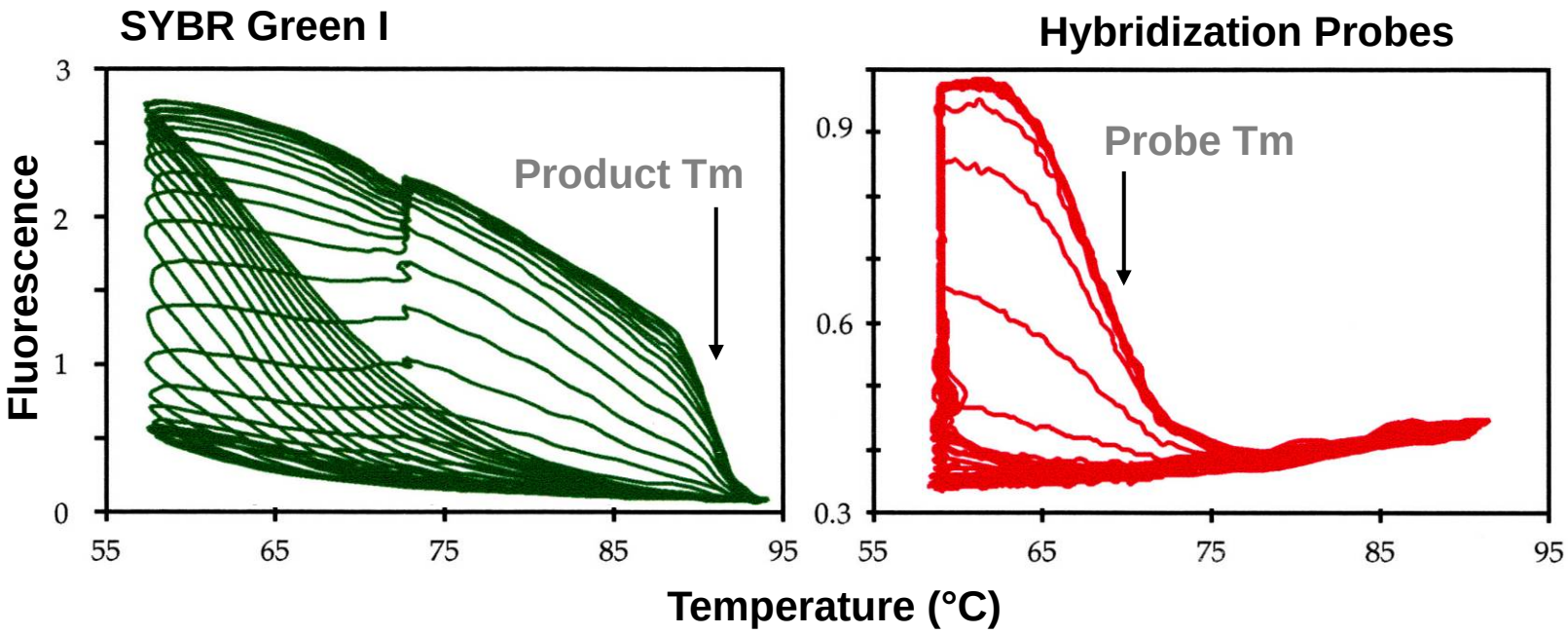
Fluorescence vs. Time

(Real Time PCR)



Fluorescence vs. Temperature

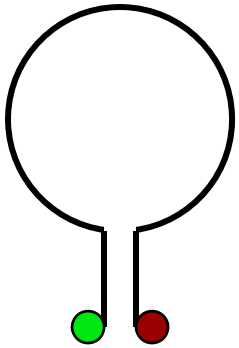
(Hybridization)



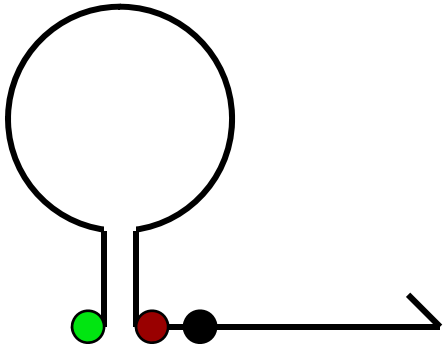
Closed-Tube Genotyping

Popular Probe Designs

Two Additions

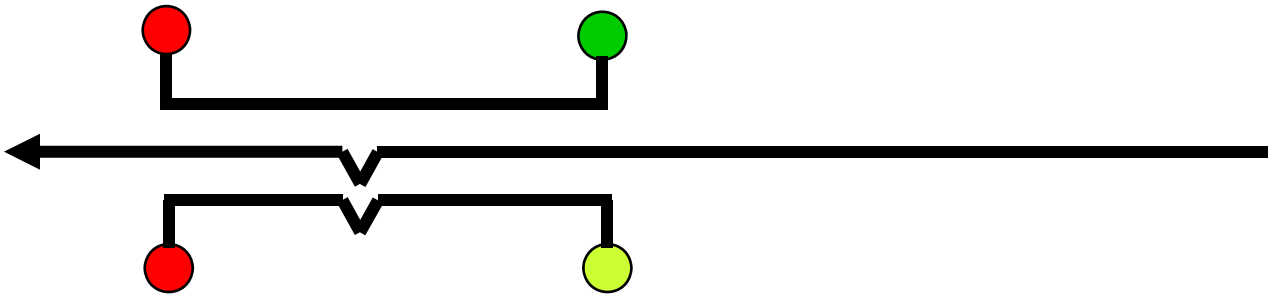


Three Additions



One probe needed for each allele

Real Time SNP Genotyping

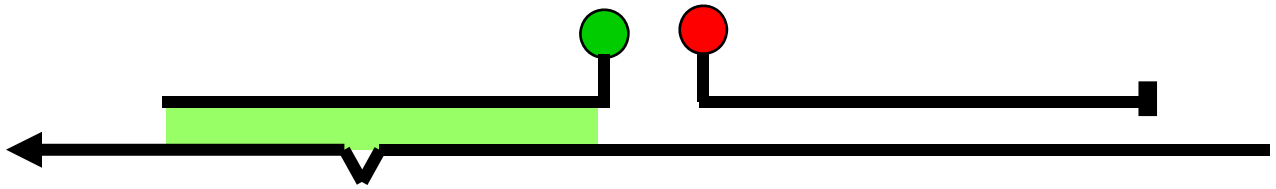


Hydrolysis Probes

(TaqMan®)

One probe required for each allele

Genotyping by Melting



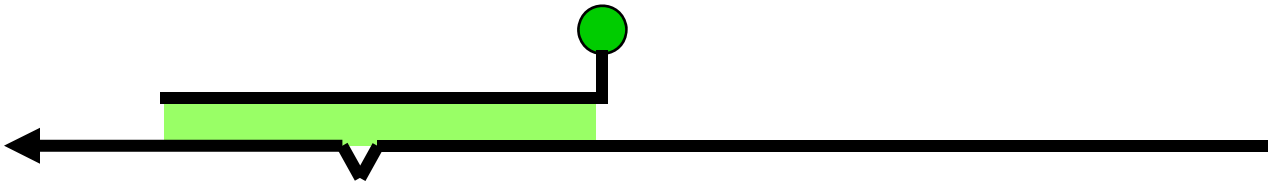
Dual Hybridization Probes

(HybProbes®)

Am J Pathol. 1998;153:1055-61

Two probes identify many alleles

Genotyping by Melting



Single Hybridization Probe
(SimpleProbe®)

Anal Biochem. 2001;290:89-97

One probe identifies many alleles

Genotyping by Melting

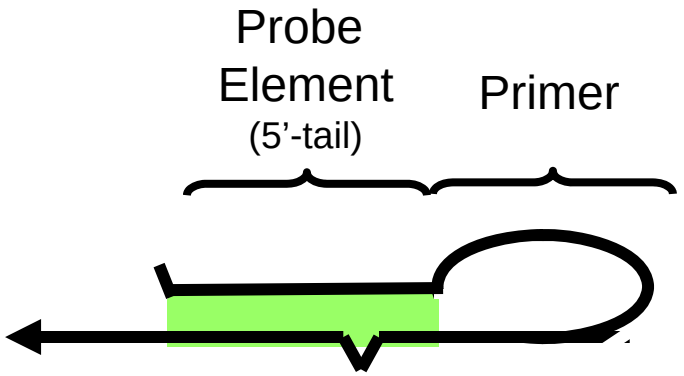
Unlabeled Probe

(Luna Probe®)

