LATE-PCR

Linear-After-The-Exponential

A Patented Invention of the Laboratory
of
Human Genetics and Reproductive Biology

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2nd Nucleic Acid Quantification Meeting, London 2003

Laboratory Co-Inventors

Cristina Hartshorn

Kenneth Pierce

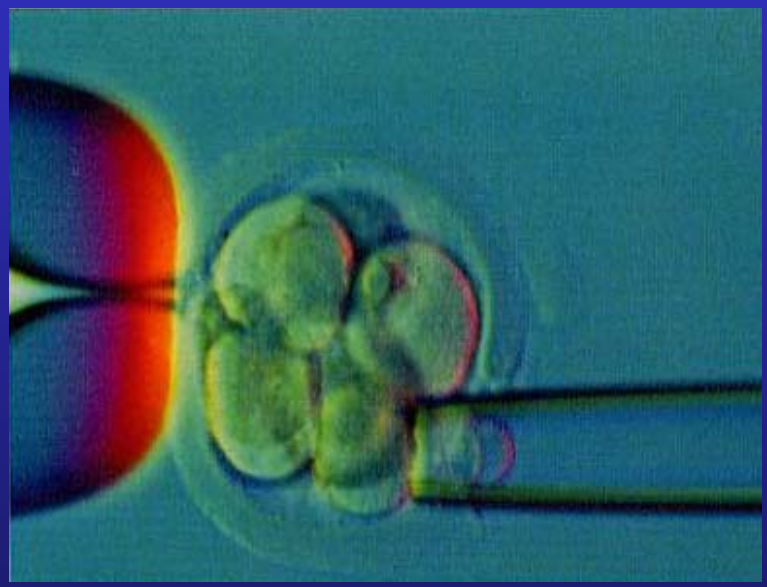
John Rice

J. Aquiles Sanchez

Lawrence J. Wangh

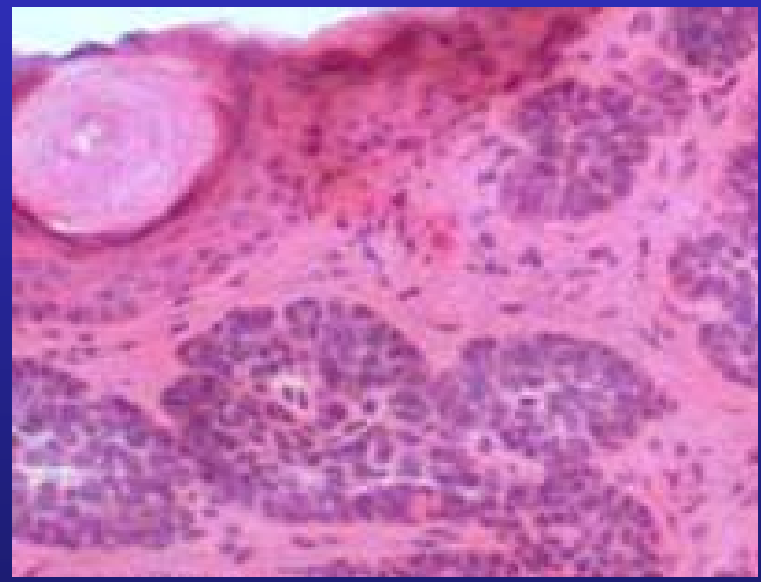
Additional Challenges of Clinical Importance

Pre-Implantation Genetic Diagnosis



Problem: Small Sample Size

Tumor Cancer Diagnosis



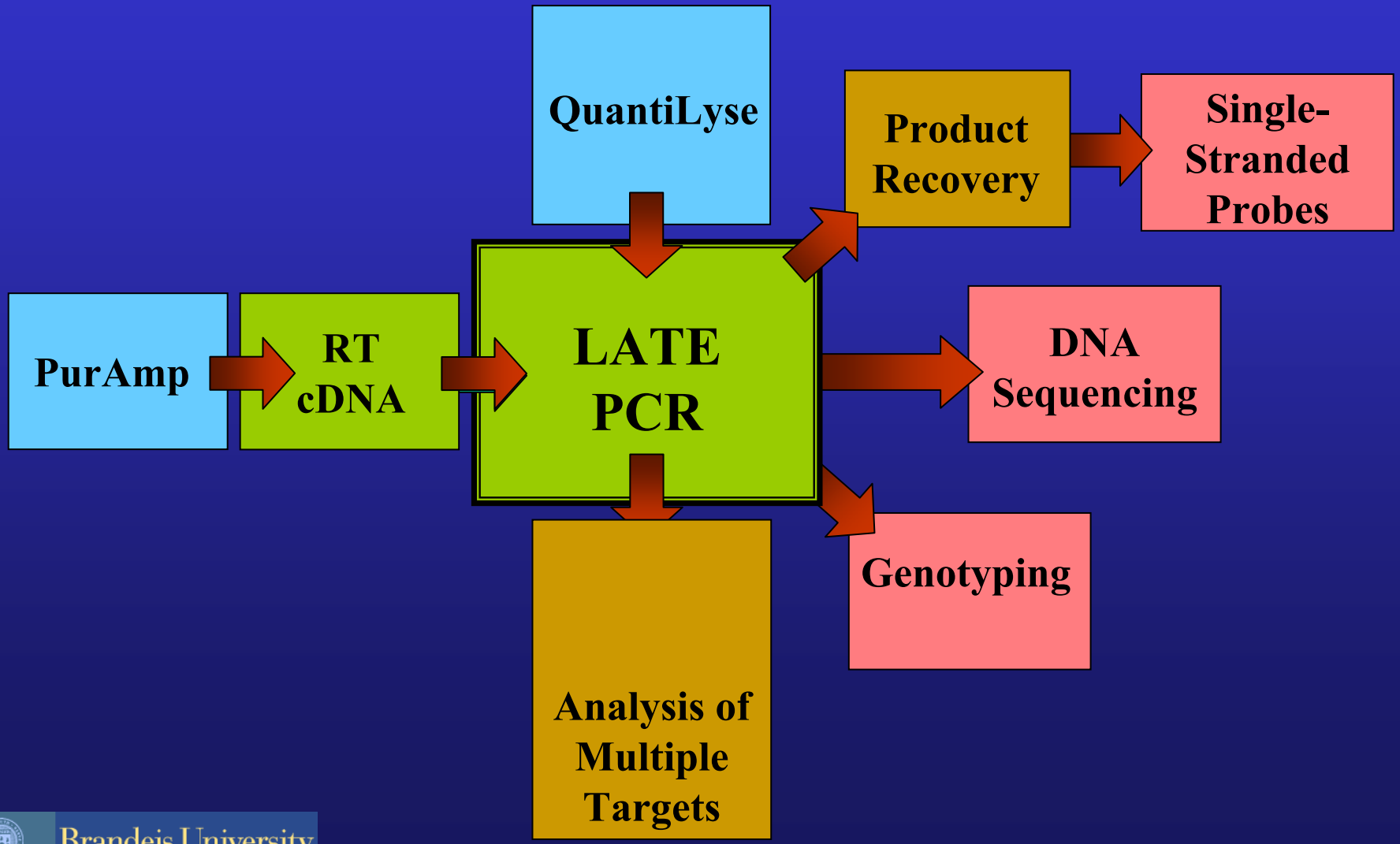
Problem: Tissue Heterogeneity

Solutions to General Problems in *In Vitro* Diagnostics:

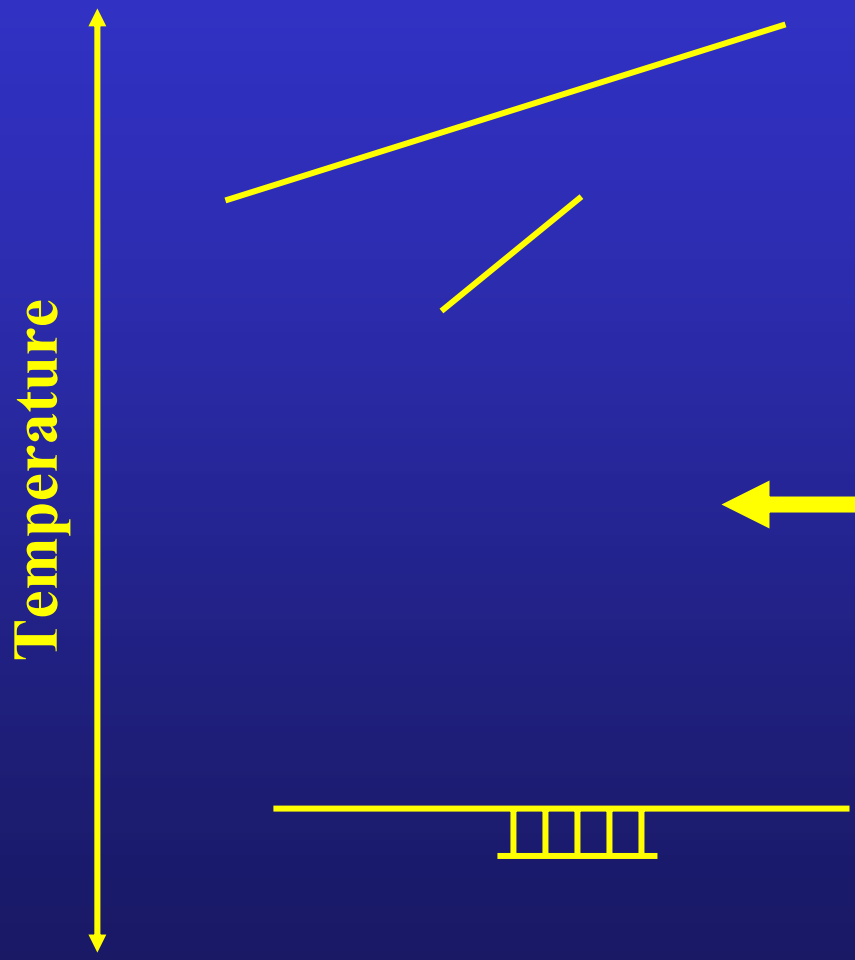
- Improved Sensitivity of Detection
- Increased Accuracy of Detection
- Increased Reliability of Detection
- Simpler Methods and Instrumentation
- Simultaneous Detection of Multiple Targets
- Broader Range of Applications
- Construction of Automated – Integrated Systems
- Reduce Time and Cost of Detection

