Extreme Molecular Diagnostics

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ARUP, Oct 22, 2019, Salt Lake City, UT

How to Innovate:

Outline

(our focus is speed)

- Current state of the art
  - Sample preparation, amplification, analysis
- Making amplification faster
  - Rapid-cycle PCR
  - Extreme PCR
- Making analysis faster
  - High speed melting
- Making sample preparation faster
  - Genomic DNA from whole blood

Rapid Targeted Molecular Assays
(Flu A/B, RSV, Strep A)

- Real-time PCR
  - 15-30 minutes
  - Multiple manufacturers

- Recombinase polymerase assay
  - Isothermal
  - Positive results in 2-5 min
  - Negative results in 6-13 min

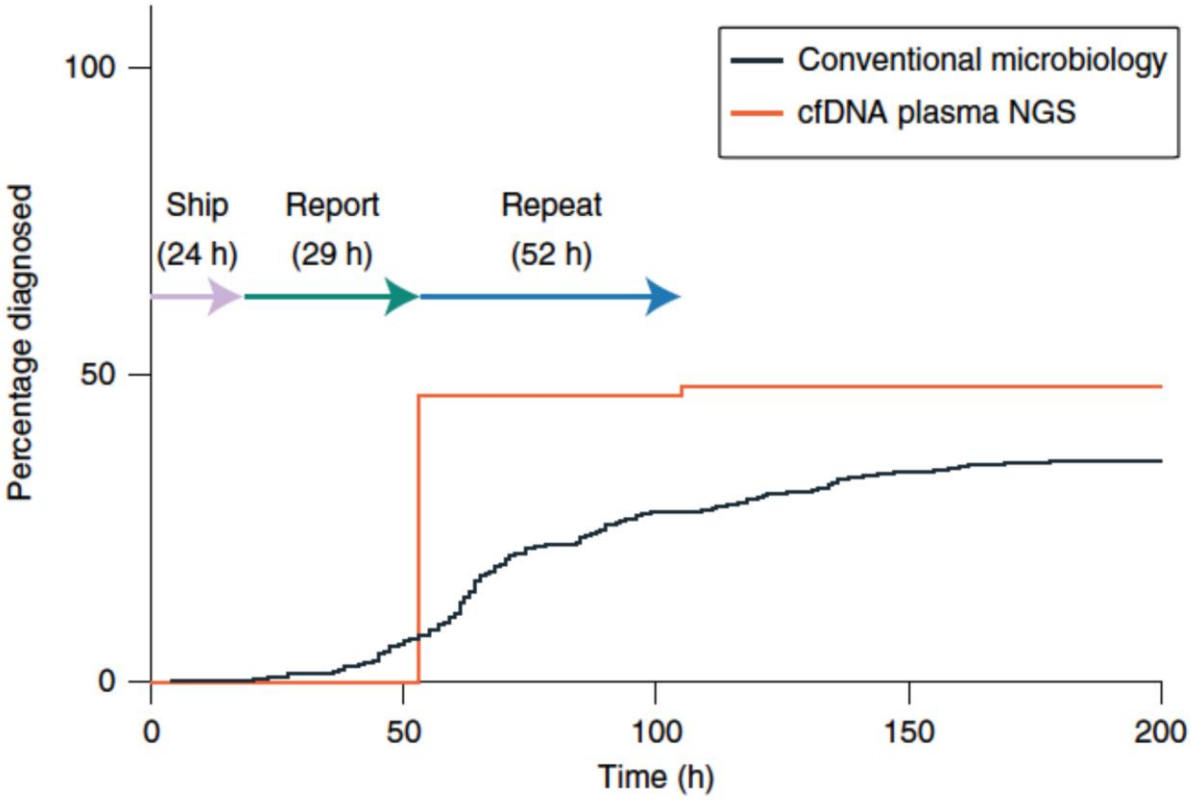
Multiplex Syndromic Tests

(FDA-approved)

Panel	Pathogens (#)	Resistance Targets (#)	Time to Result (min)
Respiratory	21		45
Blood Culture ID	24	3	60
Gastrointestinal	22		60
Meningitis	14		60
Pneumonia	26	7	60

Microbial Cell-free DNA Sequencing

Nat Microbiol 2019, 4, 663-674



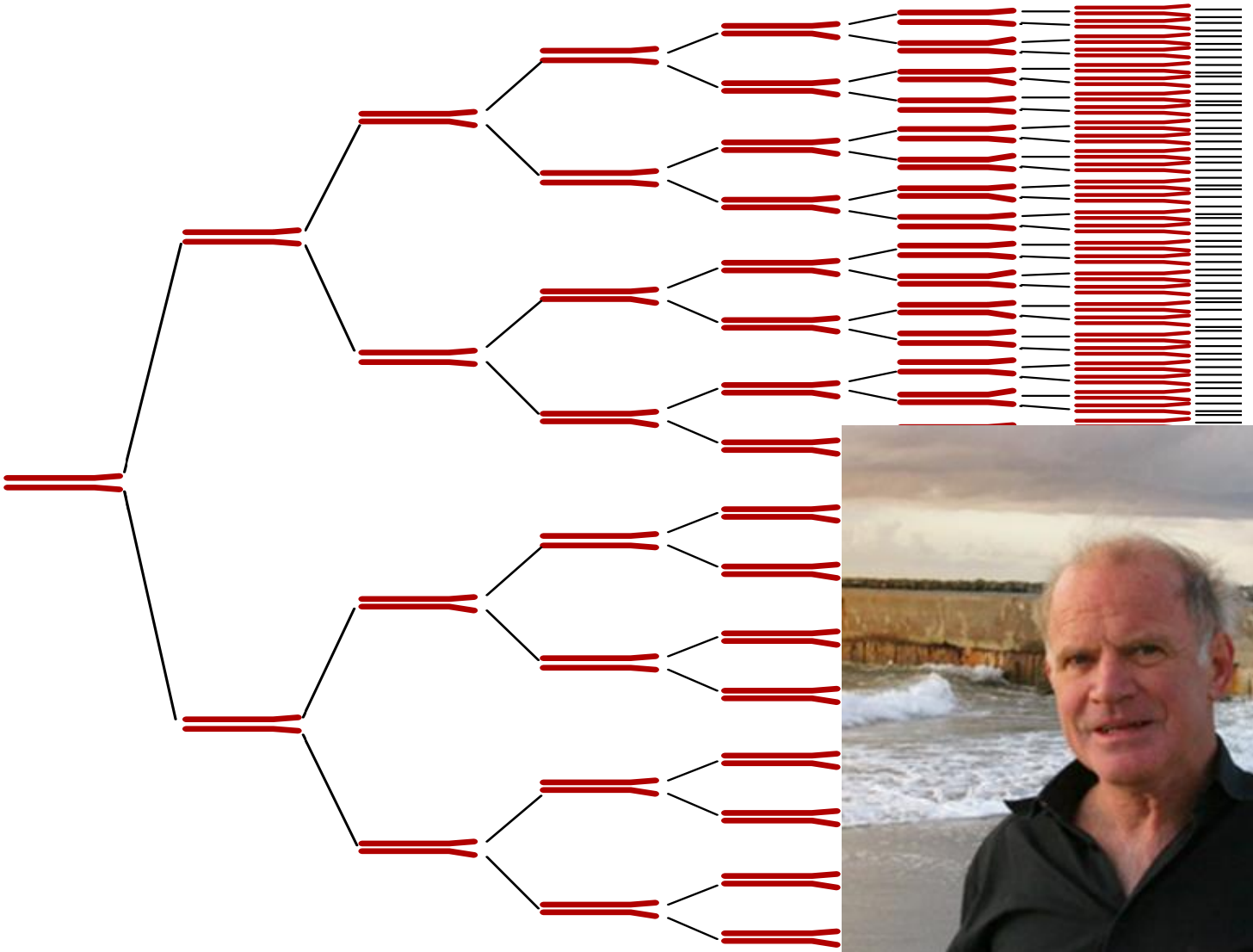
Clinical Genome Sequencing (Pediatric ICU)

Sci Transl Med (2019, 11, 6177)

- 20 hour whole genome sequencing
  - 1.5 hours of library preparation
  - 15.5 hours massively parallel sequencing
  - 1 hour of alignment and variant calling
- Automated phenotyping and interpretation
  - Phenome extraction from electronic health record
  - Match to phenomes of all genetic diseases
  - Correlate to pathogenic variants
- Guinness World Record for Fastest Genetic Diagnosis

Making PCR Faster

1985-1988: DNA replication in a test tube

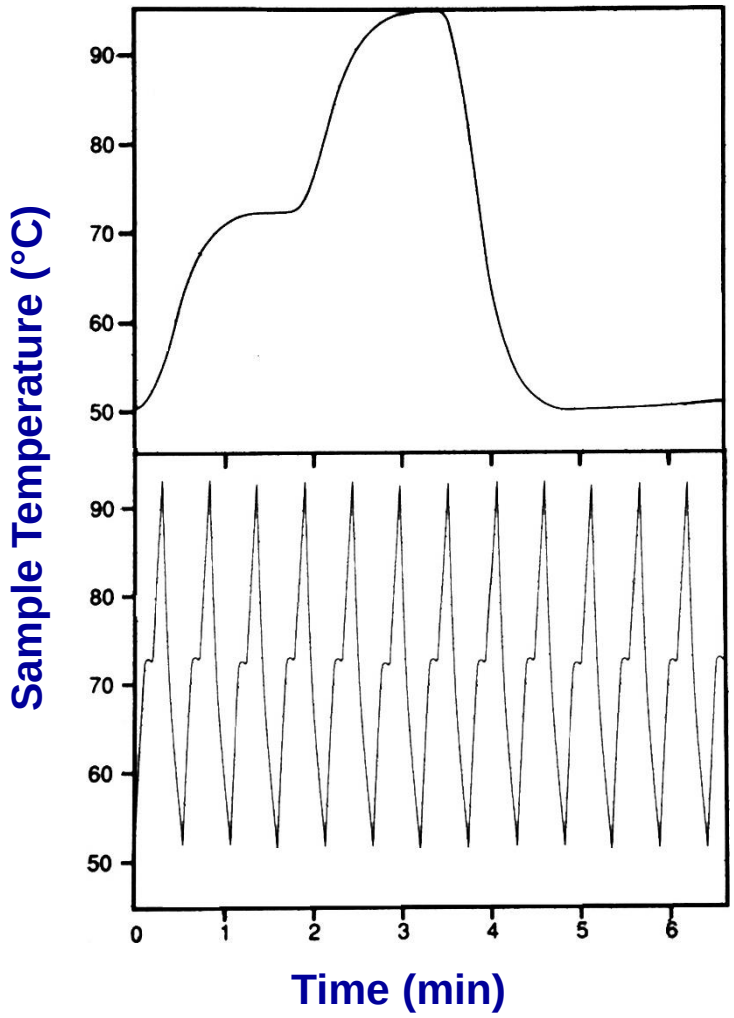


Trouble with Terminology

- “Rapid”, “Fast” are relative
- “almost instantaneous”

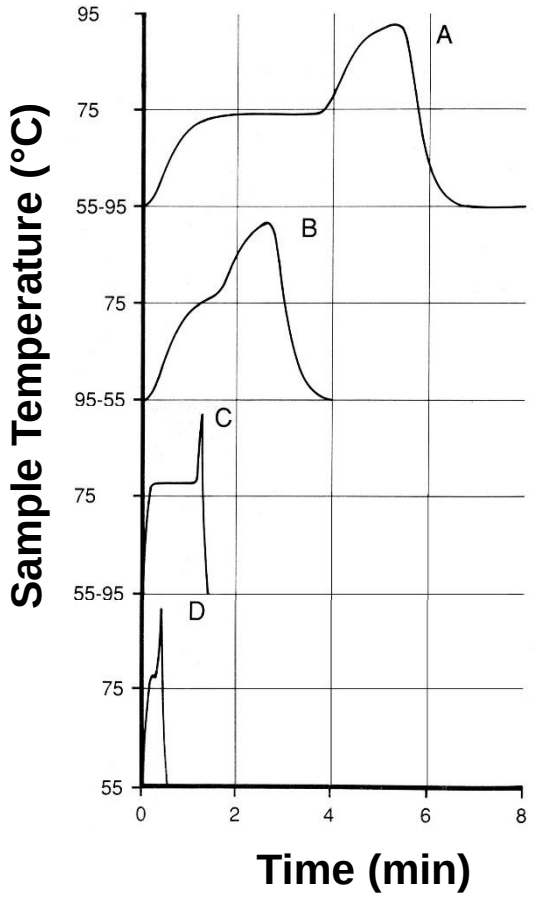
PCR Era	30 Cycles	Year
Legacy	2-4 hours	1989
Rapid Cycle	10-30 min	1991
Fast	30 min-1 hour	2000s
Ultrafast	2-10 min	2010s
Extreme	<15-60 sec	2015