Clin Chem. 2004;50:1328-35

One probe identifies many alleles

Genotyping by Melting



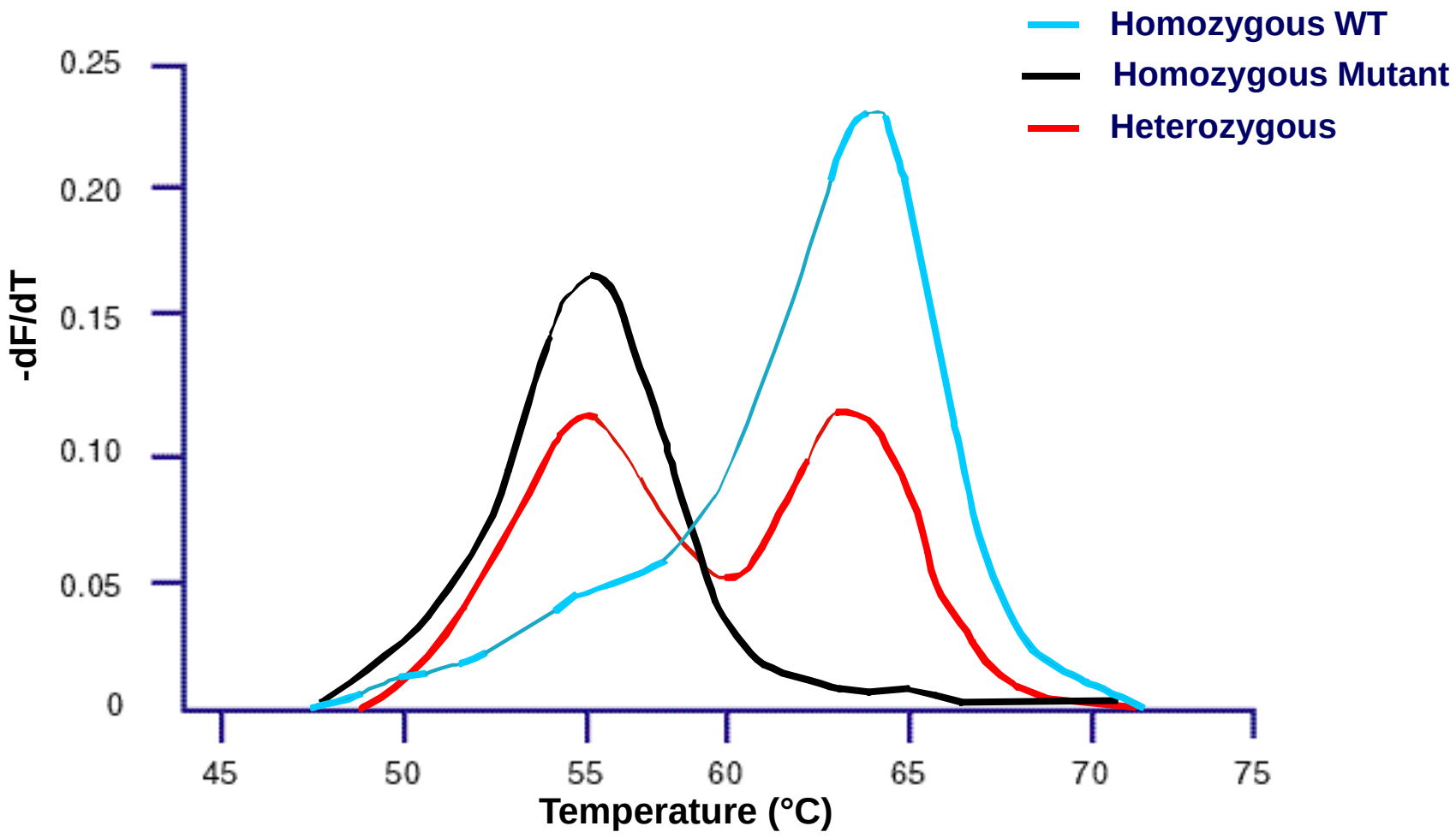
Snapback Primer

Clin Chem. 2008;54:1648-56

One probe identifies many alleles

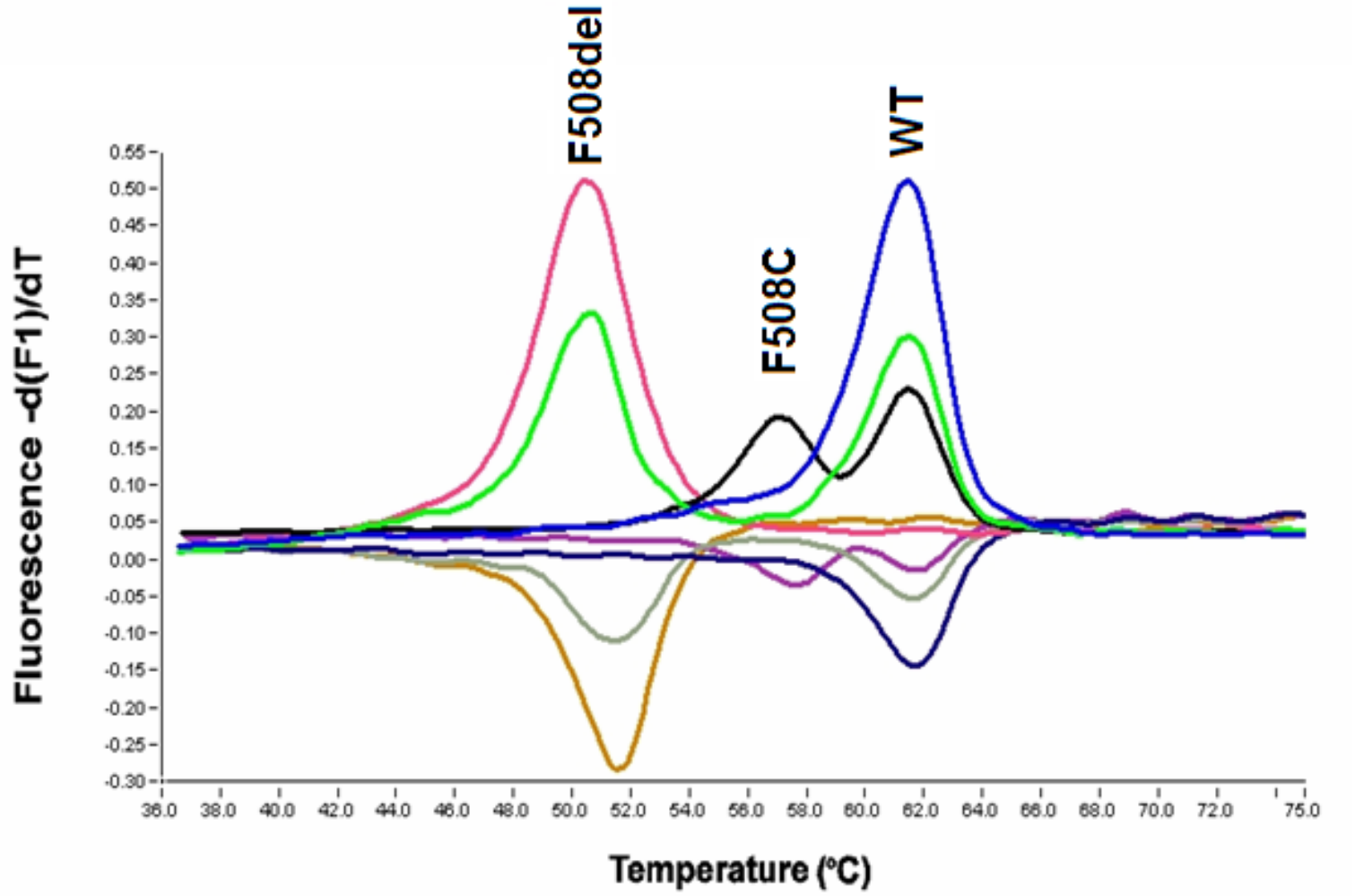
Dual Hybridization Probes

(Factor V Leiden)



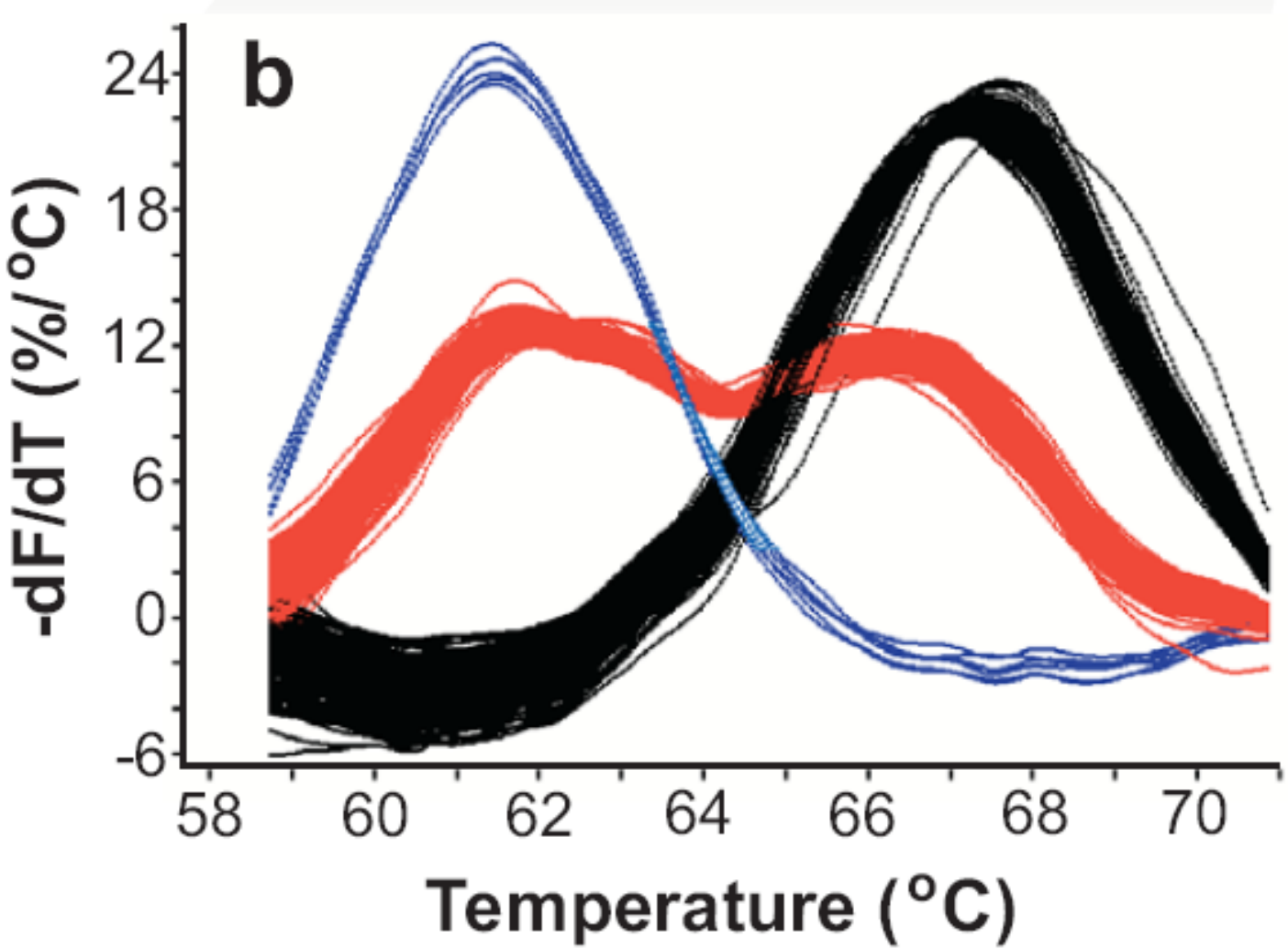
Single Hybridization Probes

(CFTR exon 10)



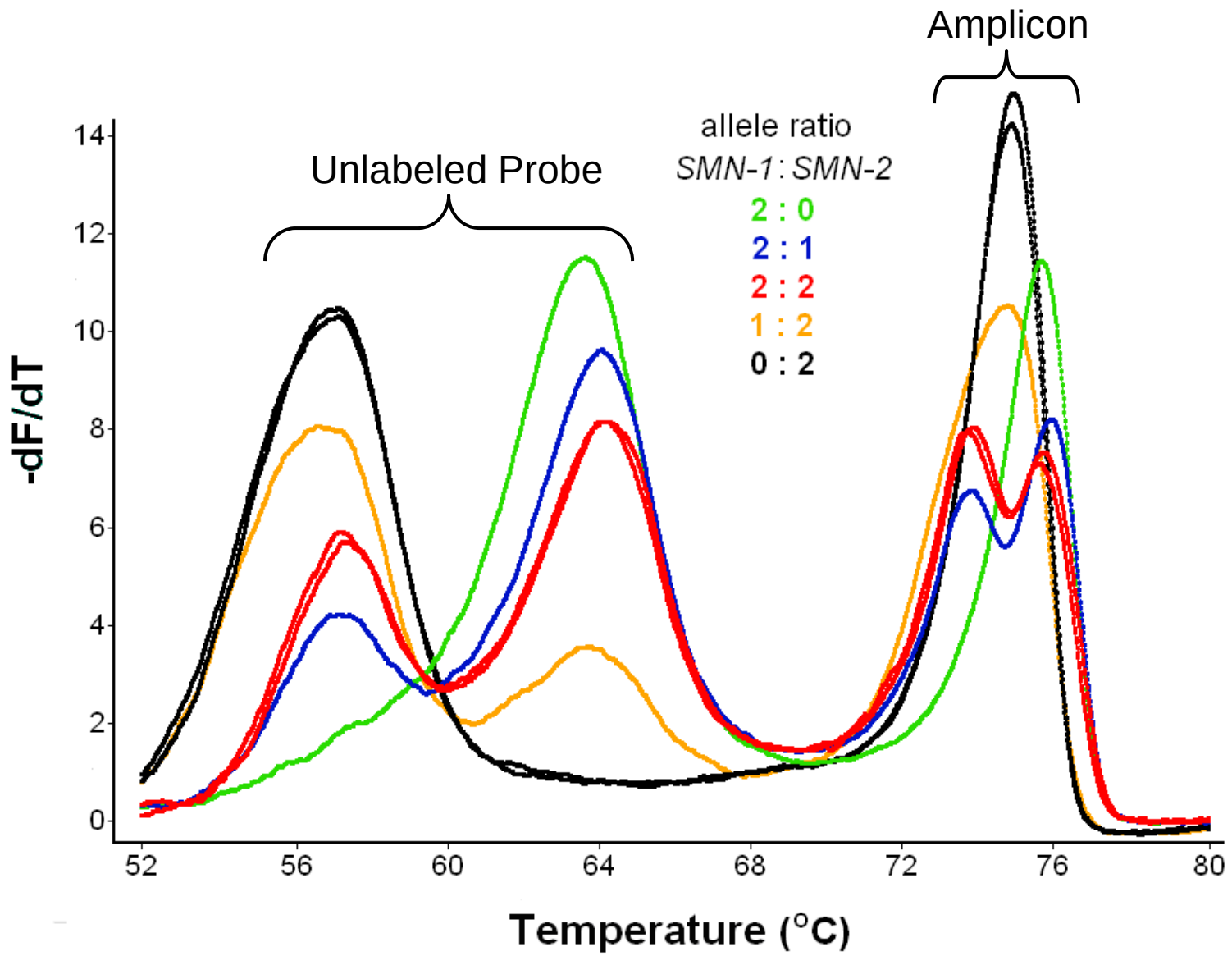
Unlabeled Probe

(Factor V Leiden)