The LATE-PCR Platform Technologies

Sample Preparation Reaction Methods Product Analysis Applications



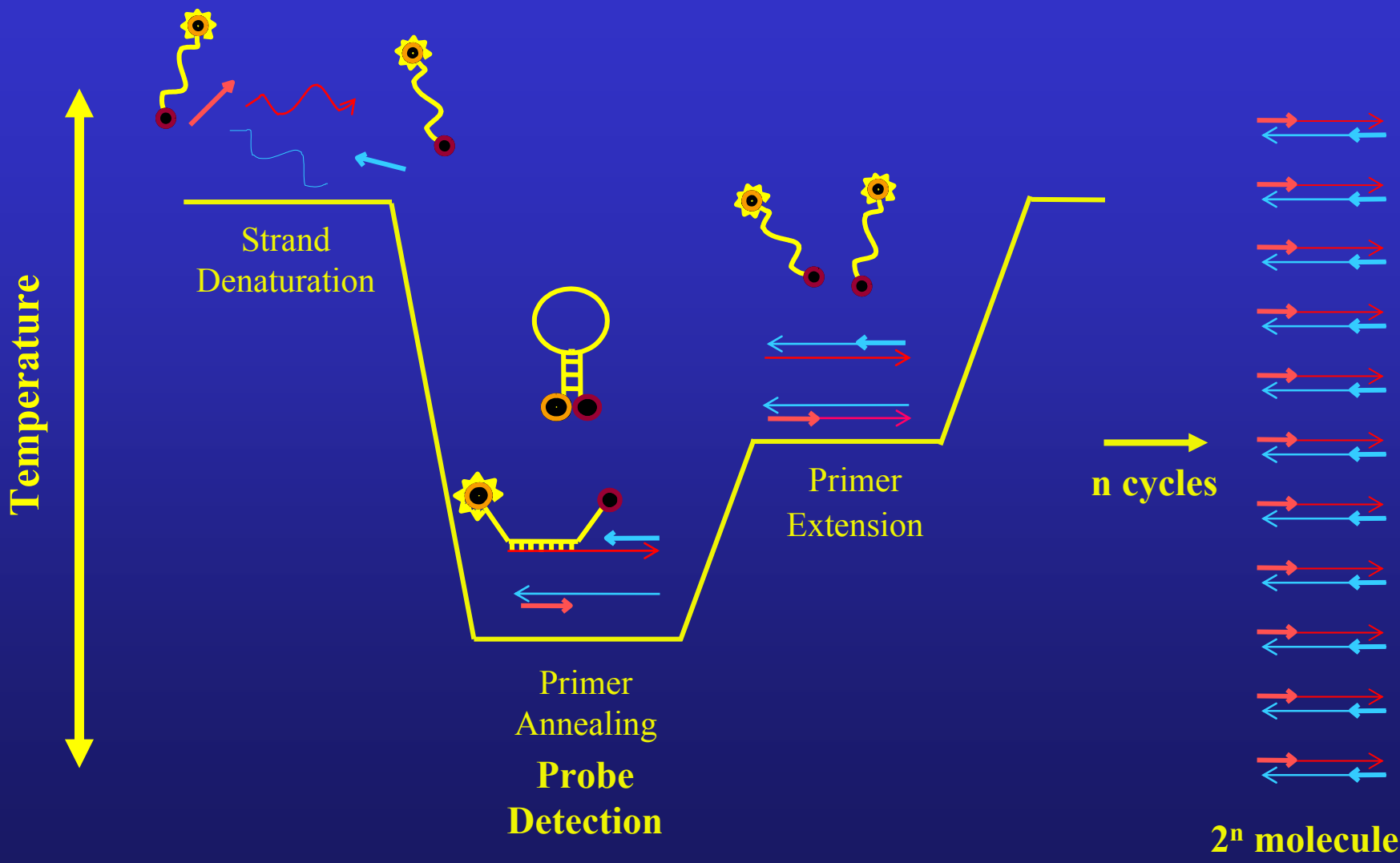
Hybridization & Melting of DNA Strands is Temperature-Dependent