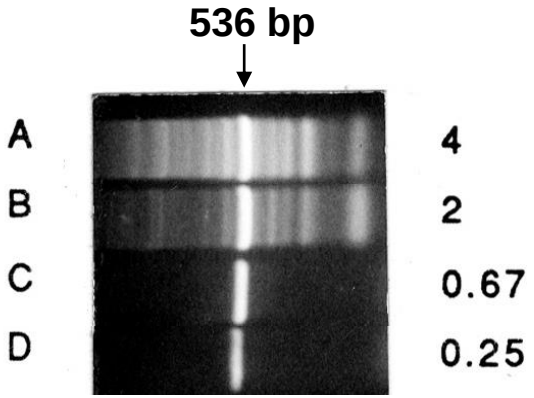
Sample Temperatures in PCR



Rapid Cycling is More Specific

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

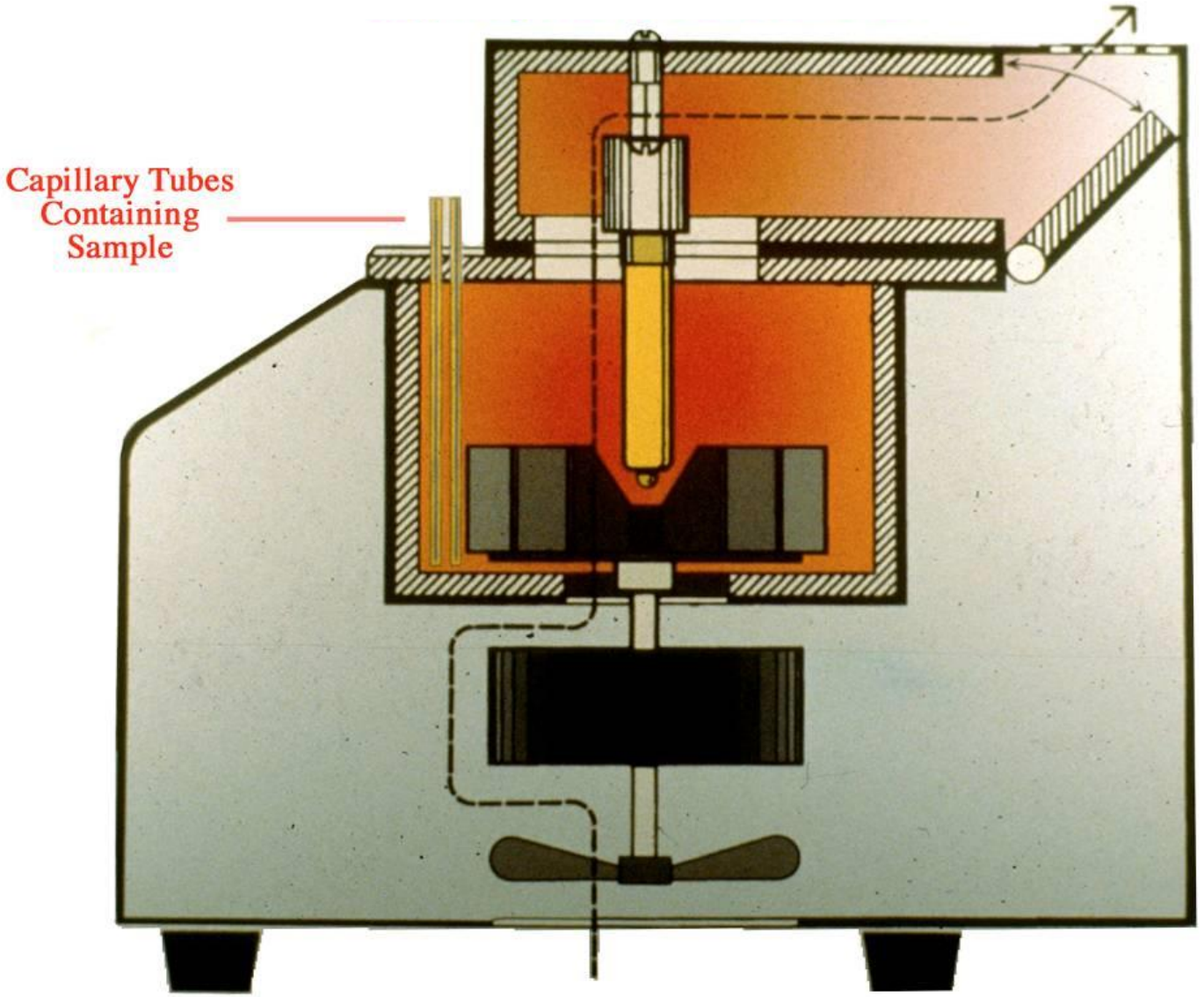




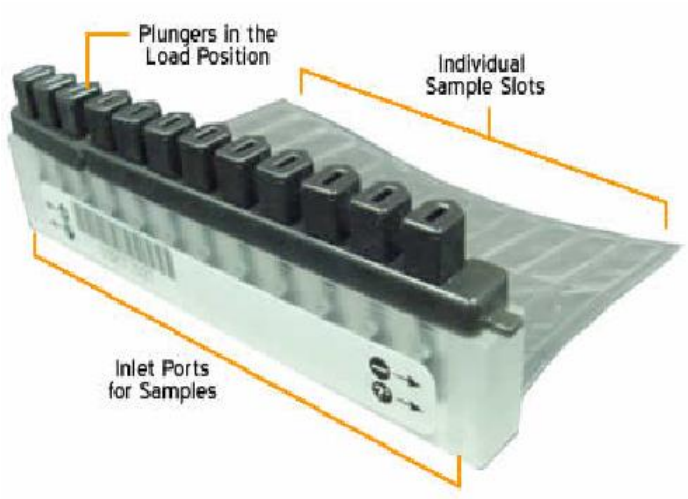
Amplification of a 536 bp β-globin fragment from human genomic DNA

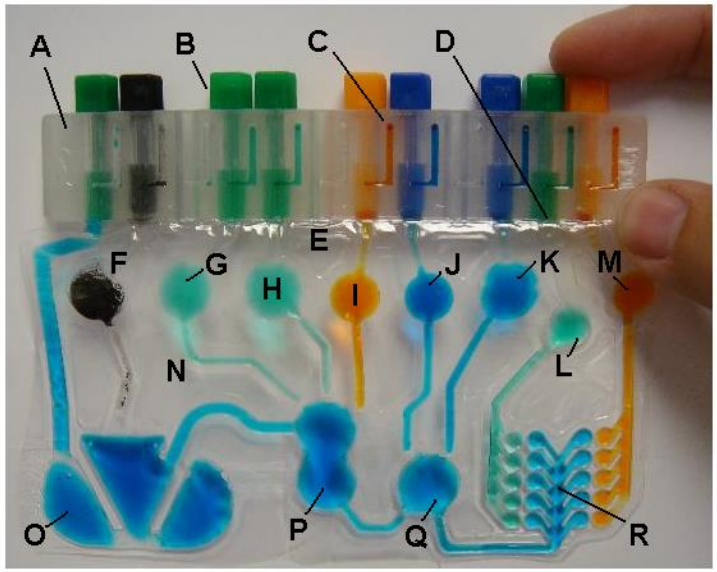
Anal Biochem 1990;***186***:***328-31,*** Biotechniques. 1991;***10***:***76-83***