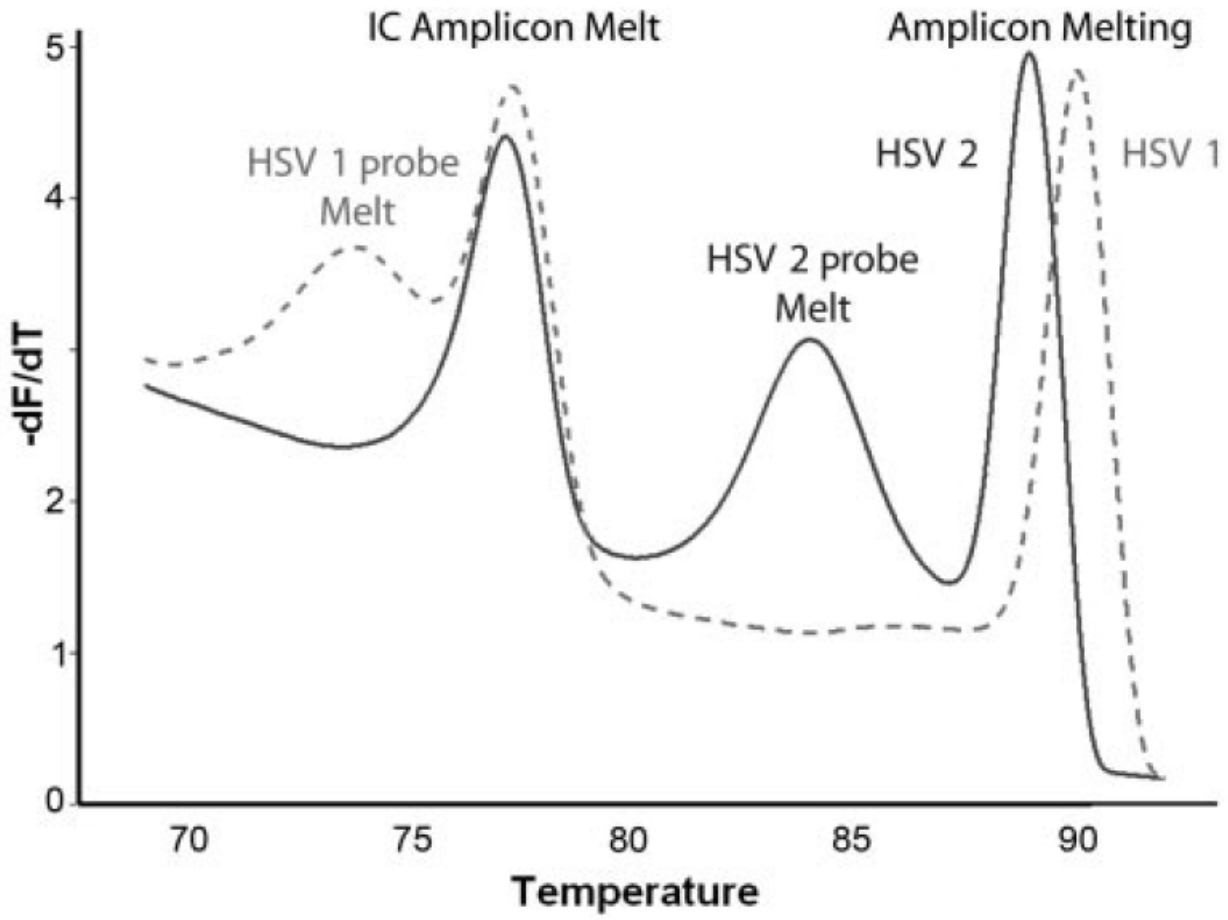


Clin Chem 2005; 51: 1770 - 1777

Relative Gene Dosage Assessment in SMA

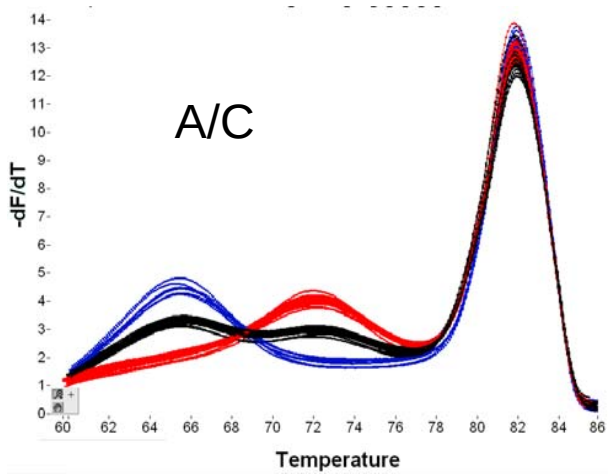


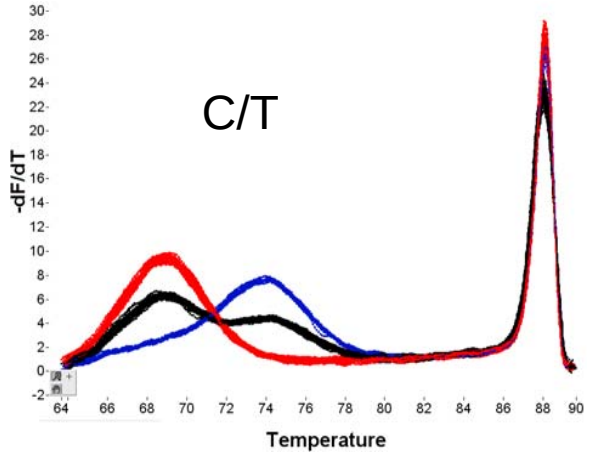
Unlabeled Probes for HSV Detection

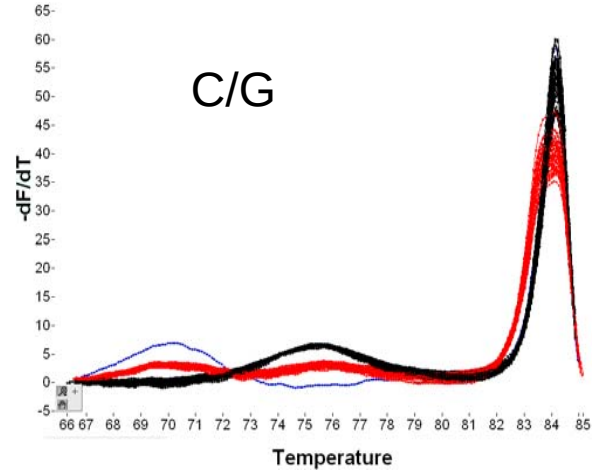


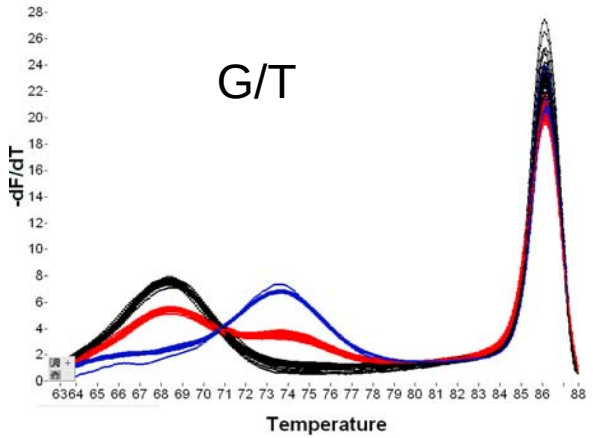
Clin Chem 53: 1847-1854, 2007

Snapback Genotyping (SNPs)



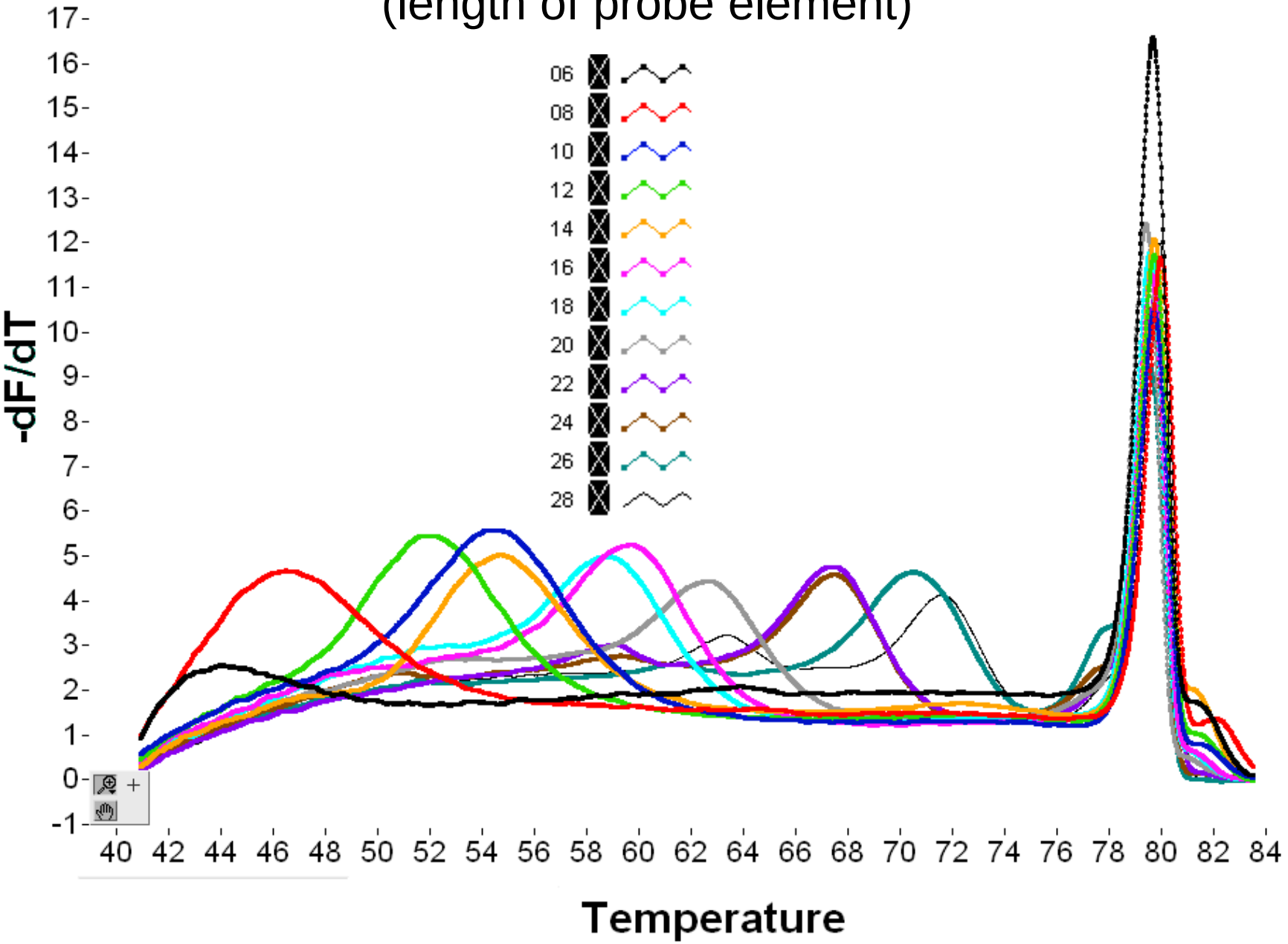






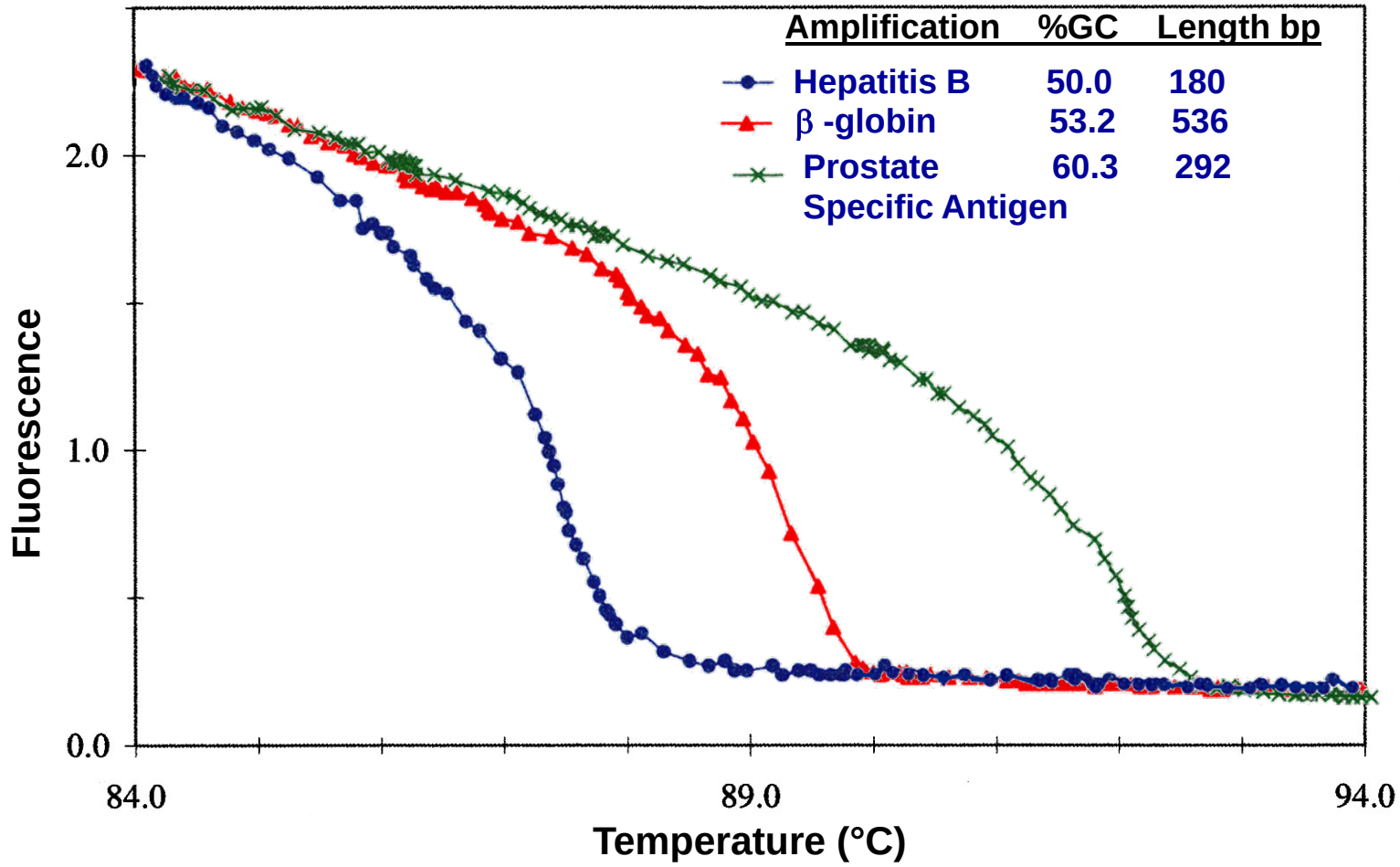
Snapback Primers

(length of probe element)



