"Forensic and Single-Molecule Assays of Mitochondrial DNA Using LATE-PCR"

Co-Investigators

Cristina Hartshorn, Ph.D.

Kenneth Pierce, Ph.D.

Arthur Reis, Ph.D.

John Rice, M.S.

Jesse Salk, B.A.

J. Aquiles Sanchez, Ph.D.

Lawrence J. Wangh, Ph.D.

Laboratory of Human Genetics and Reproductive Biology
Brandeis University, Waltham, Massachusetts

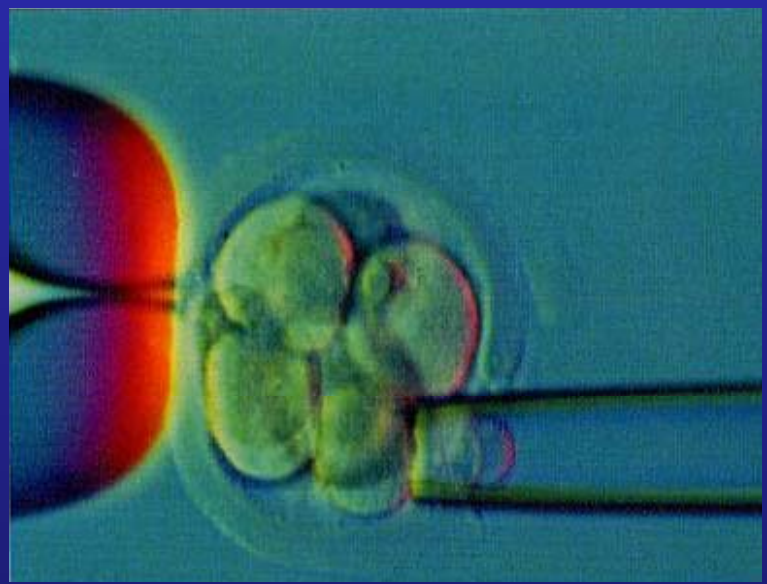
qPCR 2005, Munich, Germany, September 5 –9, 2005

Forensic Challenges

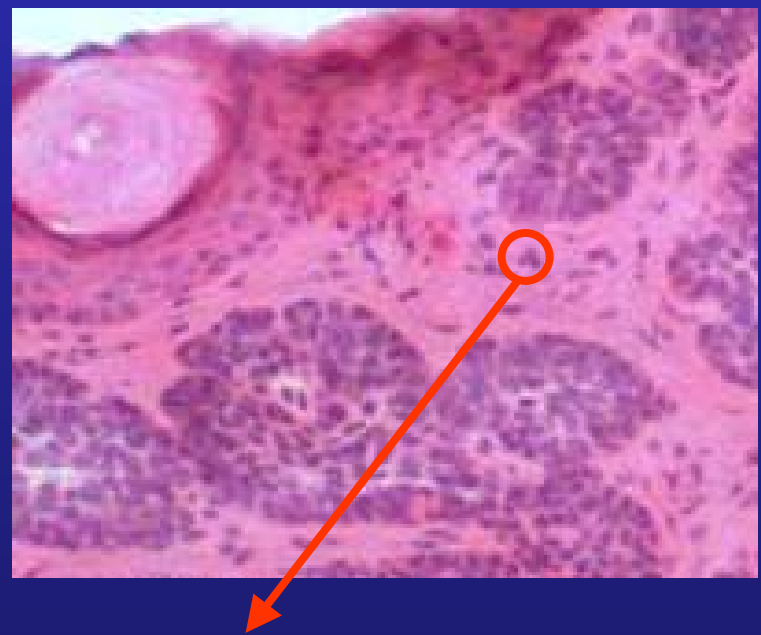
- Accuracy of Analysis
- Time: Sample to Result
- Identification/Match
- Small Sample Size
- Contamination
- Cost

The Problem of Small Sample Size is Wide Spread

Pre-Implantation Genetic Diagnosis



Tumor Cancer Diagnosis



Excision By Laser Micro-Dissection

JonBenet Ramsey Murder: 1996

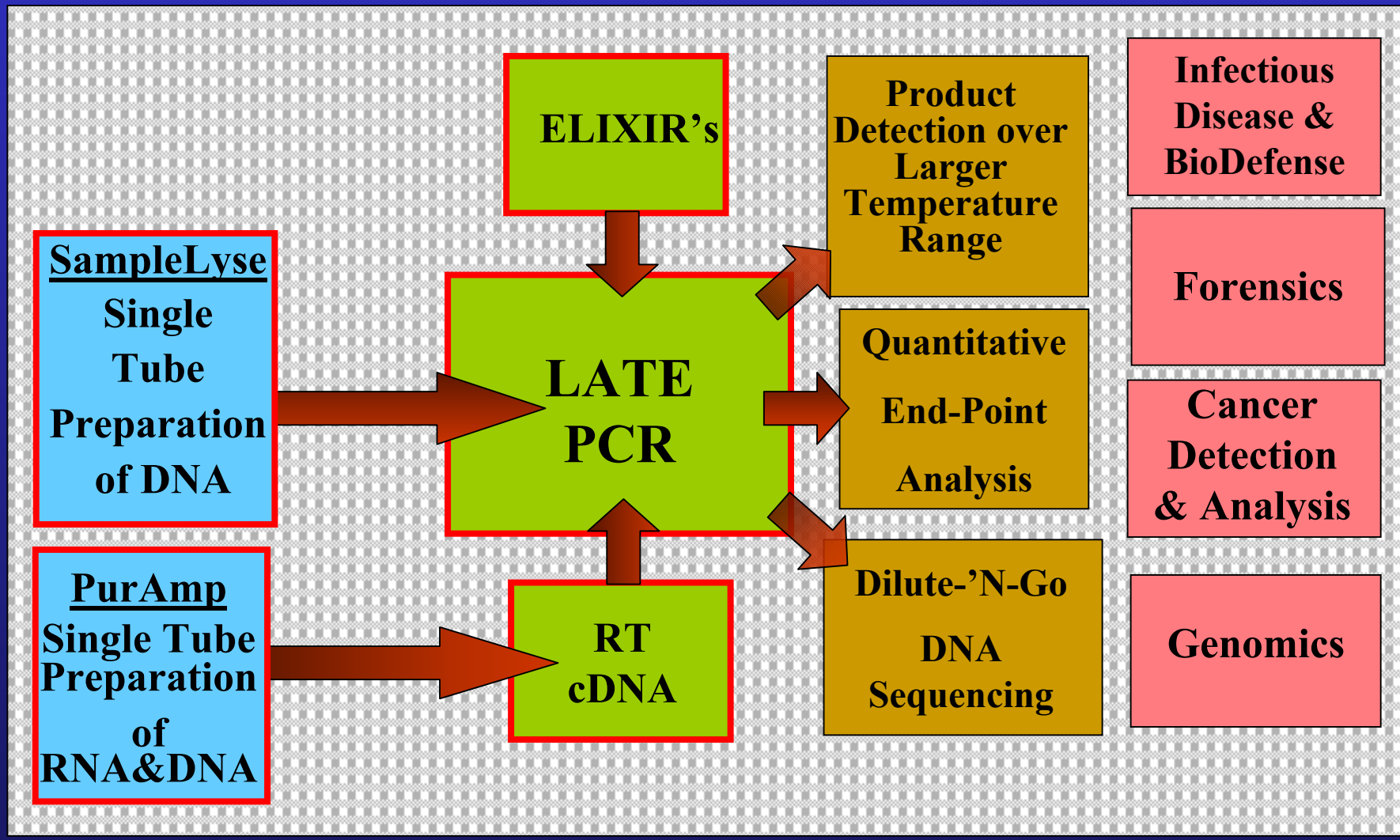
Pan Am Flight 103: 1988

Small Samples of Forensic Interest

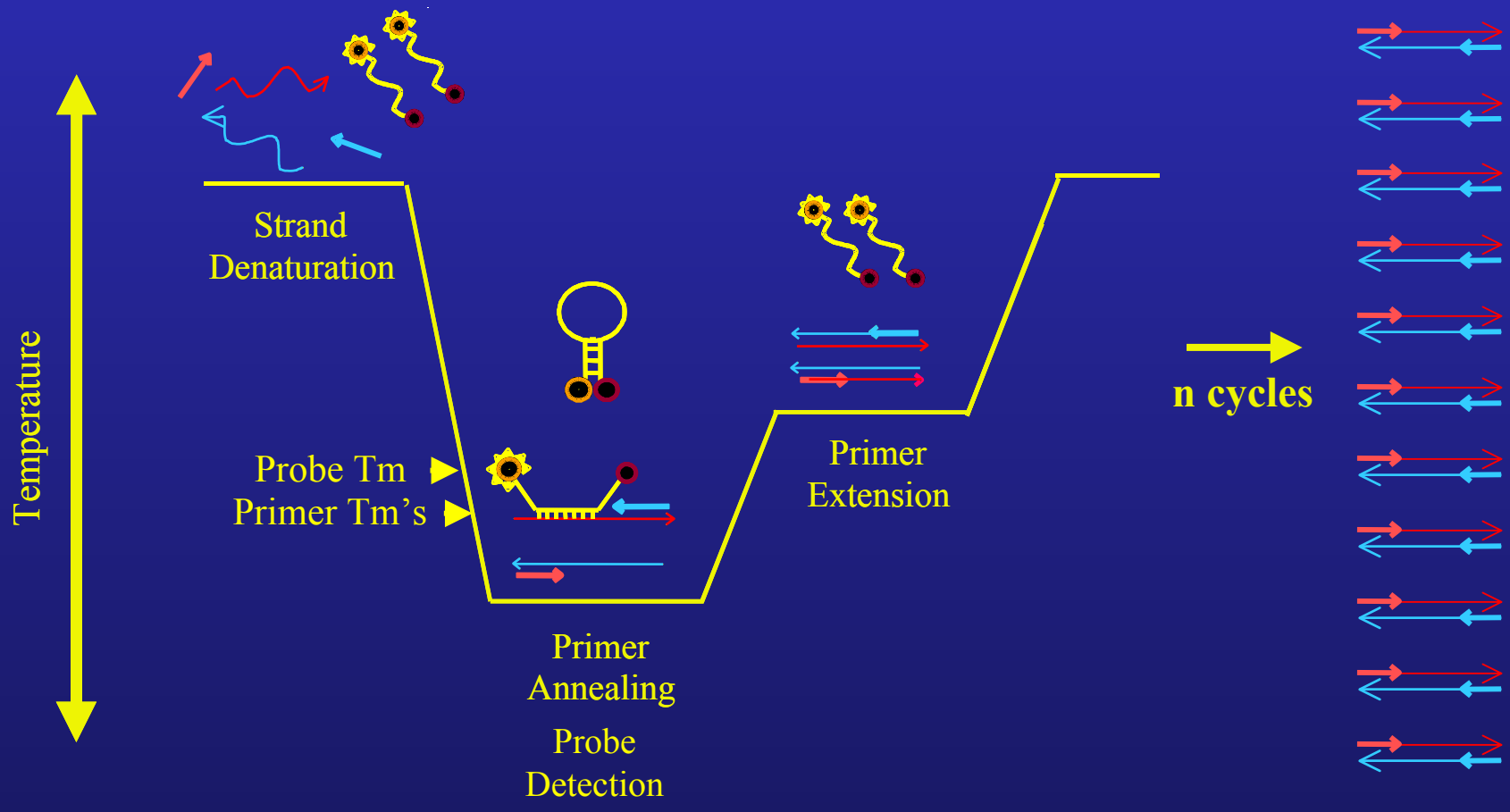
- Small Sections of Human Hair Shafts
- Single, Partial or Smudged Human Fingerprints
- Sperm
- Bone and Teeth Fragments
- Single Skin Cells

Our New Paradigm

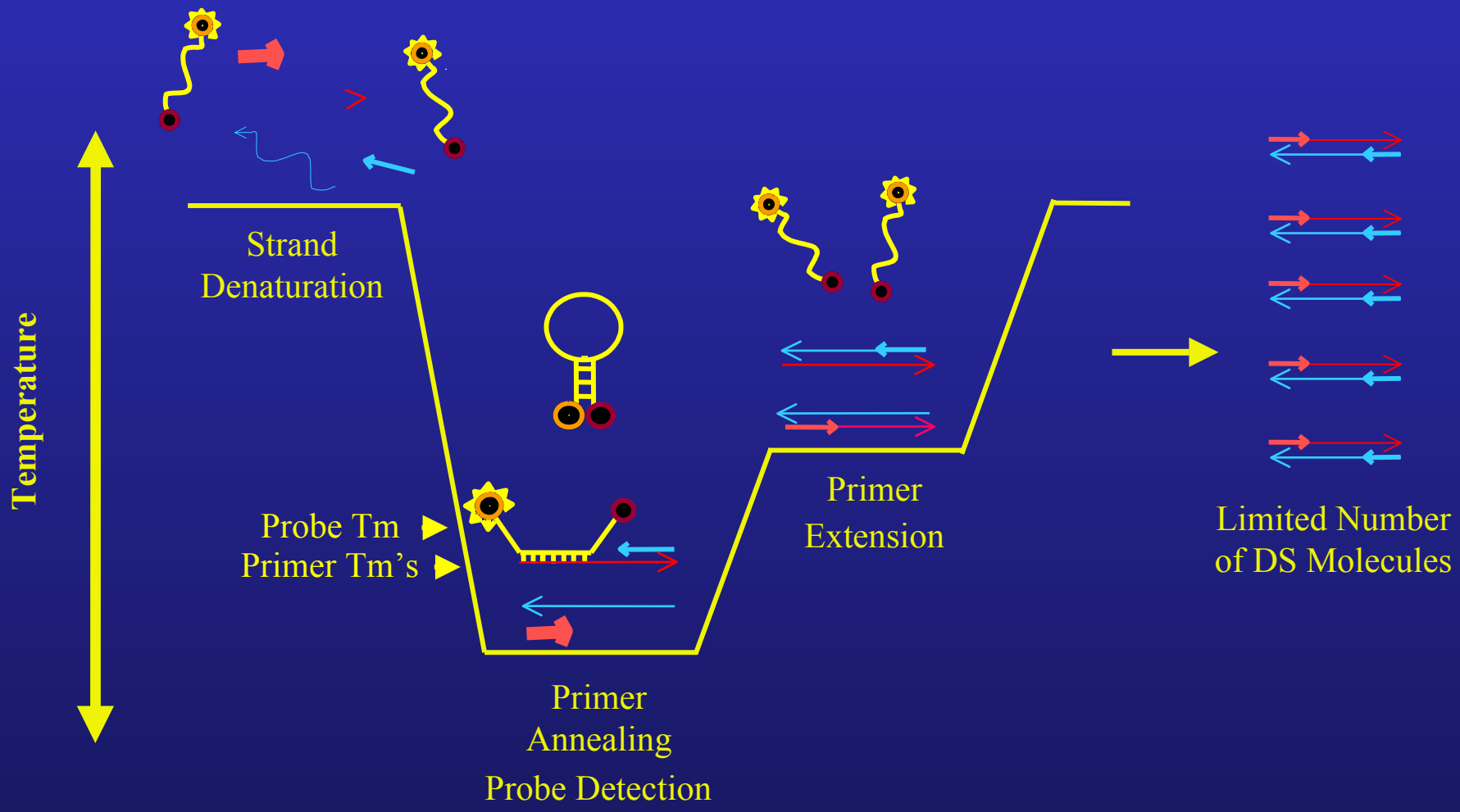
Sample Preparation | Reaction Methods | Product Analysis | Applications



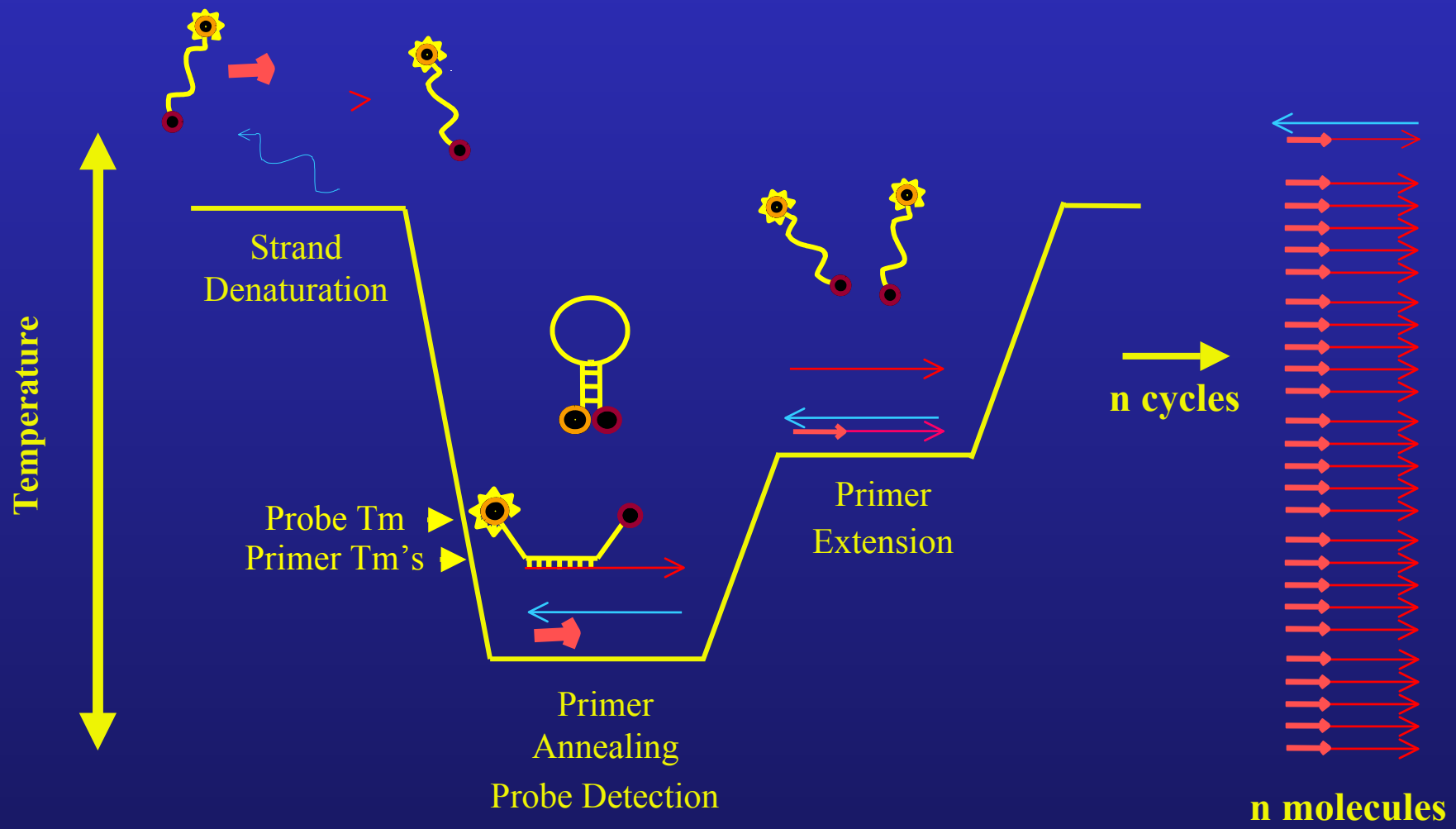
Symmetric PCR uses EquiMolar Primers with Similar Tm's: Yields dsDNA Exponentially