Rapid Cycling Instrument

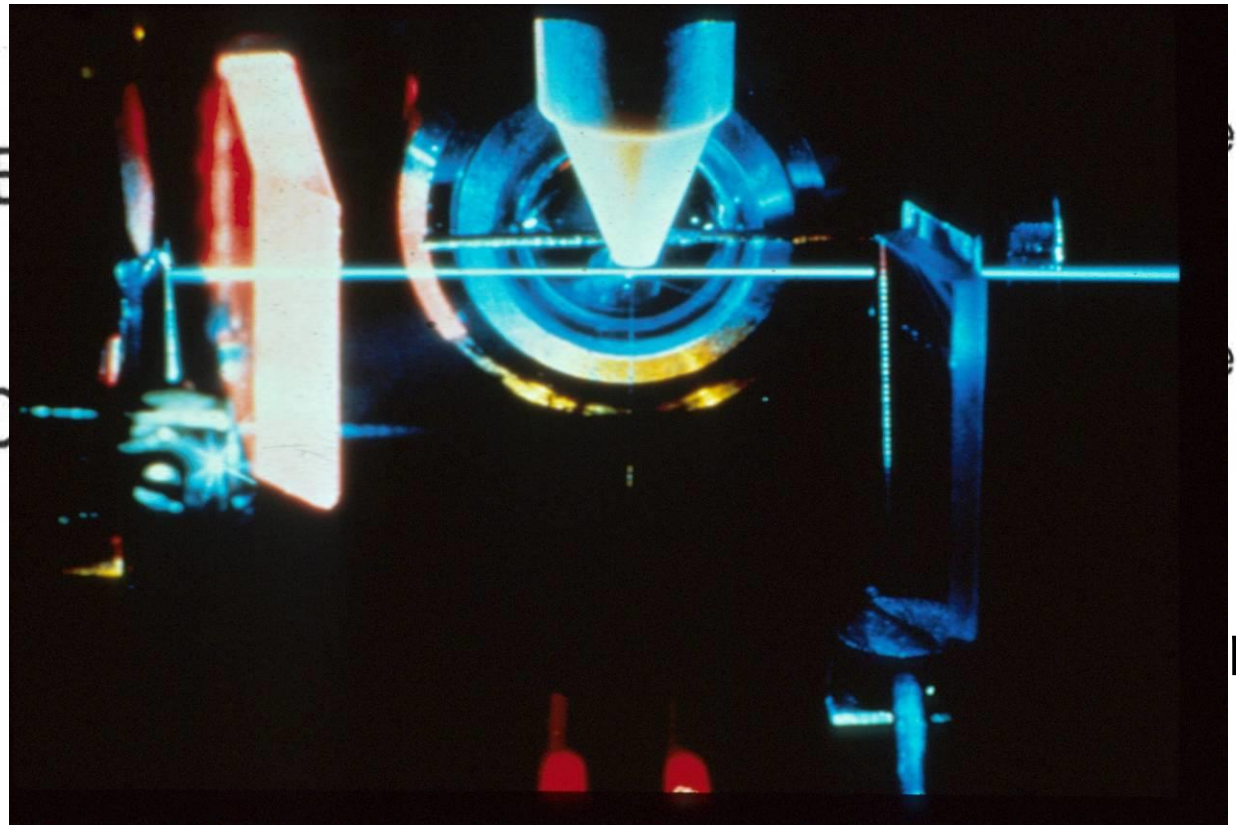


Other Containers for Rapid PCR





Monitoring PCR with Fluorescence



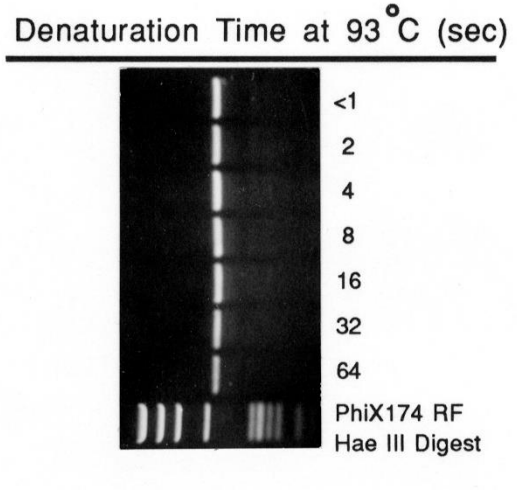
Flow Cytometry

Monitoring Fluorescence during Amplification

RapidCycler + Fluorimeter

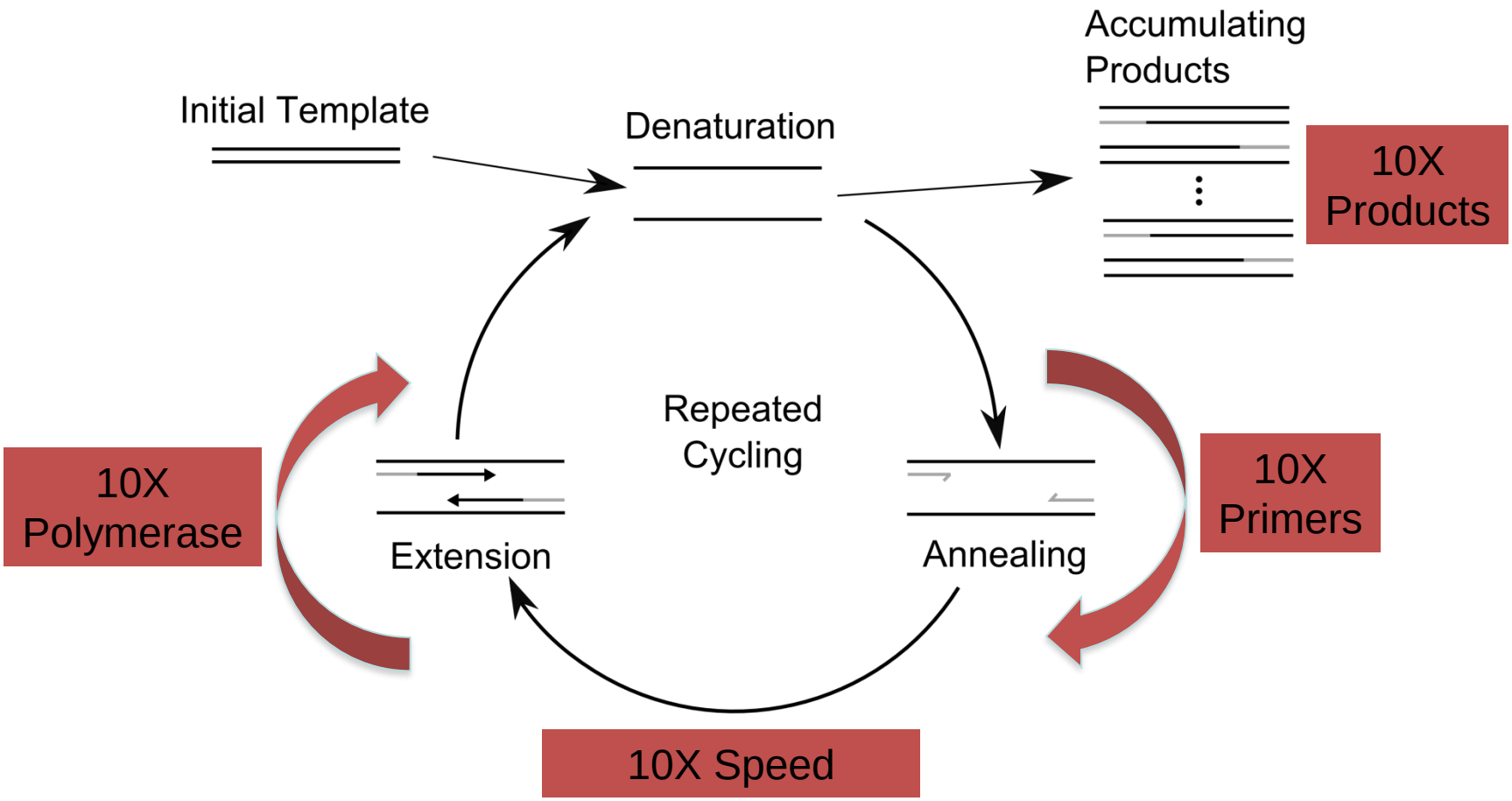
Real-Time Prototype

How long does it take to….

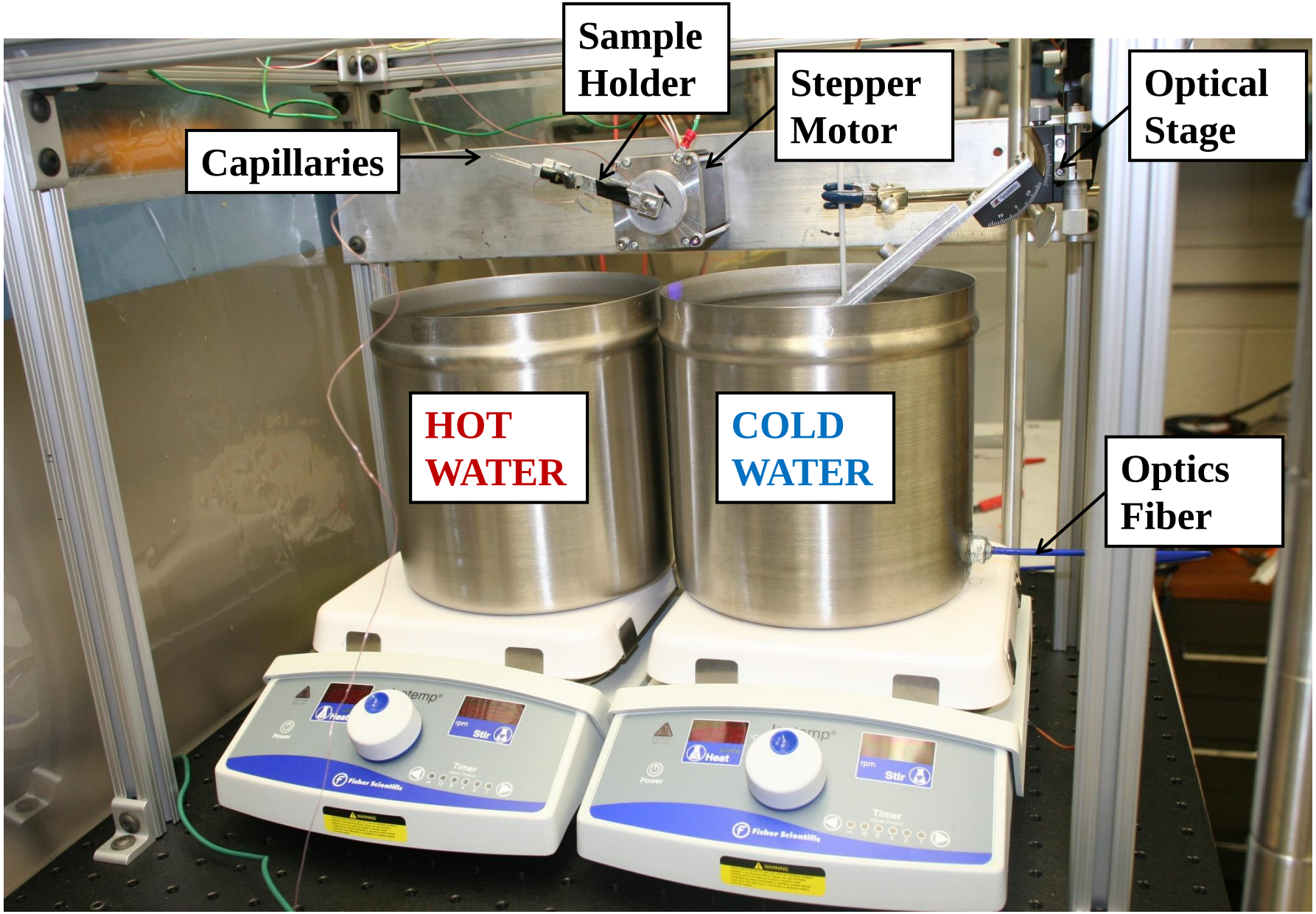


- **Denature**
  - Fast! (<1 sec)
- **Anneal**
  - Depends on the primer concentration
- **Extend**
  - Complex
  - Depends on the speed and concentration of polymerase
    - 5 ms for each nucleotide addition
    - 50 ms for binding events

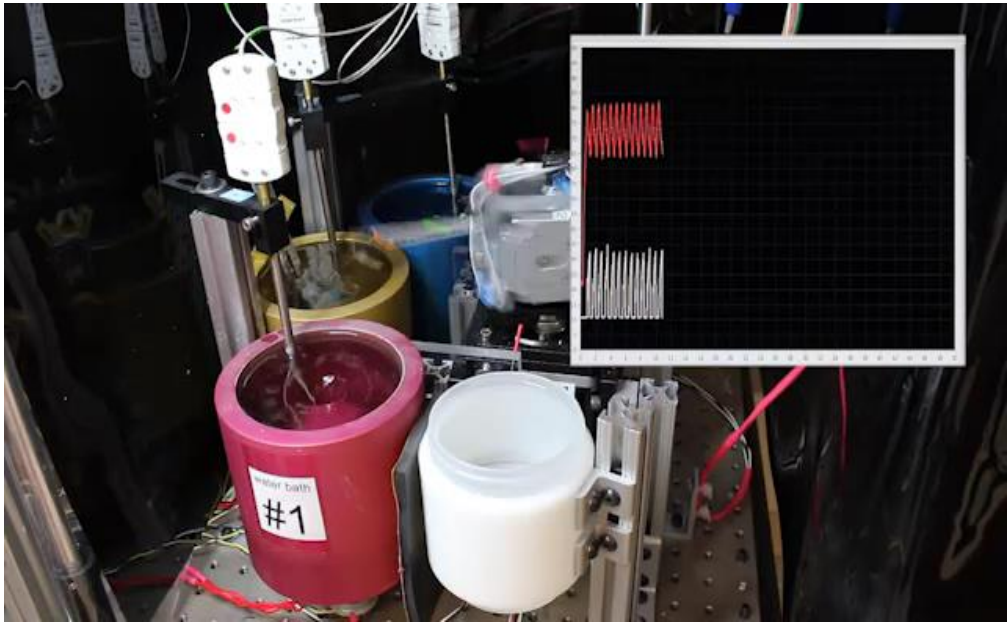
Extreme PCR


Initial Template
Denaturation
Accumulating Products
10X Products
Repeated Cycling
10X Polymerase
Extension
Annealing
10X Primers
10X Speed