Tm, the Melting Temperature,

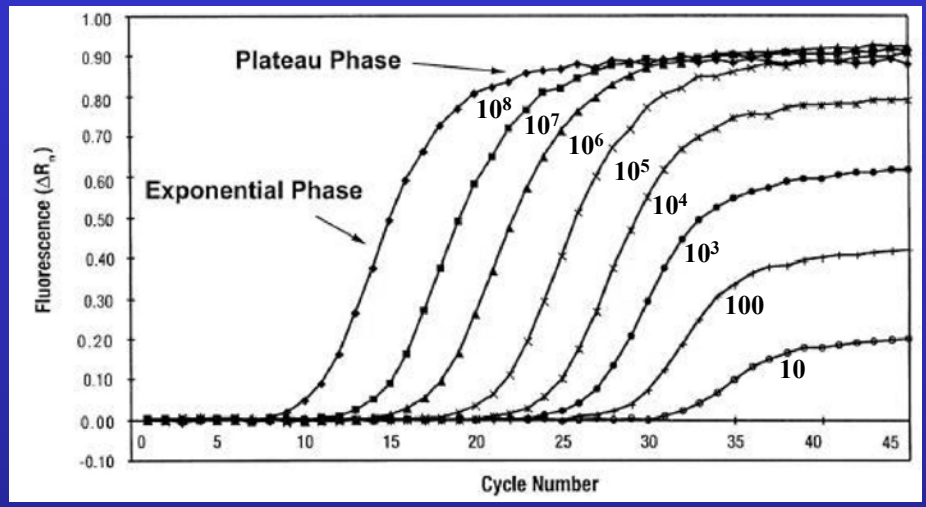
Hybridization 50% of the Time

Symmetric (conventional) PCR

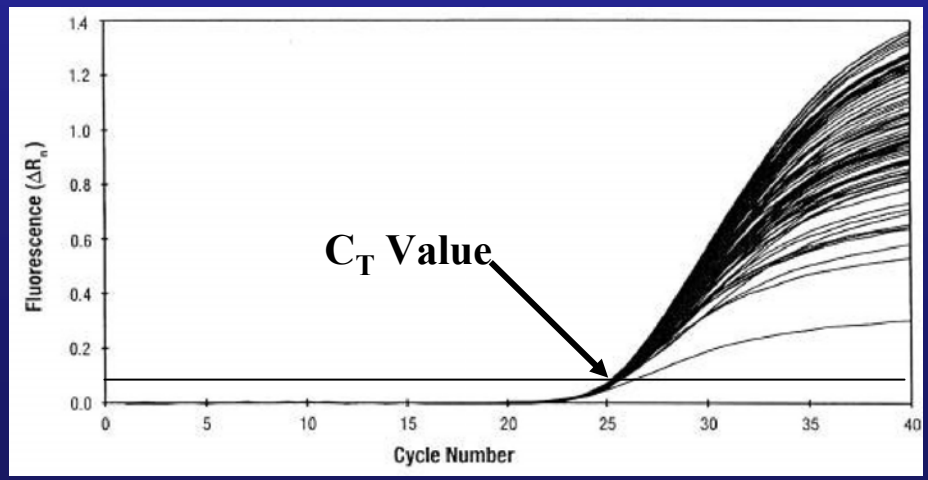


Limitations of Real-Time Symmetric PCR

Lower Sensitivity for Smaller Number of DNA Targets



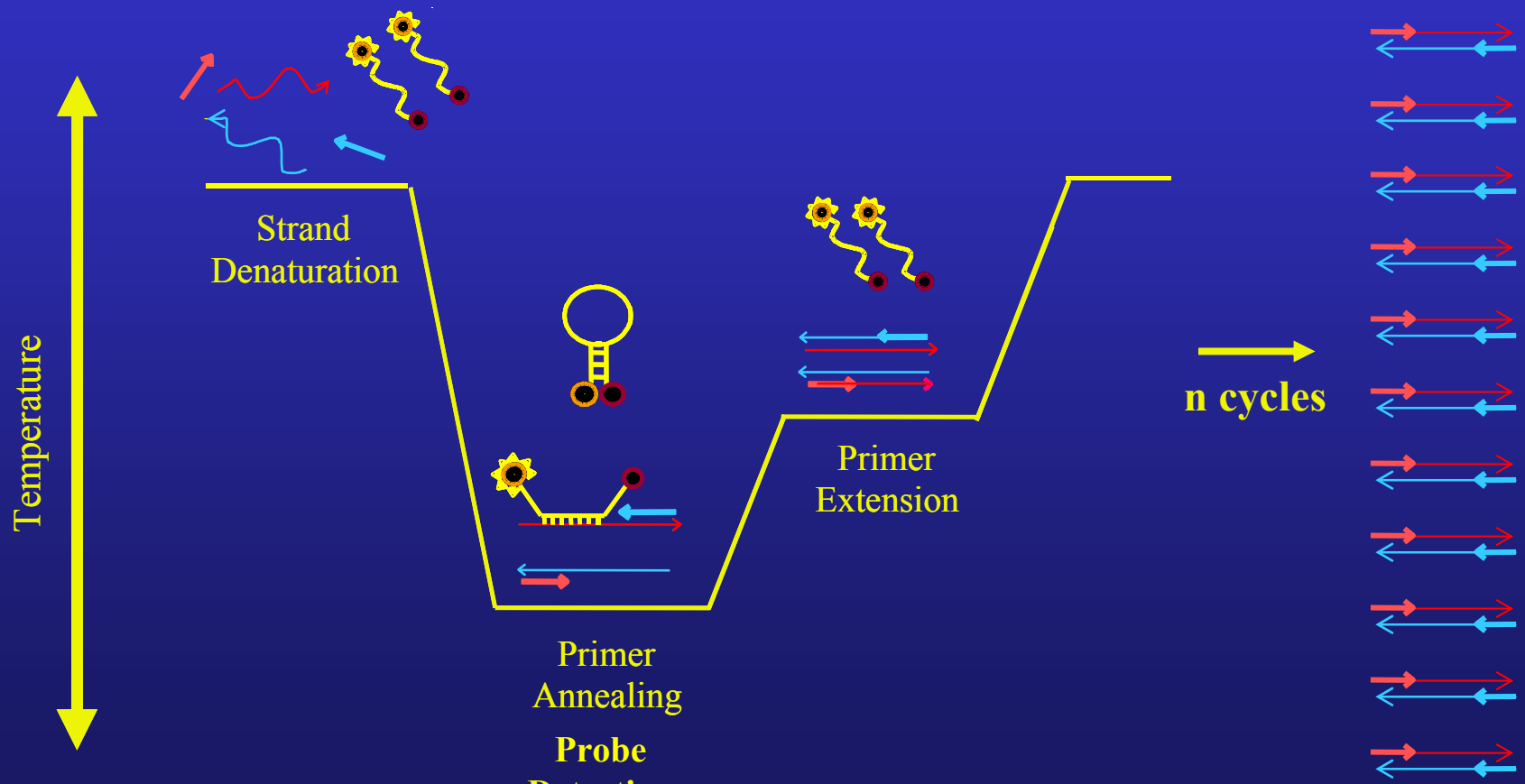
Lack of Reliability Among Replicate Samples



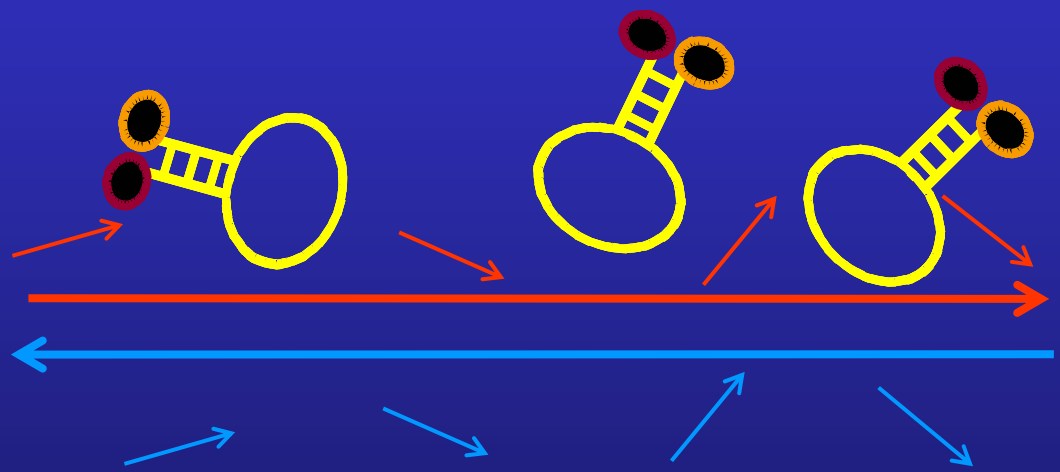
Brandeis University

Freeman *et al.*, (1999) Biotechniques 26: 122-125

Symmetric PCR Generates High Concentrations of Both Strands



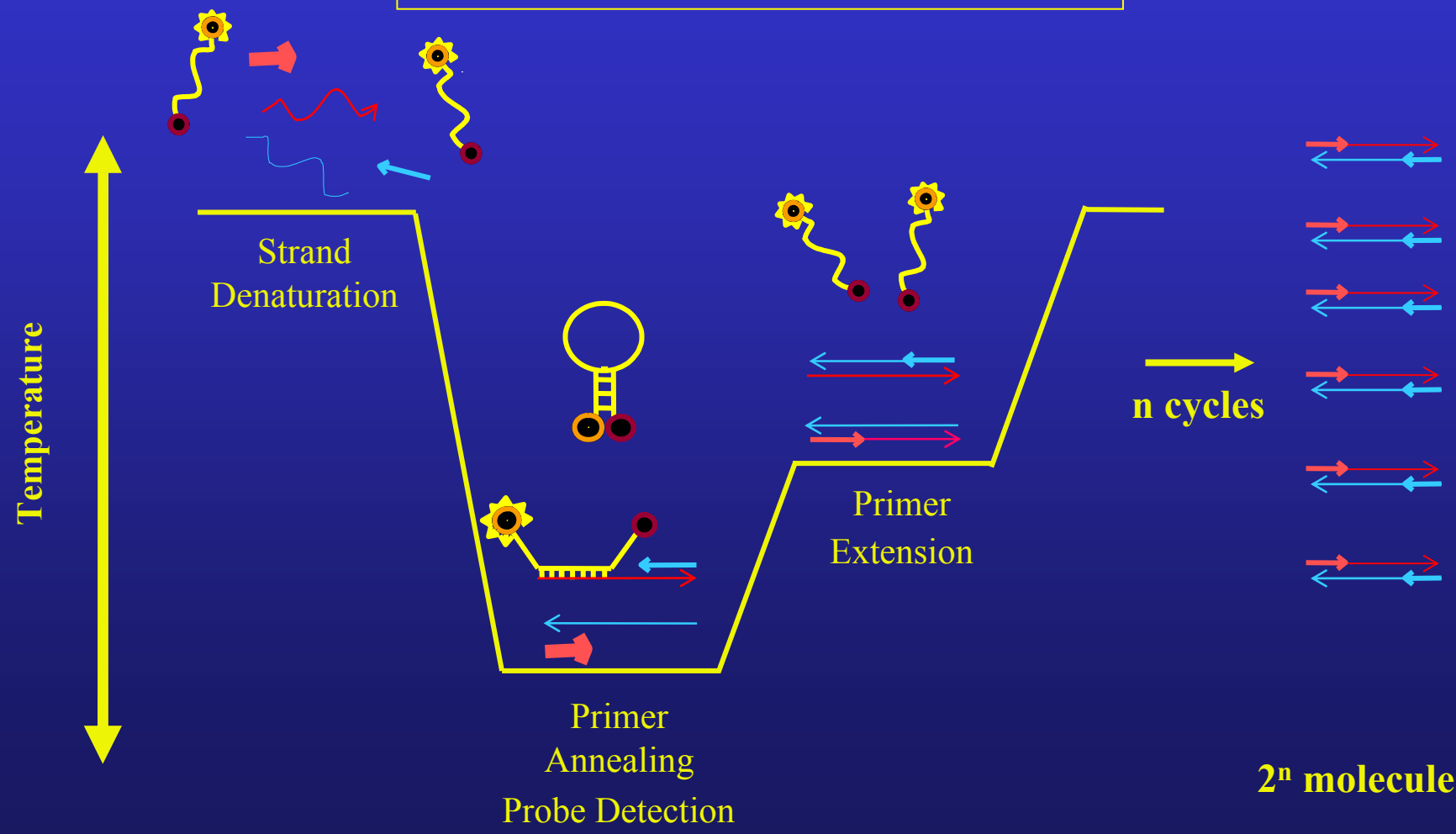
Why Does PCR Plateau?
The Problem of Amplicon Strand Reannealing