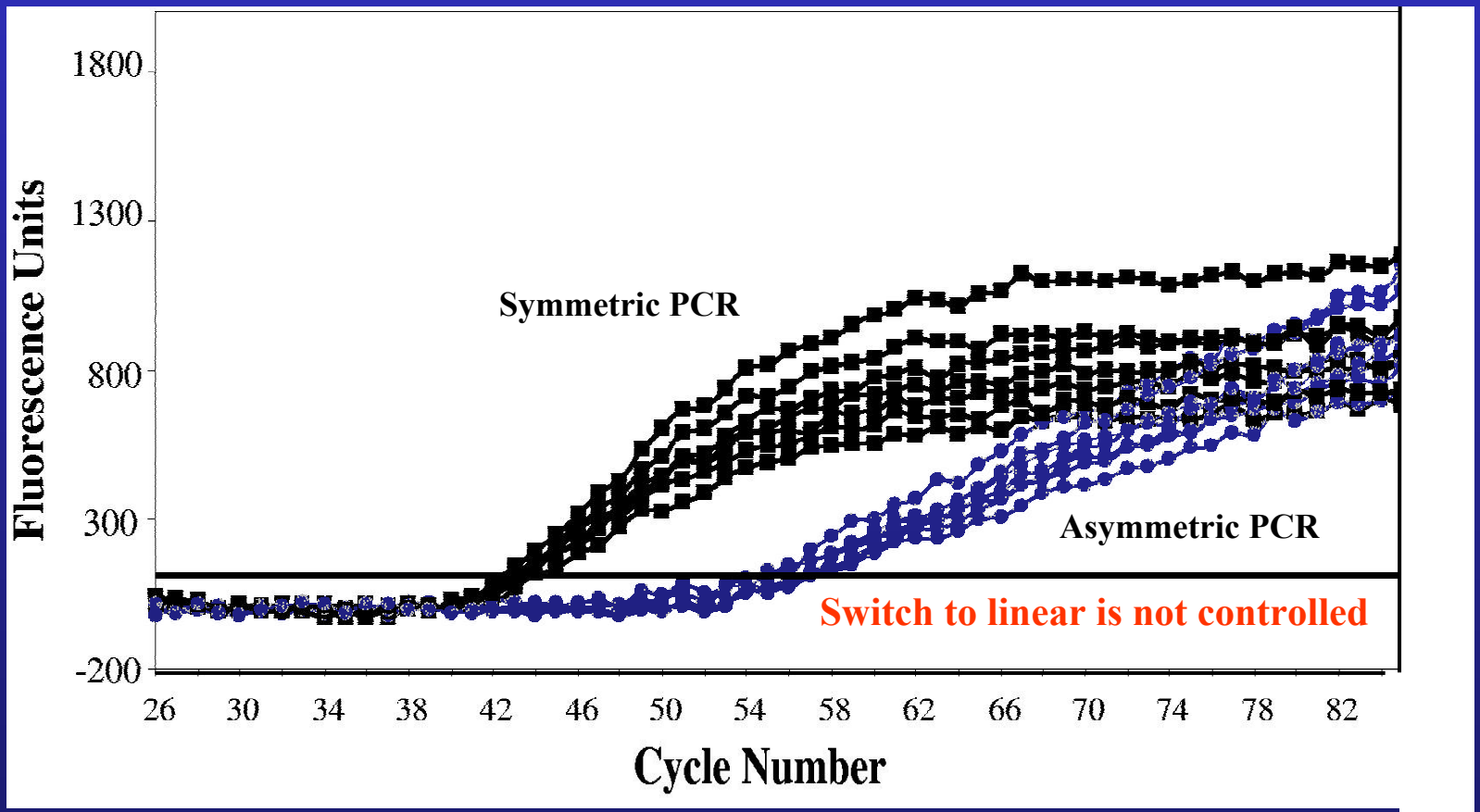
Asymmetric PCR uses nonEquiMolar Primers phase I: Yields dsDNA Exponentially



Asymmetric PCR uses nonEquiMolar Primers phase II: Yields Single Stranded DNA Linearly

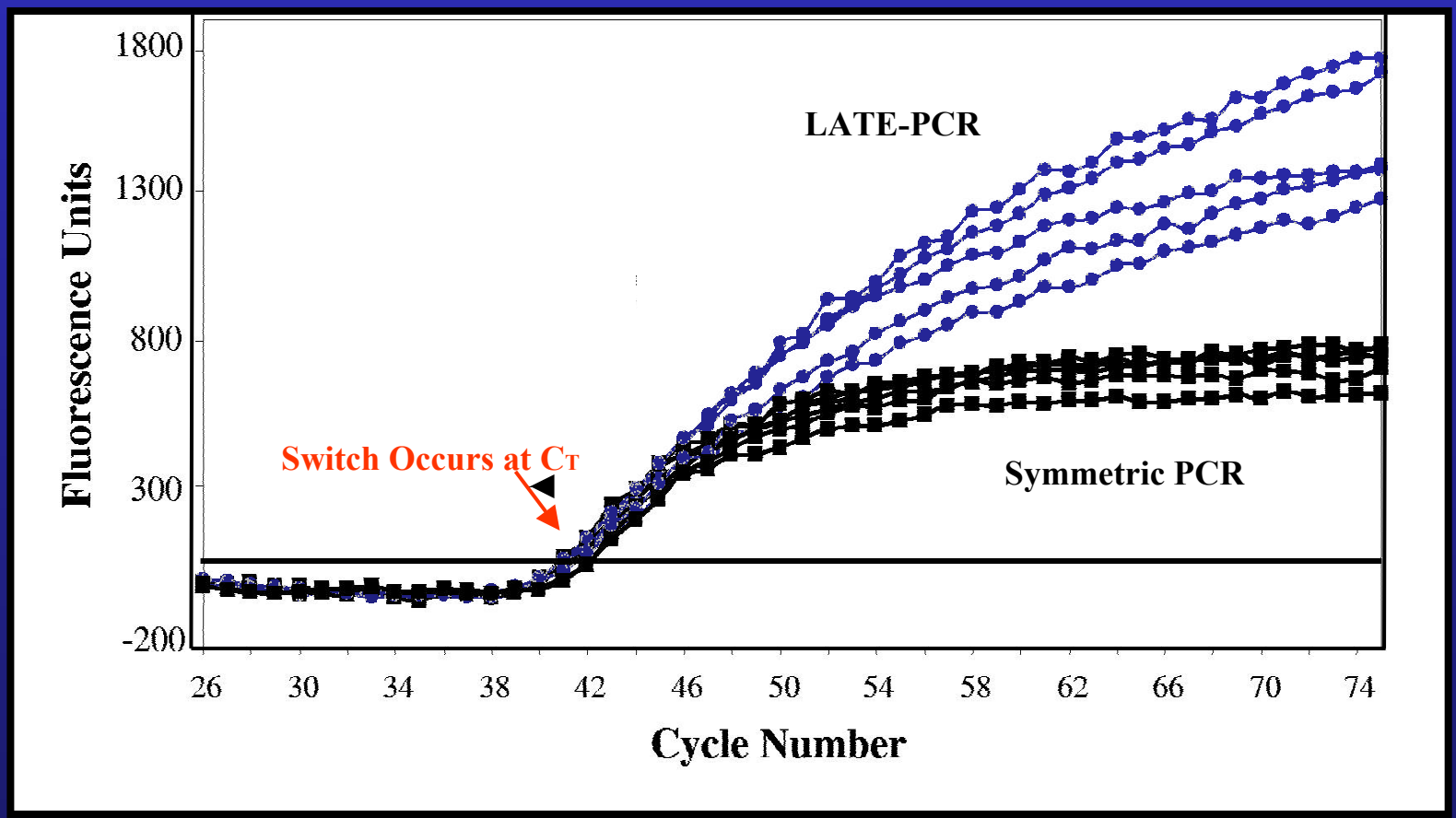


Conventional asymmetric PCR is often inefficient



Sanchez *et al.* (2004) PNAS 101:1933-1938

LATE-PCR is as Efficient as Symmetric PCR



Sanchez *et al.* (2004) PNAS 101:1933-1938

LATE-PCR: Axiom 1 $(Tm^L - Tm^X) \geq 0$

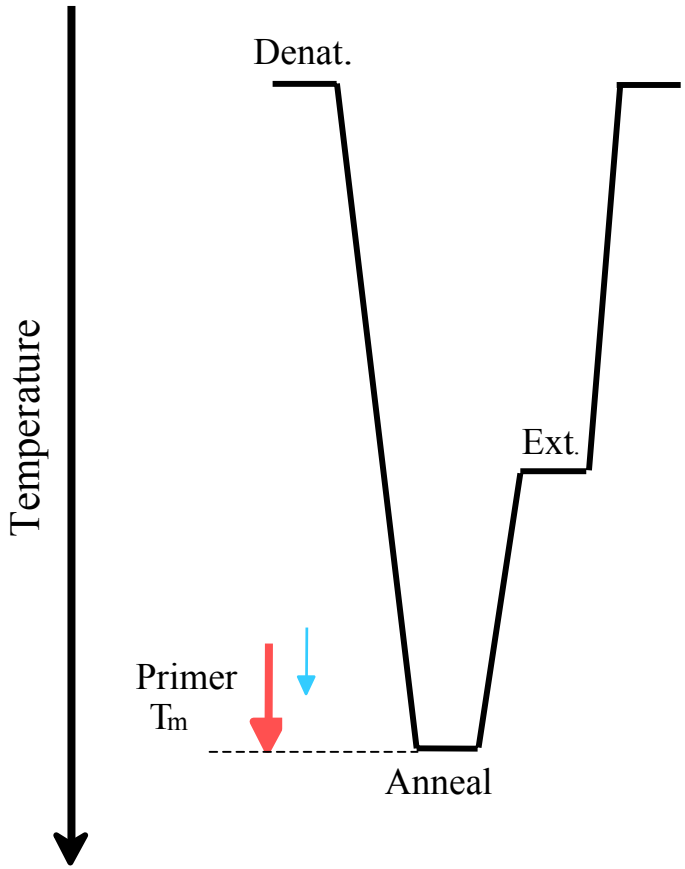
$$Tm = \Delta H/(\Delta S + R \ln (C/2)) - 273.15 + 12 \log [M]$$

LATE-PCR

Modifies Limiting Primer So That Limiting Primer T_m Is Above Excess Primer T_m

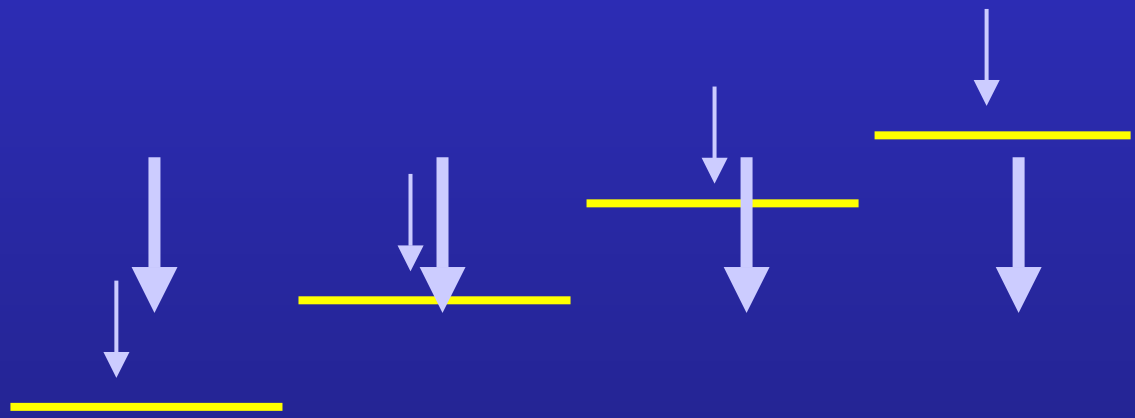
$$(T_m^L - T_m^X) \geq 0$$

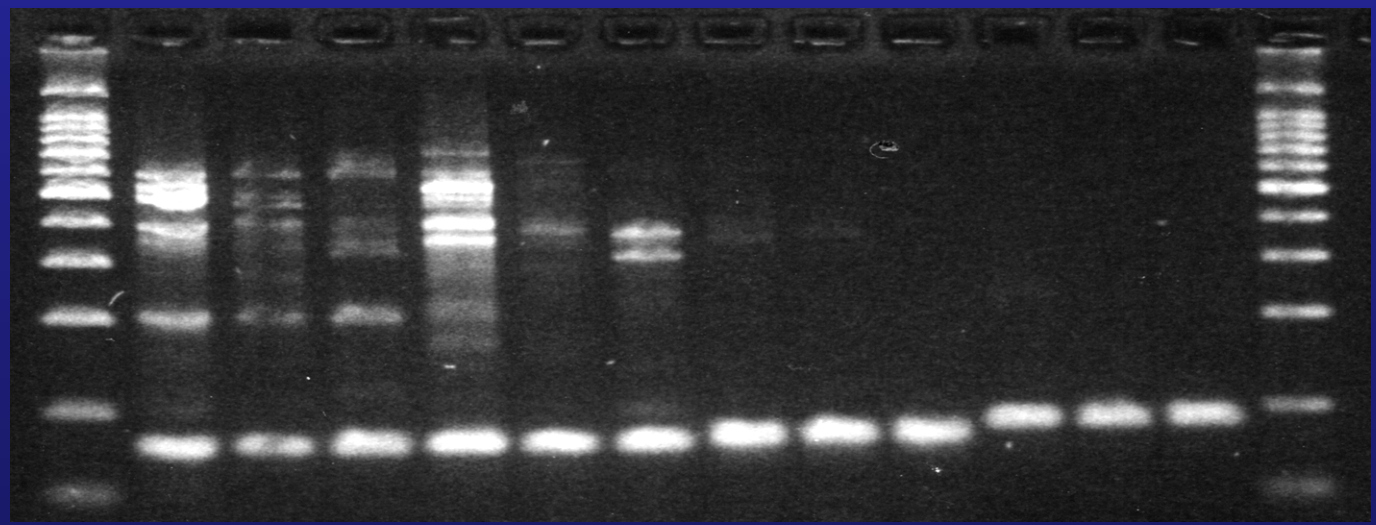
Efficient!



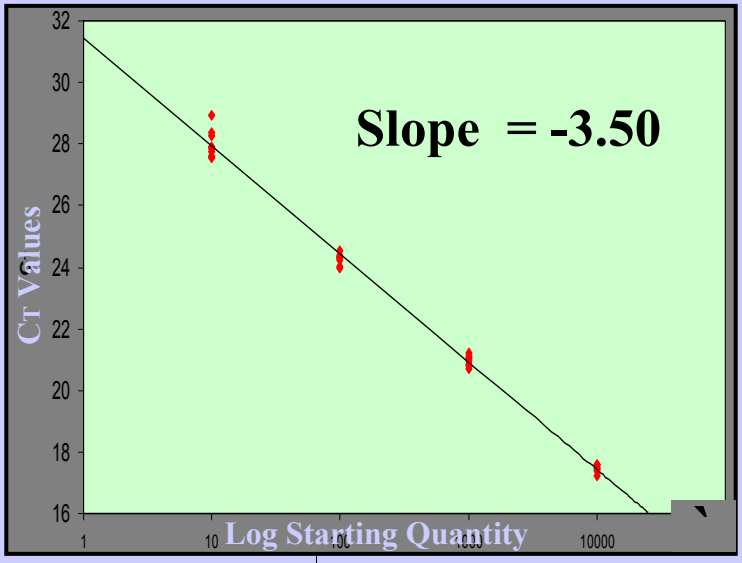
Sanchez *et al.* (2004) PNAS 101:1933-1938

LATE PCR Makes it Easier to Avoid Mispriming without Reducing Amplification Efficiency





Pierce *et al.*, PNAS, 102, 8609-8614 (2005)