Real Time PCR Extreme Alpha Prototype

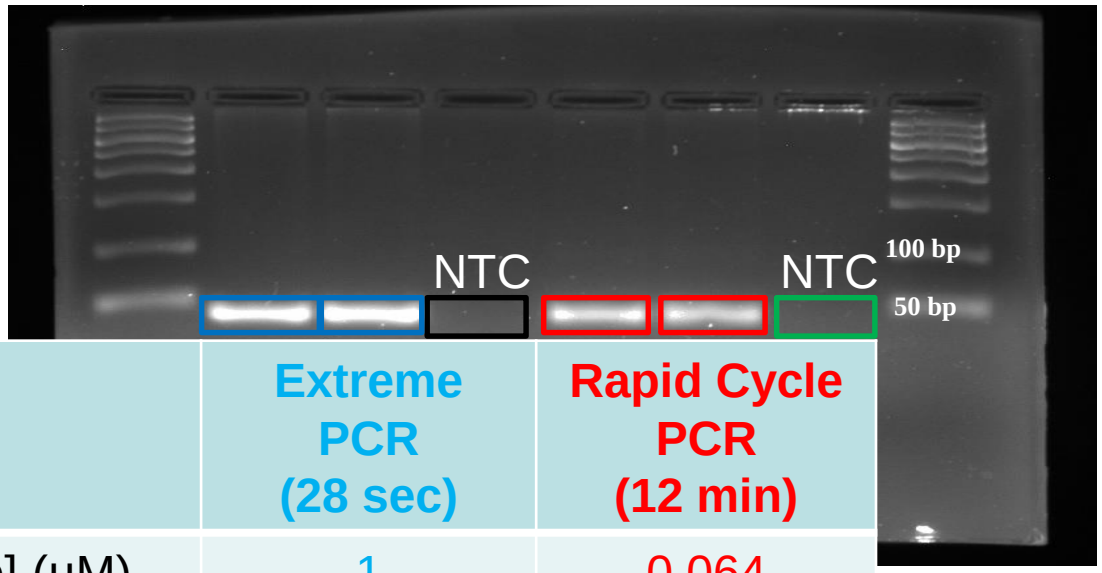


Water Bath Prototypes for Extreme Real-Time PCR

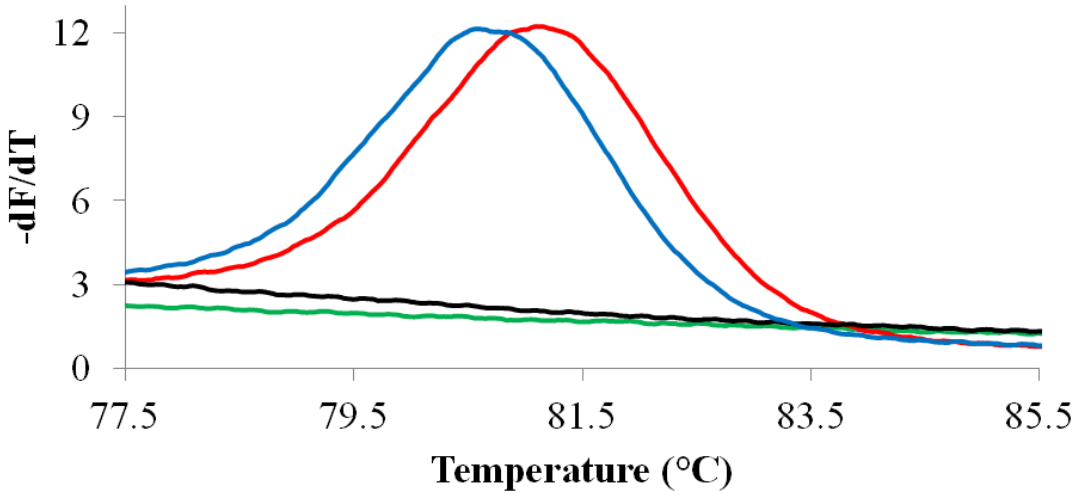


Extreme PCR compared to Rapid Cycle PCR

(45 bp human genomic target *KCNE1*)

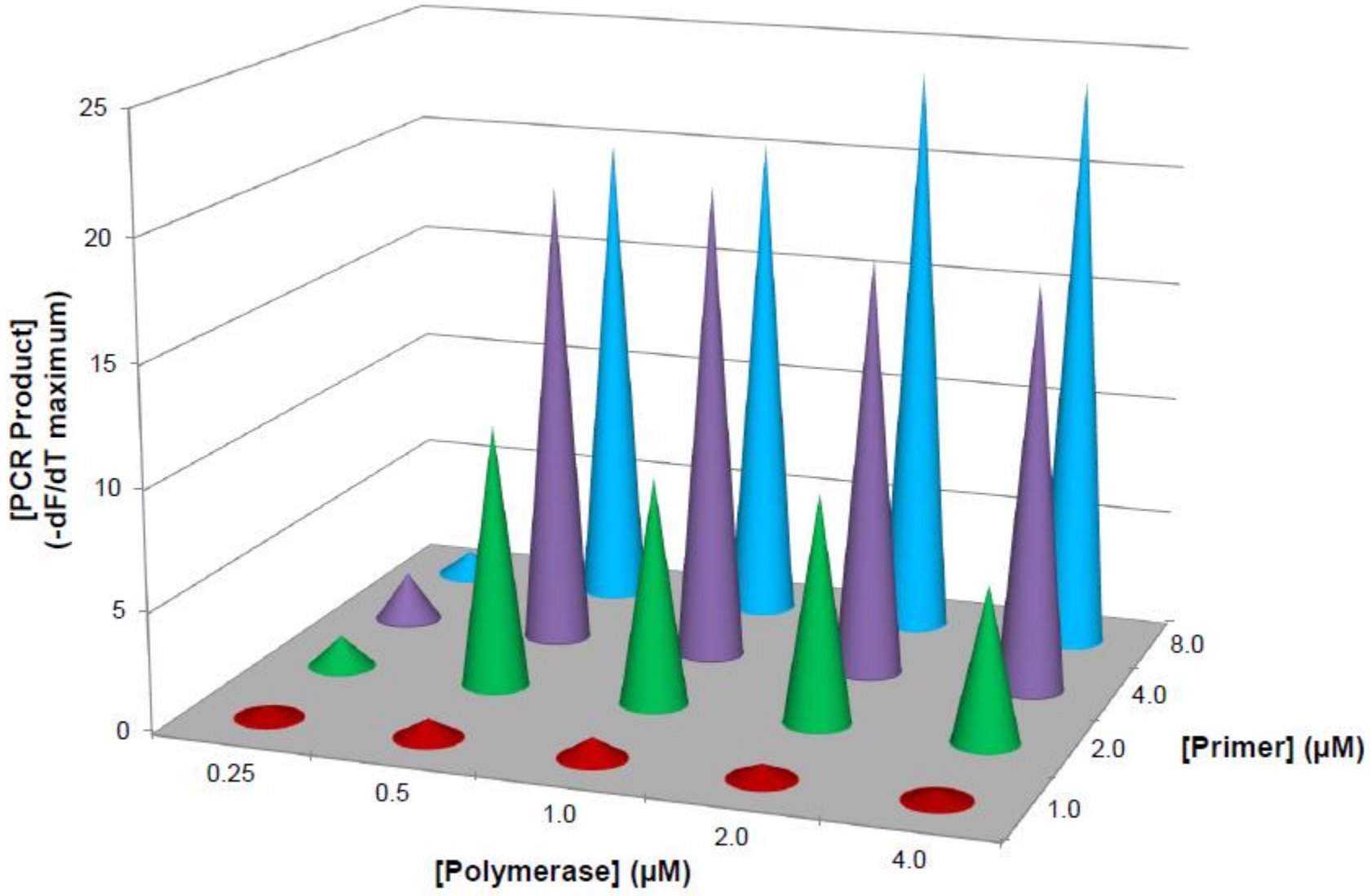


	Extreme PCR (28 sec)	Rapid Cycle PCR (12 min)
[Polymerase] (µM)	1	0.064
[Primers] (µM)	10	0.5



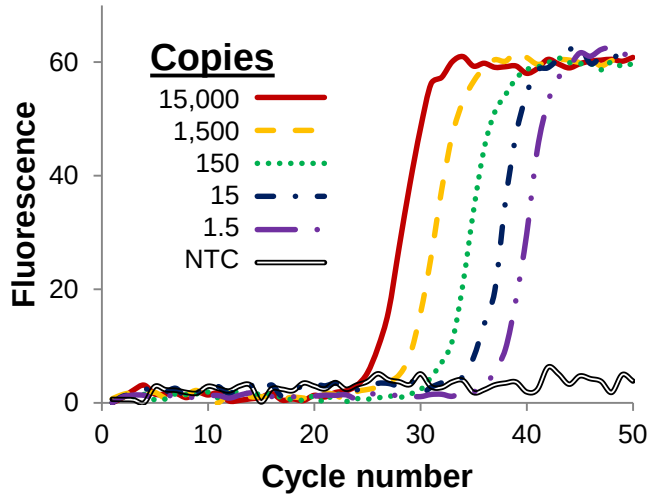
Polymerase and Primer Optimization *NQO1* (102 bp)

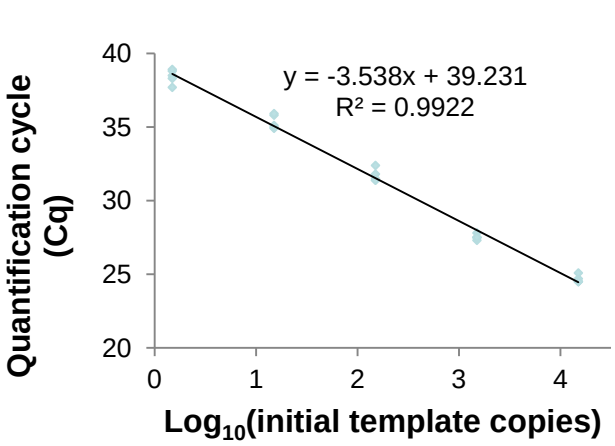
58 sec PCR (30 cycles, 1.93 sec/cycle)



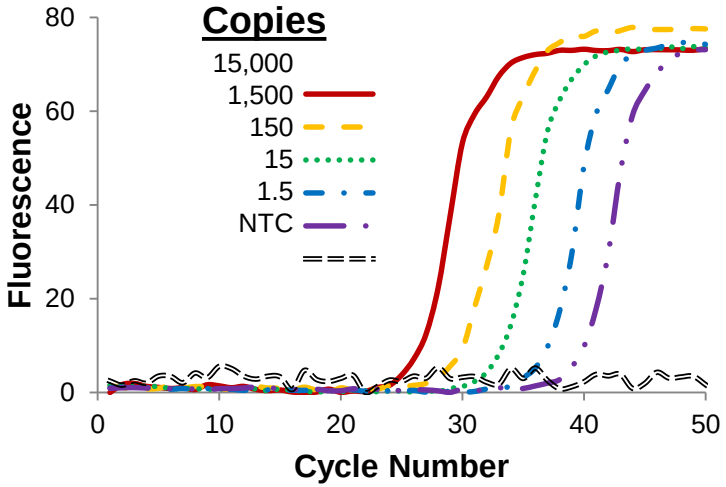
Extreme PCR Efficiency and Sensitivity

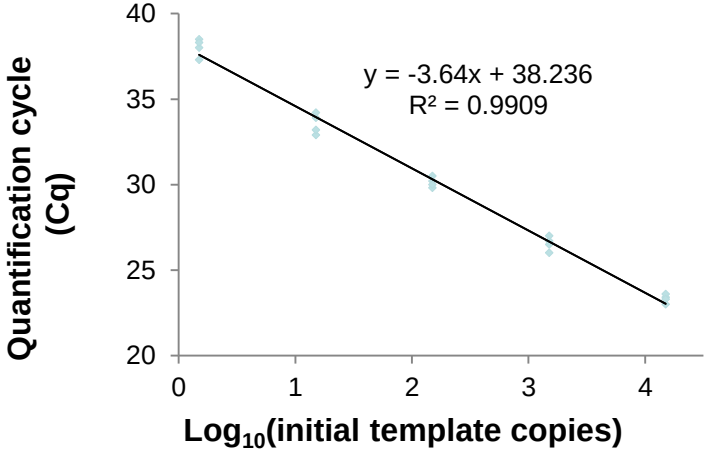
91.7% (45 bp, 28 sec PCR)





95.8% (102 bp, 58 sec PCR)