Amplicon Strand Reannealing Competes With Primer and Probe Binding

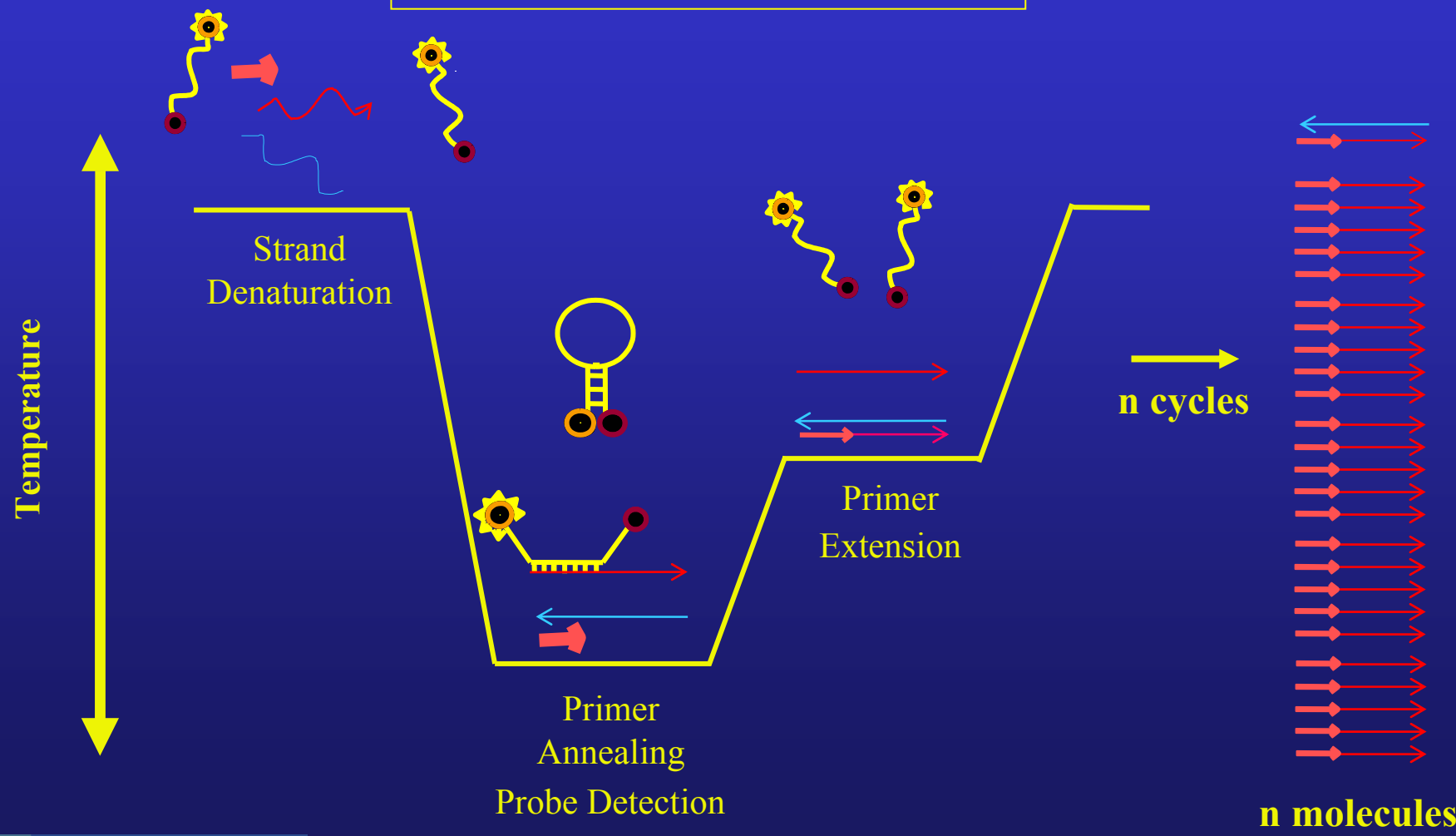
Asymmetric PCR As a Solution to Amplicon Strand Reannealing

Phase I – Exponential Amplification



Asymmetric PCR As a Solution to Amplicon Strand Reannealing

Phase II – Linear Amplification



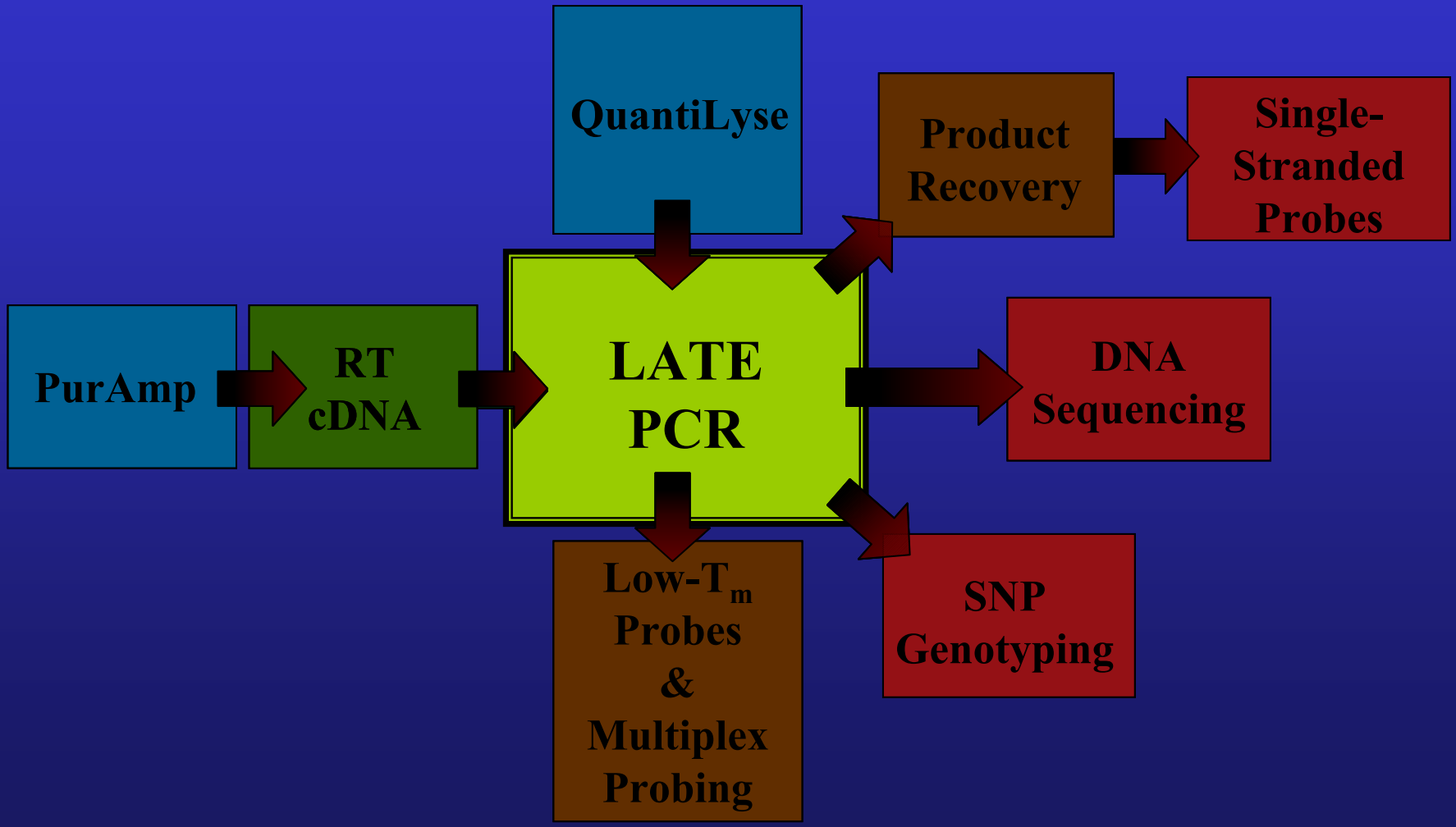
The Problem With Conventional Asymmetric PCR

"Although attractive in theory, asymmetric PCR is quite difficult to perform since the technique requires much optimization for each specific template-primer combination…. It is (also) important to find the optimal ratio between the two primers and the optimal amount of starting material…"

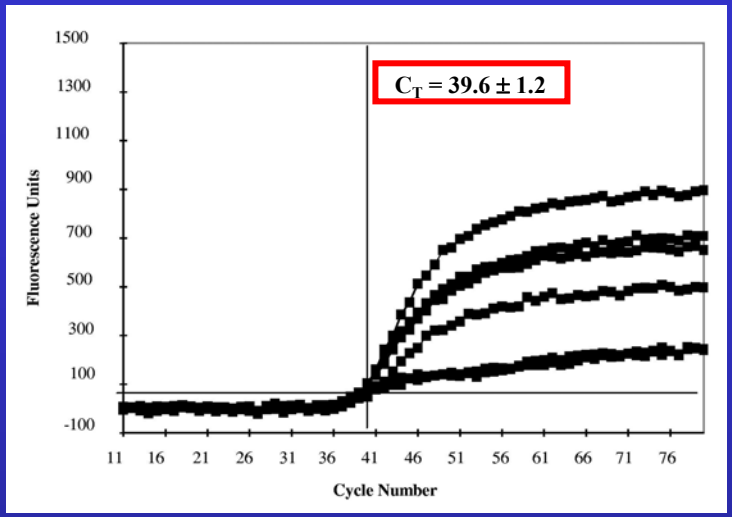
From: http://autodna.apbiotech.com/handbook/seq/seqb-18.htm

The LATE-PCR Platform Technologies

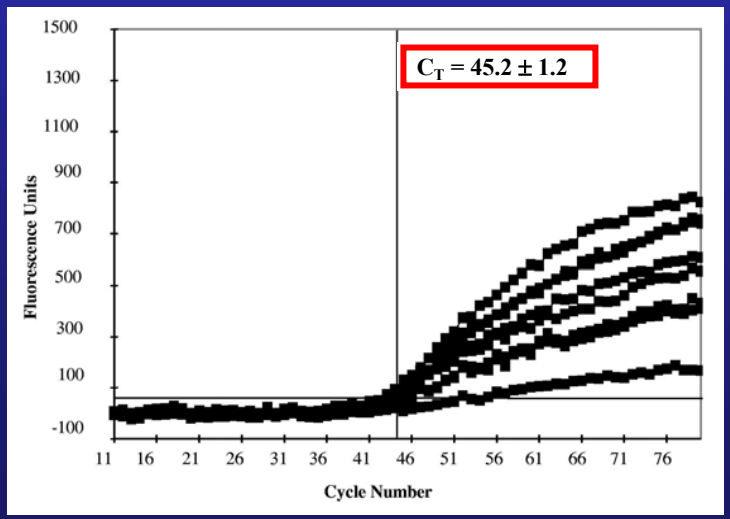
Sample Preparation | **Reaction Methods** | Product Analysis | Applications