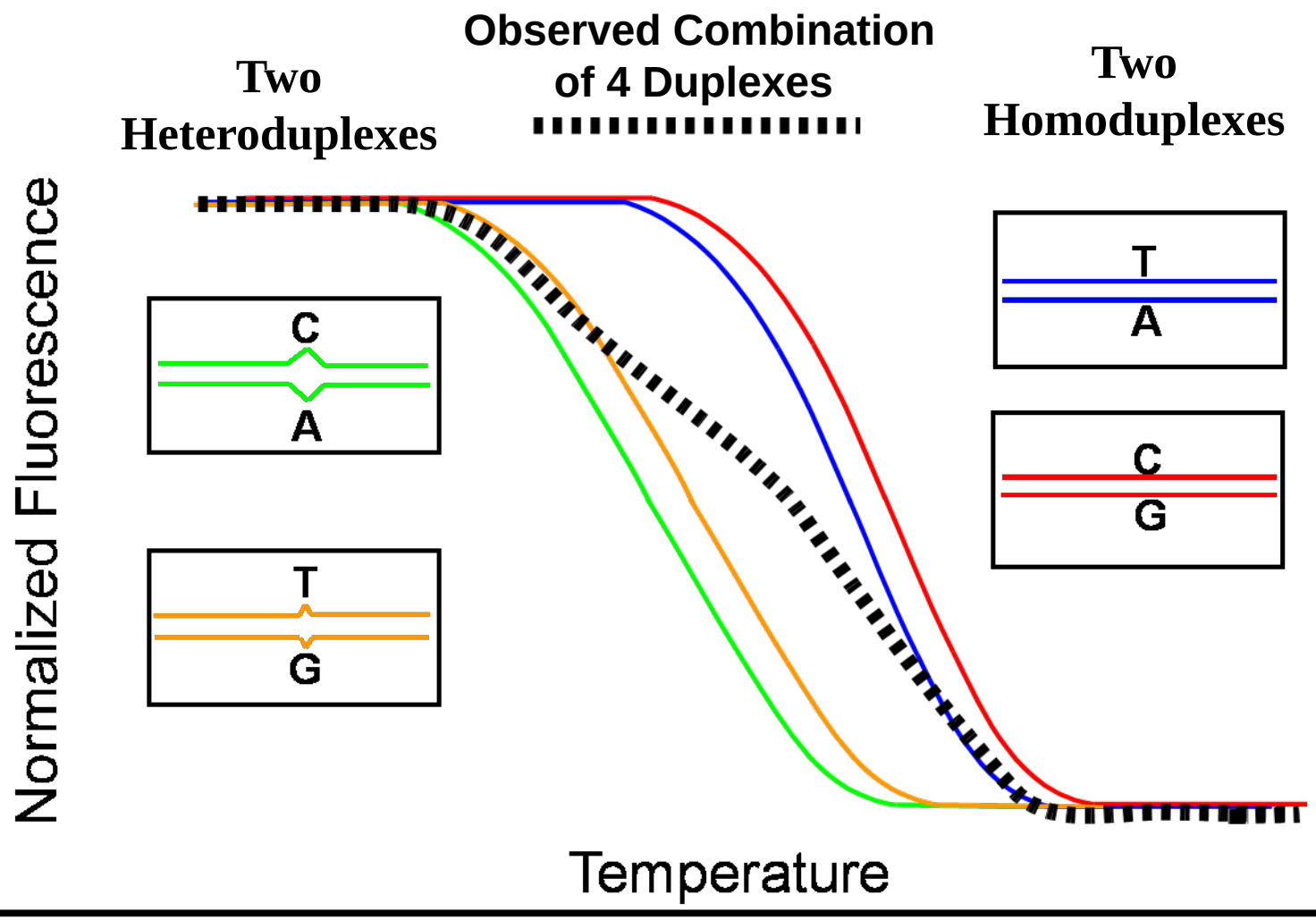
High-Resolution Melting

Melting Curves for Different Products (SYBR Green I)

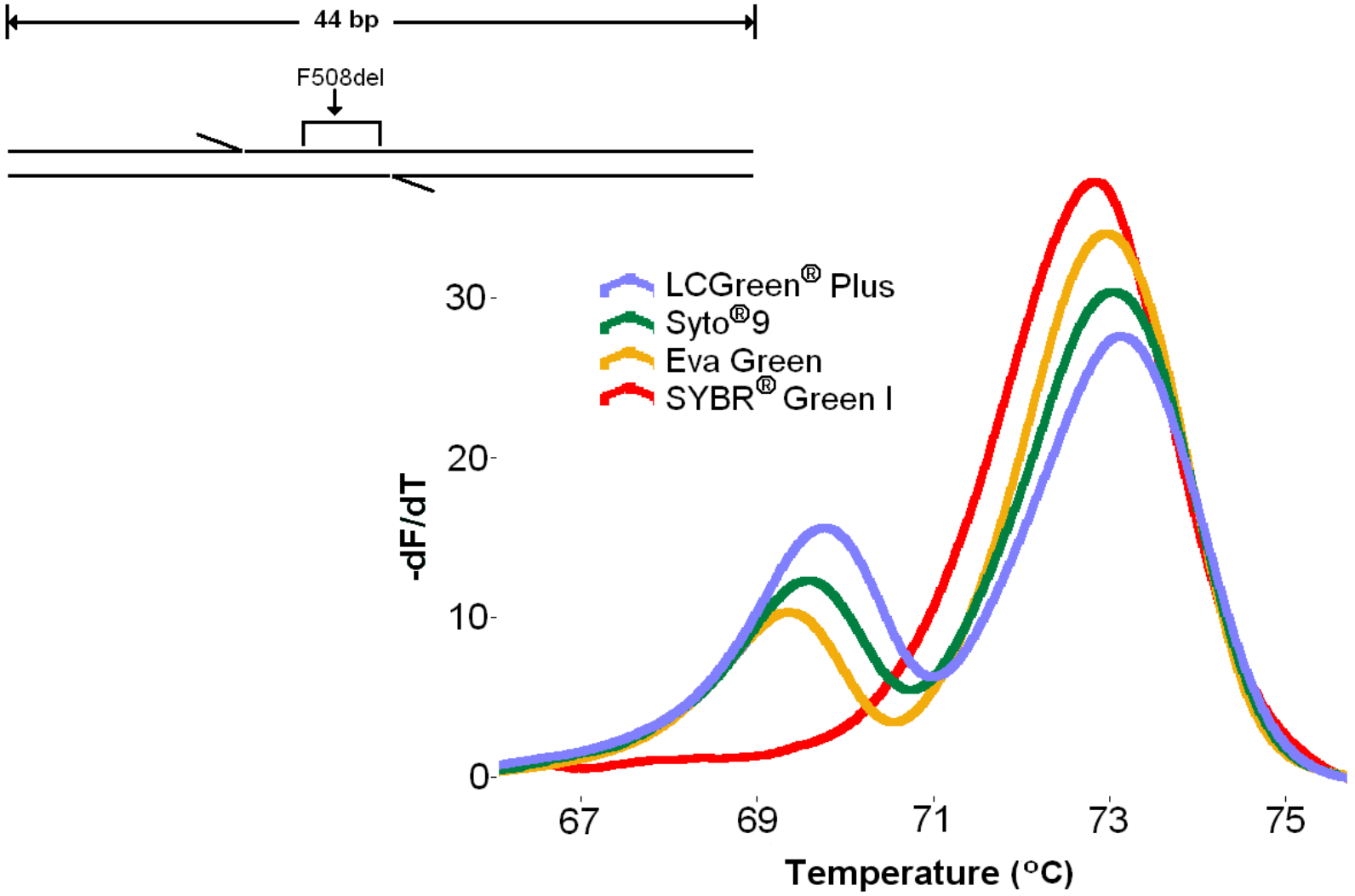


Melting Curves

Heterozygote Amplification



Heteroduplex Detection with dsDNA Dyes



Patrinos GP and Ansorge WJ, eds, Molecular Diagnostics, 2nd ed, Elsevier Chapter 18, in press, 2009

High Resolution Melting Instruments

High Resolution (>=16 bit):
- Temperature
- Fluorescence

High Data Density (>=10/°C)

Rapid Melting (0.1-0.3°C/sec)