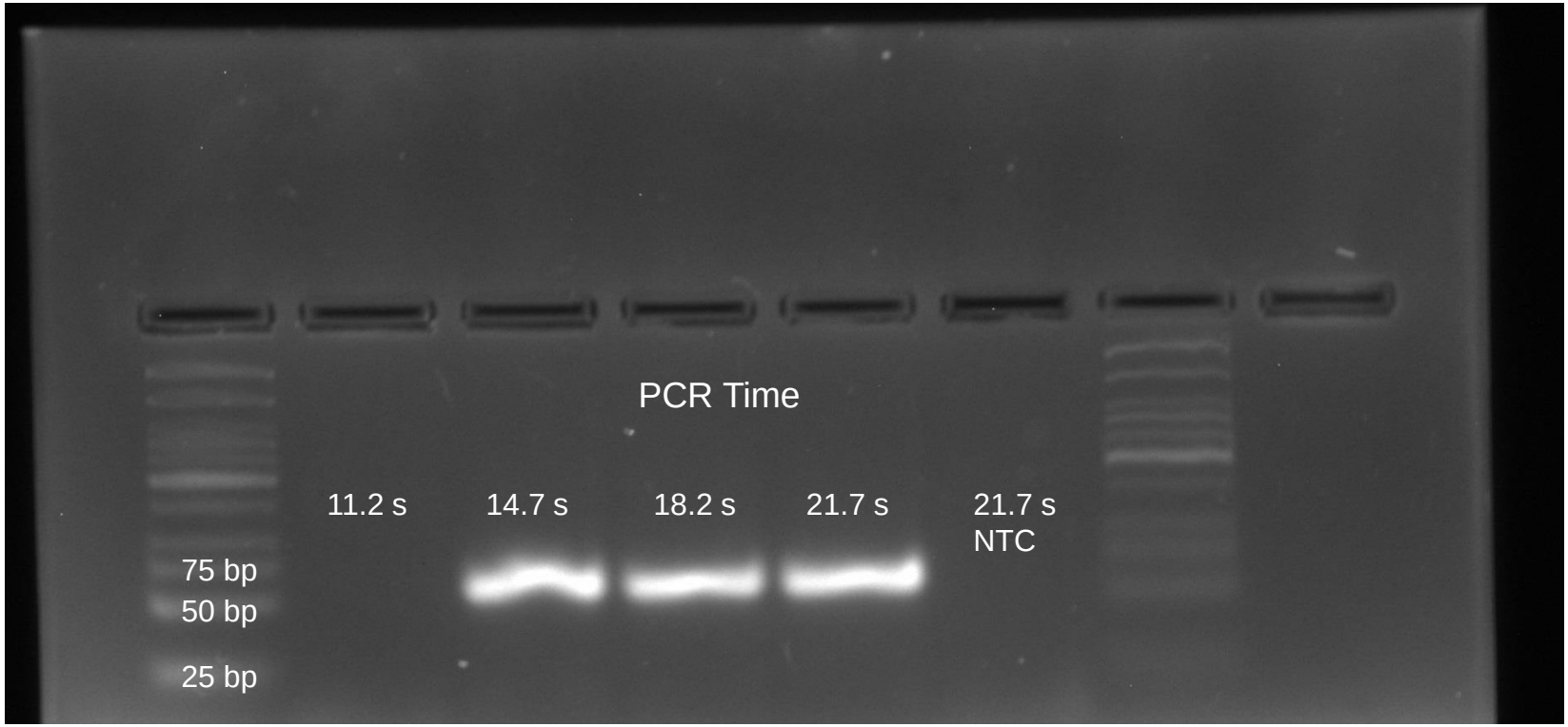




Clin Chem. 2015 Jan;61(1):145-53

14.7 second PCR

60 bp *AKAP10* (35 cycles, 0.42 sec/cycle)

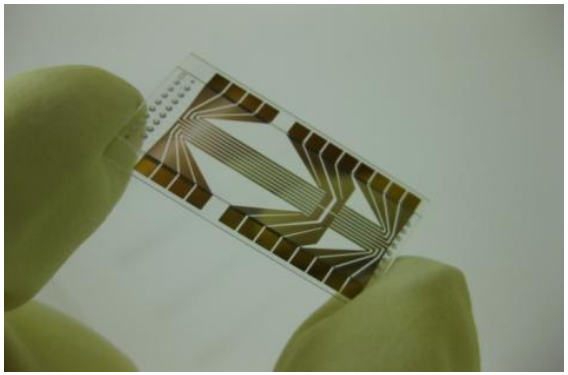


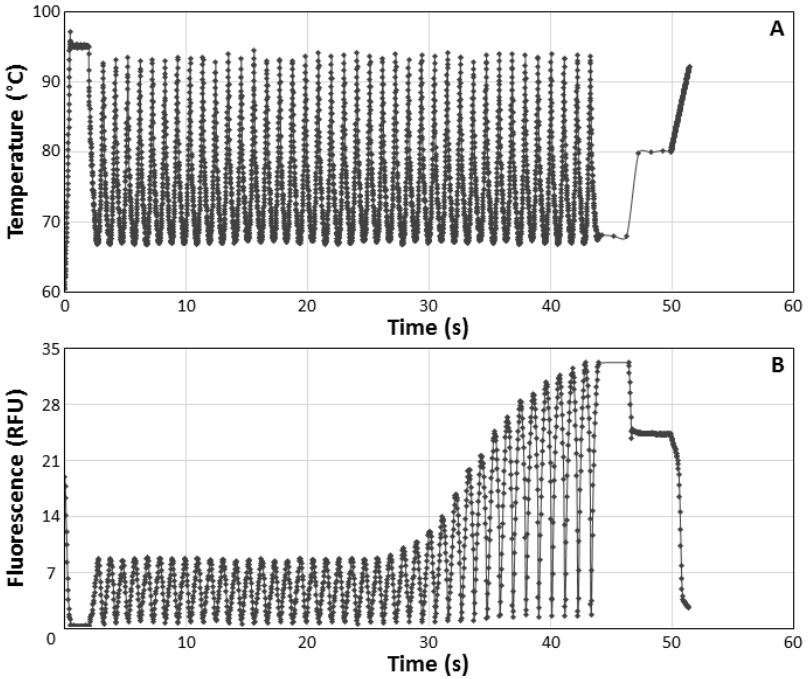
Clin Chem 2015;61:145-53

Lessons from making PCR faster

- Slow PCR is an accident of history
  - Limited instrumentation
  - Slow cycling requires low reagent concentrations
  - High reagent costs
- Science is fair
  - Never been “scooped”
  - Close calls
- The market values:
  - Numbers over quality
  - Convenience over speed
    - Capillaries
    - Water baths

Extreme PCR on a microfluidic system





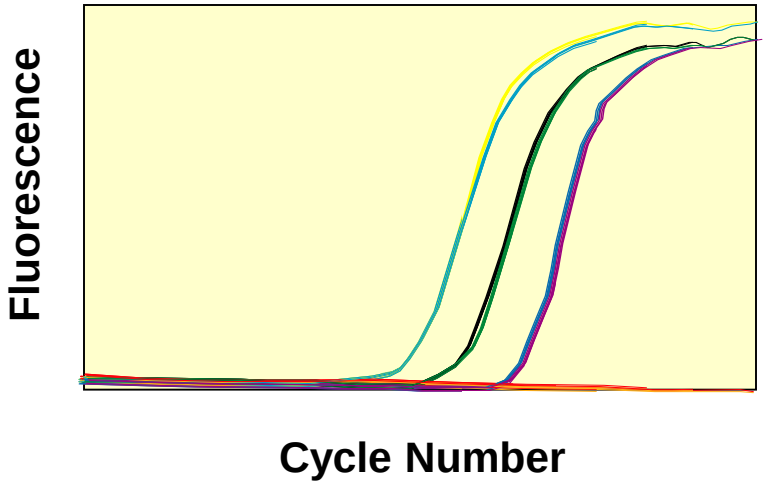
Clin Chem. 2019 Feb;65(2):263-271.

Making Analysis Faster

Nucleic Acid Analysis

- Electrophoresis
  - Separation matrix
  - Reveals size differences
- Mass Spectroscopy
- HPLC
- Sequencing by synthesis
- DNA melting
  - Solution technique
  - No additions or separations
  - Reveals melting profile differences

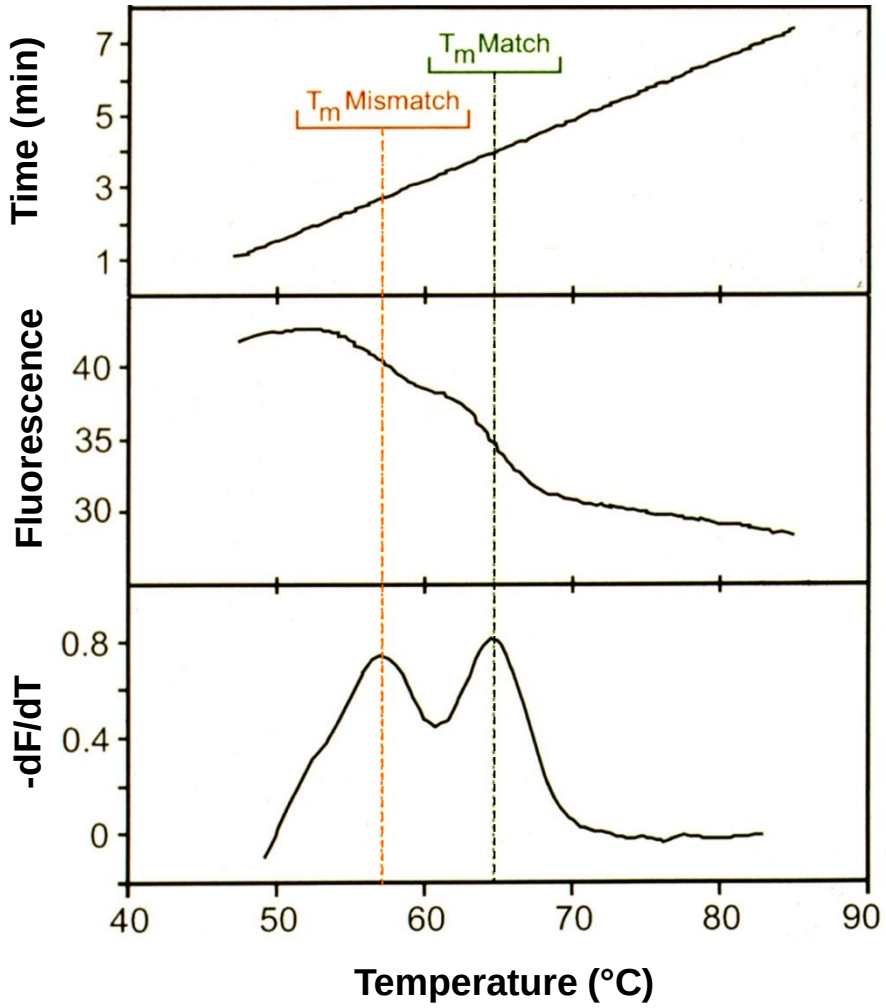
Modern melting analysis is performed after PCR

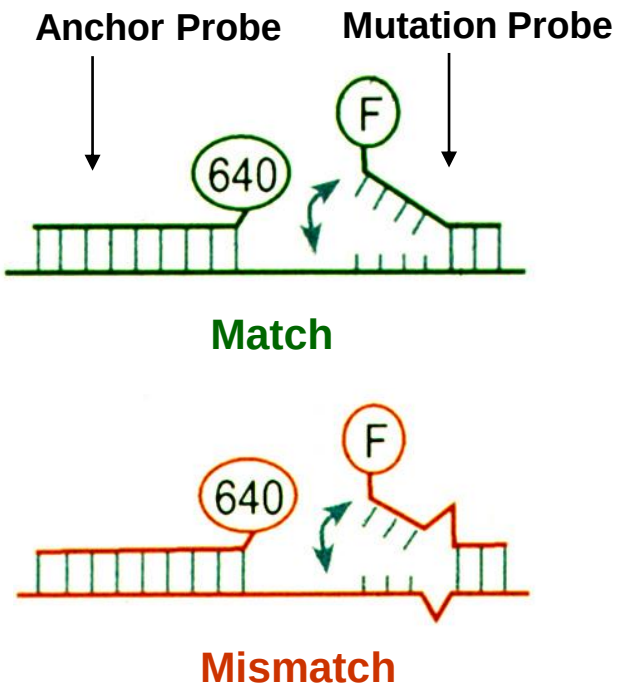


- Advances
  - Sensitivity
    - Fluorescence instead of Absorbance
  - Cost
    - Dyes vs Probes
  - Speed…..