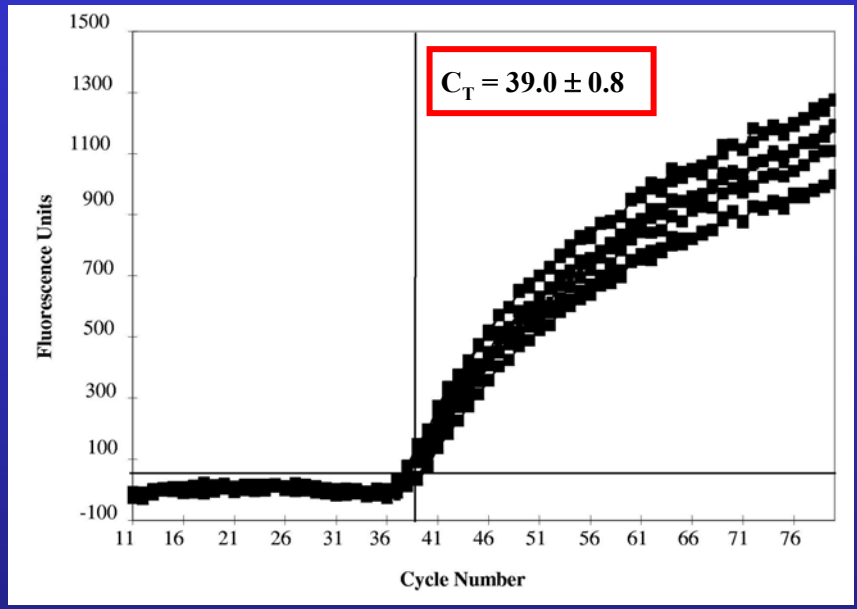
Symmetric PCR



Asymmetric PCR



LATE-PCR



- Efficient
- Sensitive (does not plateau)
- Reliable

Sanchez *et al.* (2003), In Preparation for Nature Biotechnology

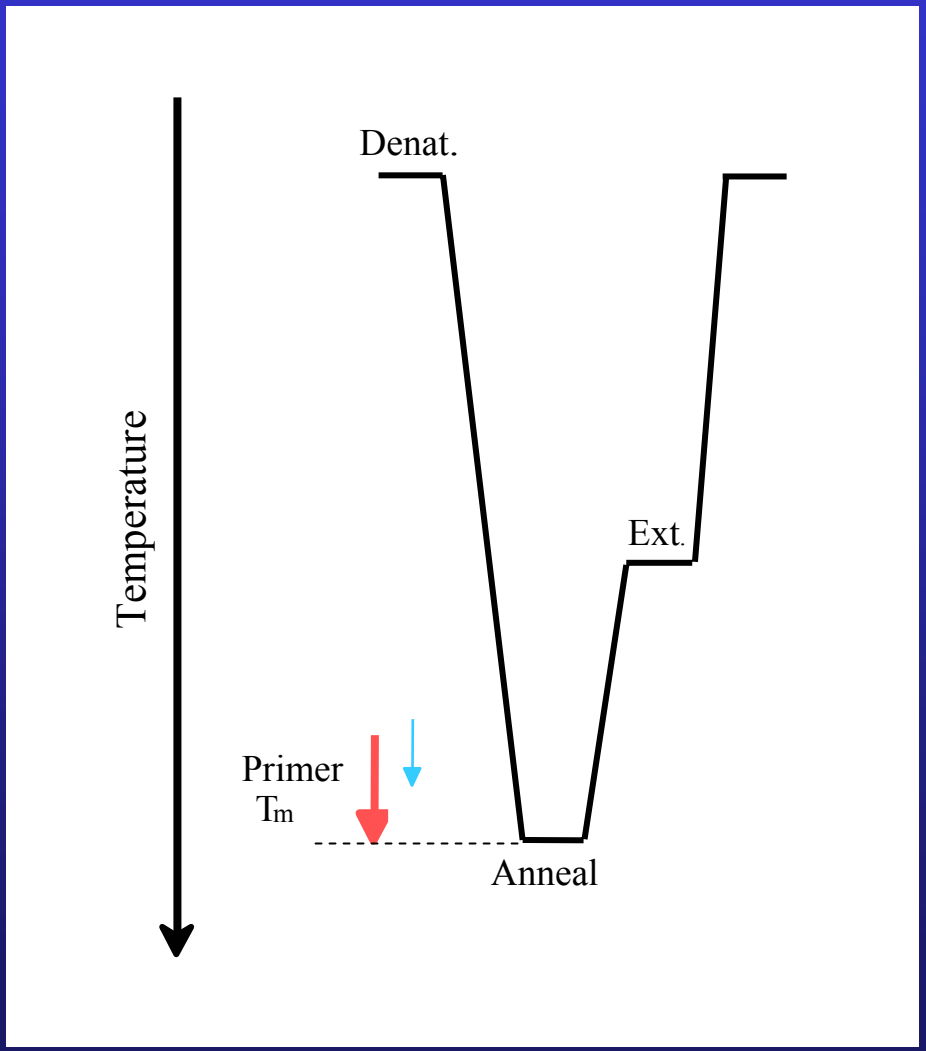
The Key to LATE-PCR Primer Design

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!



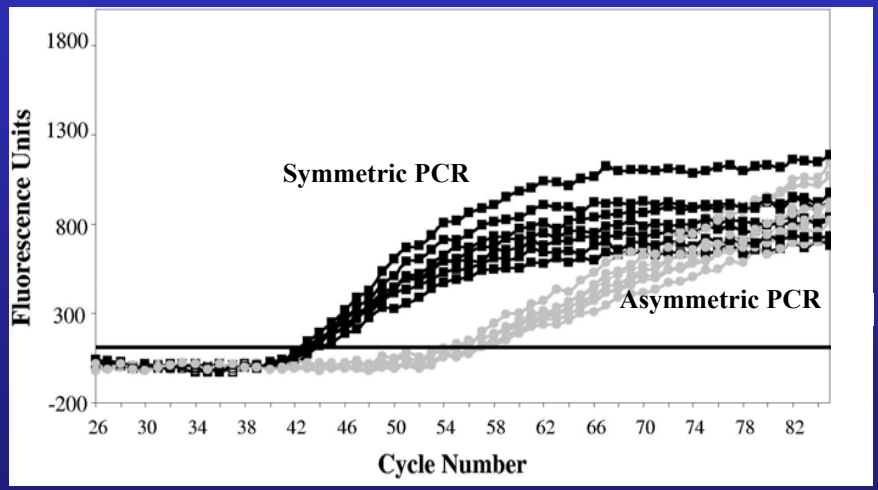
Simple Redesign of Primers Improves Amplification Efficiency and Maintains Quantitative Kinetics of Real-Time PCR

	Sequence	nM	T_m (°C)	ΔT_m
Symmetric PCR	5'-CCTTCTCTCTGCCCCCTGGT-3'	1000	64.8	**+ 0**
	5'-GCCAGGGGTTCCACTACGTAGA-3'	1000	64.3	
Conventional Asymmetric PCR	5'-CCTTCTCTCTGCCCCCTGGT-3'	**25**	**58.9**	**- 5**
	5'-GCCAGGGGTTCCACTACGTAGA-3'	1000	64.3	
LATE-PCR	5'-**GC**CCTTCTCTCTGCCCCCTGGT -3'	**25**	**64.0**	**+ 0**
	5'-GCCAGGGGTTCCACTACGTAGA-3'	1000	64.3	