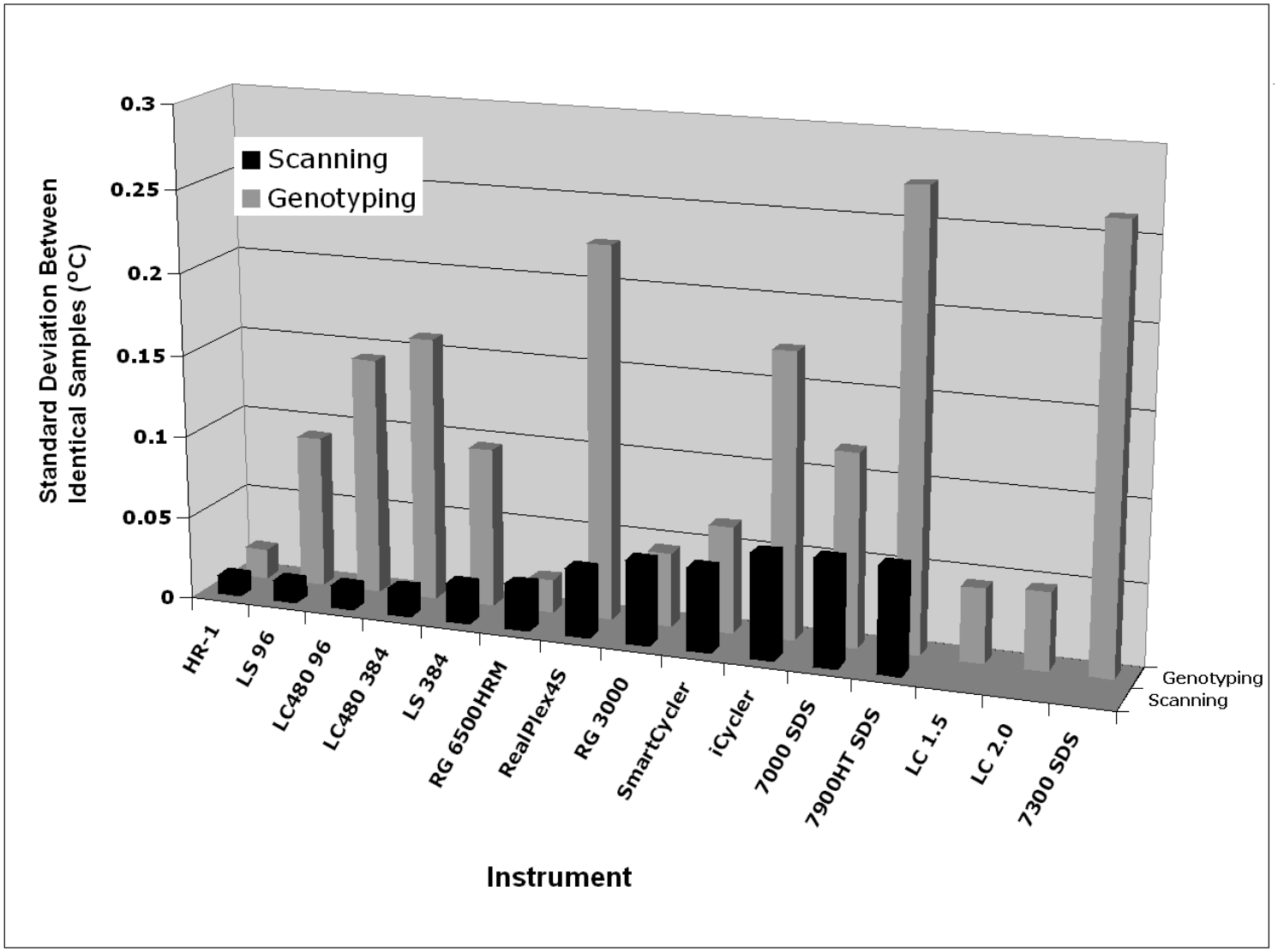
HR-1
Idaho Technology

Rotor-Gene® 6500HRM
Corbett

LightScanner®
Idaho Technology

LightCycler® 480
Roche

Instrument Comparison

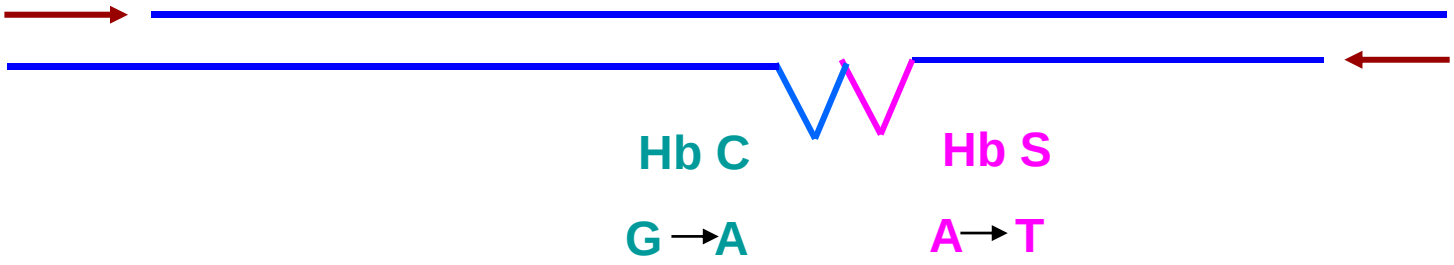


Clin Chem 52:494-503;2006, 53:150-2;2007, 53:1544-8;2007.

Beta-Globin Genotyping/Scanning

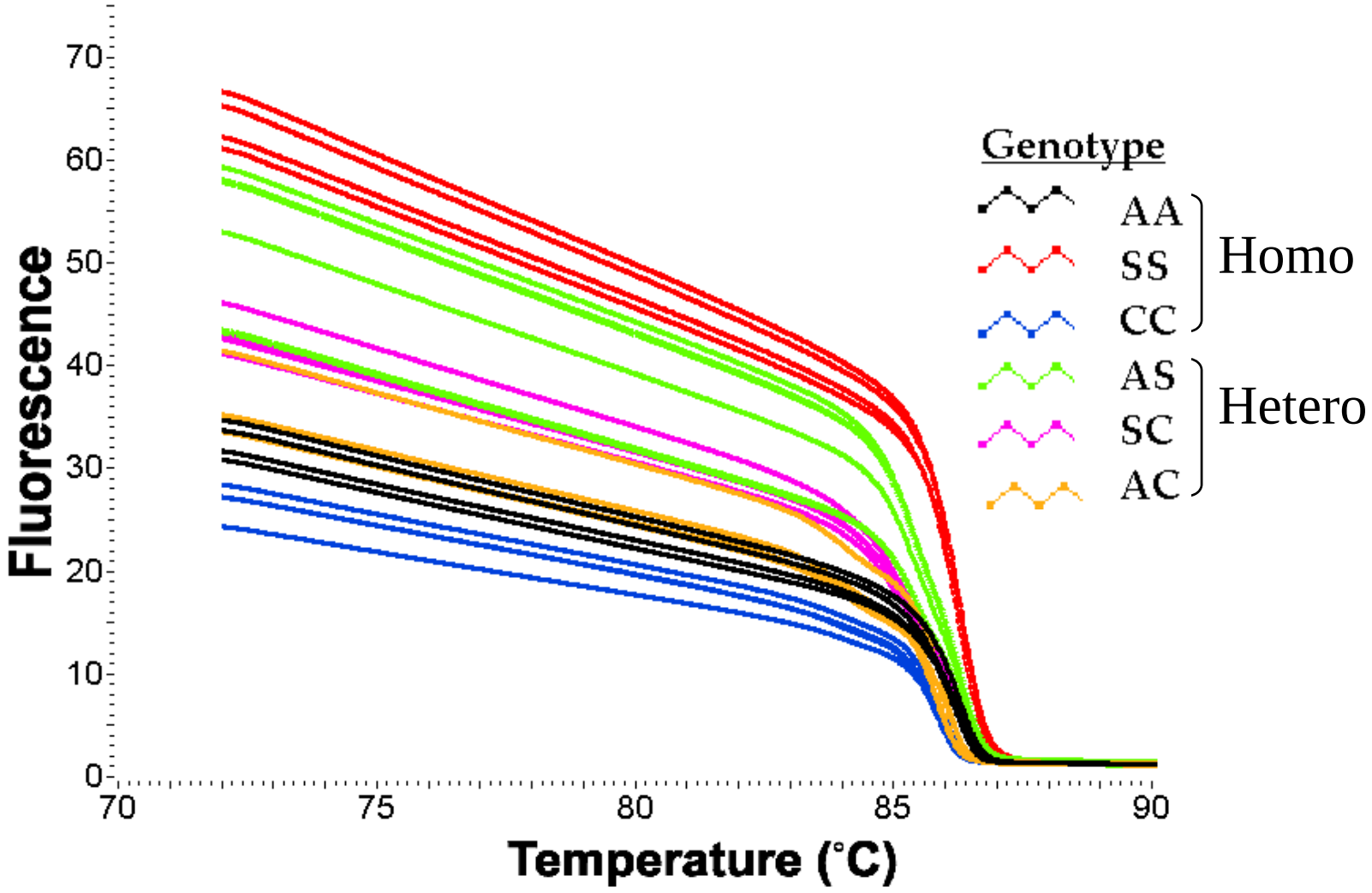
(110 bp)

Hb A = wild type

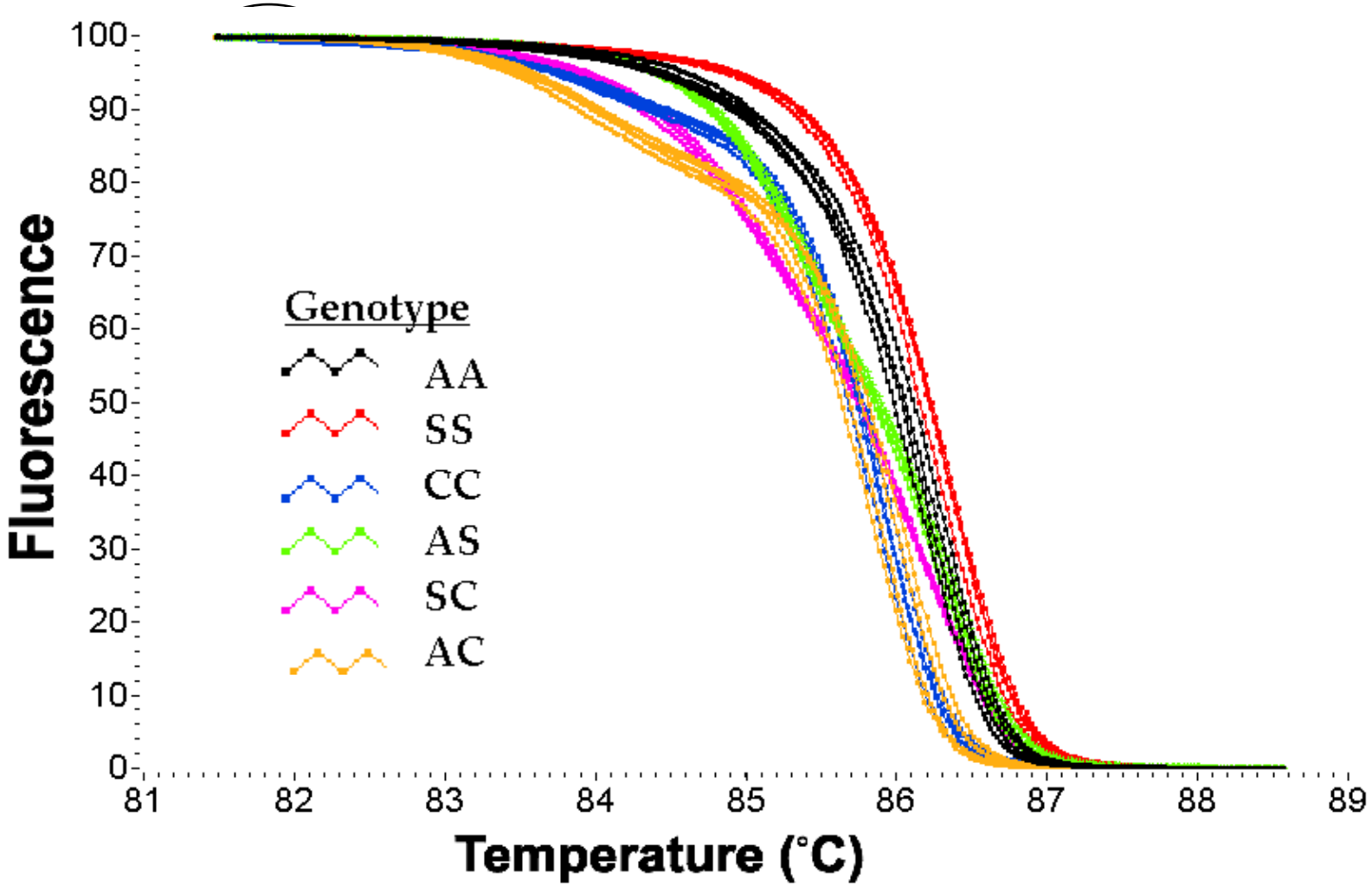


(Clin Chem 2003; 10:853-860)

Melting Curve (Original Data)

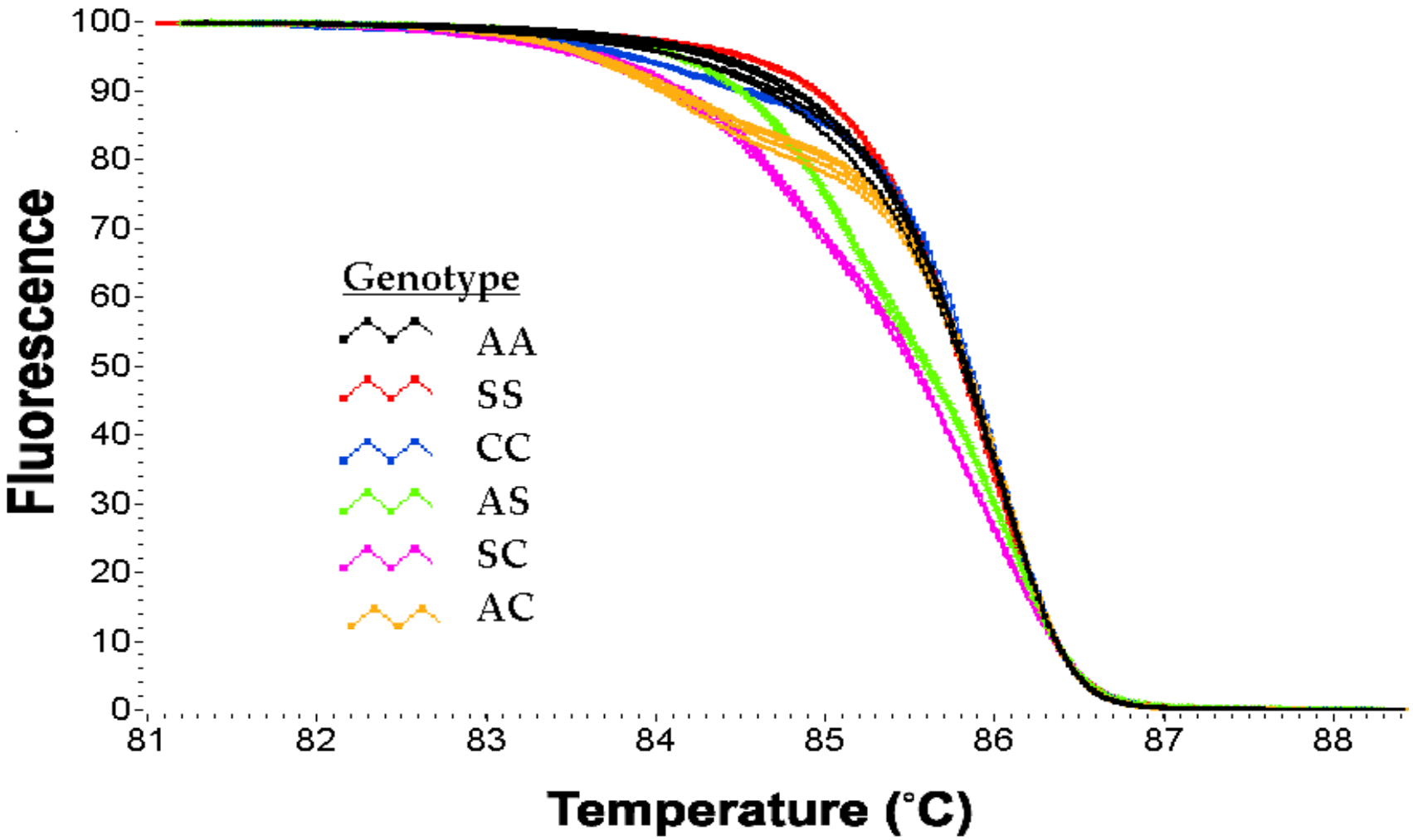


Melting Curve (Normalized)