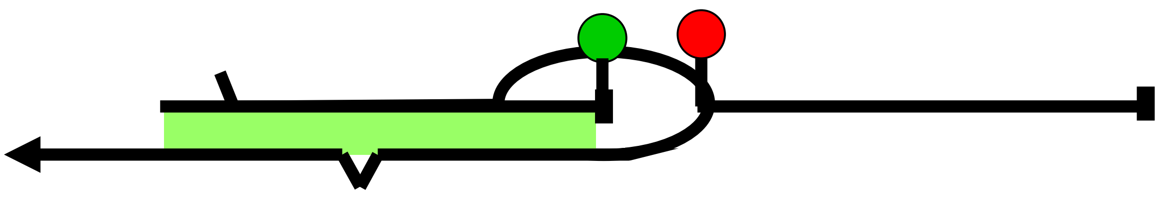
Dynamic Dot Blot for Genotyping

(labeled probes)





Genotyping by Melting



Single Hybridization Probe

Dual Hybridization Probes

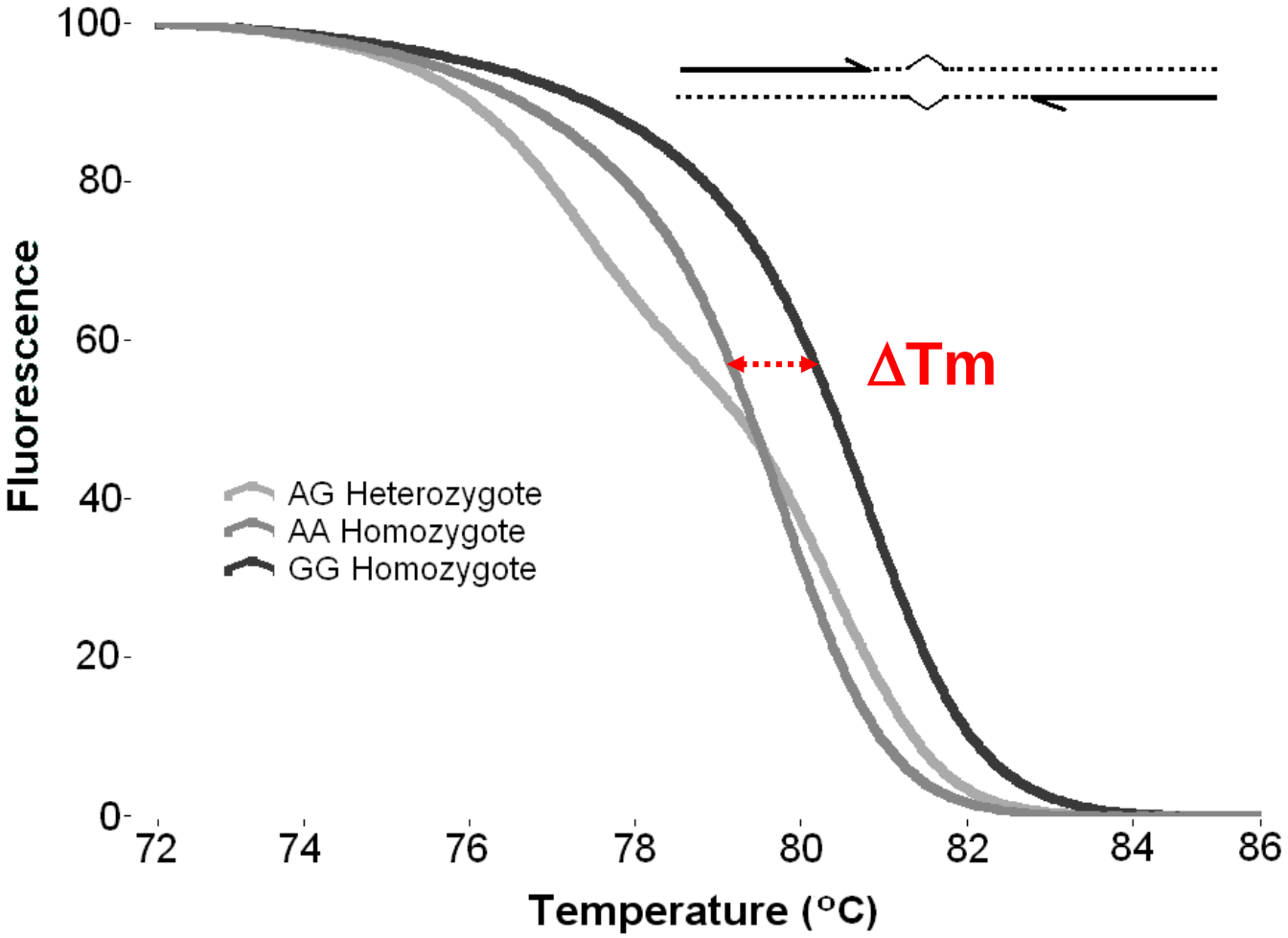
Unlabeled Probe

Clin Chem. 2008;54:1648-56

Two probes identify many alleles

One probe identifies many alleles

Genotyping by Small Amplicon Melting
(dyes)



Clin Chem 50: 1156 – 64, 2004

High Resolution Melting

(2 min)

High Resolution Melting

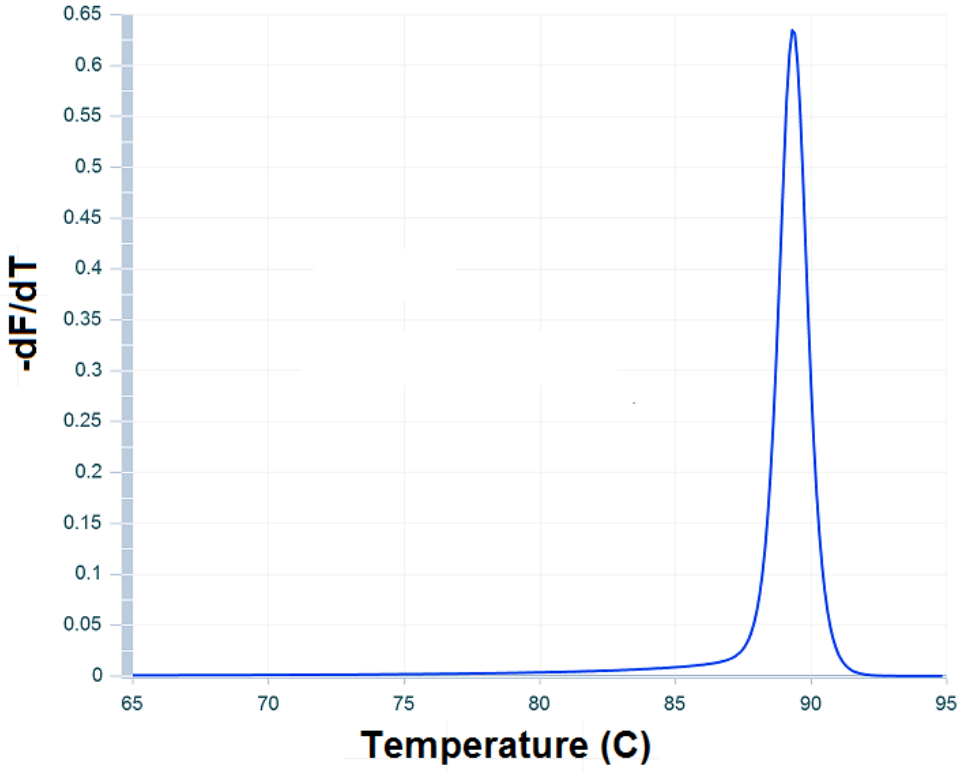
(Rates and Times)

Instrument	Recommended Setting	Measured Ramp Rate (°C/s)	Melting Time (min)
A	Step 0.04°C Hold 1 s	0.01	40
B	Ramp 0.1°C Hold 2 s	0.01	40
C	Step 0.2°C Hold 10 s	0.01	50
D	0.3% Ramp	0.005	95