Sanchez *et al.* (2003), In Preparation for Nature Biotechnology

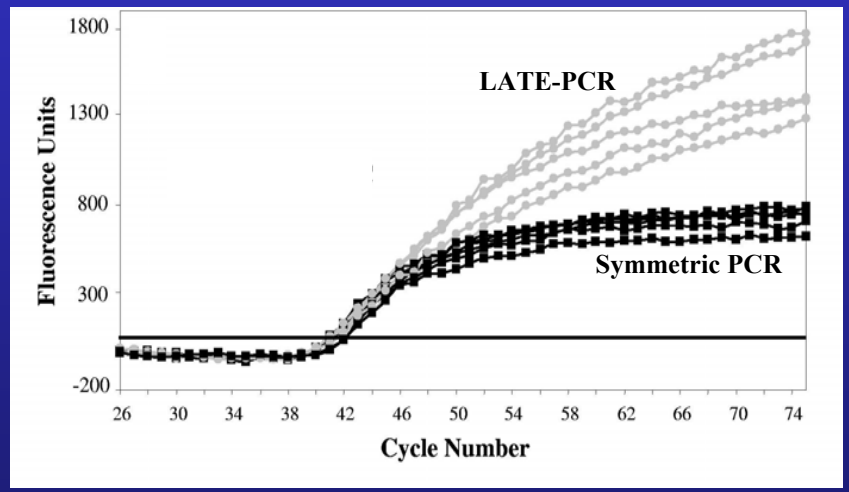
Validation of LATE-PCR Primer Design

$(T_m^L - T_m^X) < 0$



Inefficient

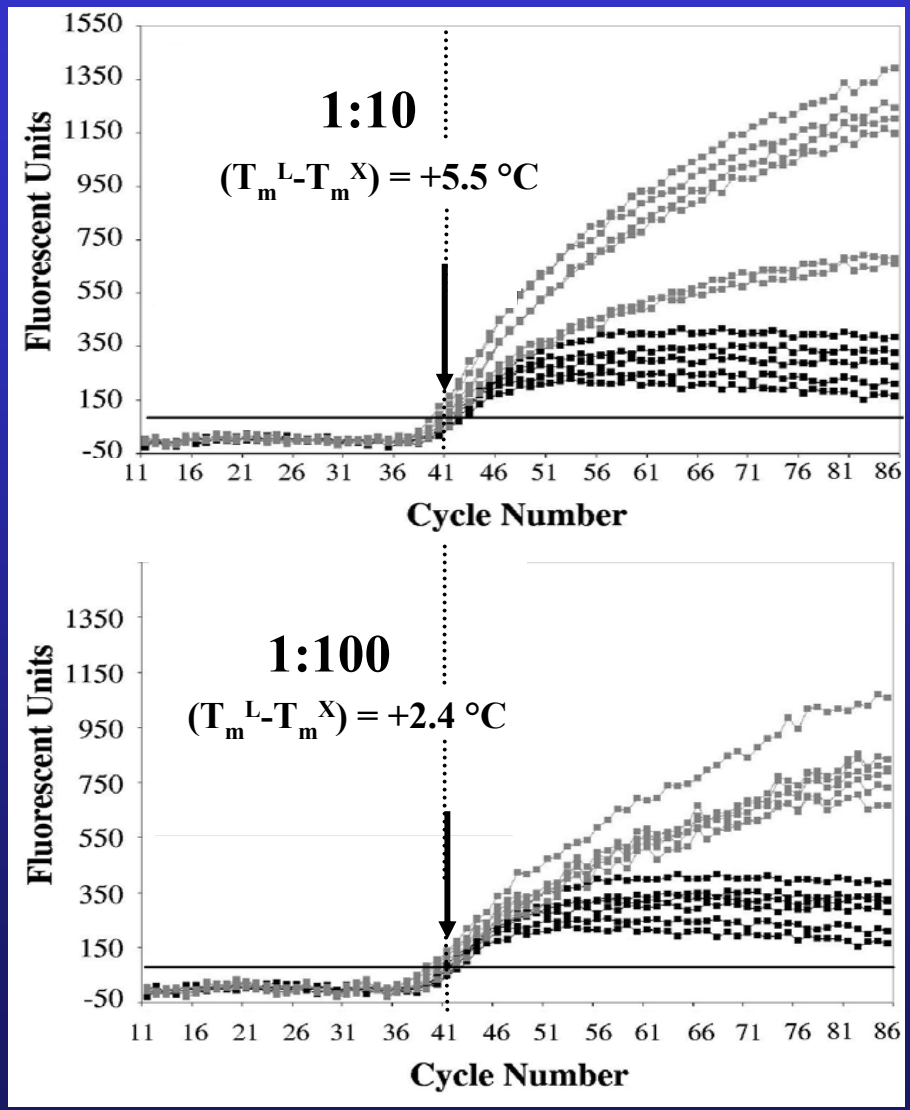
$(T_m^L - T_m^X) \geq 0$

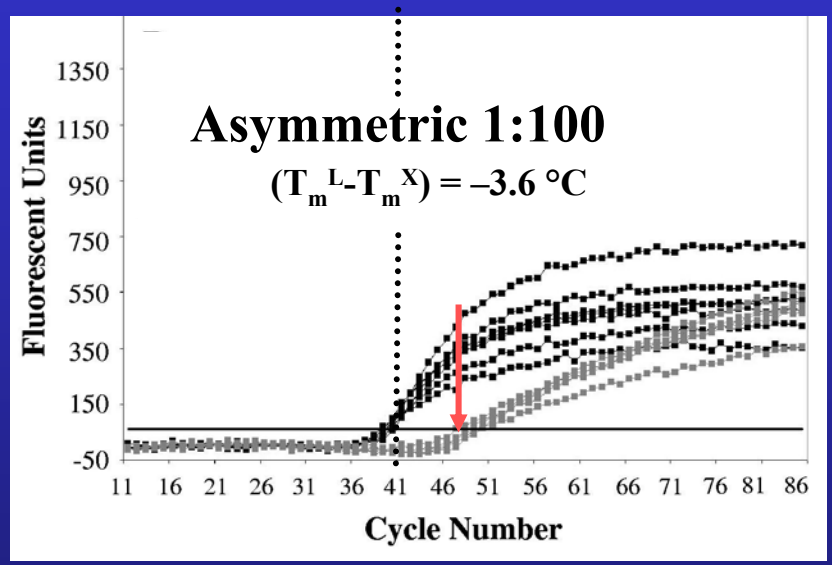


Efficient

Sanchez *et al.* (2003), In Preparation for Nature Biotechnology

LATE-PCR: Rational Design Allows A Broad Range of Primer Ratios



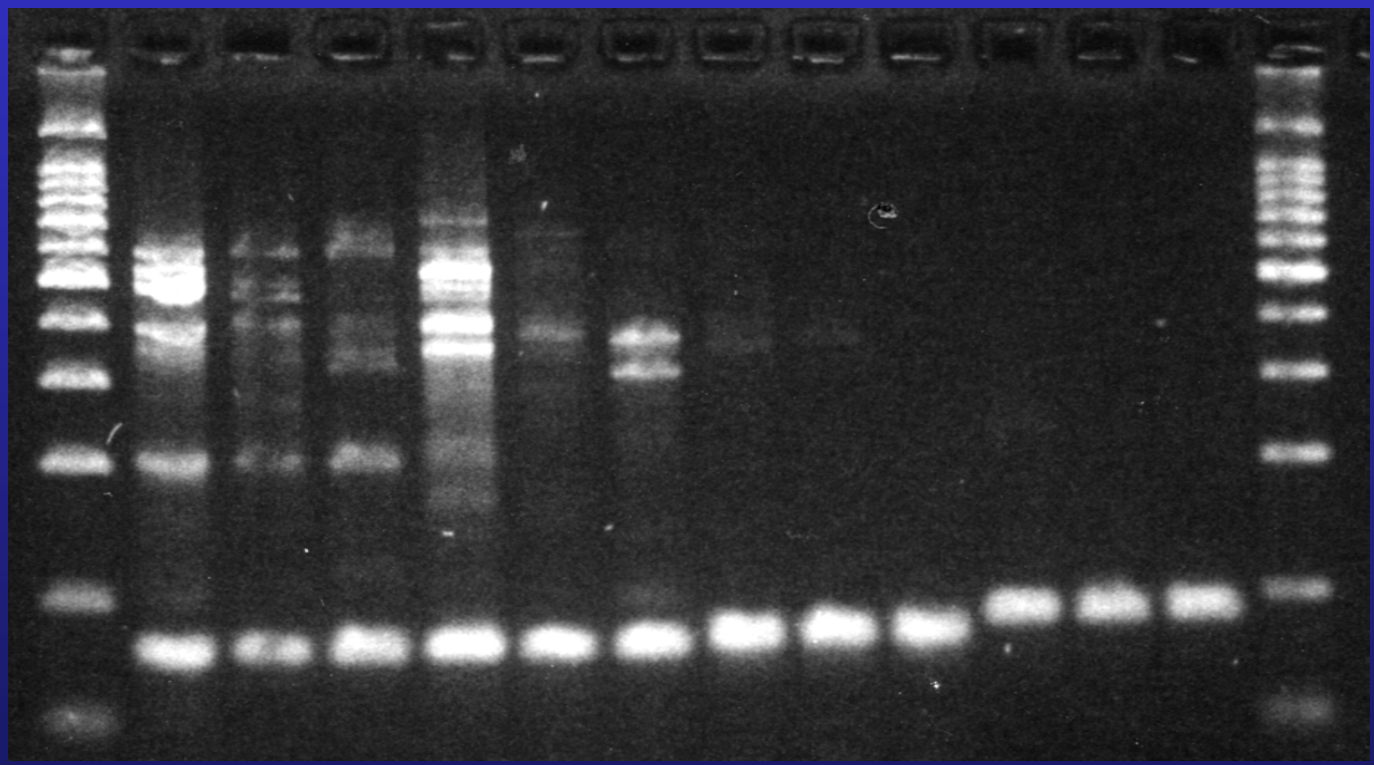


Rule 1: $(T_m^L - T_m^X) \geq 0$

Sanchez *et al.* (2003), In Preparation for Nature Biotechnology

Increasing the Value of (T_m^L-T_m^X) also Increases Amplification Specificity

-3 0 +5 +10



100 genomes, Annealing Temp. 2°C Below T_m^L

Pierce *et al.* (2003), In Preparation for Nucleic Acid Res.