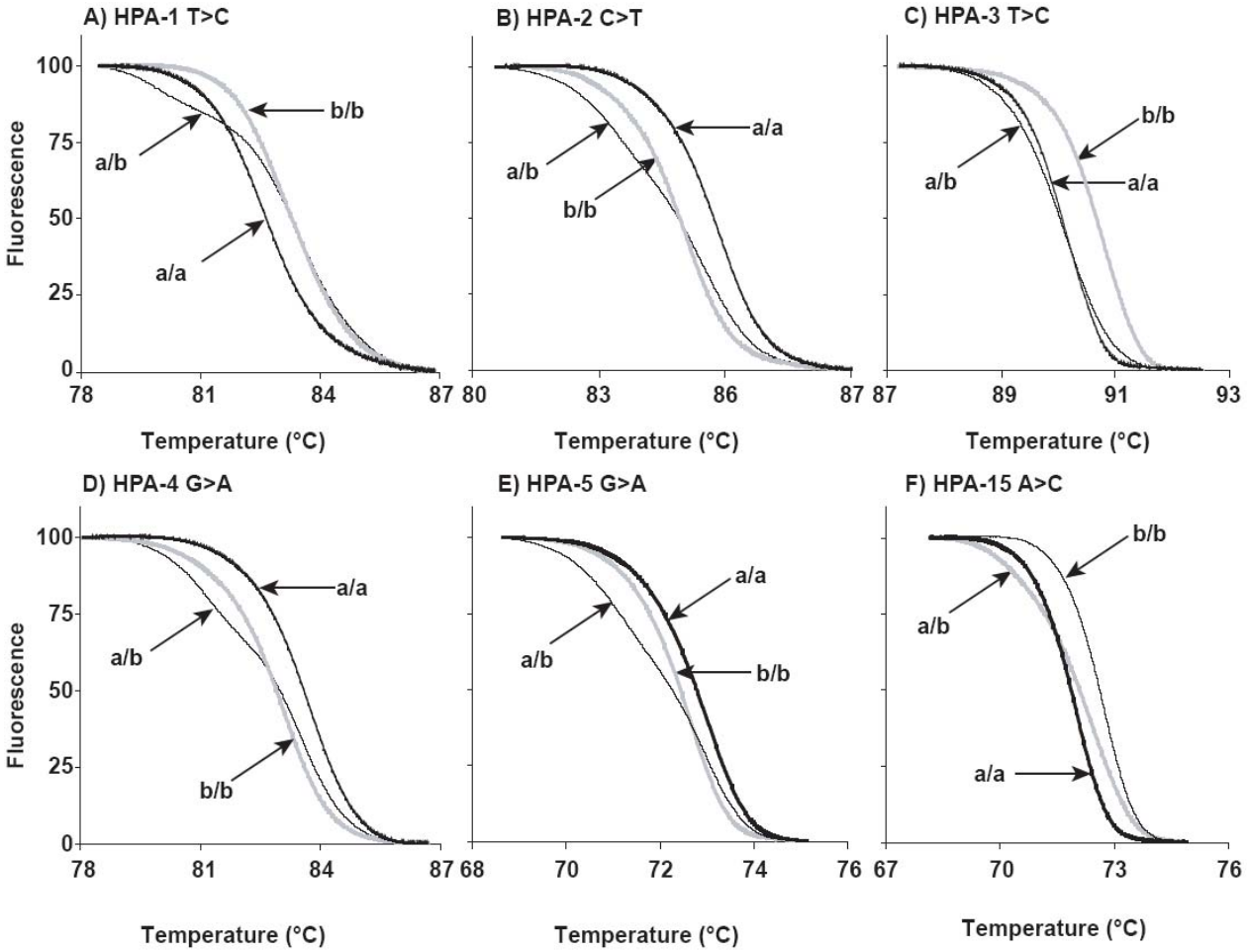


Curve Overlay

(high temperature region is matched)

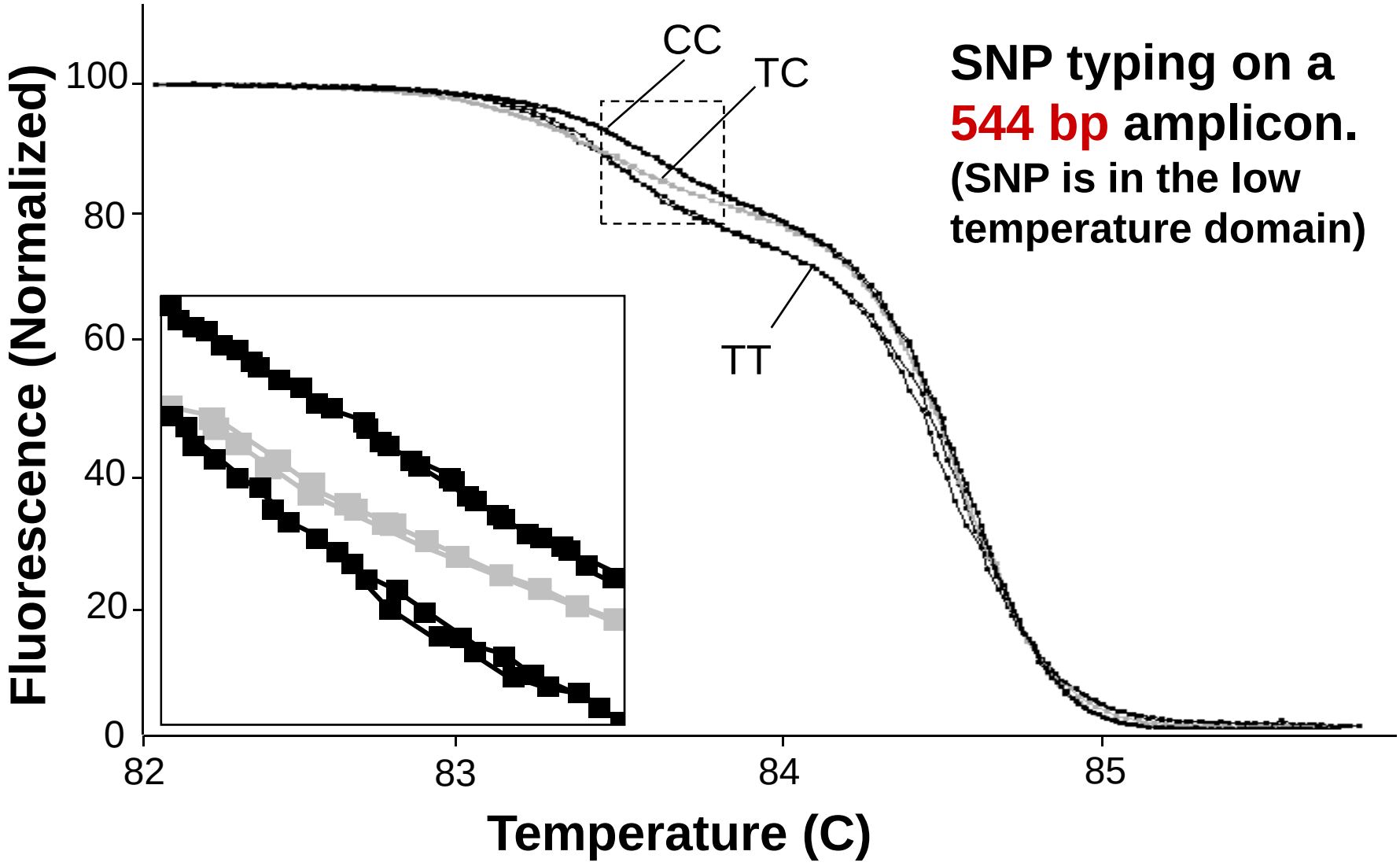


Small Amplicon Genotyping



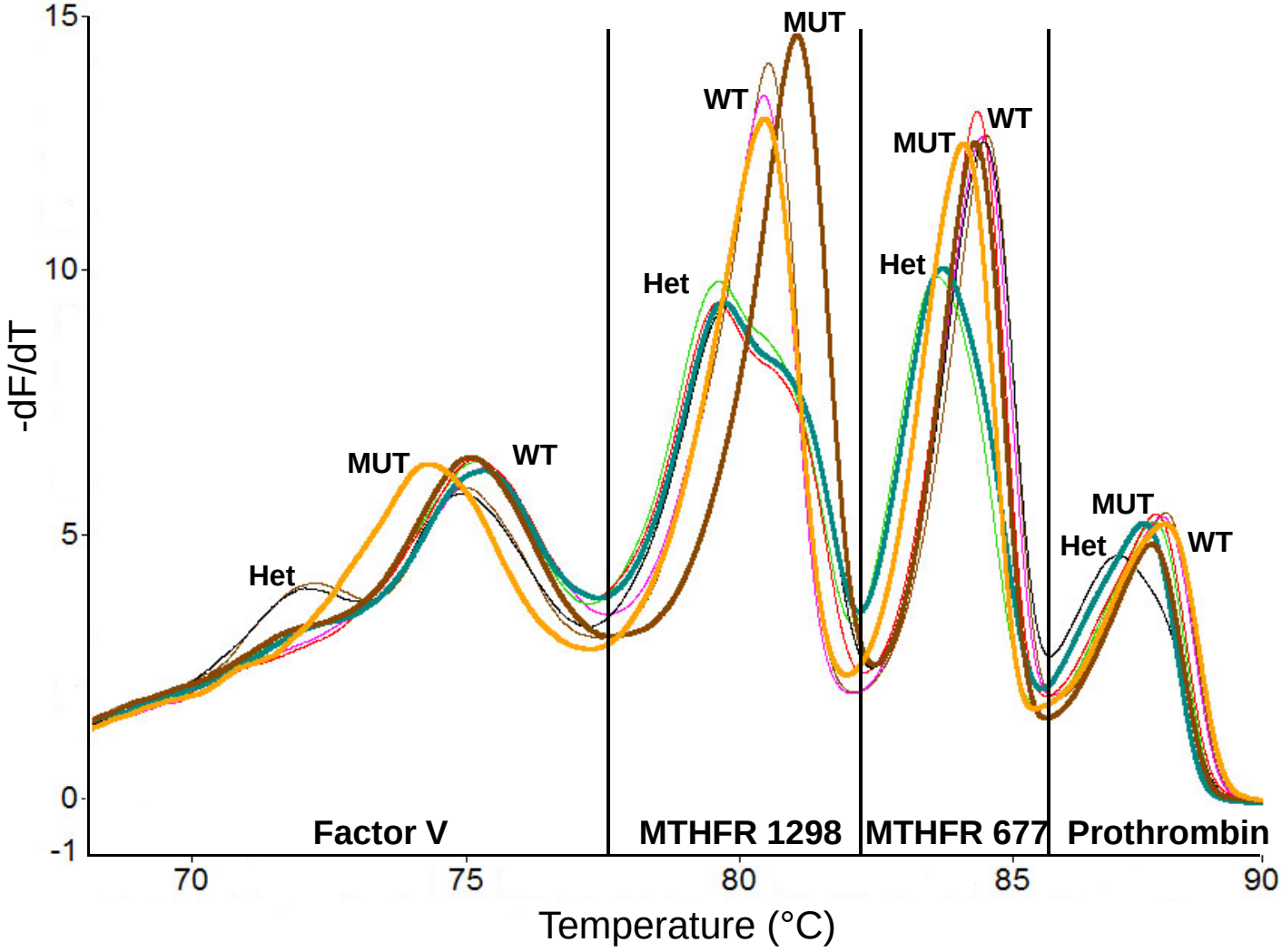
(Human Platelet Antigens - J Mol Diagn 8:97-104;2006)

Single Base Genotyping in Long Amplicons



Clin Chem 49:853-60;2003.