10,000 Genomes

1,000 Genomes

100 Genomes

10 Genomes

Fluorescence

2000

1500

1000

500

0

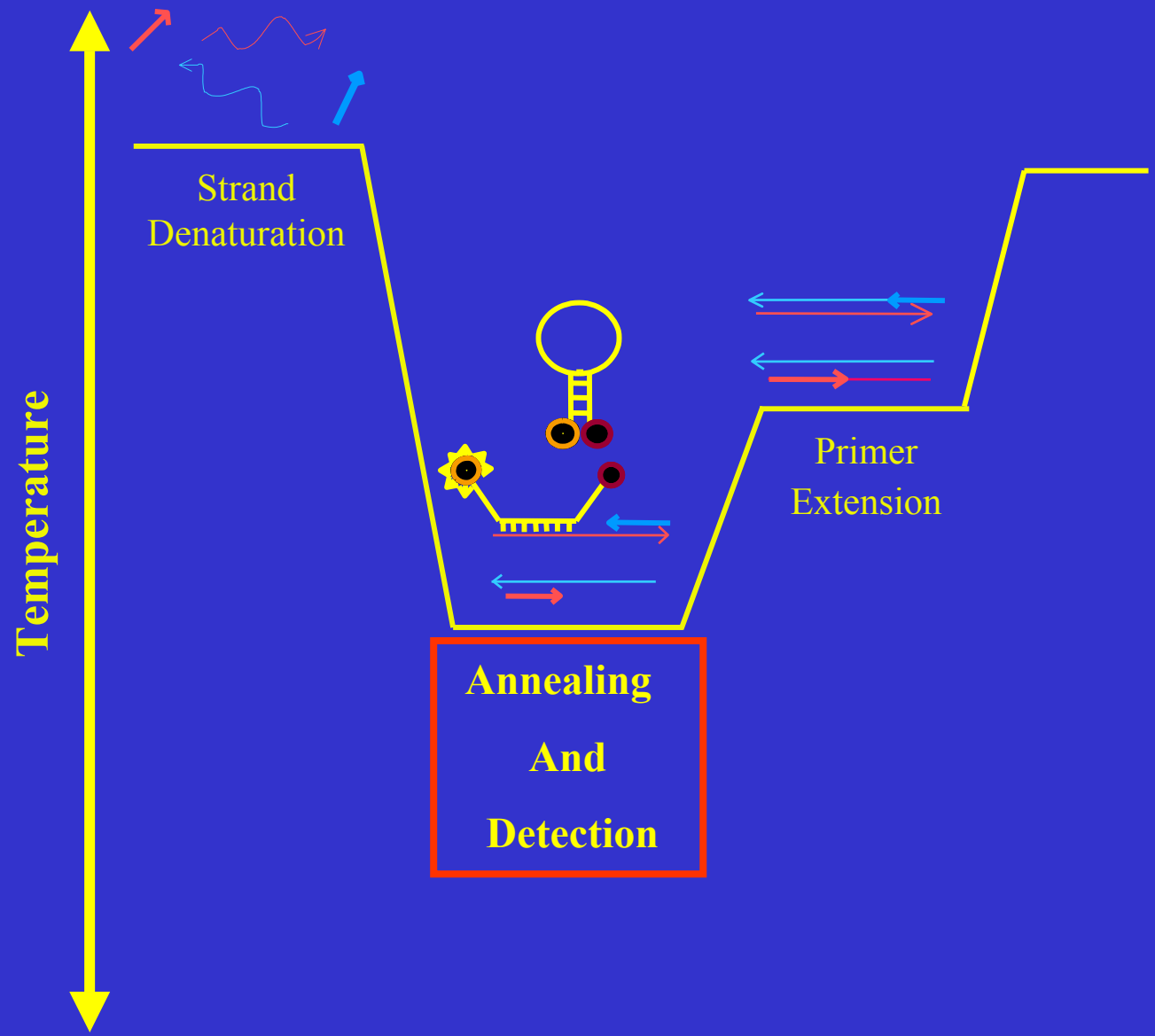
Efficient Initial Amplification

C_T Values

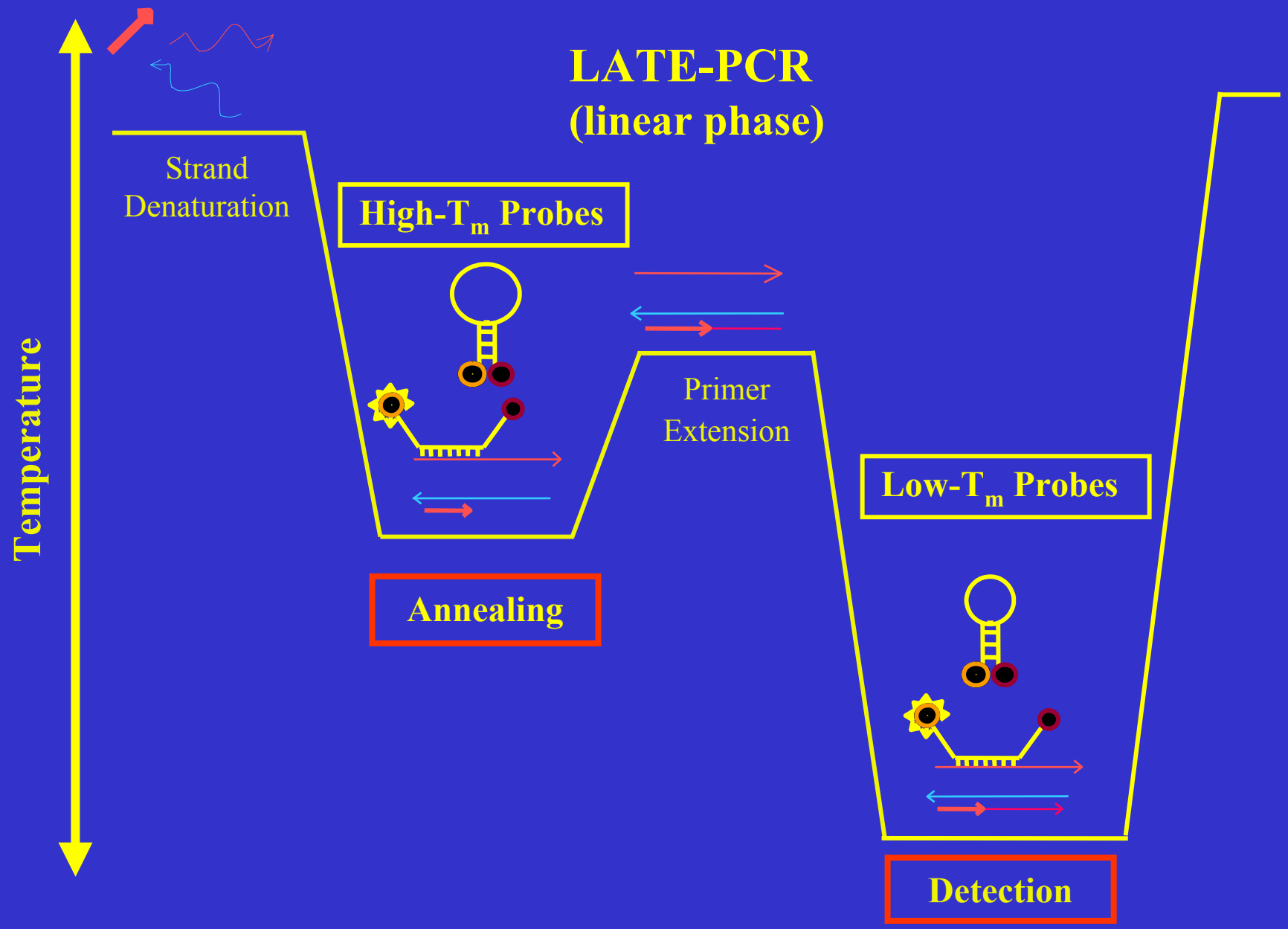
20 30 40 50 60 70 80

Cycle Number

Symmetric PCR: Annealing and Detection In One Step



LATE-PCR Uncouples Annealing and Detection



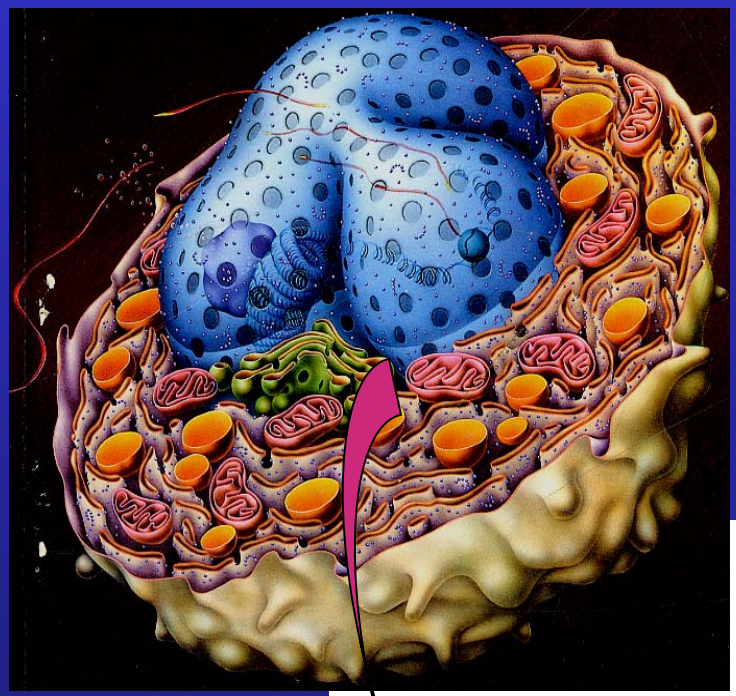
LATE-PCR APPLICATIONS

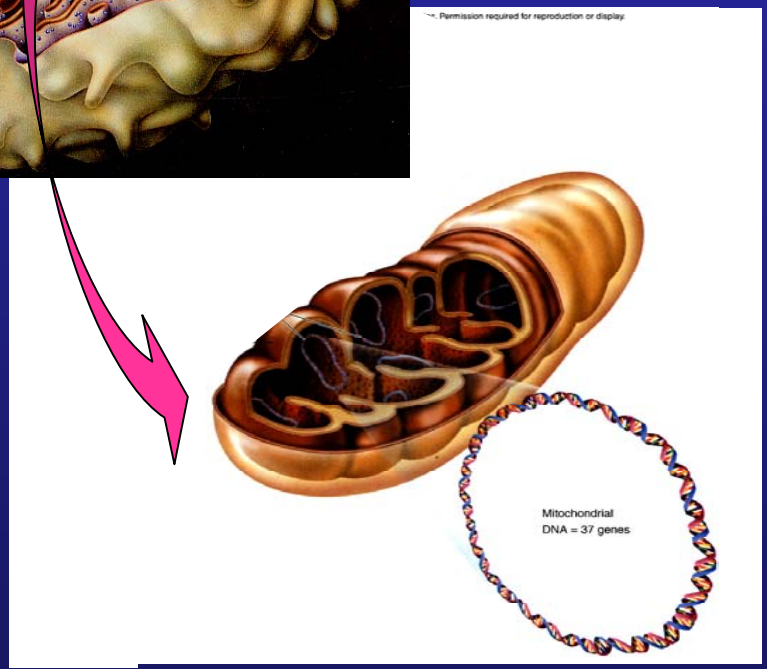
Forensic Assays

Mitochondrial DNA

Dilute-‘N-Go Sequencing

100 - 1,000 Mitochondria per Cell and 1 - 10 Genomes per Mitochondrion





Features of Mitochondrial DNA

No crossing over

No DNA repair

Maternal inheritance

Many copies per mitochondrion and per cell

High exposure to oxygen free radicals

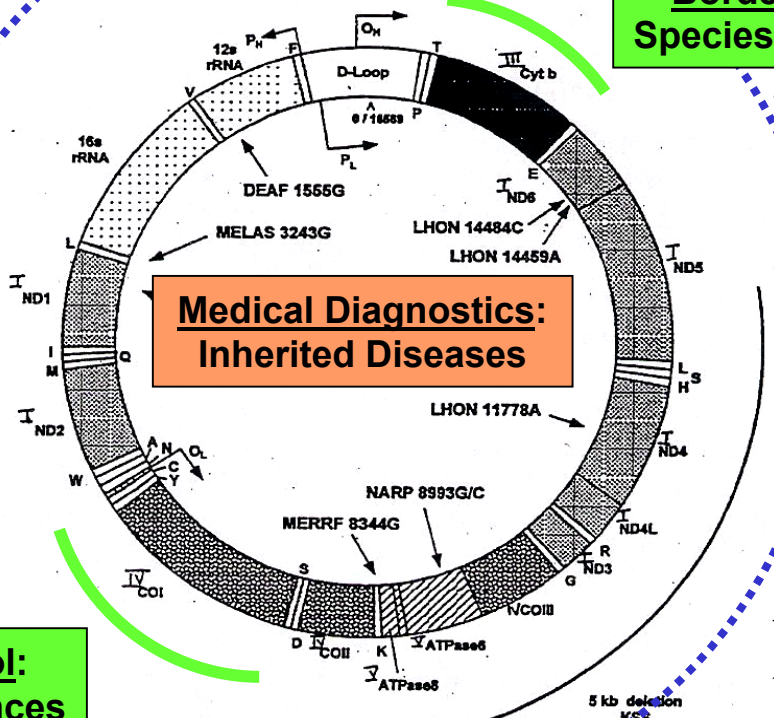
No histones

No introns

The Frequency of Sequence Differences Varies Across the Genome

Forensics: Differences Among Individuals

Border Control: Species Differences



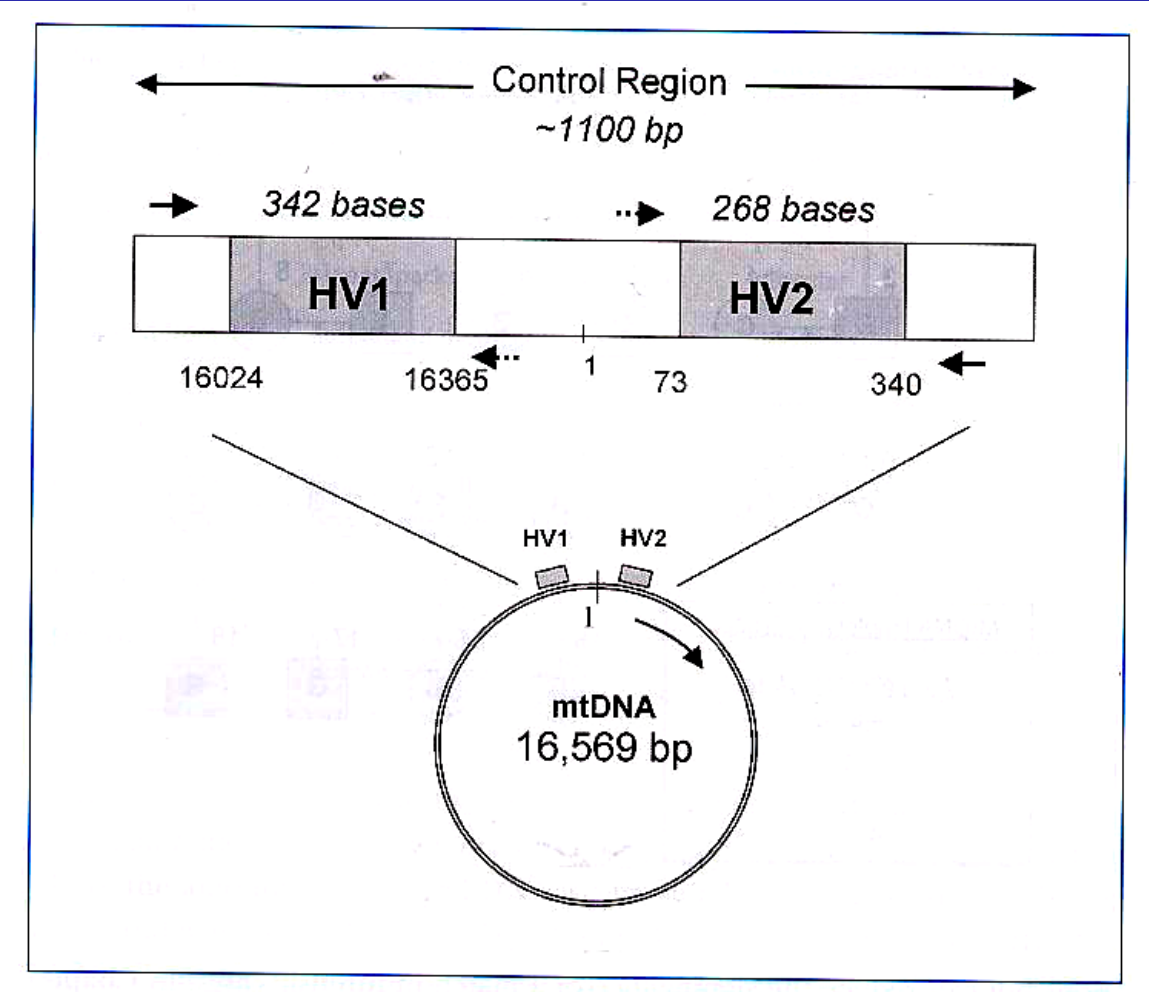
Medical Diagnostics: Inherited Diseases

Border Control: Species Differences

Health Care: Aging and Cancer, changes within a lifetime

Forensic identification of Individuals is based on the Control Region

There are >250 Known Sequences Differences within both HV1 and HV2
The population frequencies of many of these variants are known.



Our Strategy

- **Four Monoplex Assays that Expand Forensic Regions:**
  HV 1 H & L : 15910-16458 (549 vs. 342 bases)
  HV 2 H & L : 7-470 (464 vs. 268 bases)

. **Sample to Sequence in Fewest Number of Steps**

. **Begin with Small Sample Sizes**

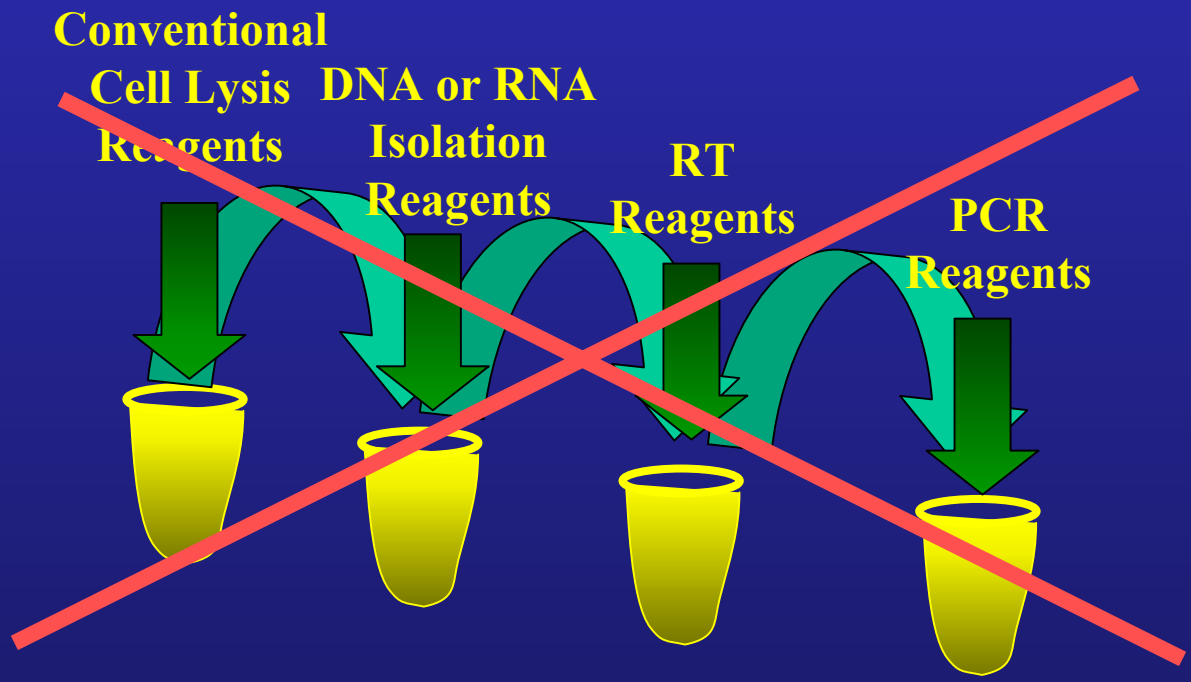
. **Heteroplasmy at Single Cell Resolution**

Summary of mt DNA Experiments

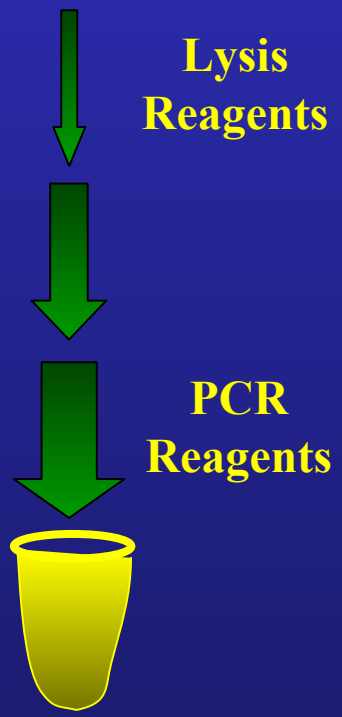
- Human Blood Lymphocytes (HBL)
- Human 10 and 5 mm Hair Shafts
- Single Human Fingerprints
- Single HBL mtDNA Molecules

Single-Tube Sample Preparation and Amplification

Conventional Approach



The Solution

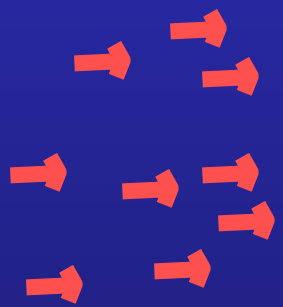


Done!