The Problem of Product Amplicon Strand Competition

Rule 2: $(Tm^A - Tm^X) \leq 18^oC$

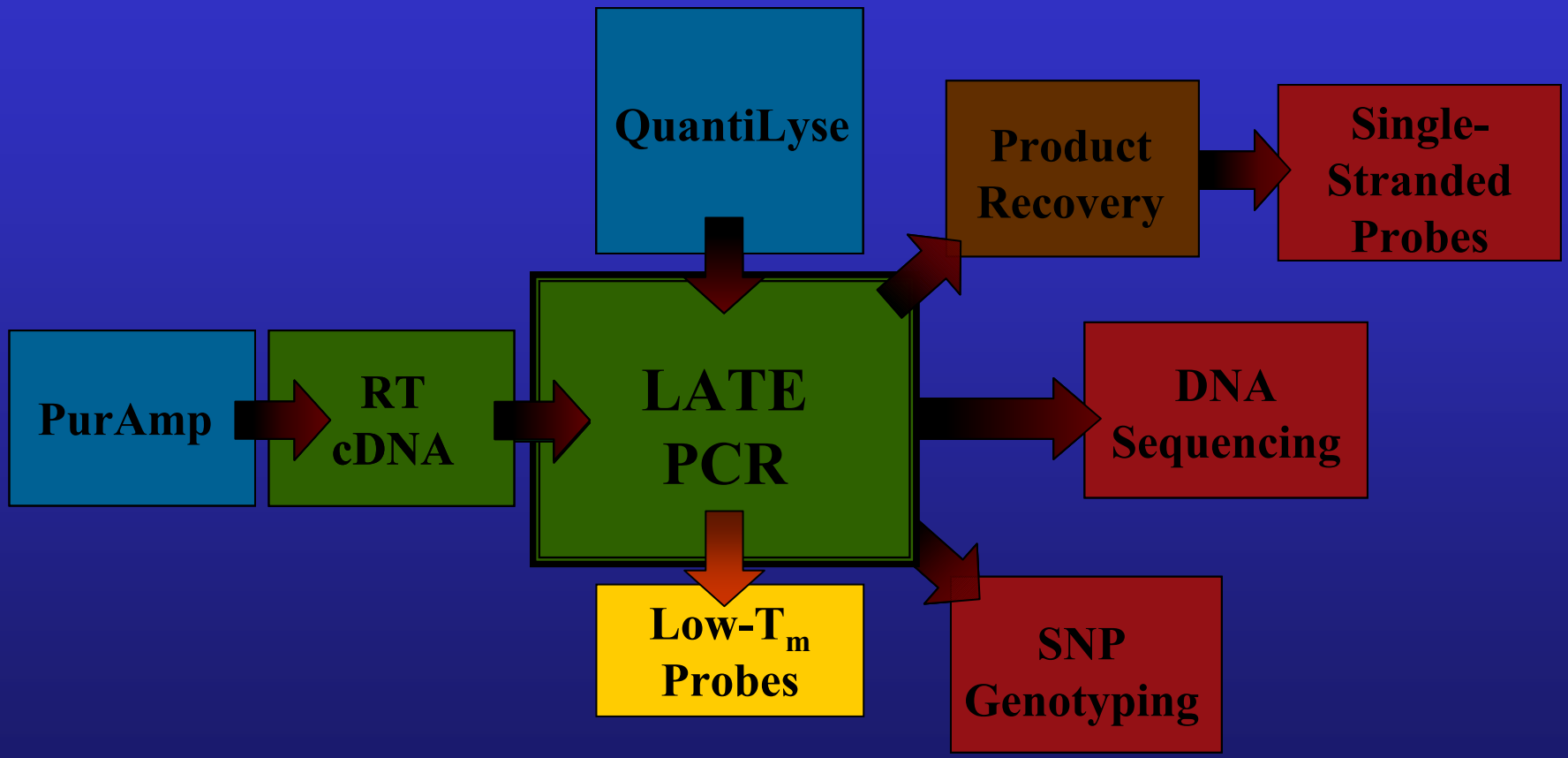
Benefits of LATE-PCR Primer Design

- Increased Efficiency
- Improved Sensitivity (No Plateau)
- Flexible Use of Primer Ratios

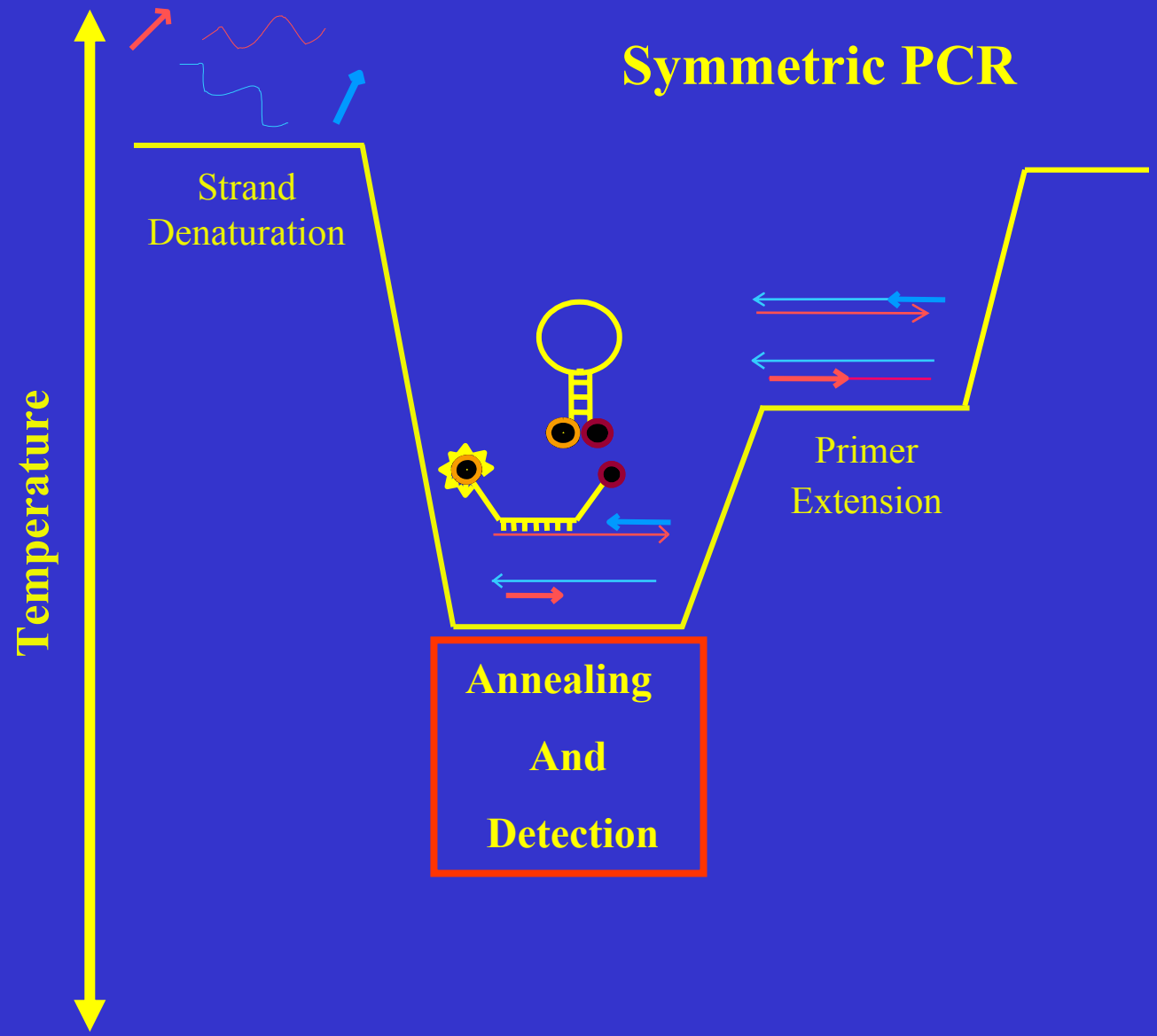
LATE-PCR Provides a Rational Framework for Efficient and Reliable Amplification of Single-Stranded DNA

The LATE-PCR Platform Technologies

Sample Preparation | Reaction Methods | **Product Analysis** | Applications



LATE-PCR Uncouples Annealing and Detection



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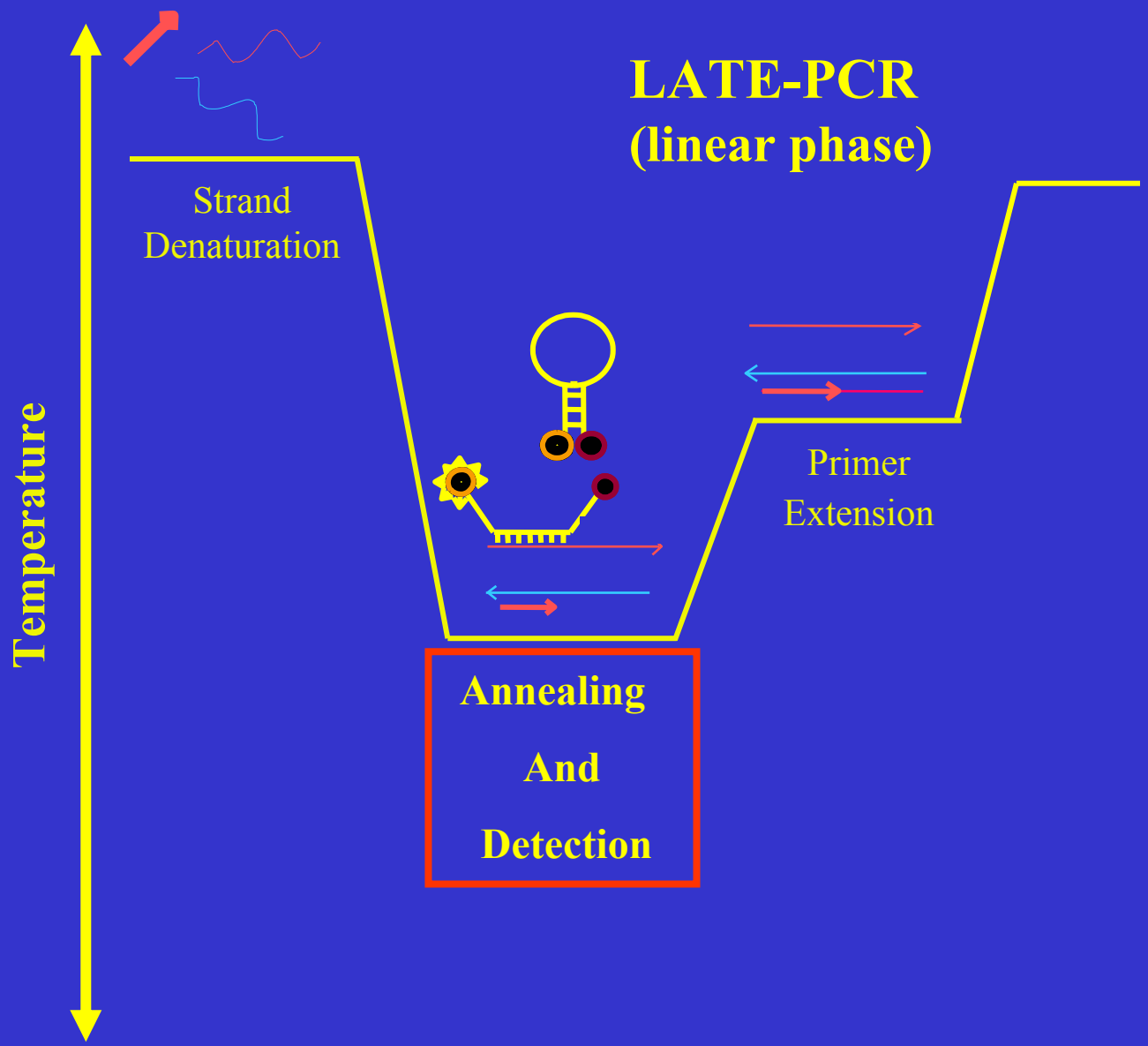