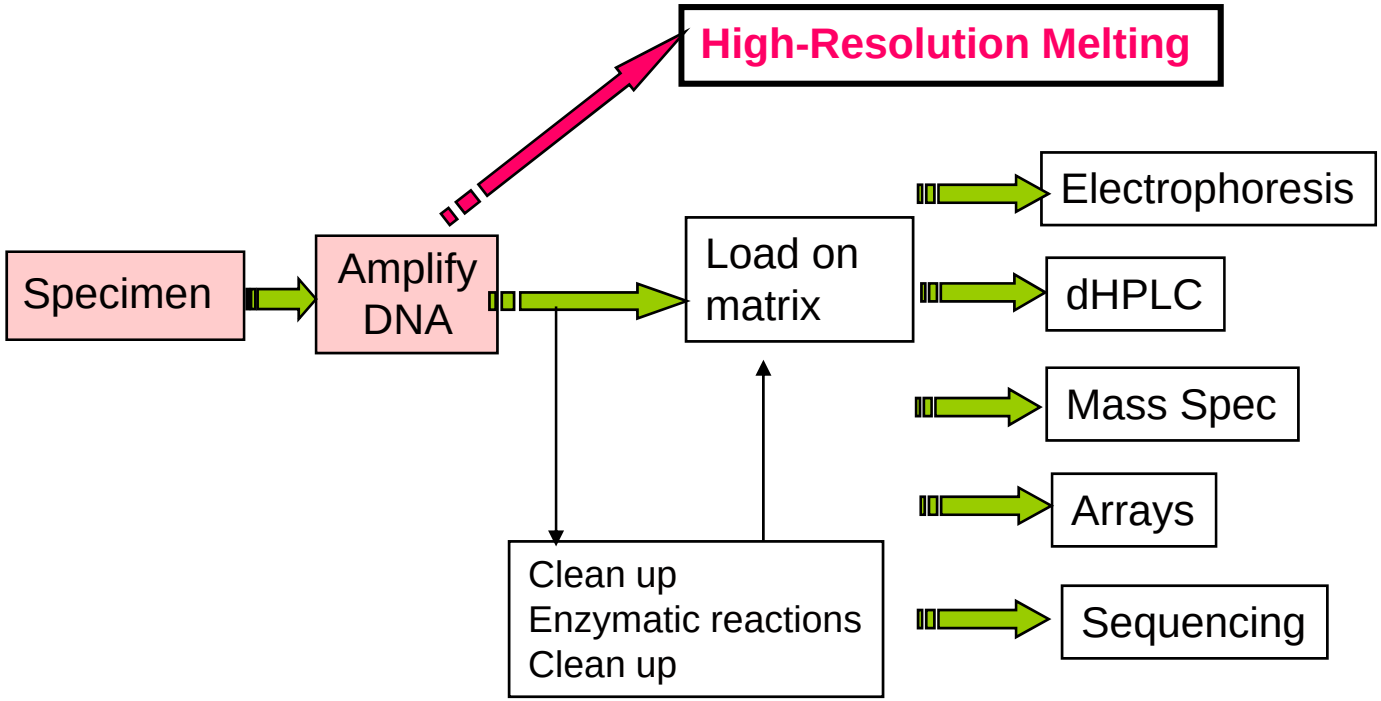
Coagulation Quadraplex



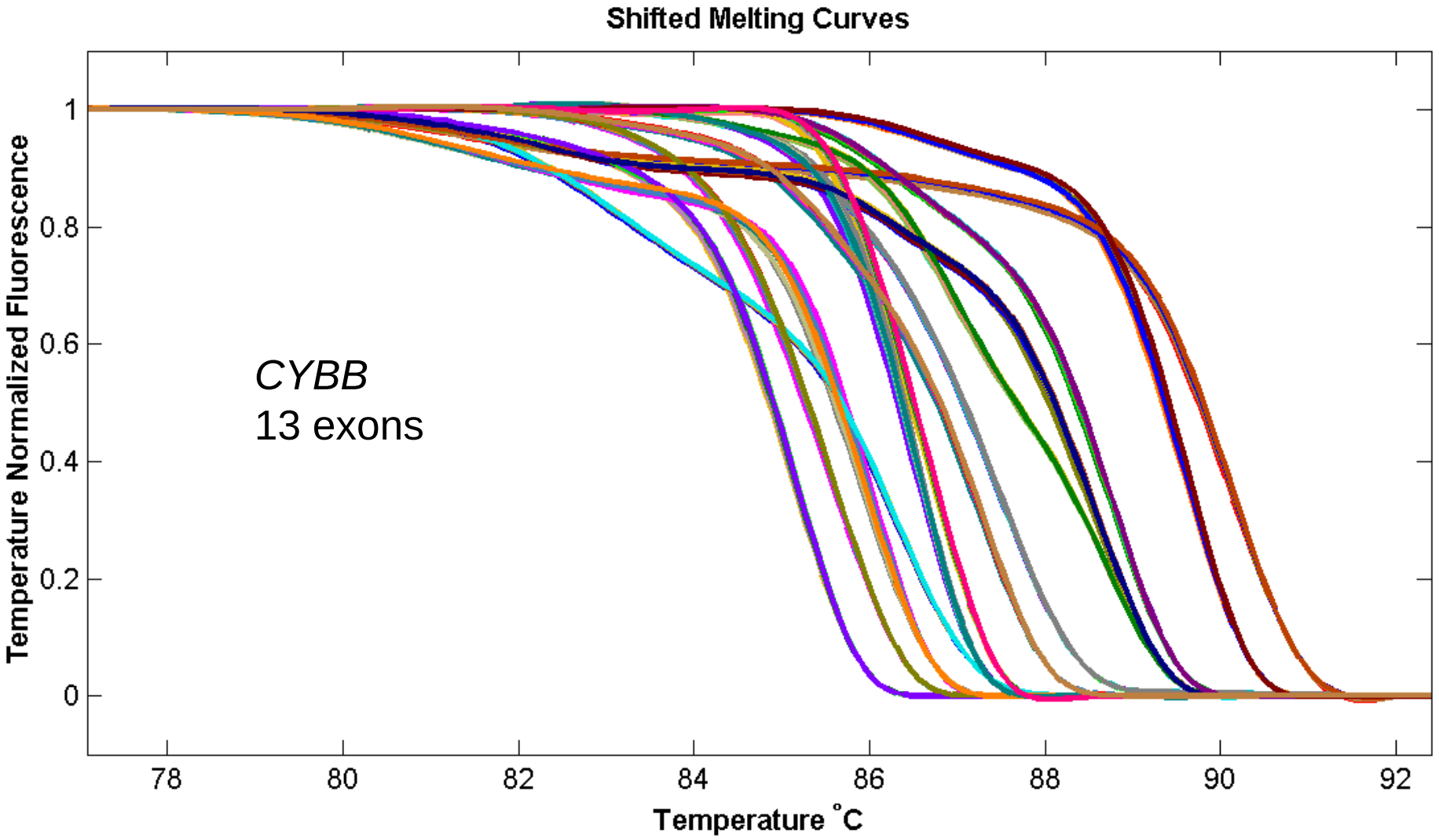
Clin Chem 54:108-15;2008.

Scanning for Heterozygotes

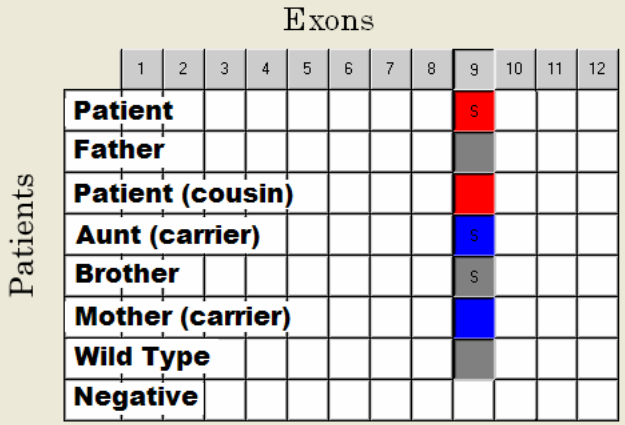
- All use PCR first

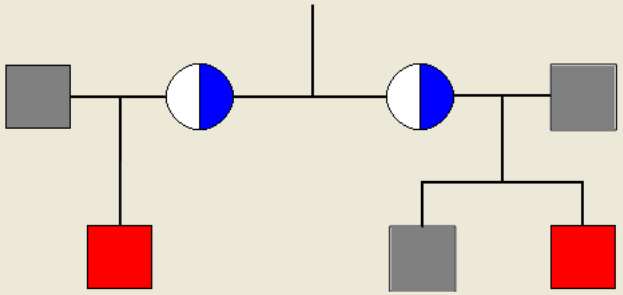


X-linked Chronic Granulomatous Disease



Chronic Granulomatous Disease Family Study

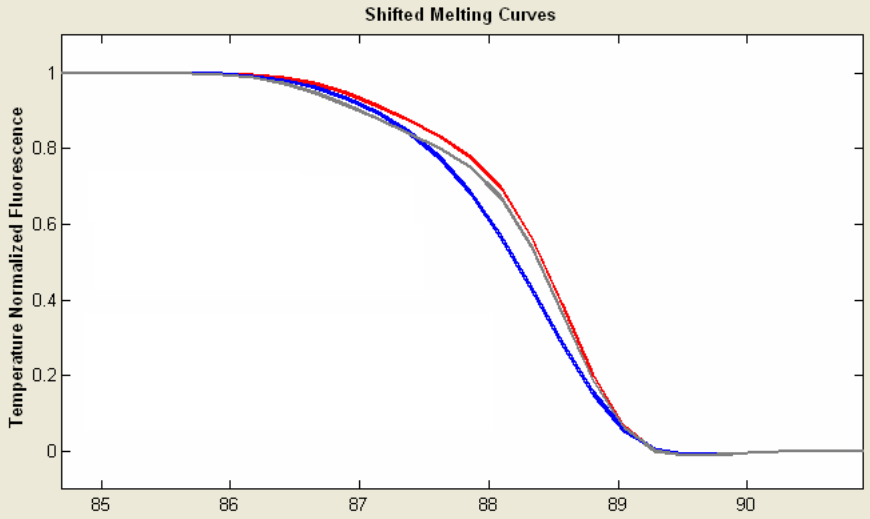


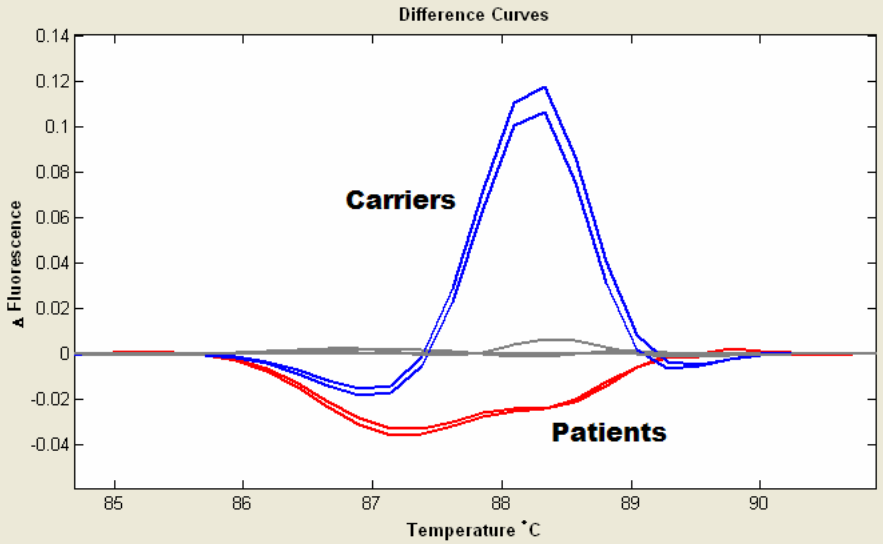


Missense Mutation

TGGCACCCTTTTACA
↓
A

pro to his





Hi-Res Melting for Genetic Disease Scanning

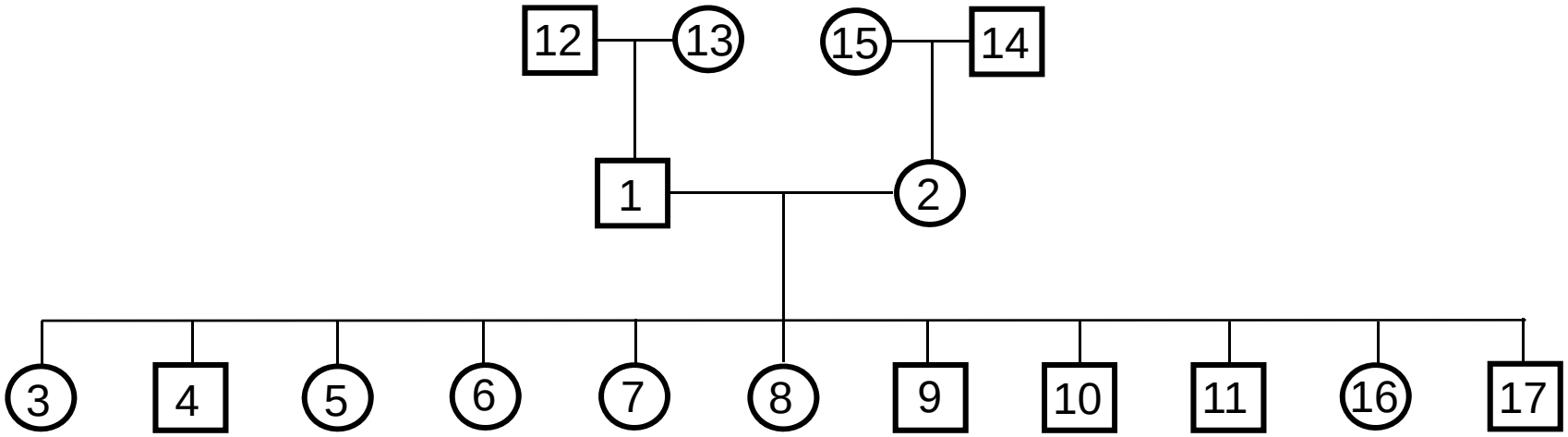
- Published studies (19)
  - BRCA1/2, CFTR, F8, NF2, ApoB…
  - Sensitivity 99.3% (n=839)
  - Specificity 98.8% (n=2,659)