Clin Chem. 2014 Jun;60(6):864-72

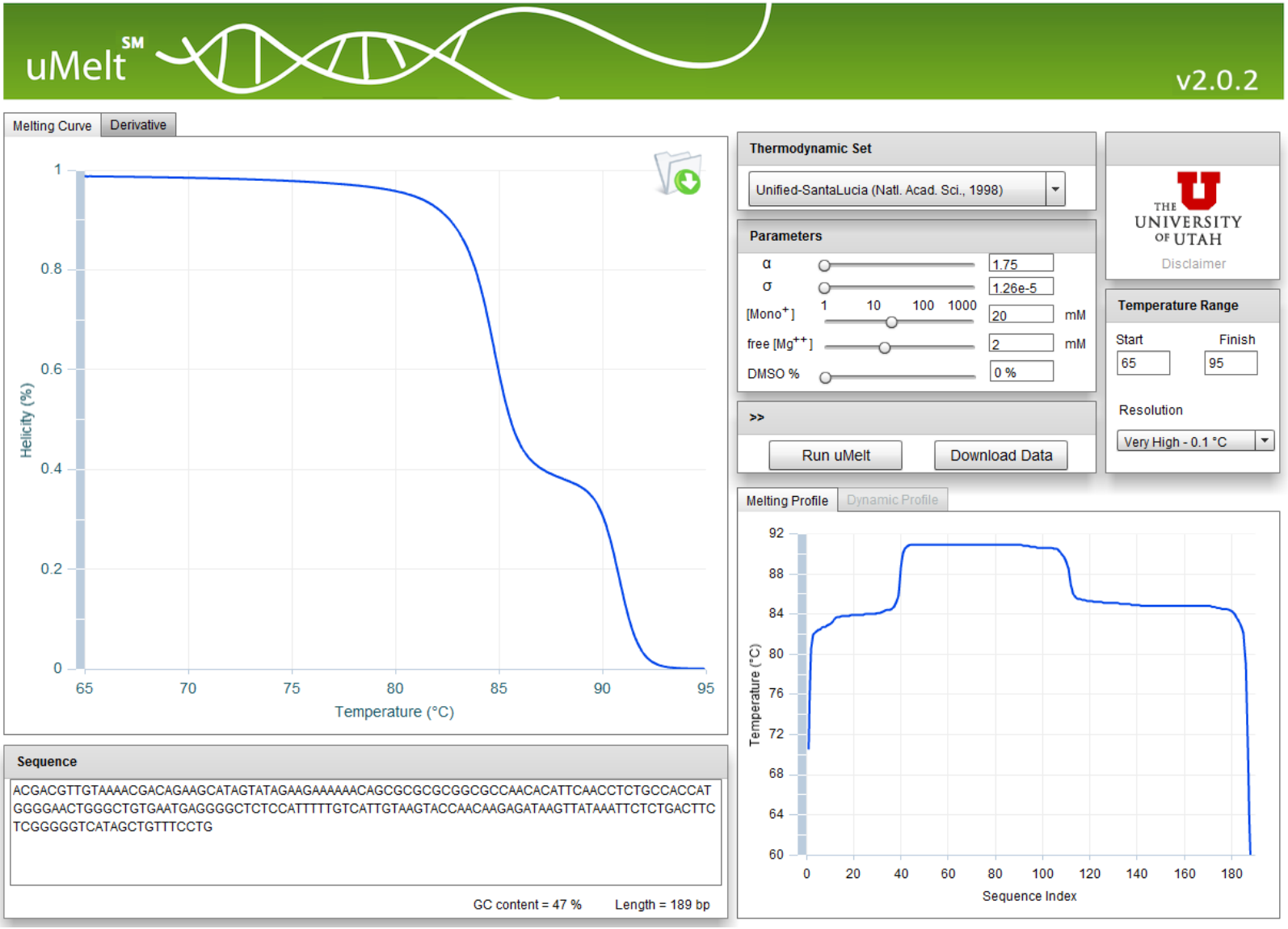
Amplicon Melting as PCR Quality Control

- Each PCR: single transition

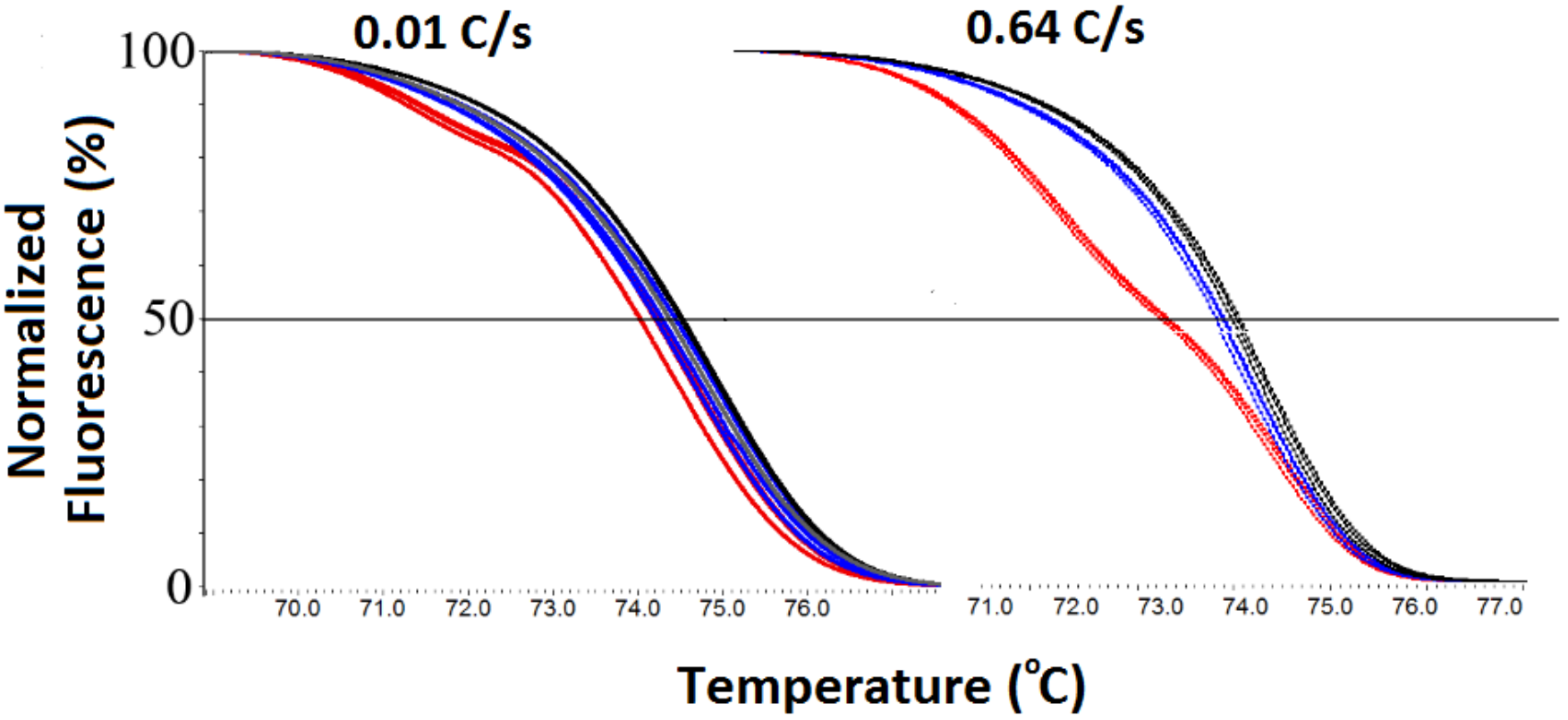


Melting Curve Prediction

(uMelt: dna.utah.edu)

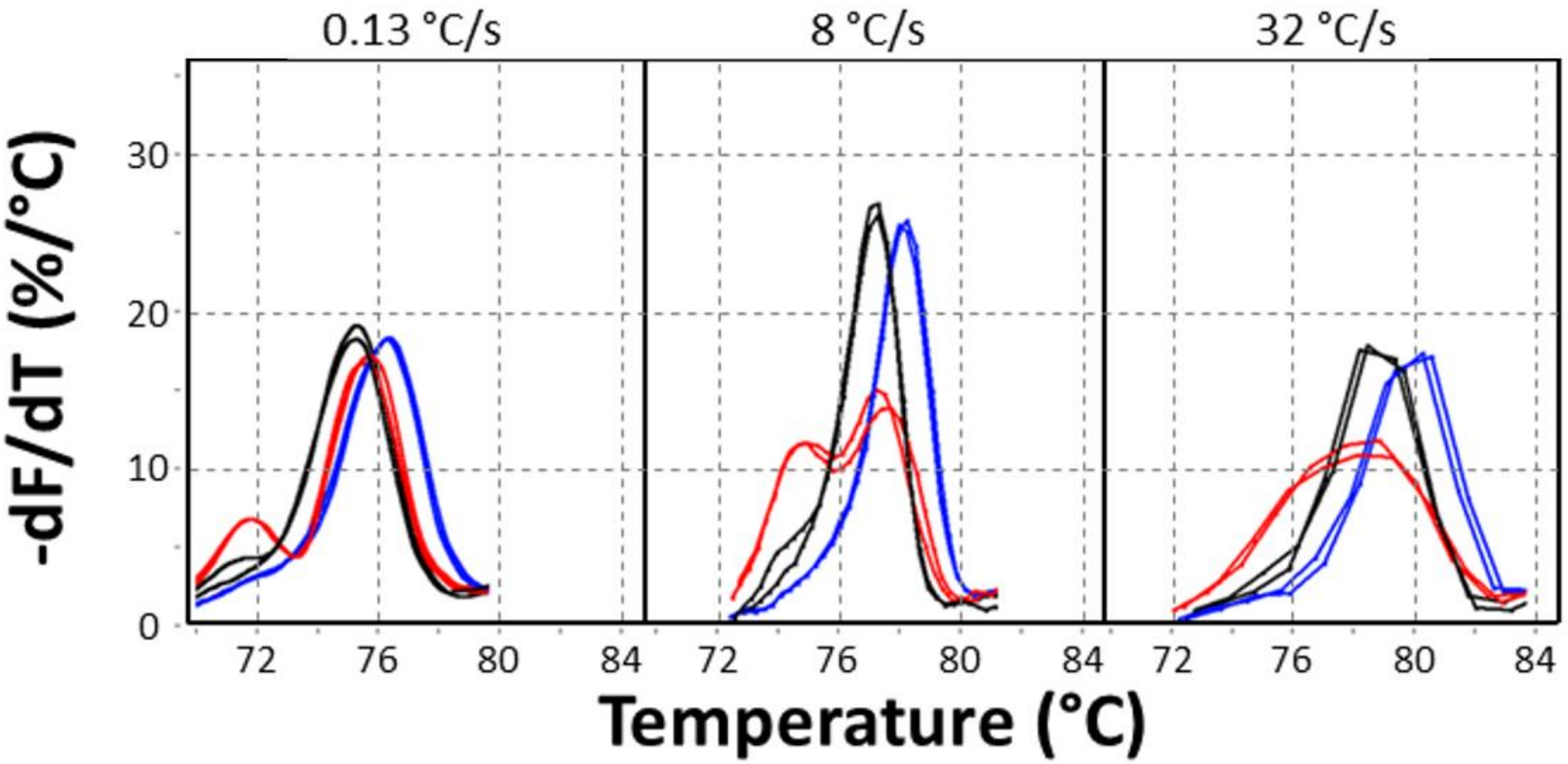


Faster SNV Melting Rates Improve Genotype Resolution



Anal Biochem 2017;539:90-95

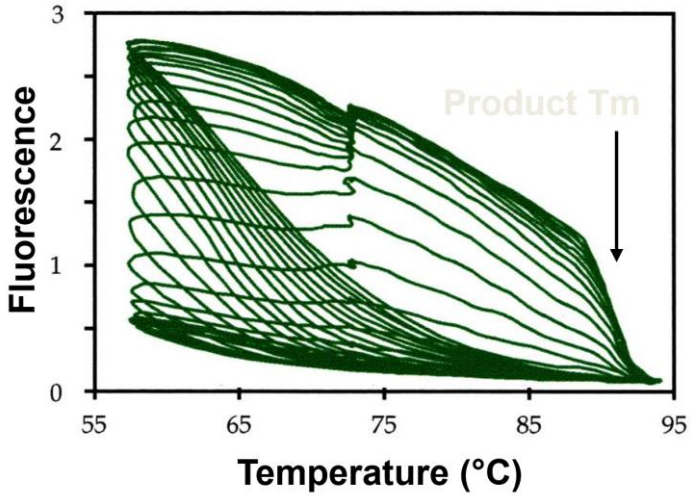
Microfluidic High Speed Melting



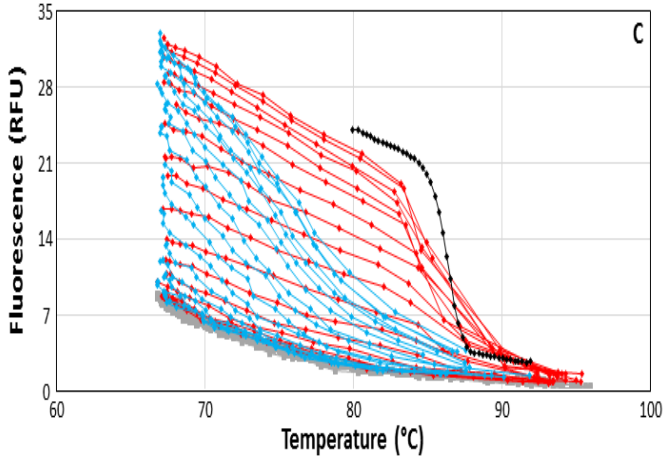
Clin Chem 2017;63:1624-32

Rapid Cycle vs Extreme PCR

1996 – Rapid Cycling
(28 seconds/cycle)



2018 - Microfluidics
(1.05 seconds/cycle)



Making Sample Preparation Faster

Nucleic Acid Preparation

- Depends on the matrix
  - Blood, chicken, anthrax, woolly mammoth
- Depends on the target
  - RNA, DNA
- Some sample types require no purification
  - Swabs (respiratory/pharyngeal)
  - Thermal cycling only

Genomic DNA from Blood

- DNA release from histones
  - Chaotropes
  - Enzymes
- 30 min – 2 hours
  - Most manual kits
  - Most automated systems
- 15 min
  - Single tube digestion
  - Temperature control

DNA Extraction from Blood with NaOH

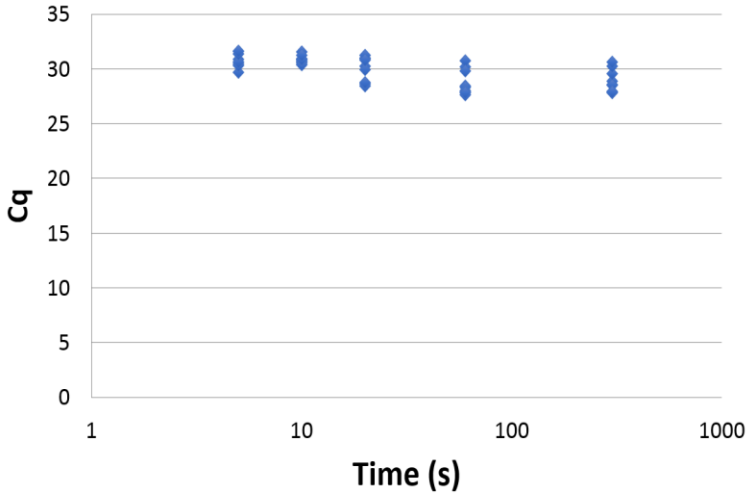
(lye for lysis)

Rapid, Simple Alkaline Extraction of Human Genomic DNA from Whole Blood, Buccal Epithelial Cells, Semen and Forensic Stains for PCR

BioTechniques 25:588-592 (October 1998)