Cleanup of LATE-PCR Products by Dilution

0.1

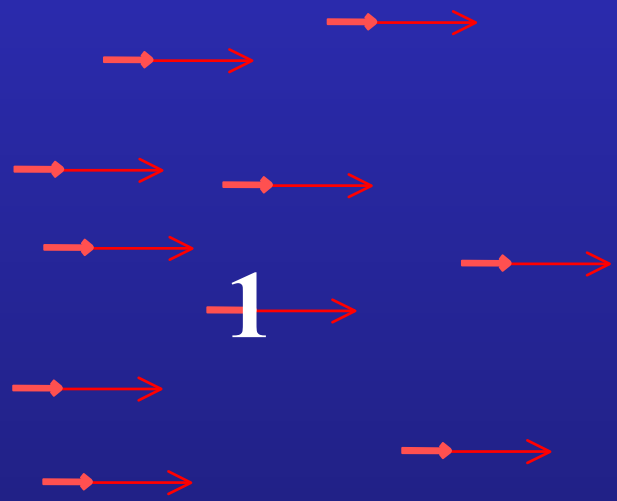
dsDNA
molecules



Unincorporated
Excess Primer

dNTP

Unincorporated
dNTPs



1

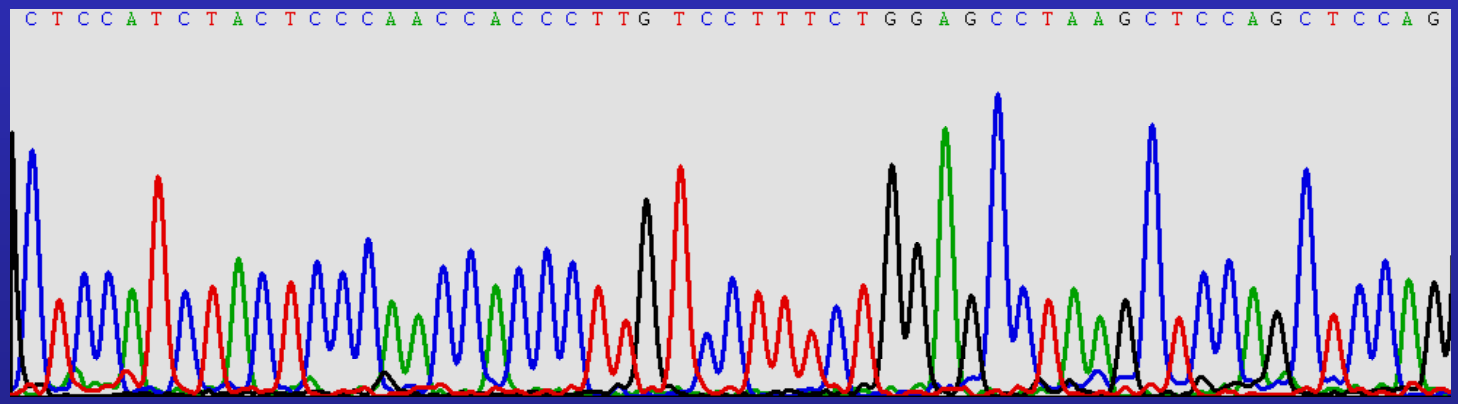
ssDNA
molecules

Sequencing of the p53 Gene Amplified with LATE-PCR From Single-Cells

Prep Time

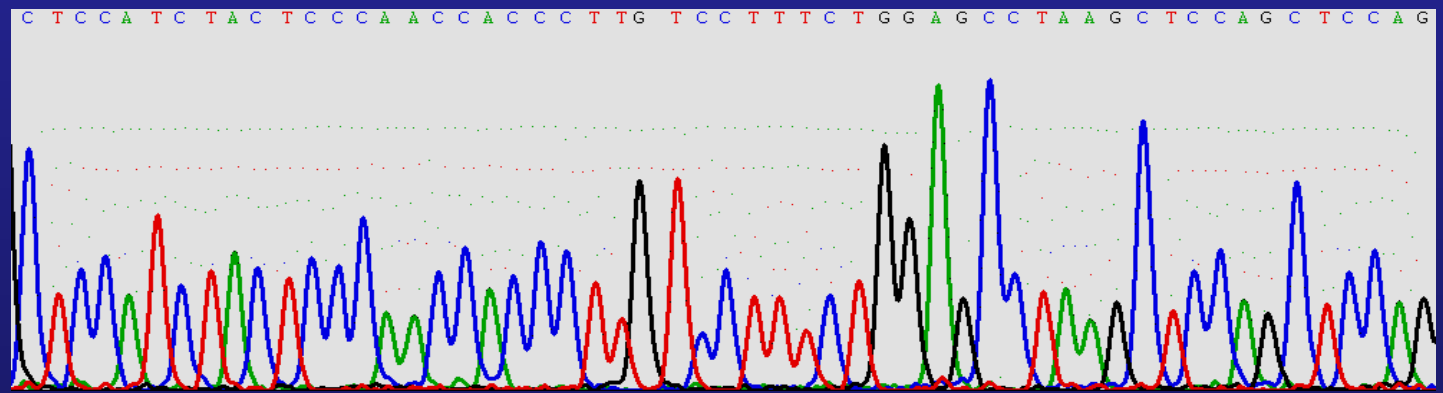
Seconds

After LATE-PCR and Direct Dilution into Cycle-sequencing Mix



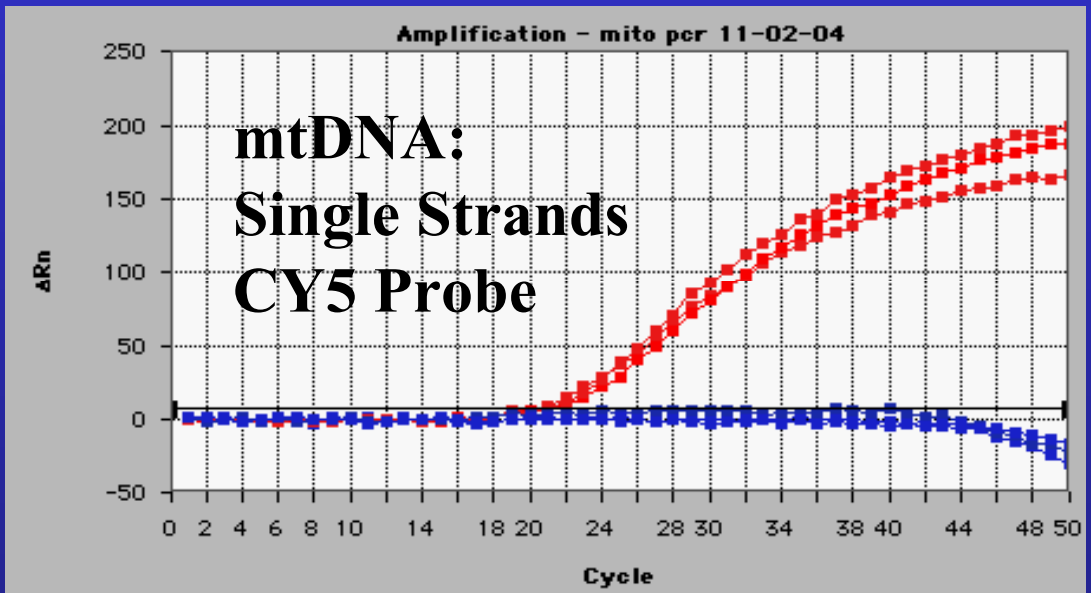
After Symmetric PCR and Conventional Preparation Methods