Patrinos GP and Ansorge WJ, eds, Molecular Diagnostics, 2nd ed, Elsevier Chapter 18, in press, 2009

Scanning Summary

- Inexpensive (PCR reagents + dye)
- Rapid (PCR time + melting)
- Detects “all” heterozygous variants
- Most heterozygous variants have unique melting curves (93%)
- Detects minor allele fractions better than sequencing
- Does not detect large insertions or deletions
- Requires good PCR

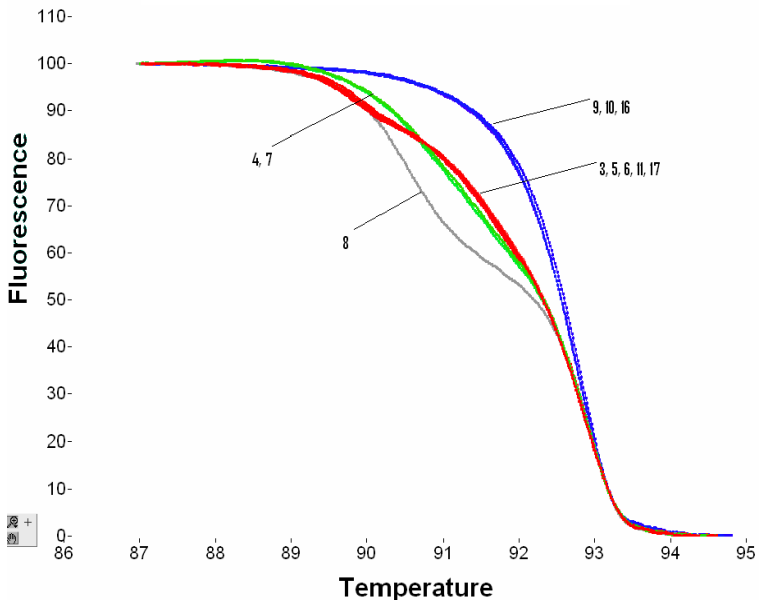
Hi-Res Melting of HLA



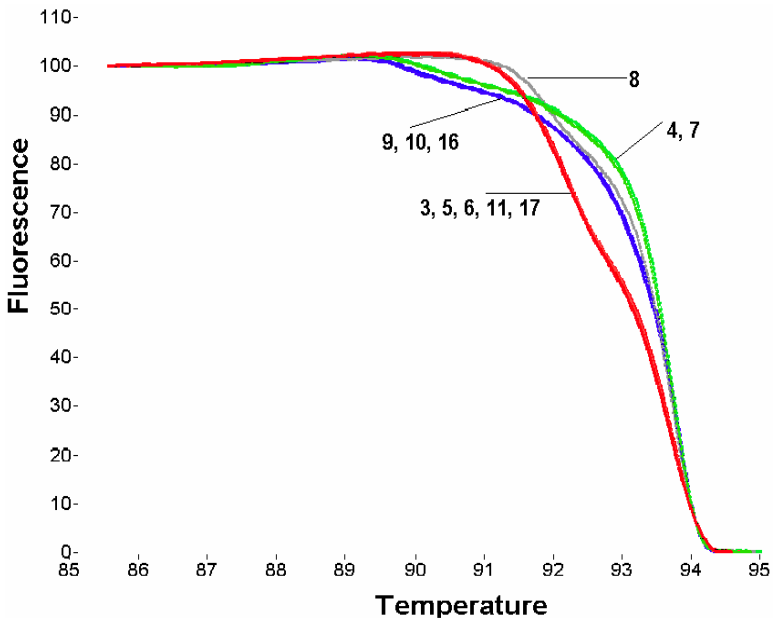
Match instead of genotype

HLA-B

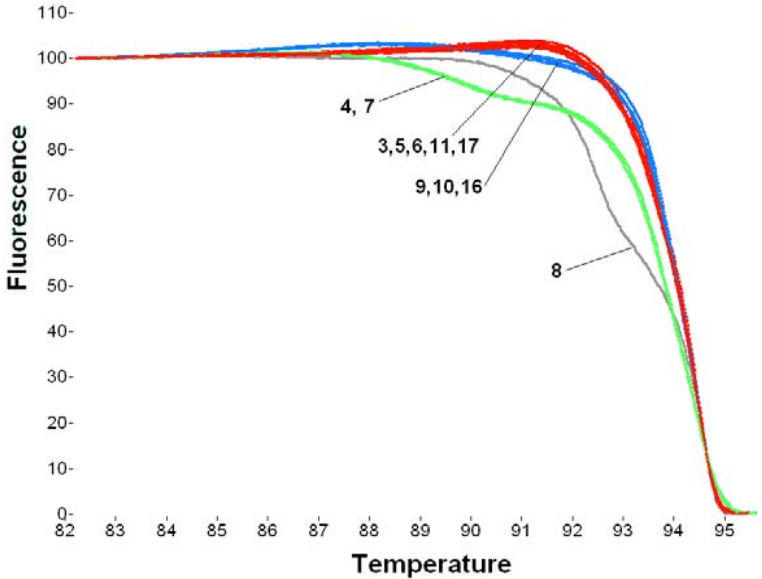
Exon 2

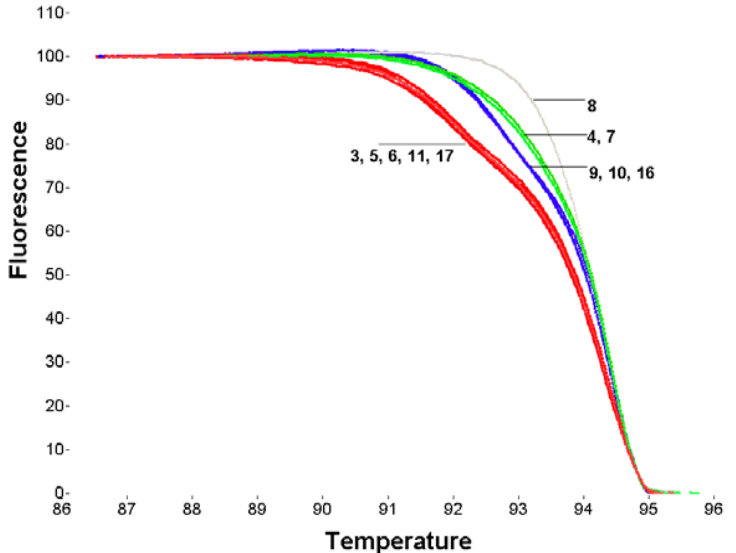


Exon 3

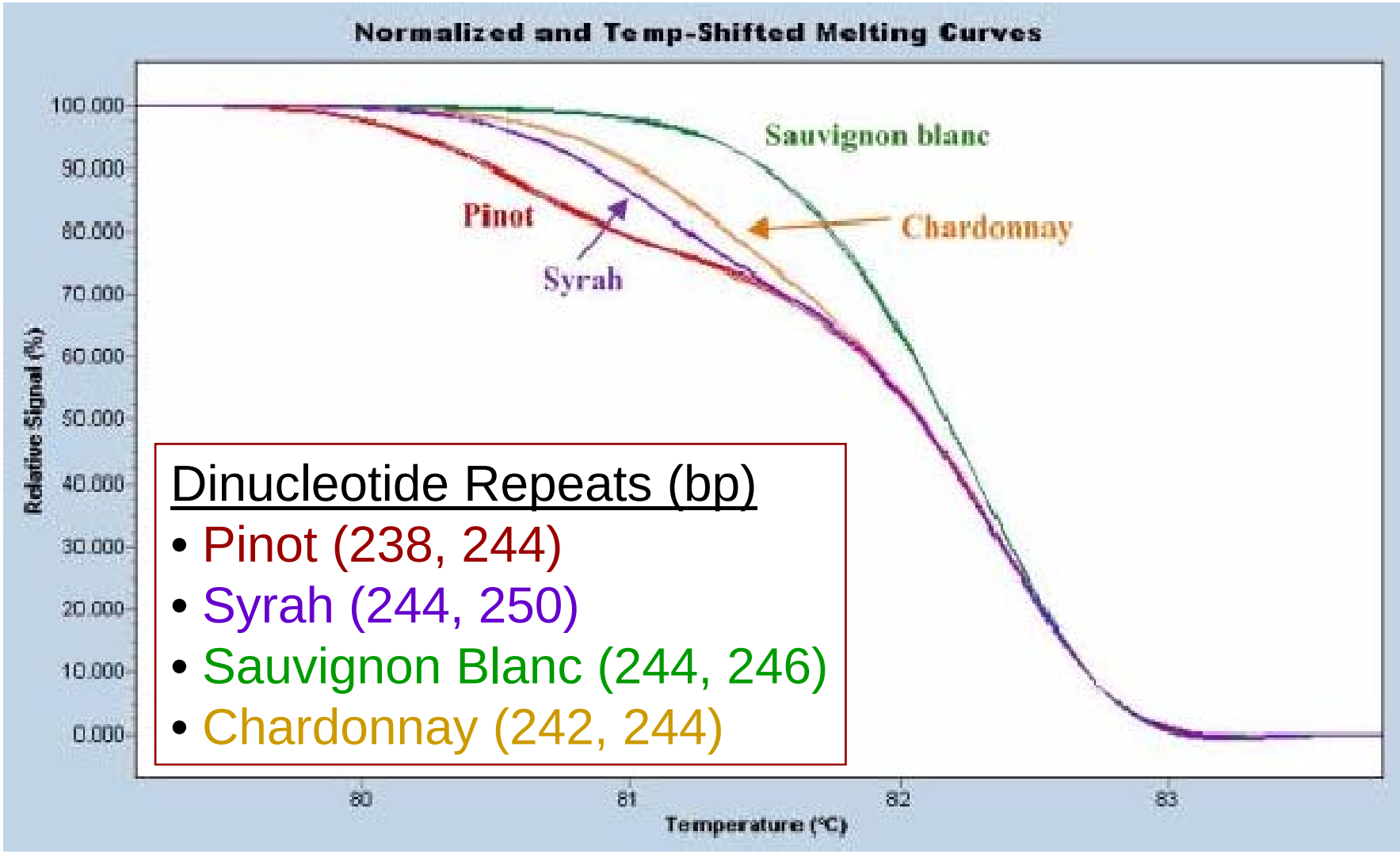


HLA-C





Variety Identification of Grapevines



John Mackay et al., Linnaeus Laboratory, Plant Methods 2008, 4:8.

Summary

- PCR can be fast
- You don’t need labeled probes for homogenous genotyping
  - Unlabeled Probes
  - Snapback Primers
- High Resolution Melting
  - Small amplicon genotyping
  - Heterozygote Scanning
  - Sequence Matching
    - HLA
    - Repeated sequences

Thanks!

Idaho Technology
NIH
ARUP
Roche Applied Science
University of Utah

http://dna.utah.edu