Quantitative DNA release from blood with NaOH

Fast

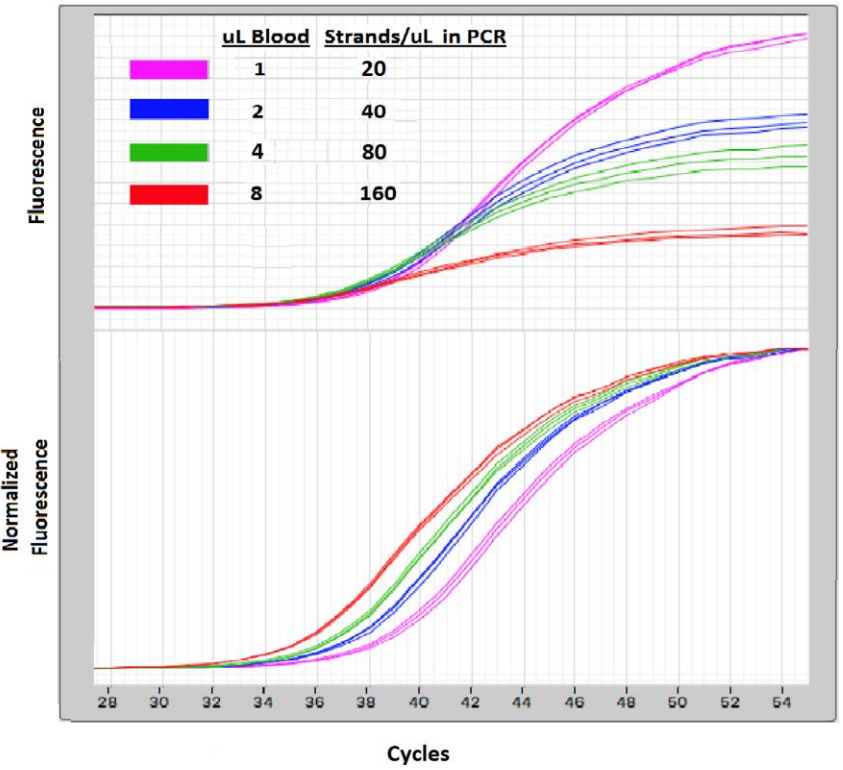


Complete

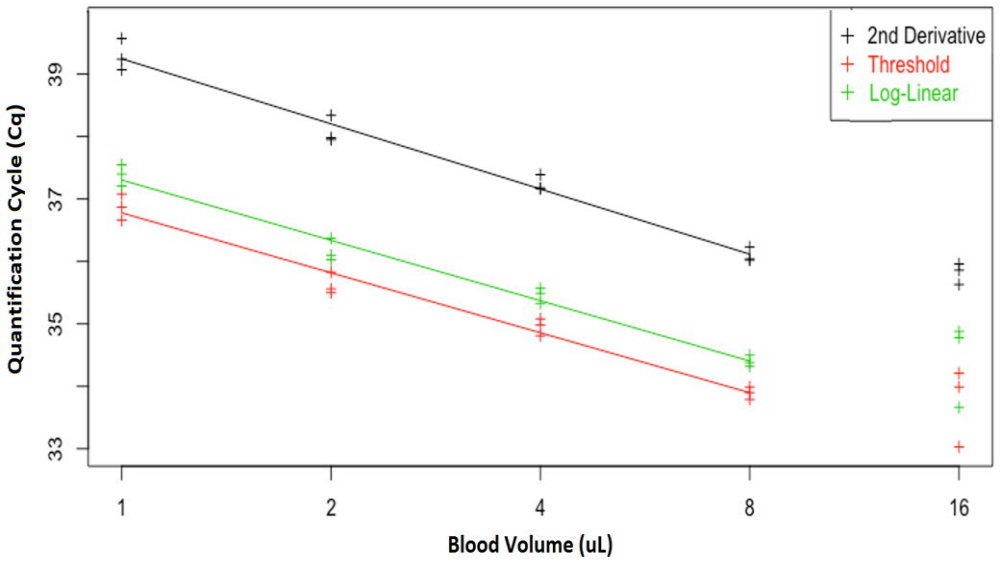
- Limiting dilution analysis
  - WBC
    - 0.2 cells/well = 0.8 strands/well
  - 58/96 wells positive
    - 0.93 strands/well
  - 115% recovery
  - 84 – 146% recovery (95% confidence)

Real-time monitoring of NaOH-treated whole blood

Inhibition of fluorescence
with constant efficiency

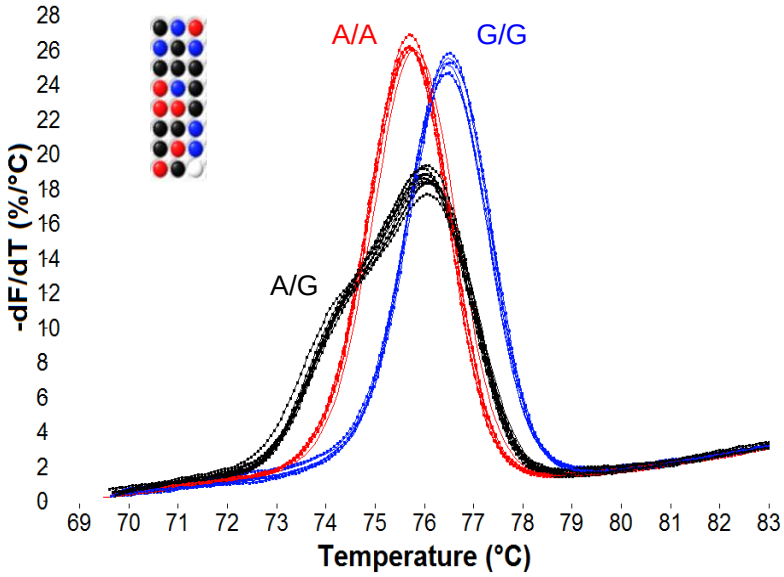


Eventual inhibition of efficiency



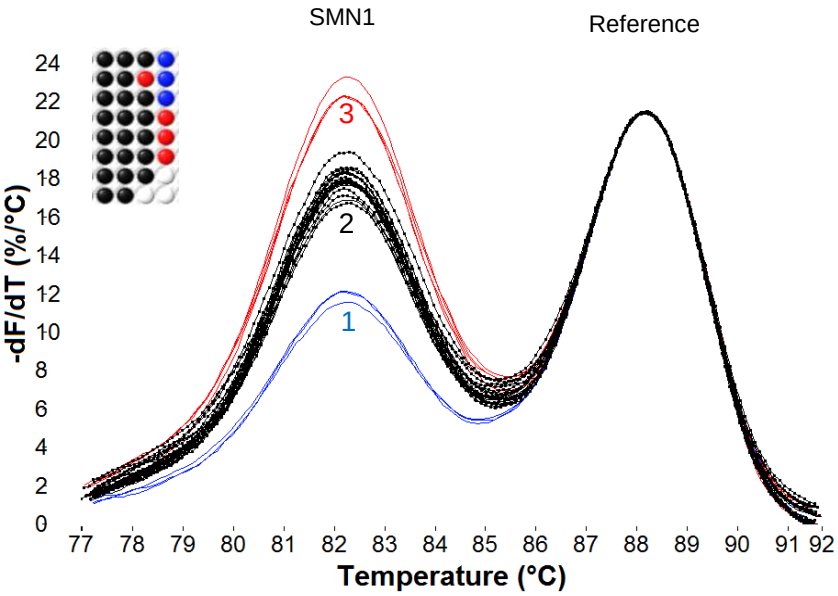
Melting analysis from NaOH-lysed whole blood

Small Amplicon Genotyping
(rs1024116)



Clin Chem **50**:1156-64;2004

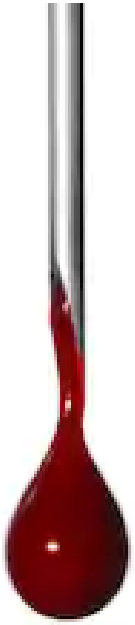
Copy Number
(SMA – spinal muscular atrophy)



Clin Chem **61**:724-33;2015

Clinical lab tests from a single drop of blood

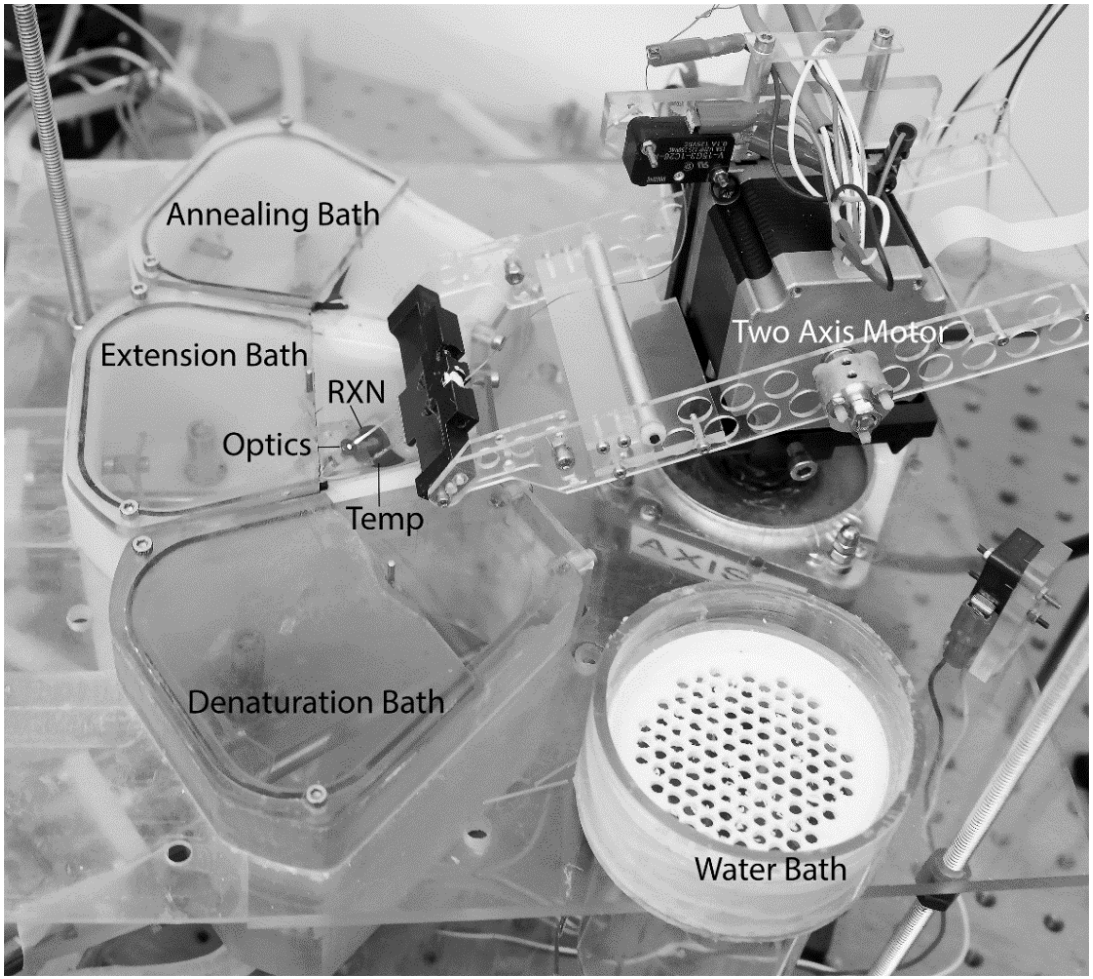
Blood drop = 46 +/- 5 µL



- 5,000 WBC/µL
- 20,000 PCR templates/µL
- 25-fold dilution in NaOH
  - 800 templates/µL
- 10-fold dilution into PCR
  - 80 templates/µL
- Five µL PCR
  - 400 templates

Can we go from a finger prick to real-time detection in < 1 min?

- Human blood
- Single copy gene



Testing Times

(from the physician/patient viewpoint)

	Reference Labs	Point-of-Care
Pre-analytical	>12 hours	Fast!
Analytical	(varies)	(varies)
Post-analytical	~8 hours	Fast!

- Point of care eliminates most pre- and post analytical steps
- Rapid testing has limited value for reference labs
- Rapid testing is critical for point-of-care value

Summary

- Extreme PCR
  - Increase speed 200X
  - Efficient, sensitive, and specific
- High Speed Melting
  - Increase 100-1000X over conventional melting
- Extreme sample preparation
  - In seconds

- Faster is better (PCR and melting)
- Chemicals and enzymes are fast, people and their machines are slow

Thanks!

BioFire / bioMerieux

- Kirk Ririe
- Randy Rasmussen

NIH

ARUP

Roche Applied Science

Canon

State of Utah

University of Utah

- Mark Herrmann
- Jared Farrar
- Luming Zhou
- Rob Pryor
- Adam Millington
- Felix Ye

Website: https://www.dna.utah.edu