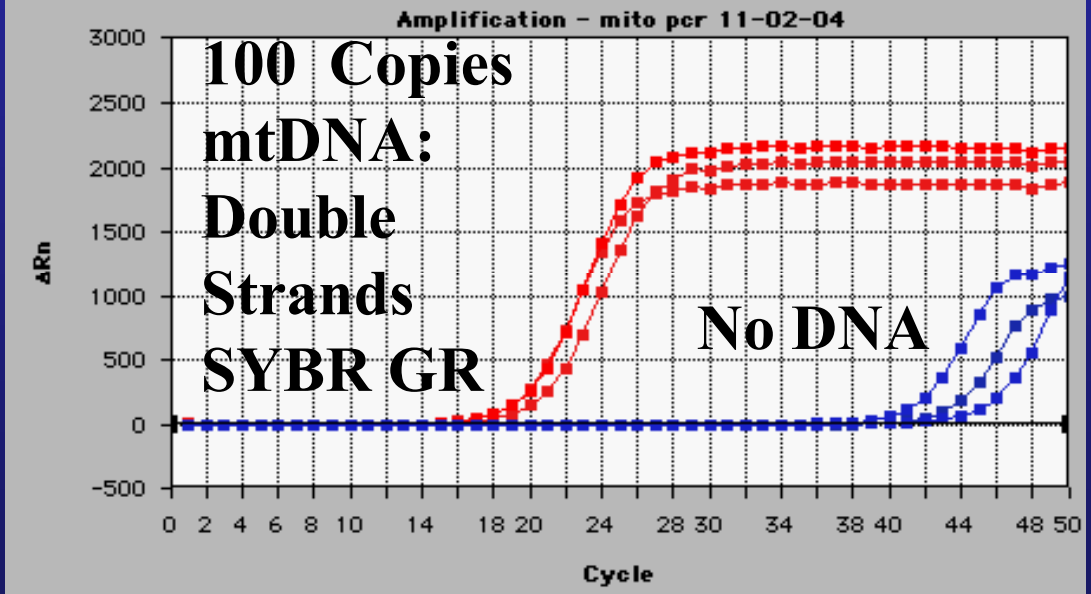
Hours

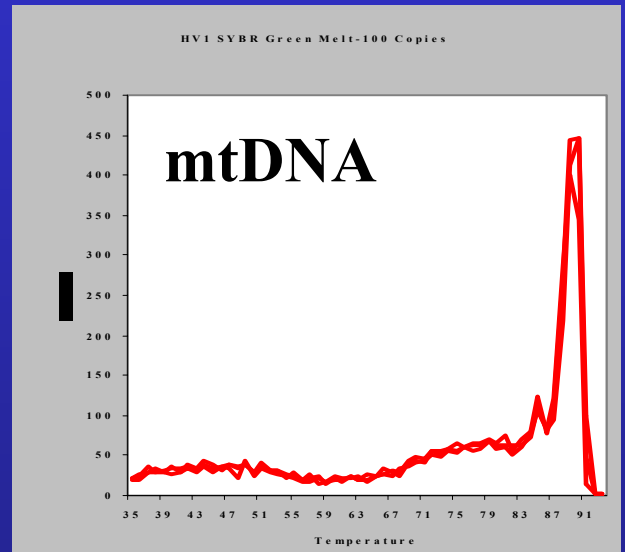


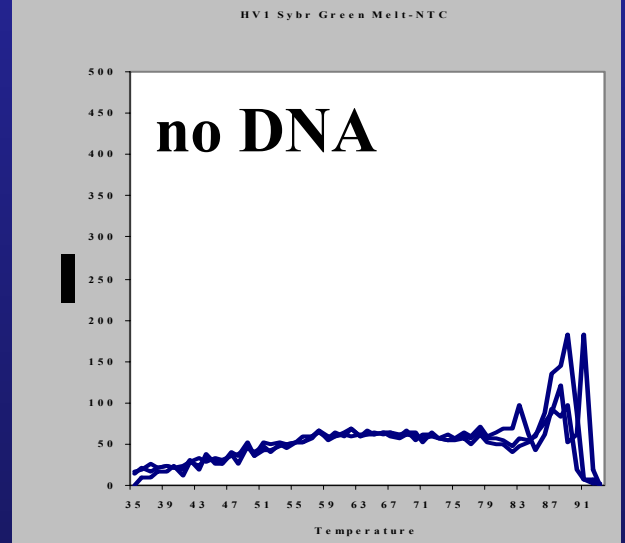
Blood Lymphocyte HV1

SYBR Melt Analysis



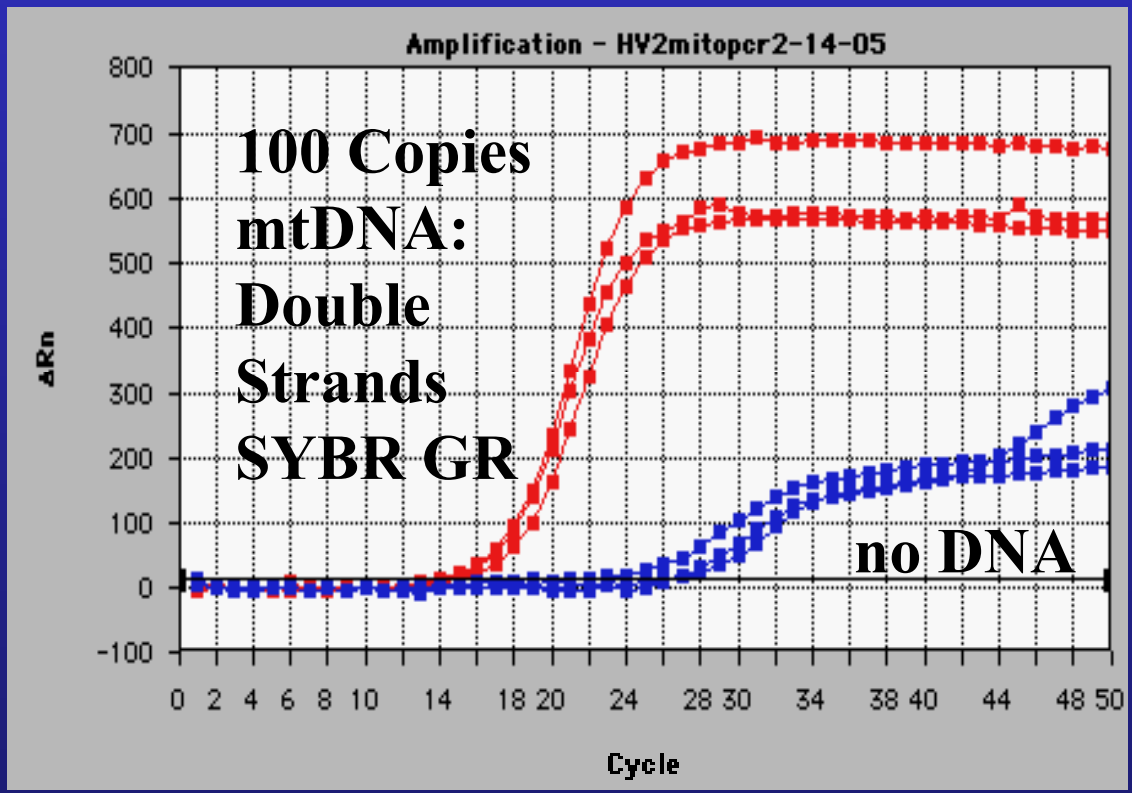


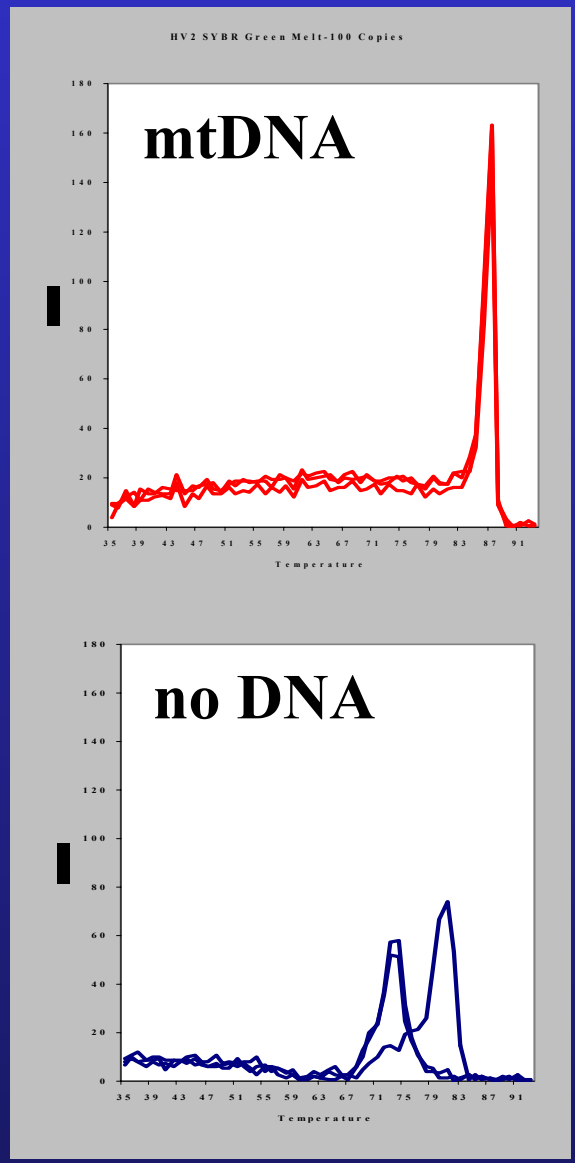




SYBR Melt Analysis

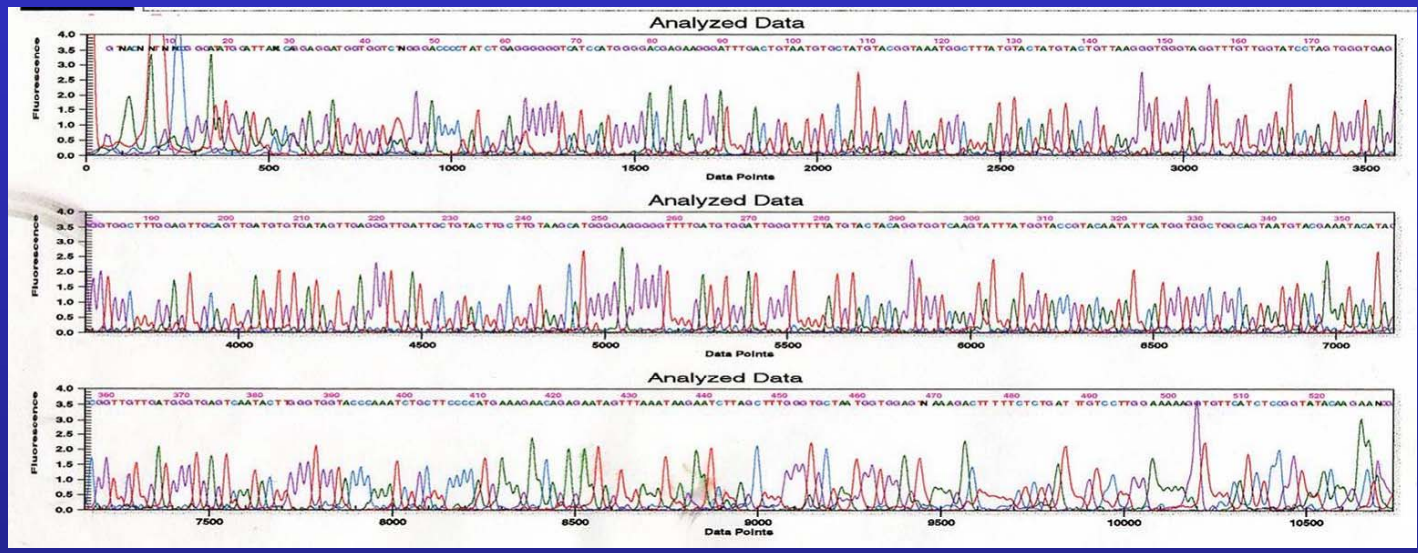
Blood Lymphocyte HV2



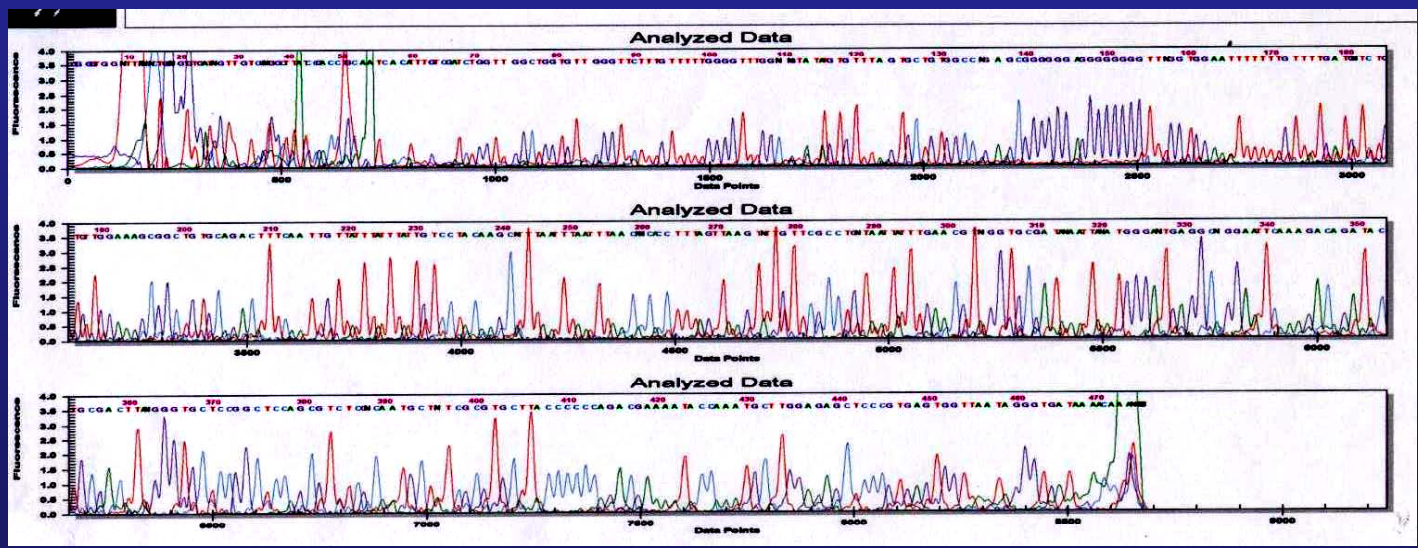


Dilute-'N-Go Sequencing for HBL mtDNA

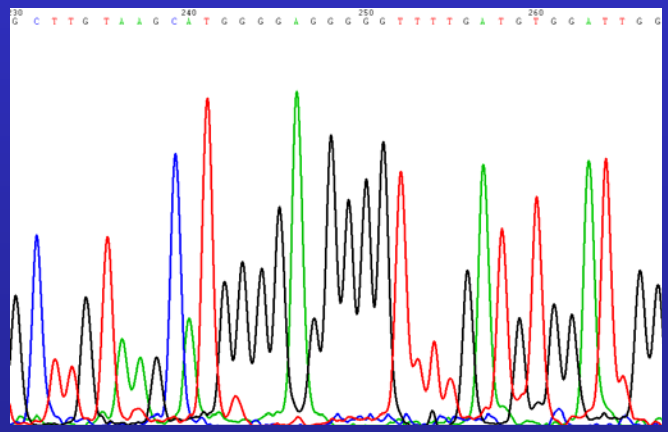
HV1 H



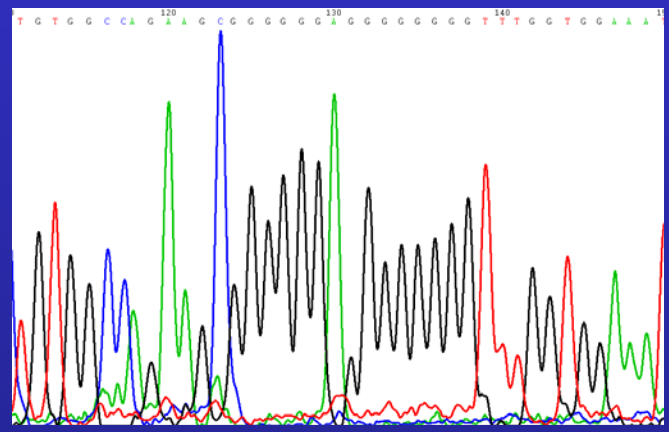
HV2 H



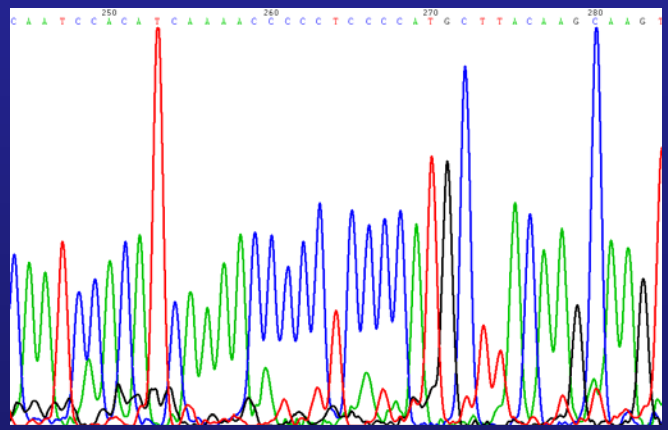
mt DNA Monoplex Assays of Human Blood Lymphocytes: Homo Polymeric Regions



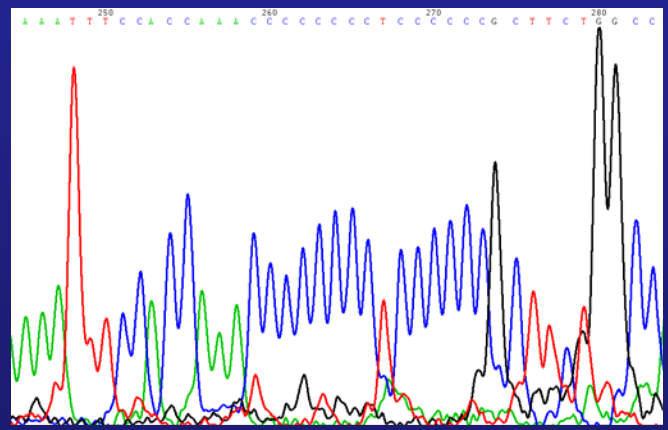
HV1 H (16209-16169)



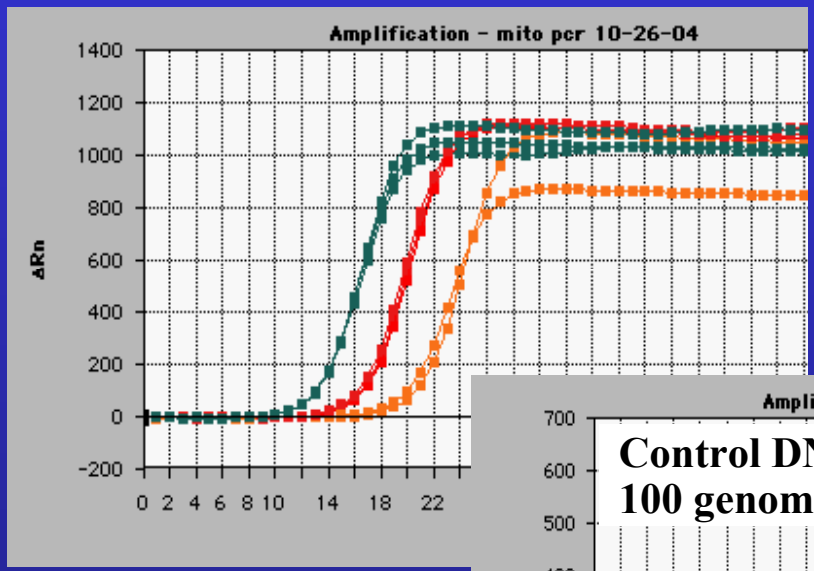
HV2 H (326-289)



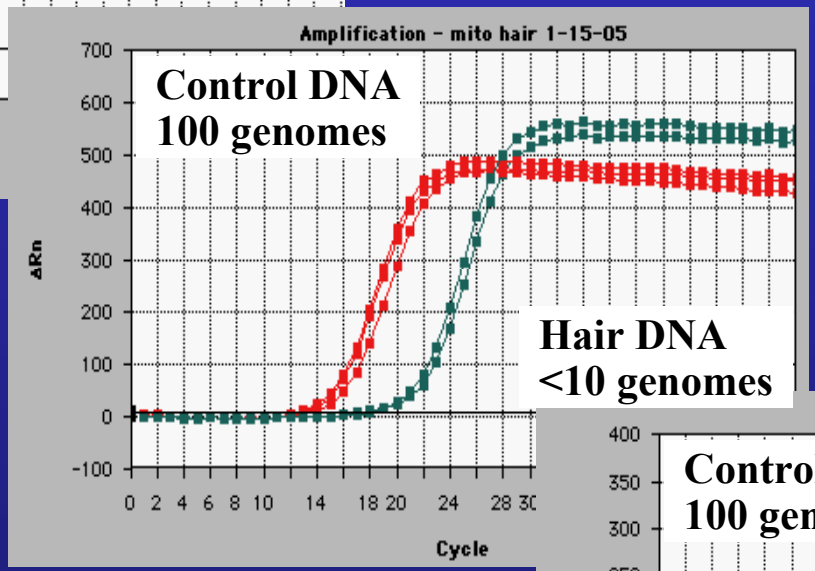
HV1 L (16169-16209)



HV2 L (289-326)



HV1 in control DNA: 1000, 100, 10 genomes

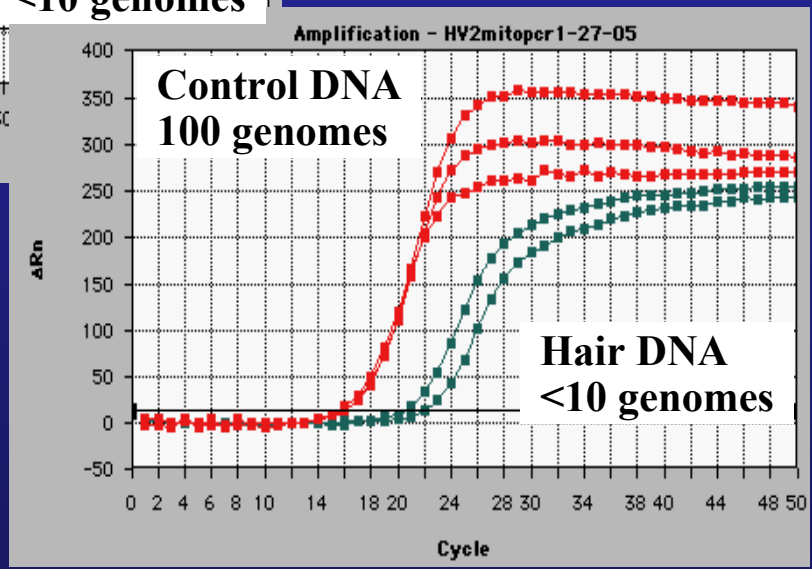


HV1

HAIR STRAND 1

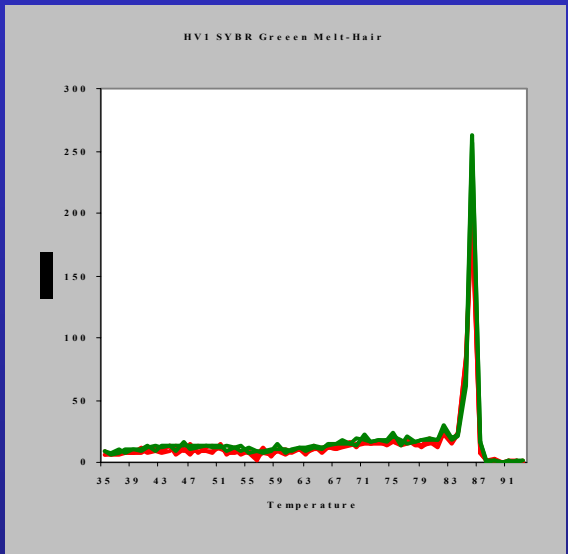
(10 mm)

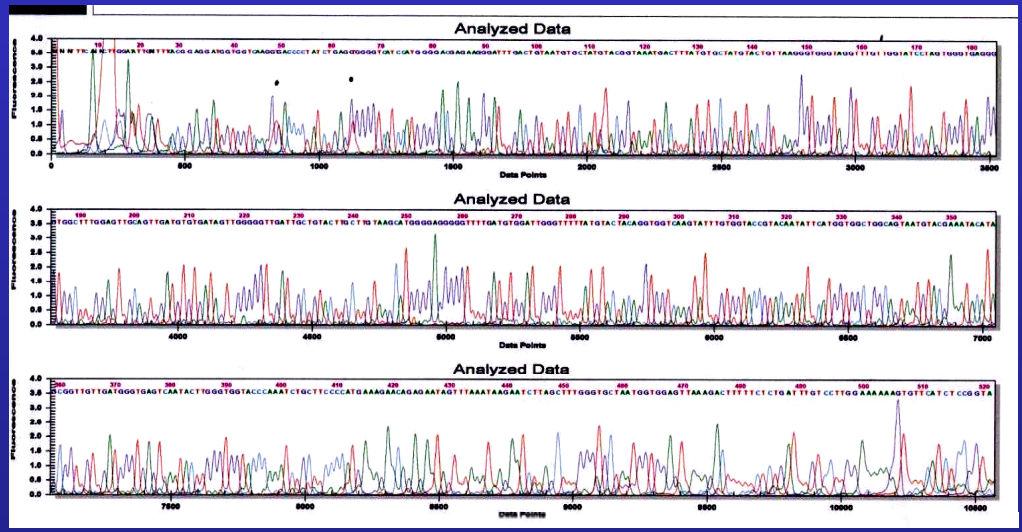
HV2



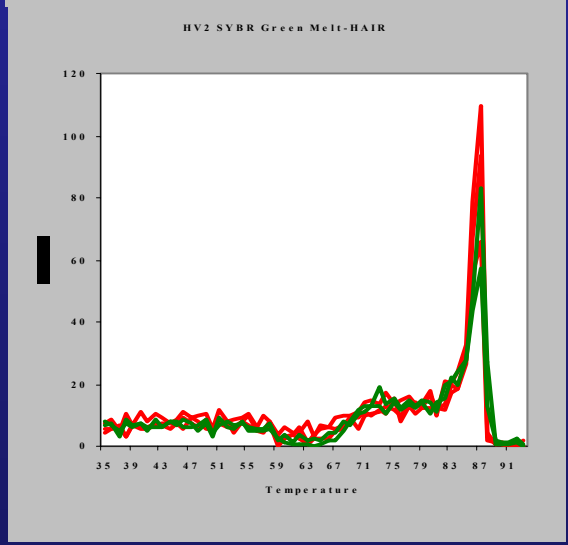
Melt and Sequencing Analysis of Hair 1 Samples

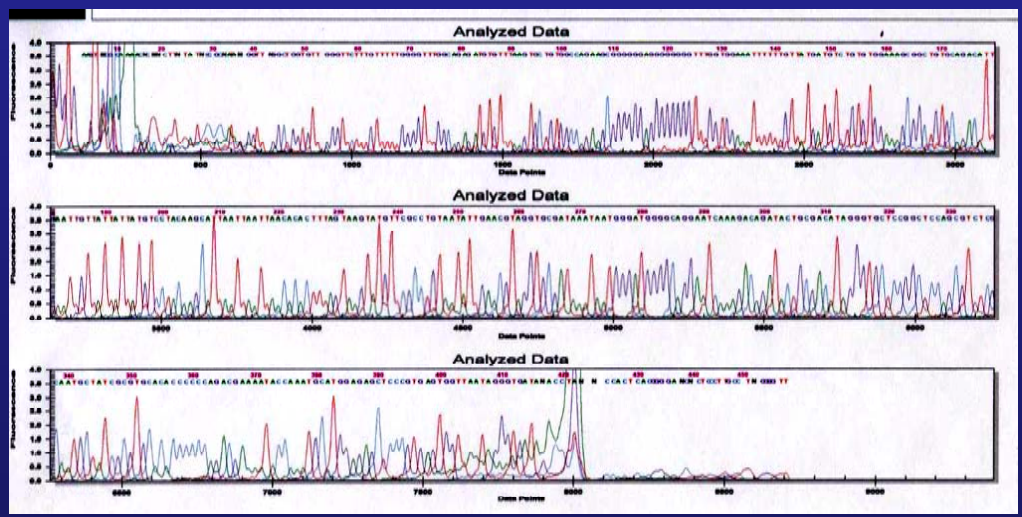
HV1 H



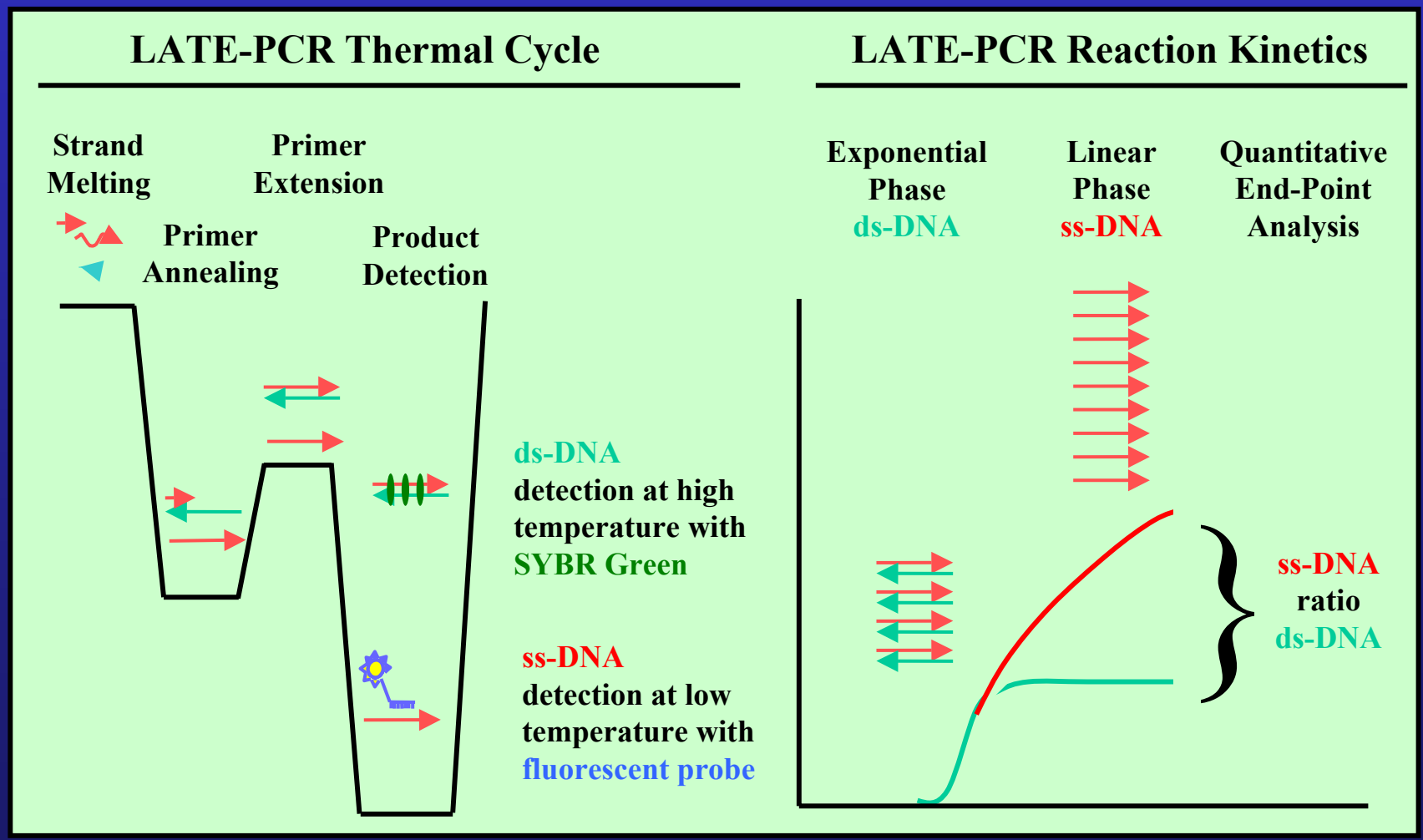


HV2 H



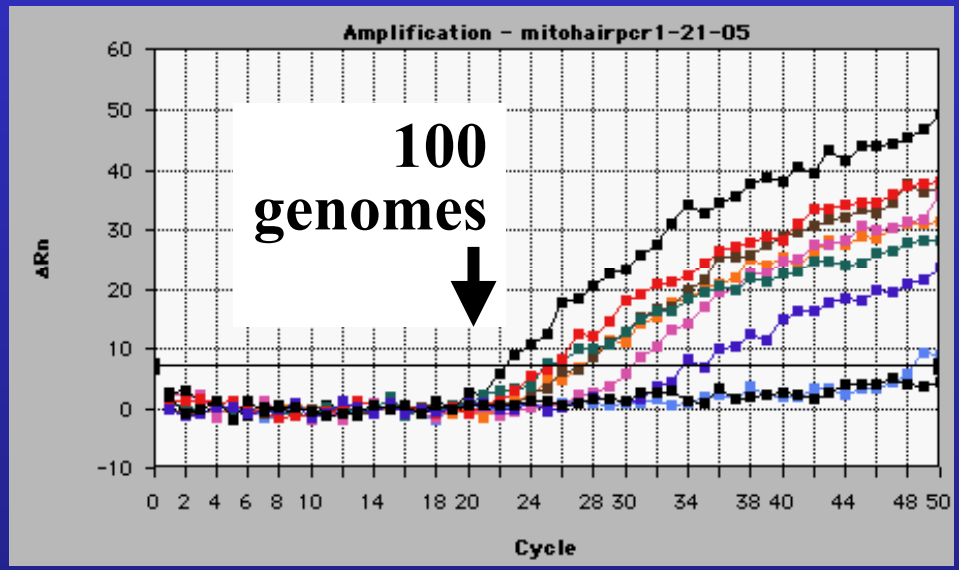


The Logic of LATE-PCR for mtDNA

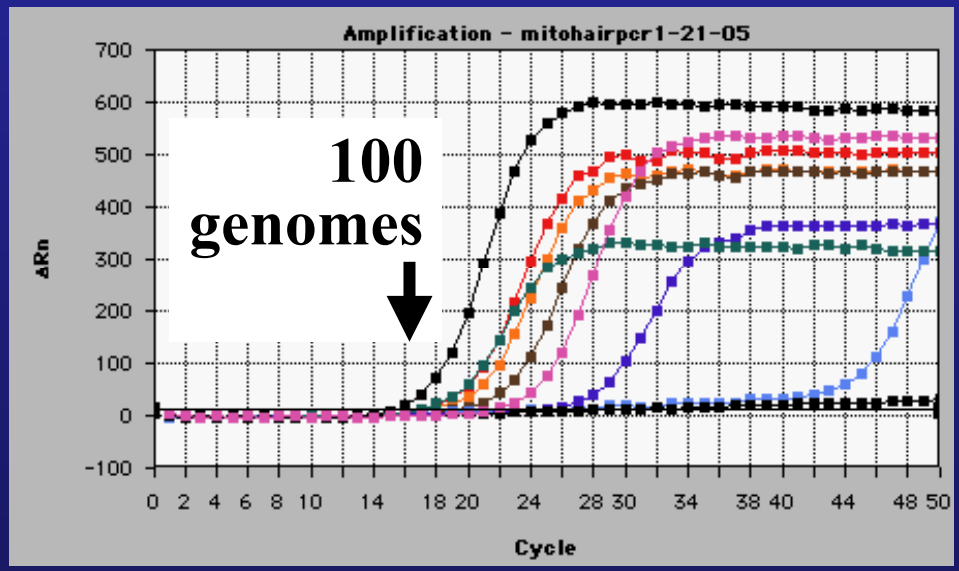


LATE-PCR HV1 H Analysis of 5 mm Hair 2 Samples

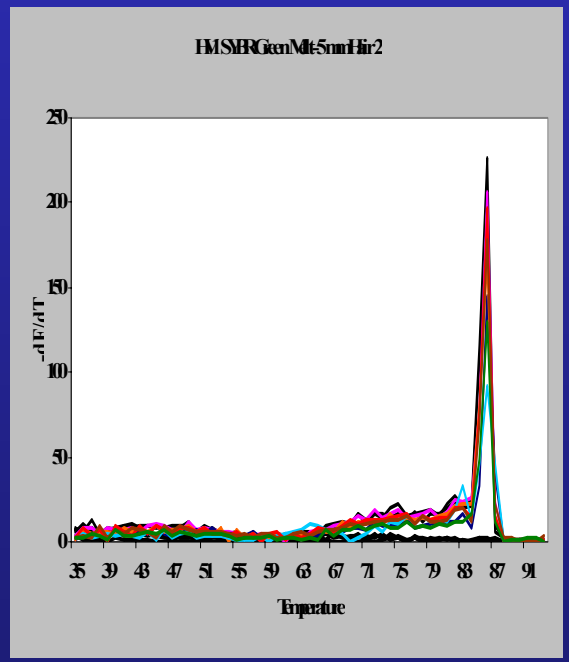
ssDNA



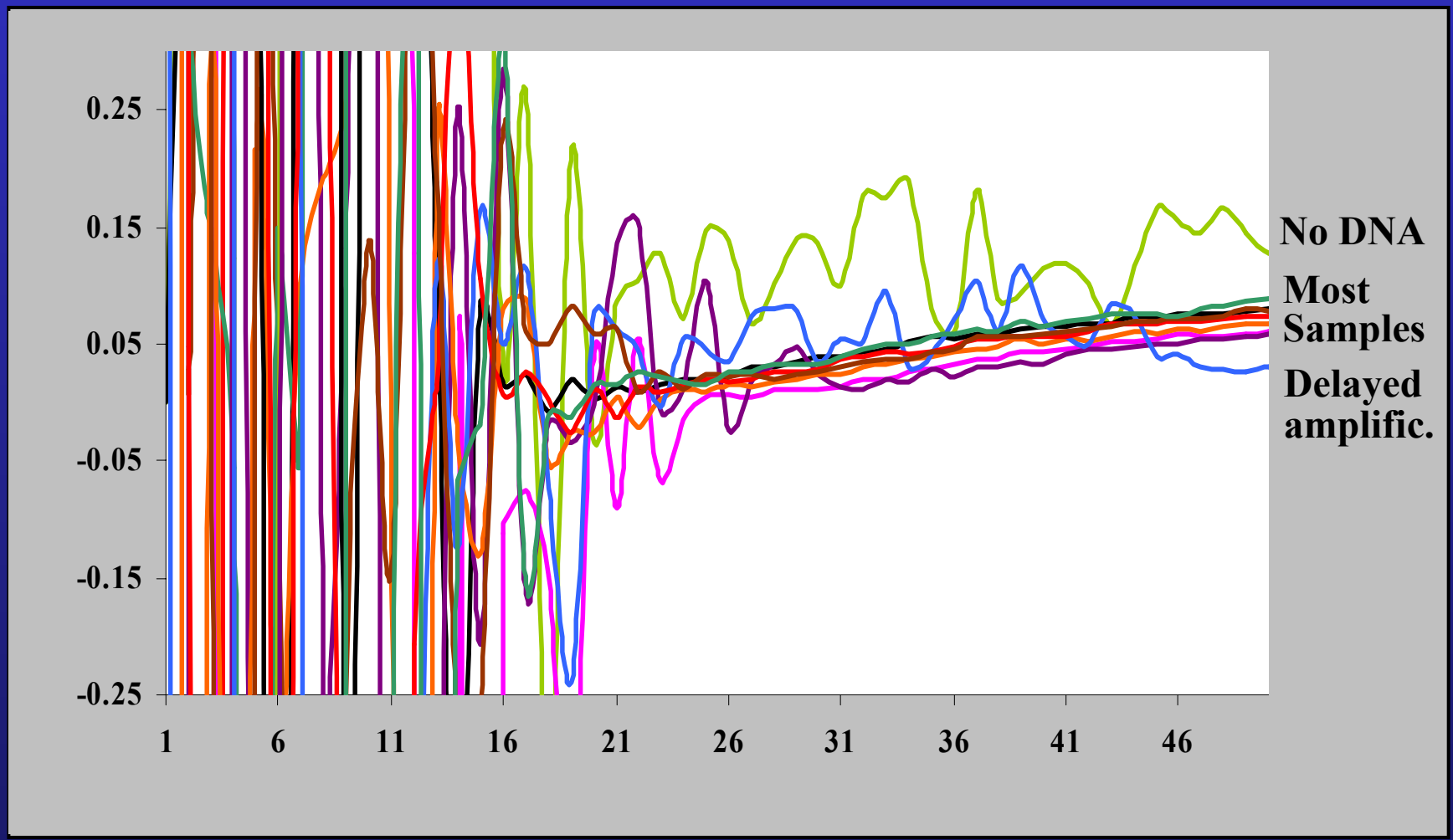
dsDNA



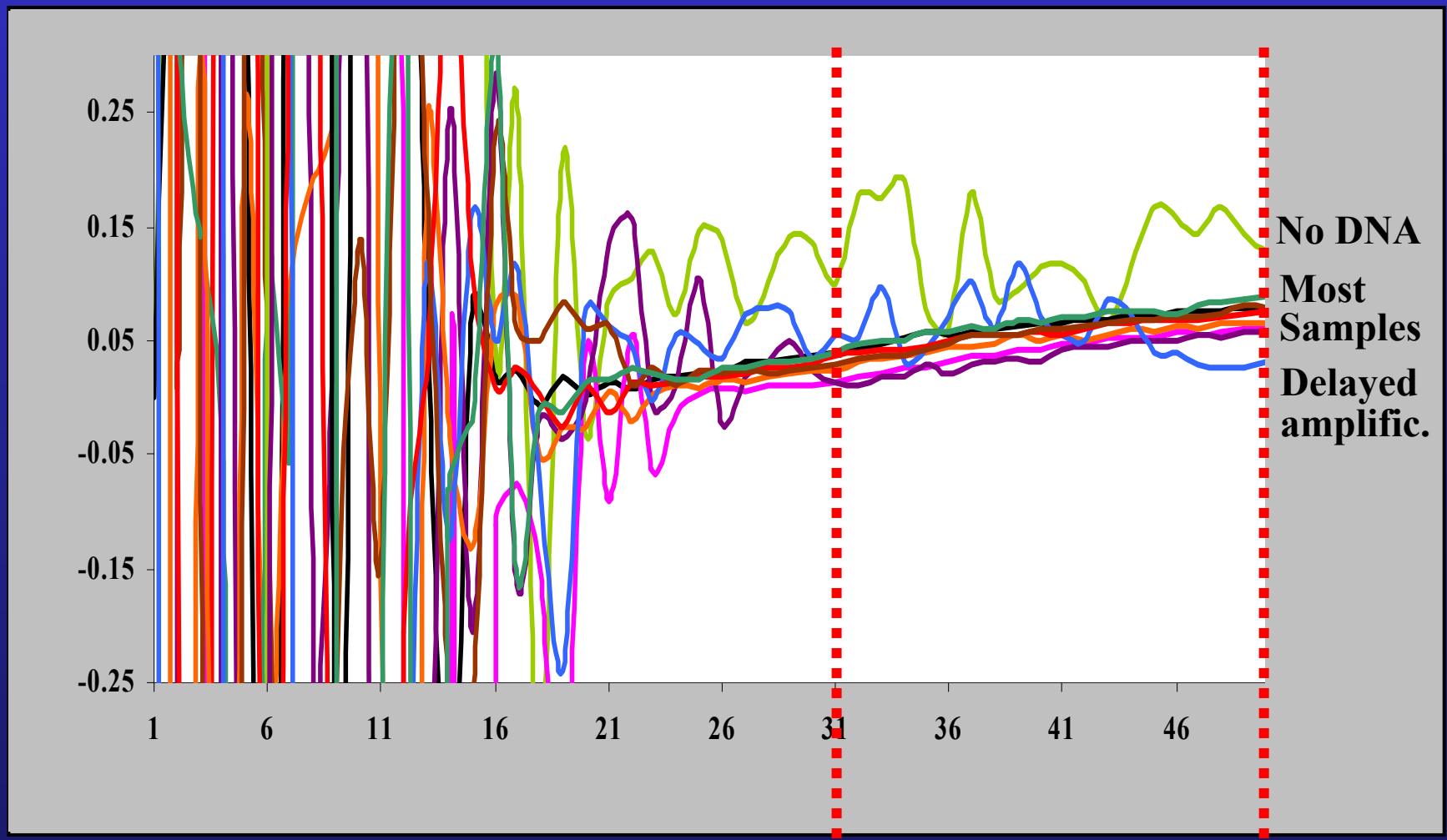
Melt Analysis



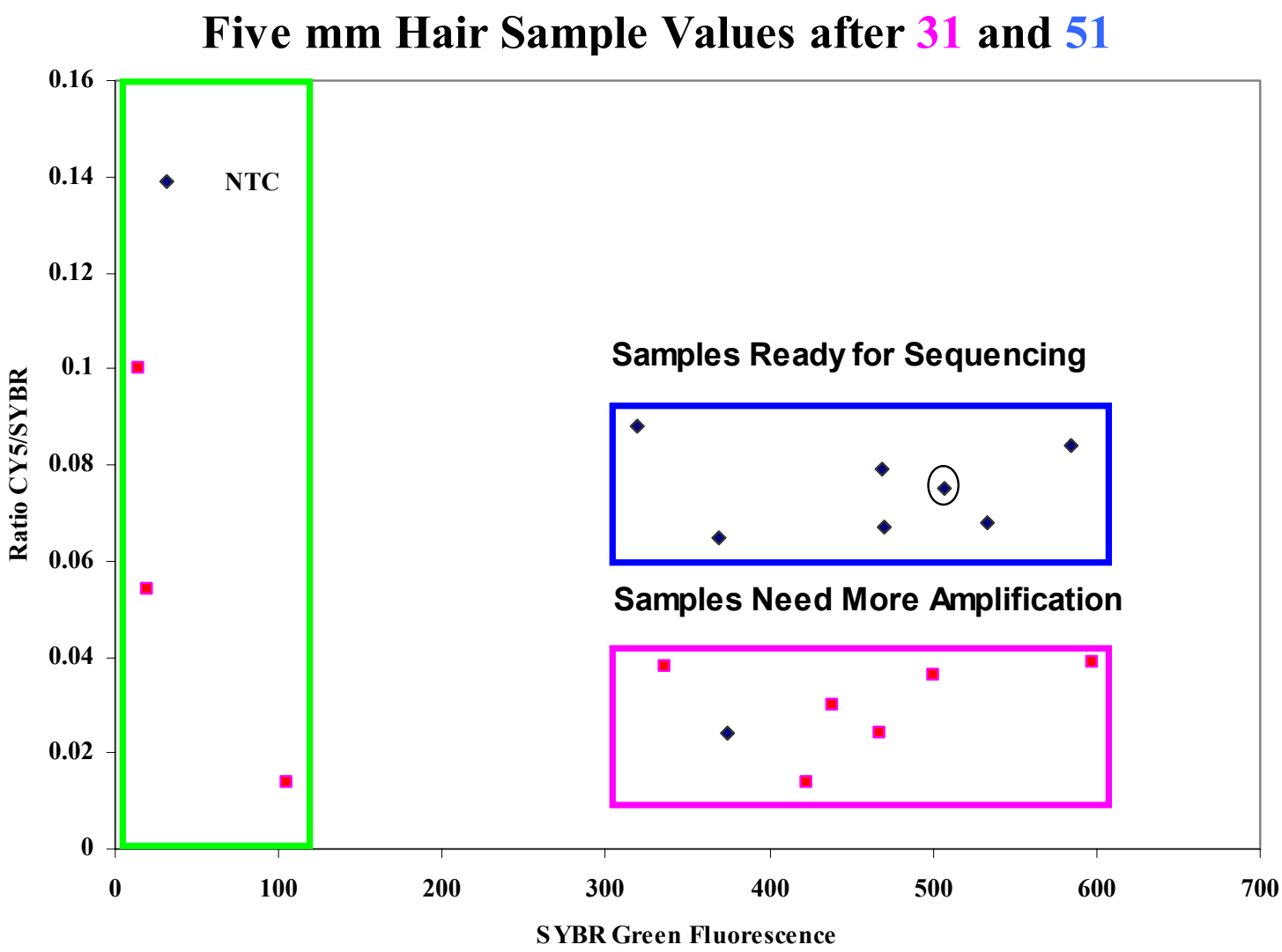
Ratio Analysis of ss-DNA / ds-DNA Hair 2



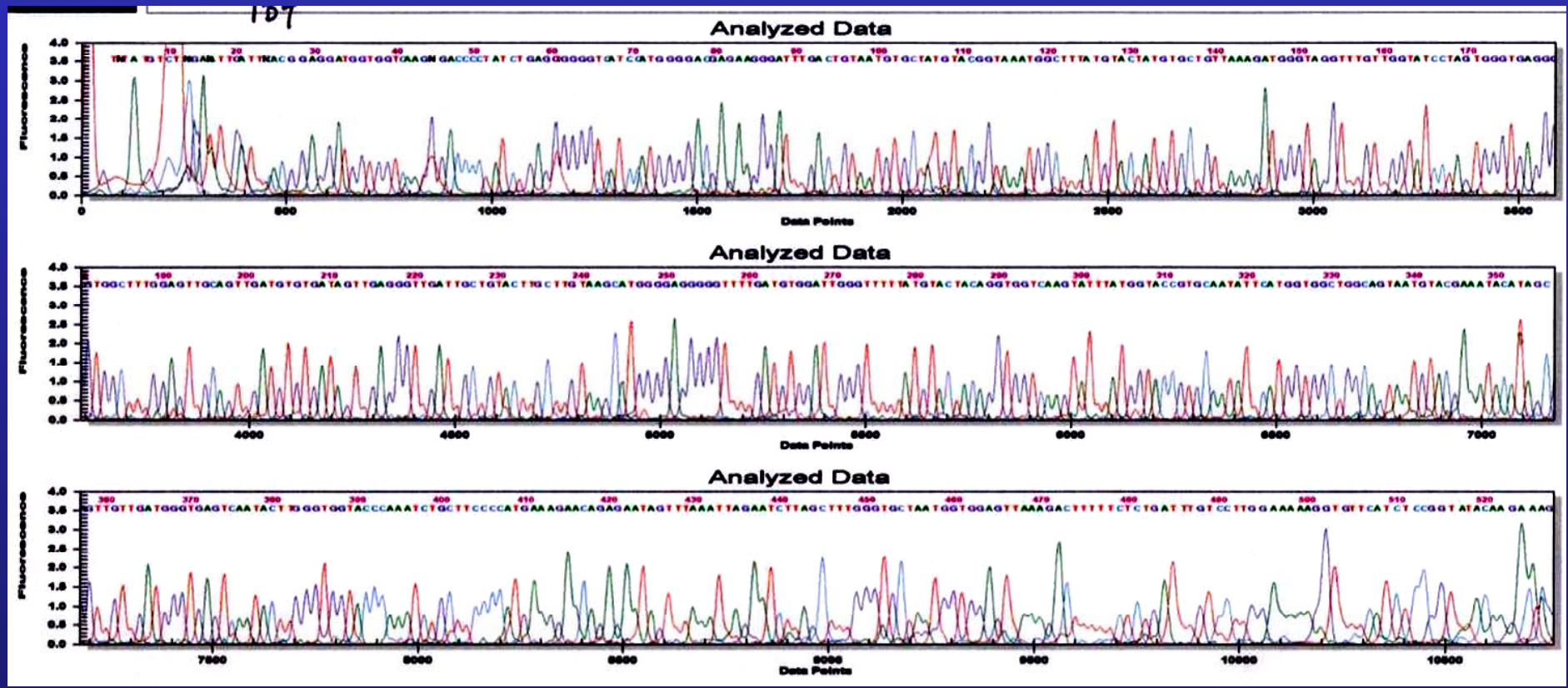
Ratio Analysis of ss-DNA / ds-DNA Hair 2



Quantitative End Point Analysis



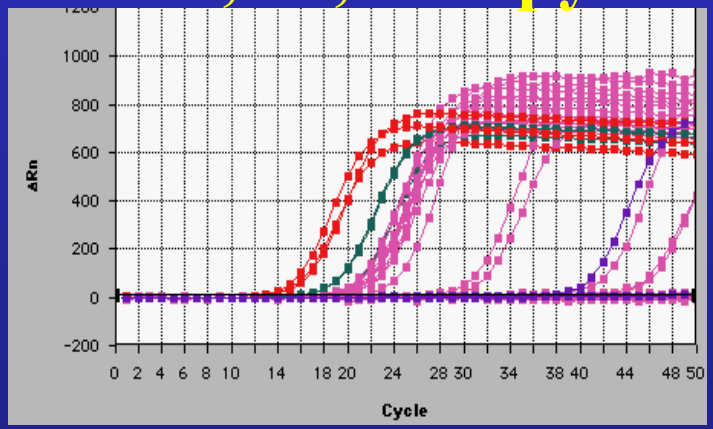
HV1 H Sequence via Dilute-'N-Go Method



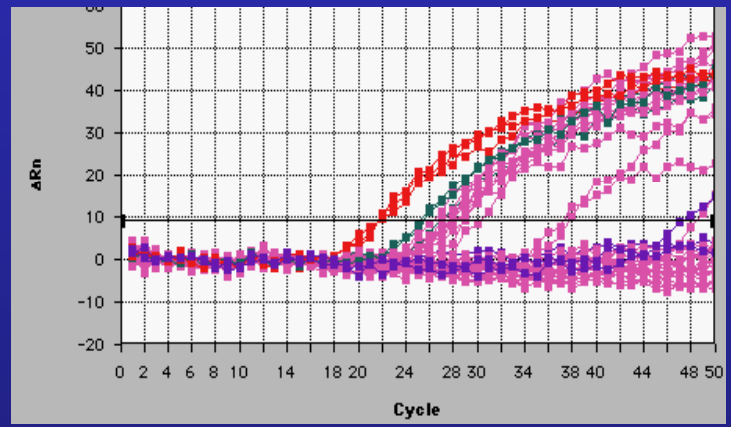
1/30,000th of total sample available from 5mm of hair

Single mtDNA Molecule HV1 H Amplification of Human Blood Lymphocytes

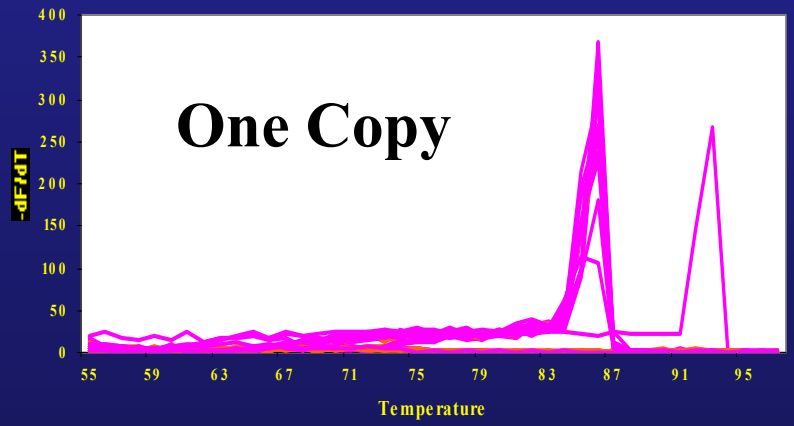
100, 10, 1 Copy

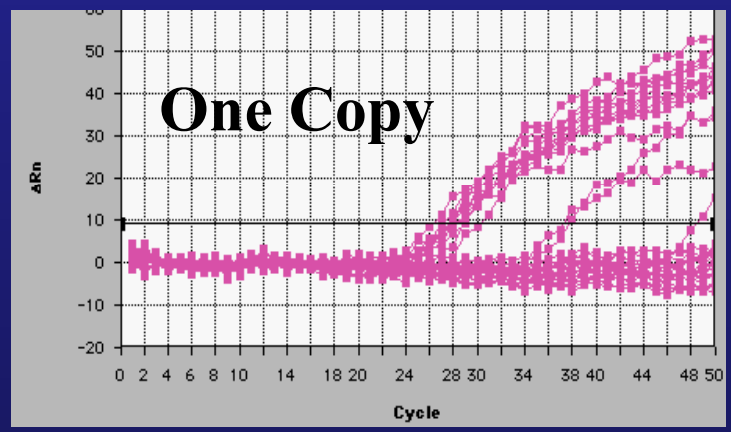


Single Strands



SYBR Melt Curves

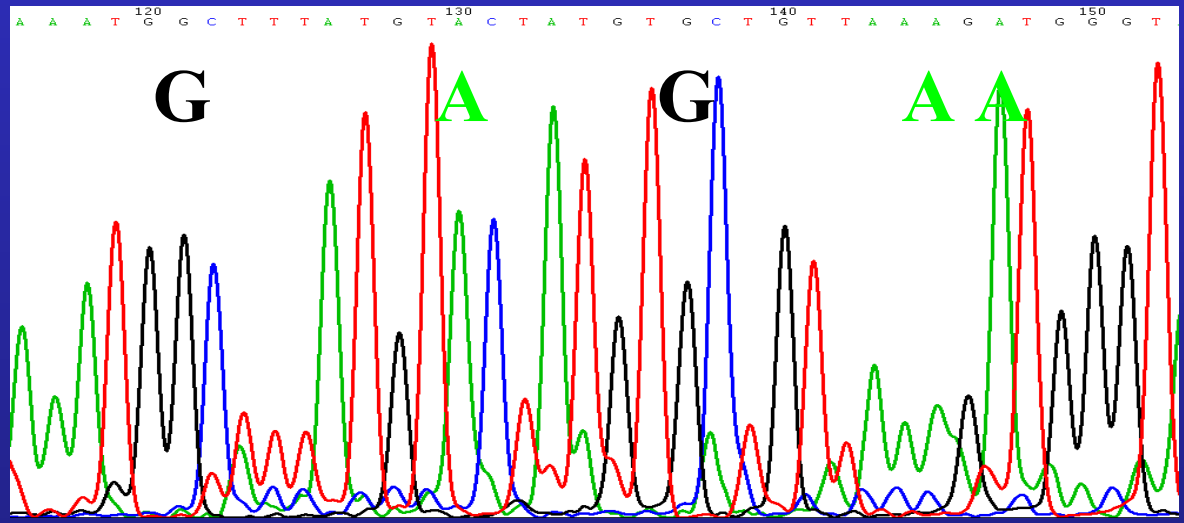




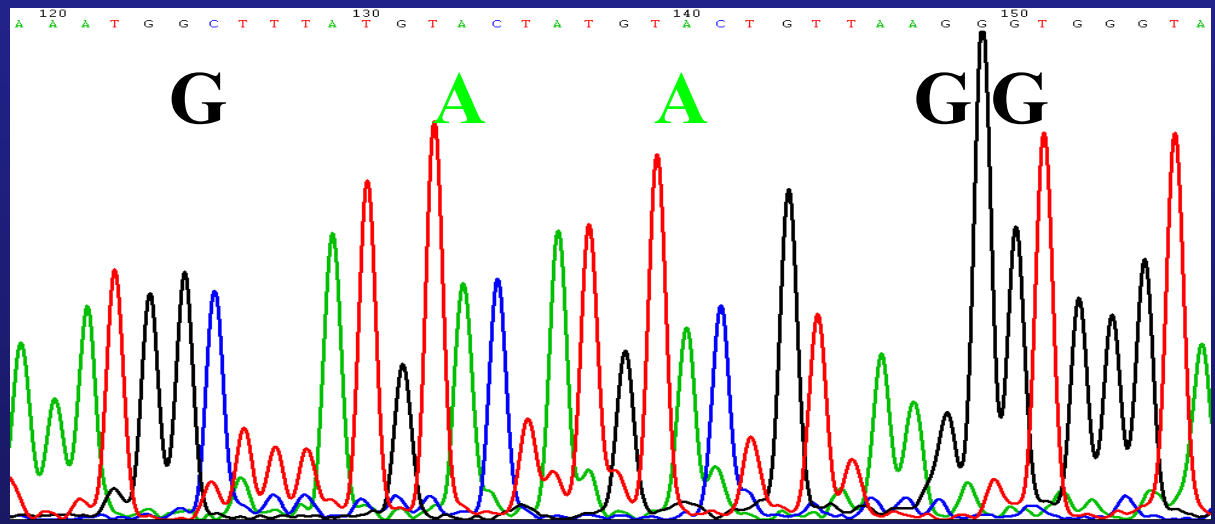
Single mtDNA Molecule HBL Sequences

Sample

C2



D8

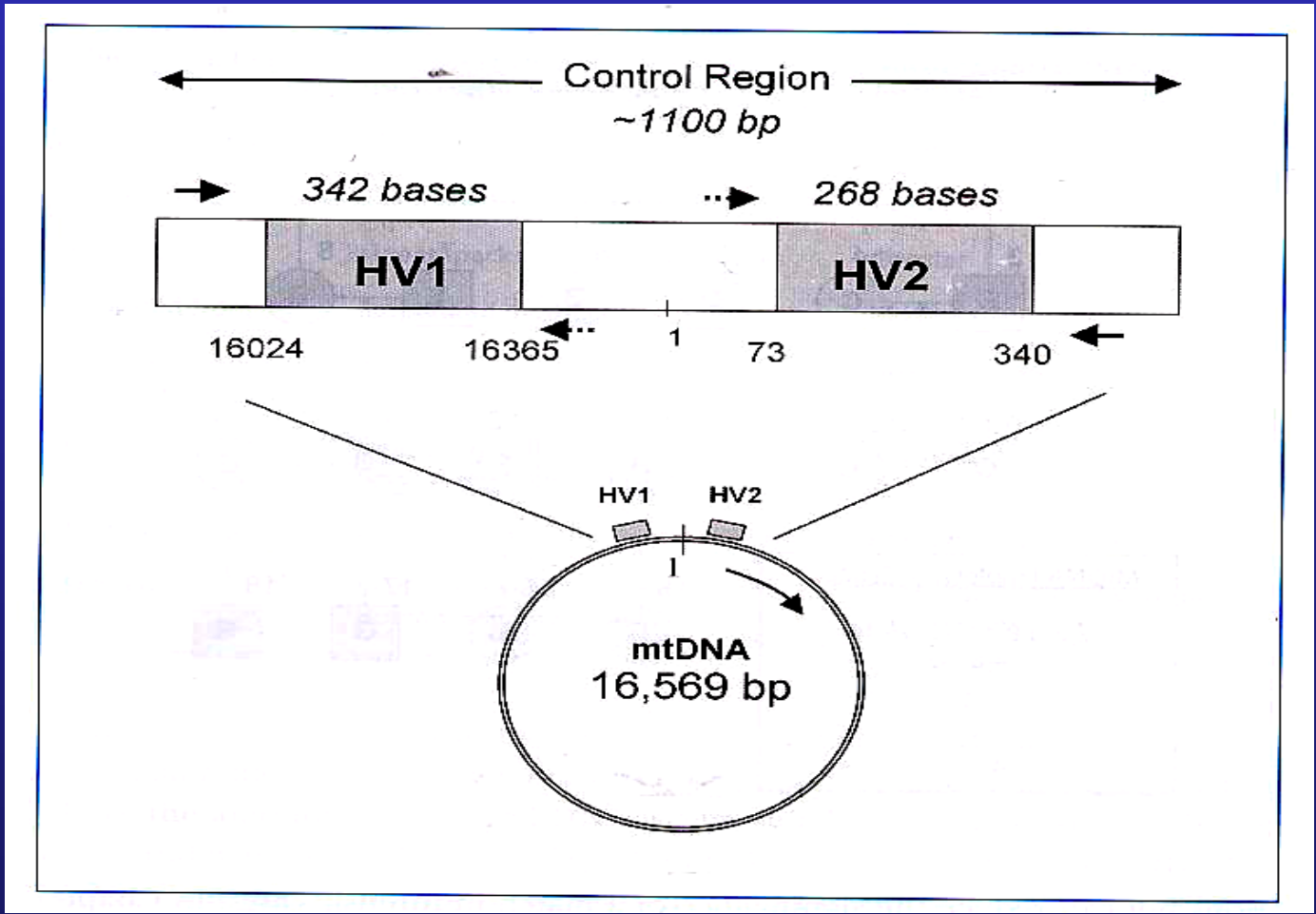


Sequencing Results: Coverage and Accuracy

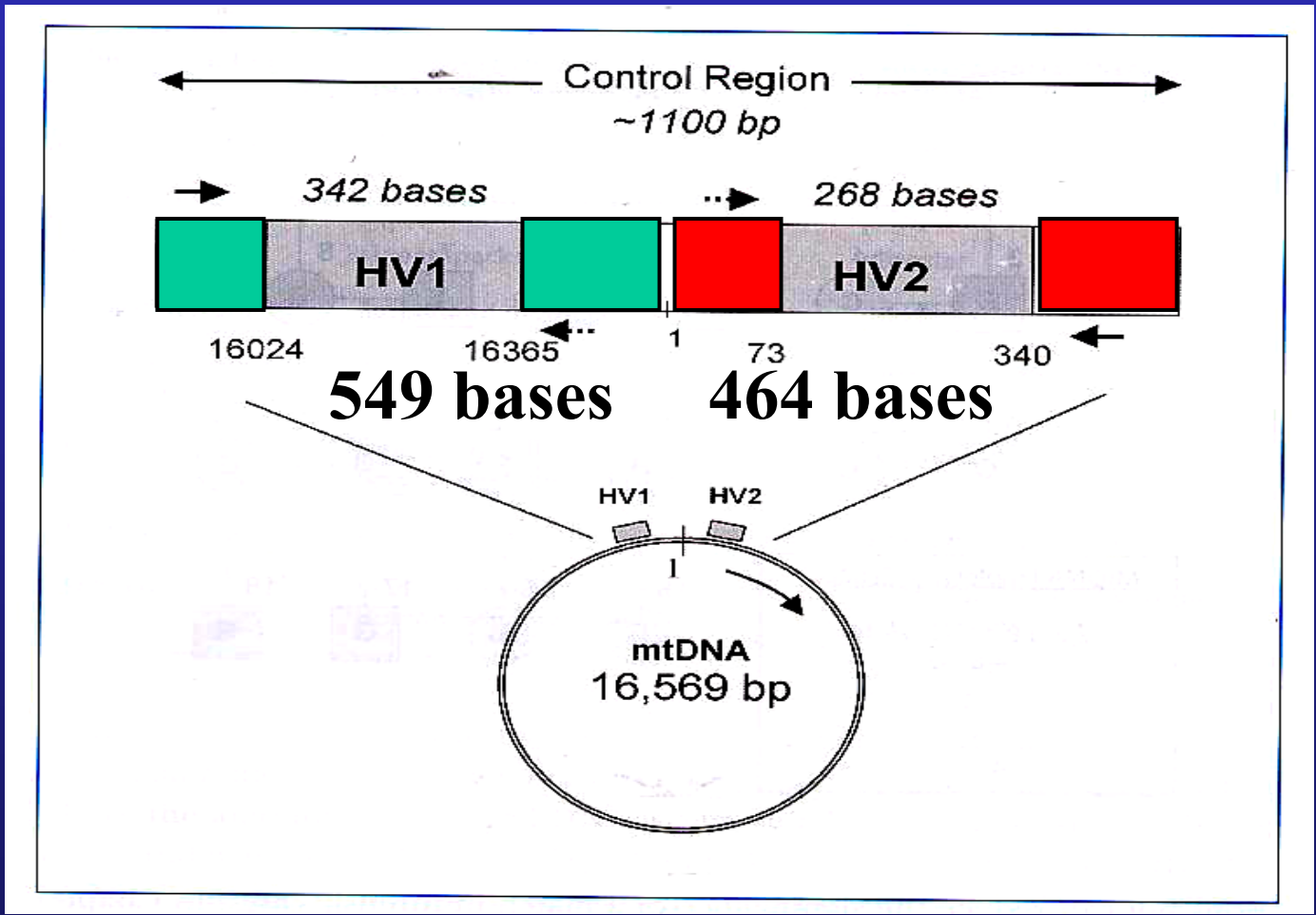
ASSAY	mtDNA REGIONS	
	HV1 (#)	HV2 (#)
• Current Forensic	16024-16365 (342)	73-340 (268)
• Expanded Forensic	15998-16400 (403)	30-407 (378)
• Theoretical (this work)	15910-16458 (549)	7-470 (464)
• Observed	15910-16450 (541)	8-455 (448)

(Lymphocyte Monoplexes: HV 1 H & L, HV 2 H & L)

Current HV 1 and HV 2 mtDNA Coverage



Extended HV 1 and HV 2 mtDNA Coverage

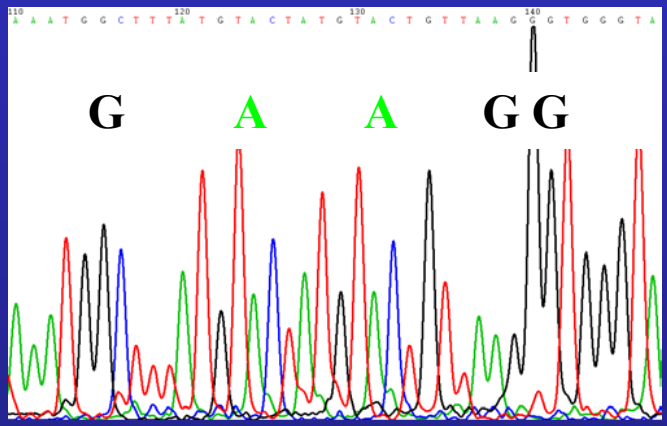


HV1 and HV2 mtDNA SNP Sequence Comparison of Hair Shaft 1 vs. Revised Anderson Standard Sequence

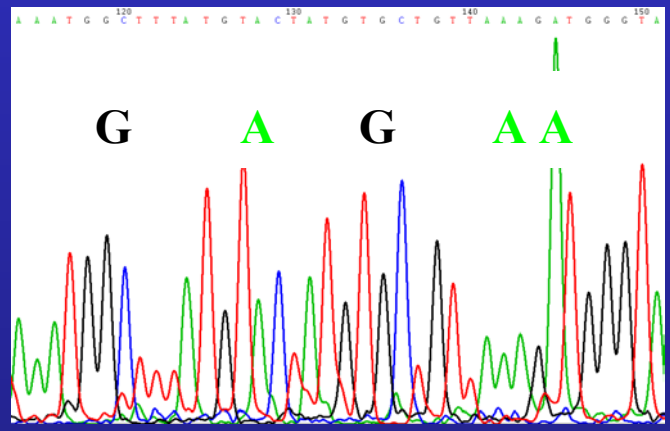
Base Number	Observed Base	Anderson Base	MITOMAP Ref.
HV1 H			
16320	A	G	YES
16311	G	A	YES
16224	G	A	YES
16136	G	A	YES
HV2 H			
310+1	G	-	YES
309+1	G	-	YES
263	C	T	YES
152	G	A	YES
146	G	A	YES
73	C	T	YES

Single Nucleotide Polymorphisms for mtDNA HV1 H Region 16325-16288

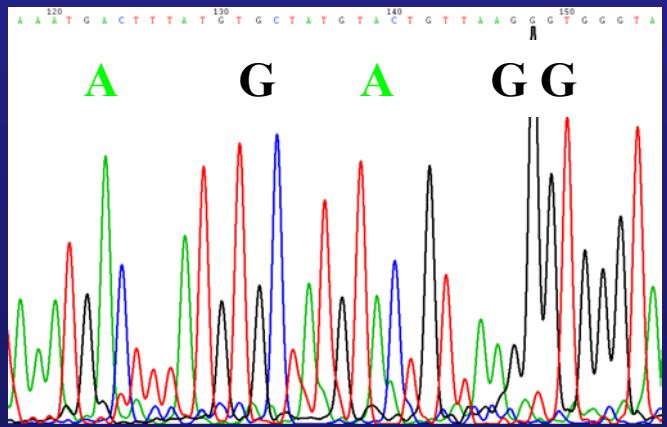
Human Blood Lymphocytes



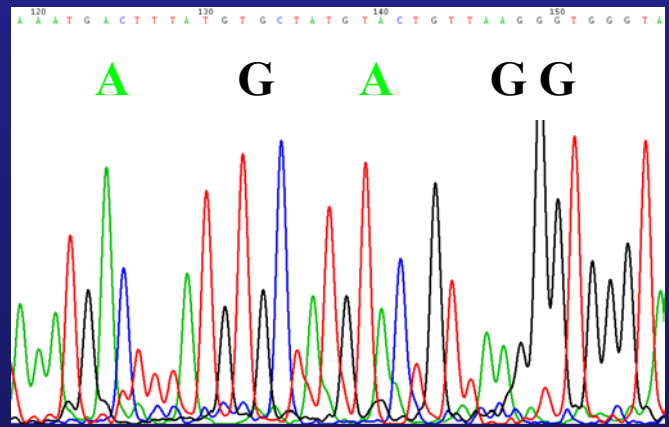
Human Hair Shaft 2 (5mm)



Human Hair Shaft 1 (10mm)



Human Thumbprint



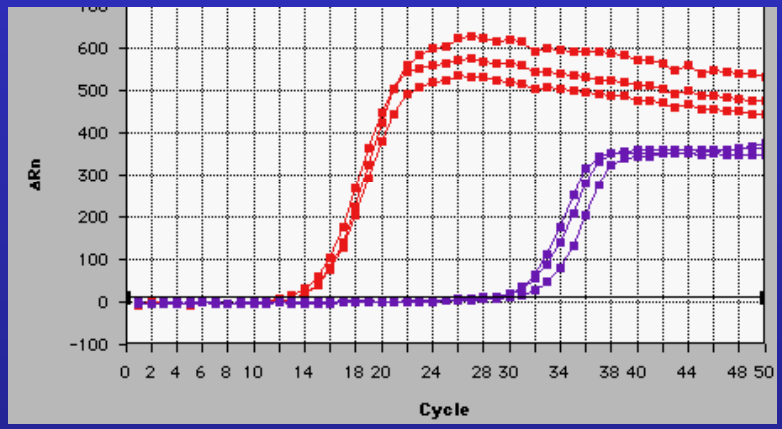
Fingerprint Assay

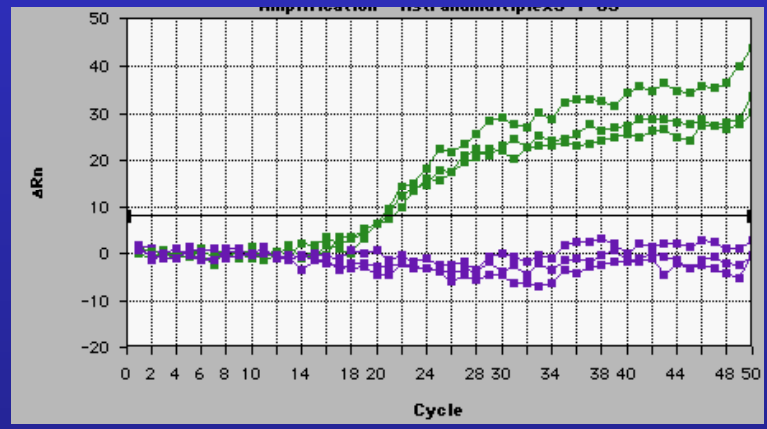
- Single Thumbprint on Glass Slide vs. Glass Slide Control
- Both Washed with 50 ul of Lysis Buffer on Cotton Swab
- Cell Lysis (100 ul) of Swab and Buffer
- One Micro-Liter of Lysis Sample Used per Late-PCR Reaction

HV1 H Strand SNP Sequence Comparison of Hair Shaft 1, 5mm Hair Shaft 2, Thumbprint, and Revised Anderson Standard

Base	Hair Shaft 1	Hair Shaft 2	Thumbprint	Anderson	MITO
16320	A	G	A	G	YES
16311	G	A	G	A	YES
16304	A	G	A	A	YES
16296	G	A	G	G	YES
16294	G	A	G	G	YES
16136	G	A	G	A	YES
16126	A	G	A	A	YES

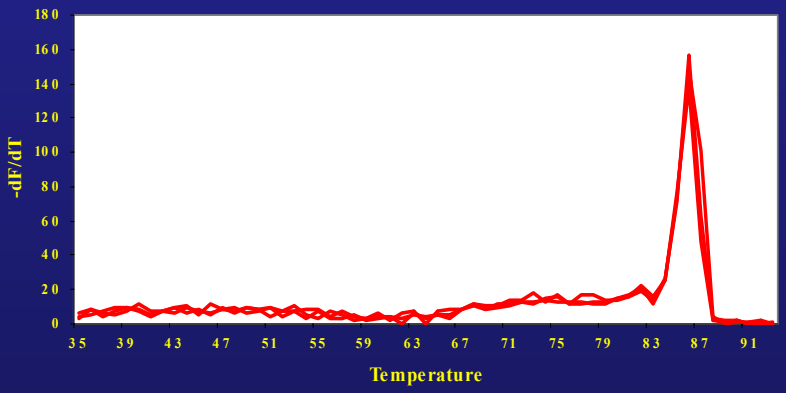
Duplex Assay for HV1H and HV2 H of Human Blood Lymphocytes



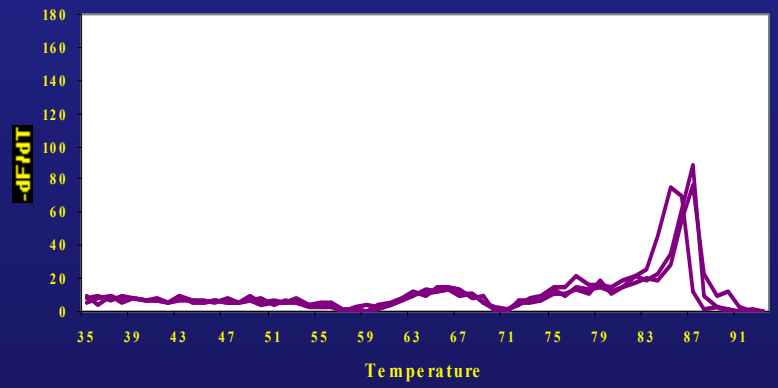


Melt Curves

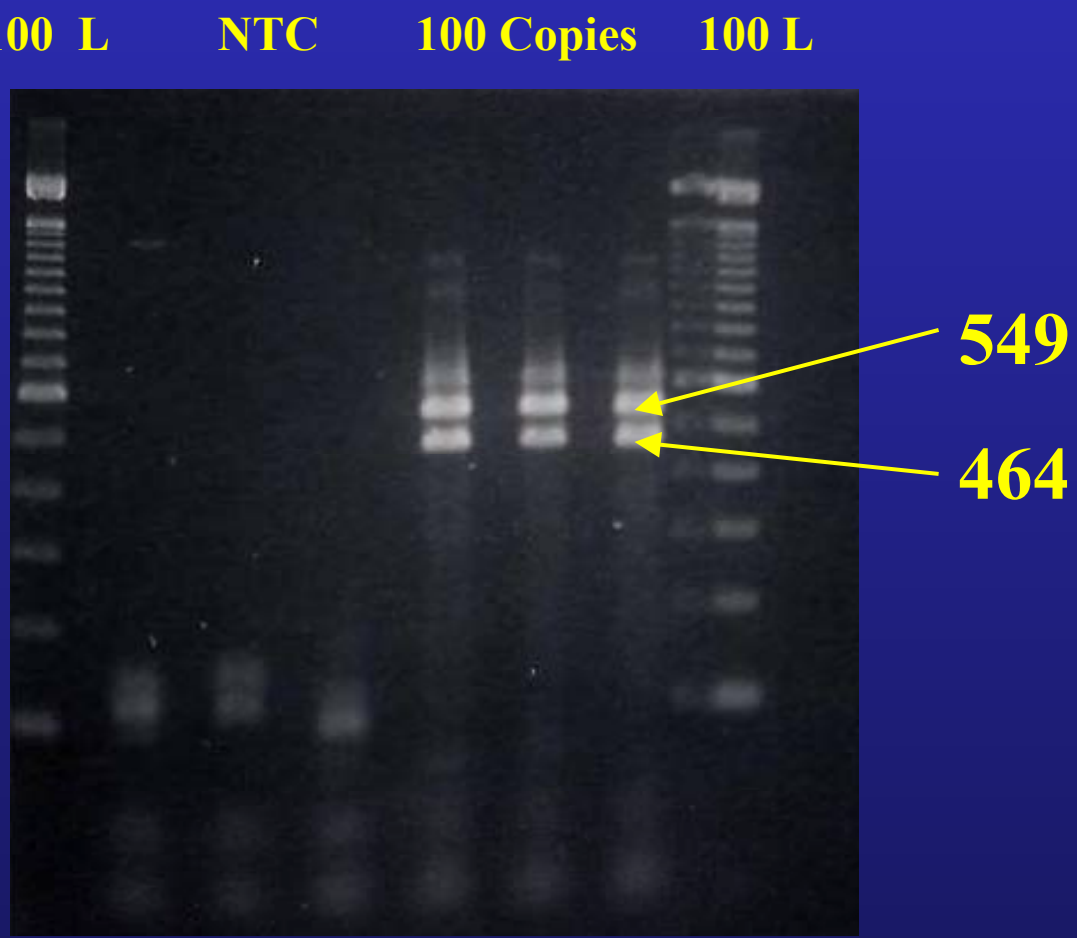
H Strand Duplex 100Copy Standard



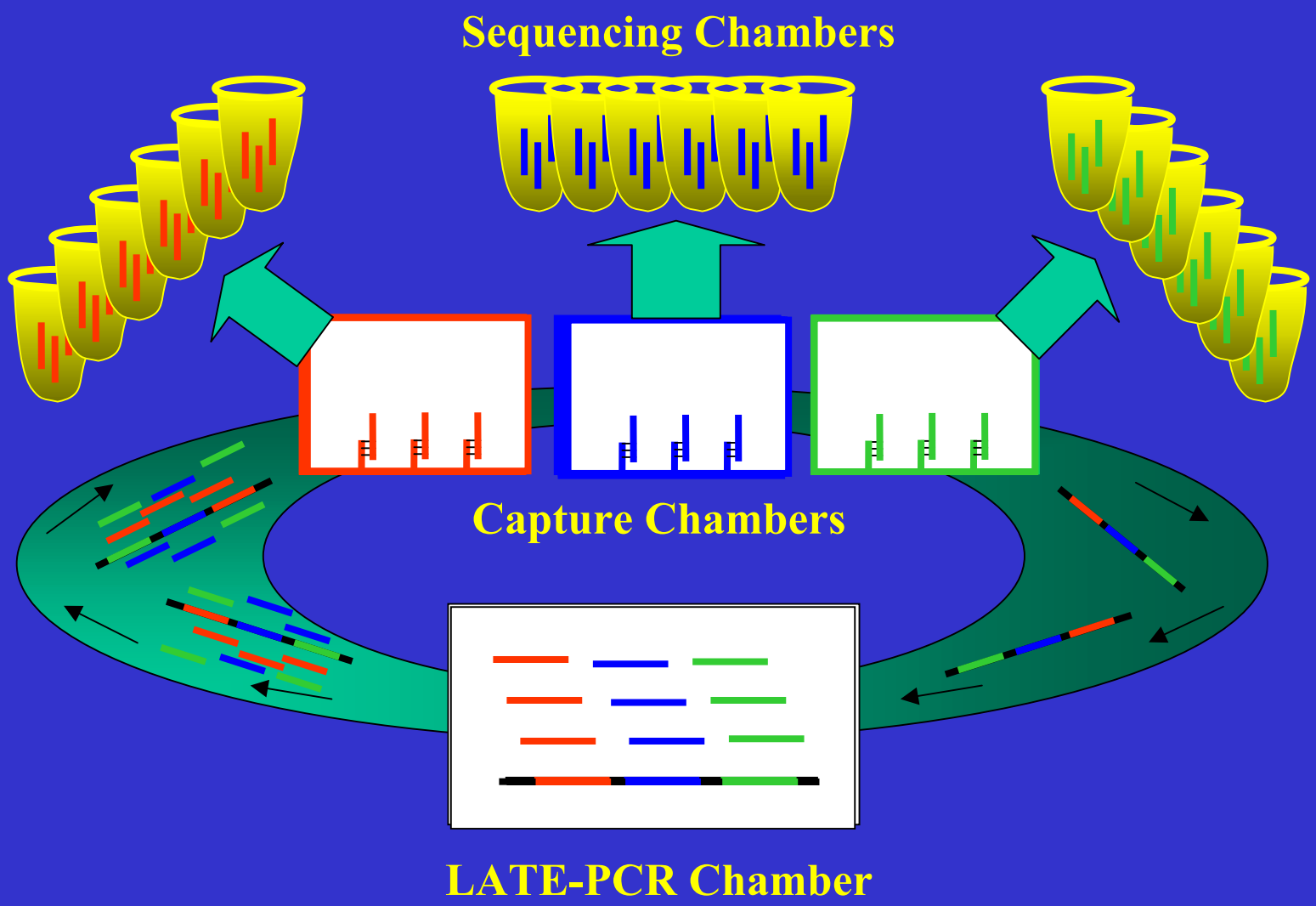
H Strand Duplex NT Controls



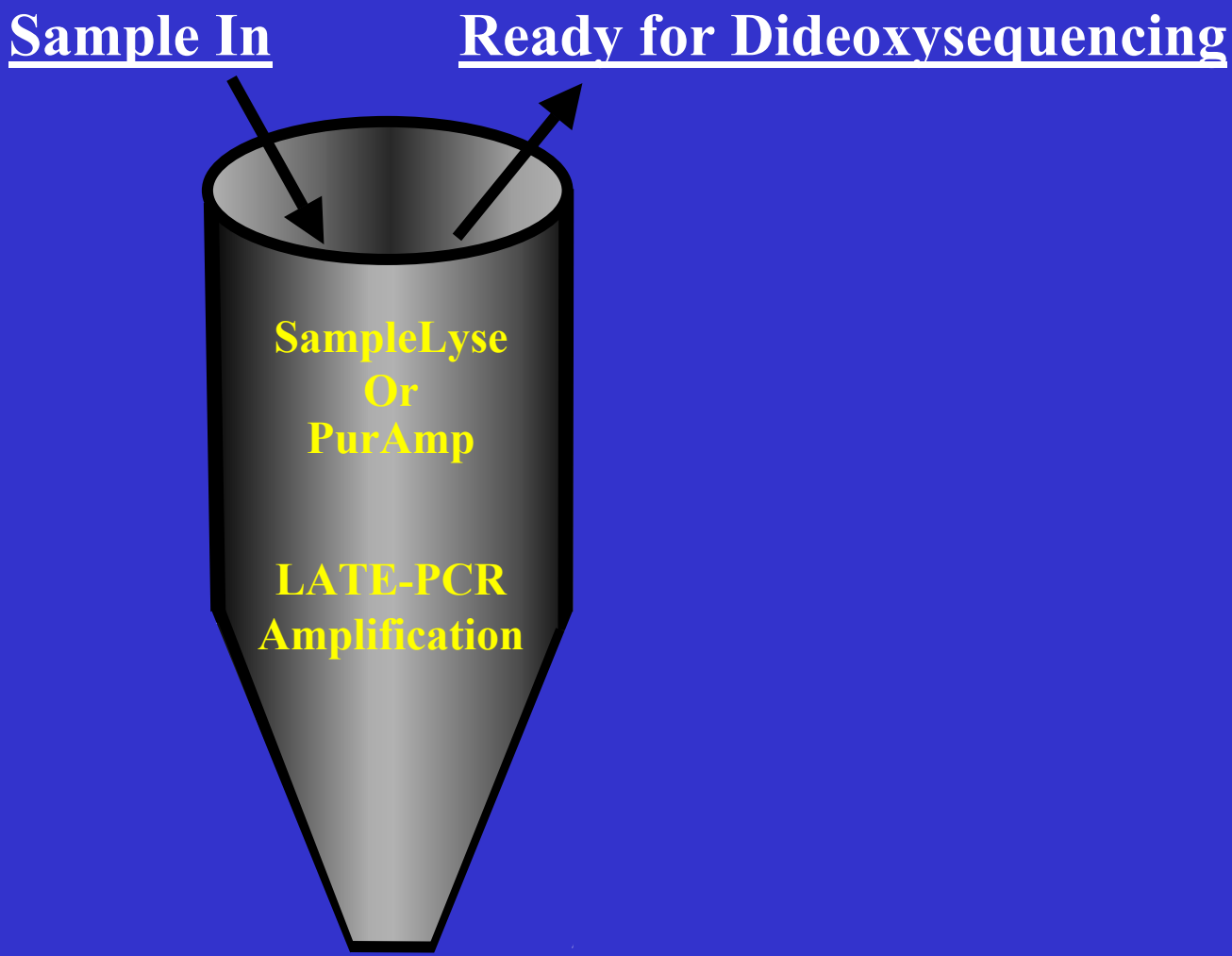
Duplex Assay for HV1H and HV2 H Agarose Gel



Automated Product Recovery and Parallel Sequencing in a Closed System



LATE-PCR: "Sample-to-Sequence" in a Single Tube



Forensic Challenges

- Accuracy of Analysis
- Time: Sample to Result
- Identification/Match
- Small Sample Size
- Contamination
- Cost

A Whole System Approach