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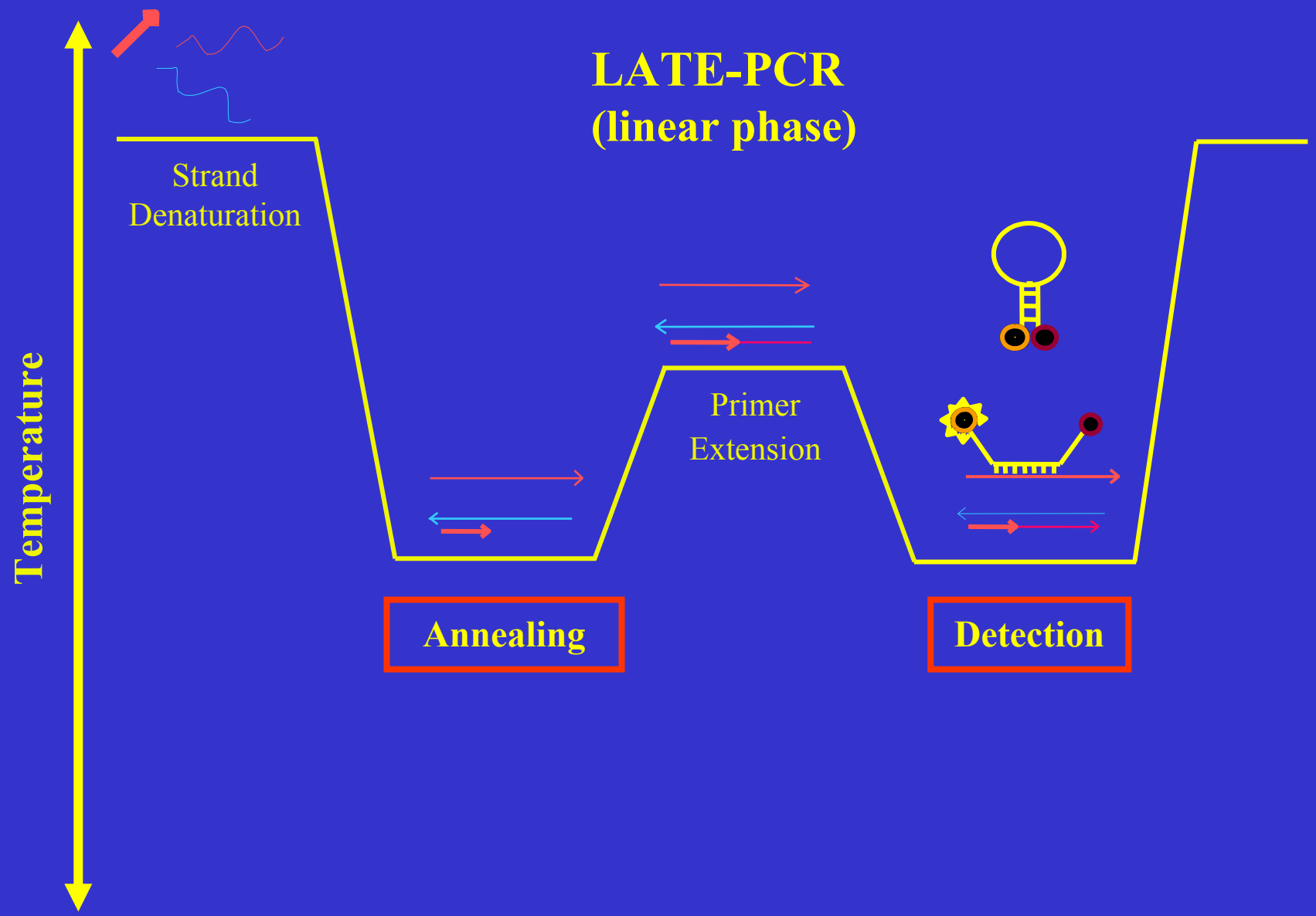
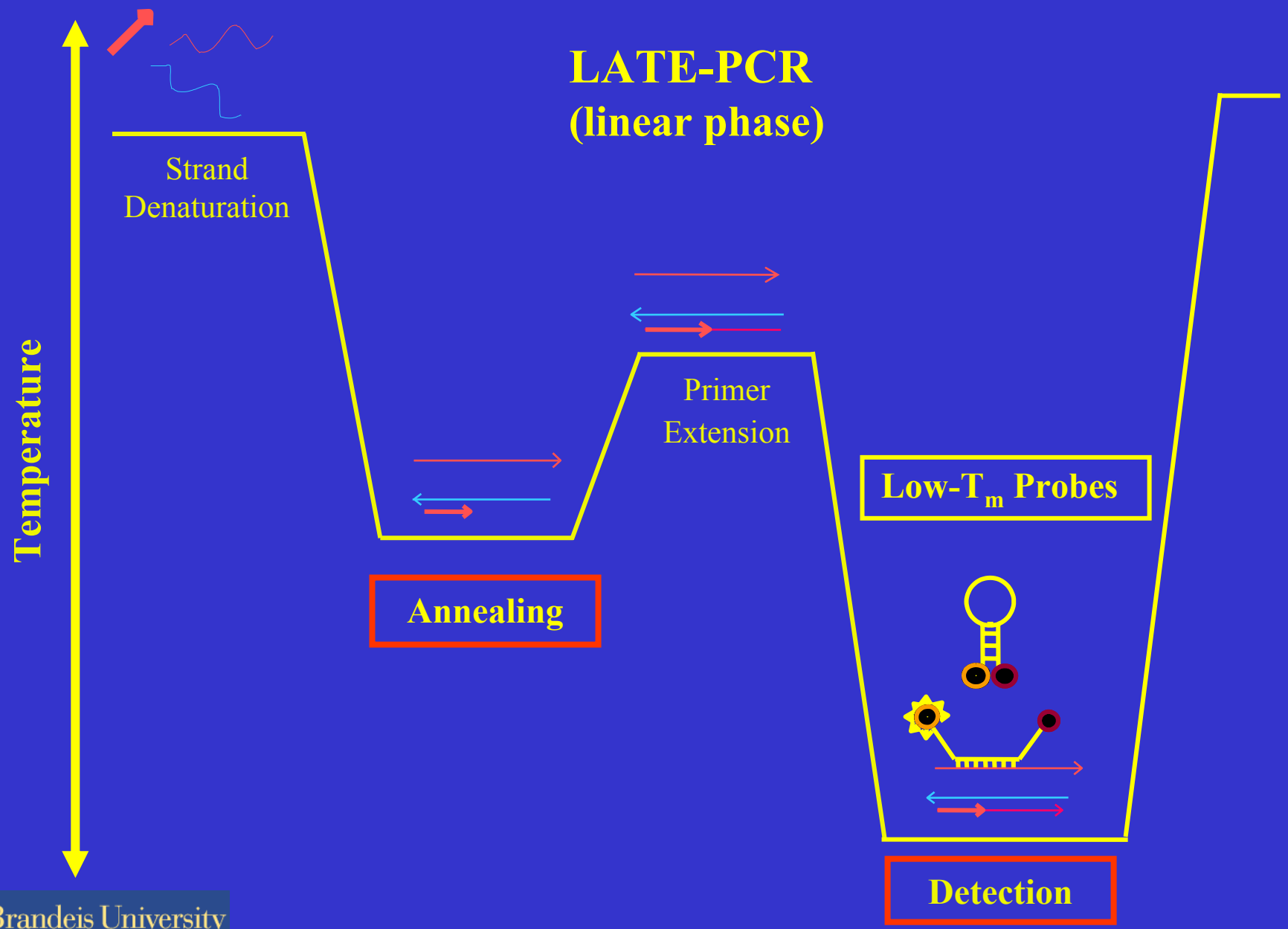
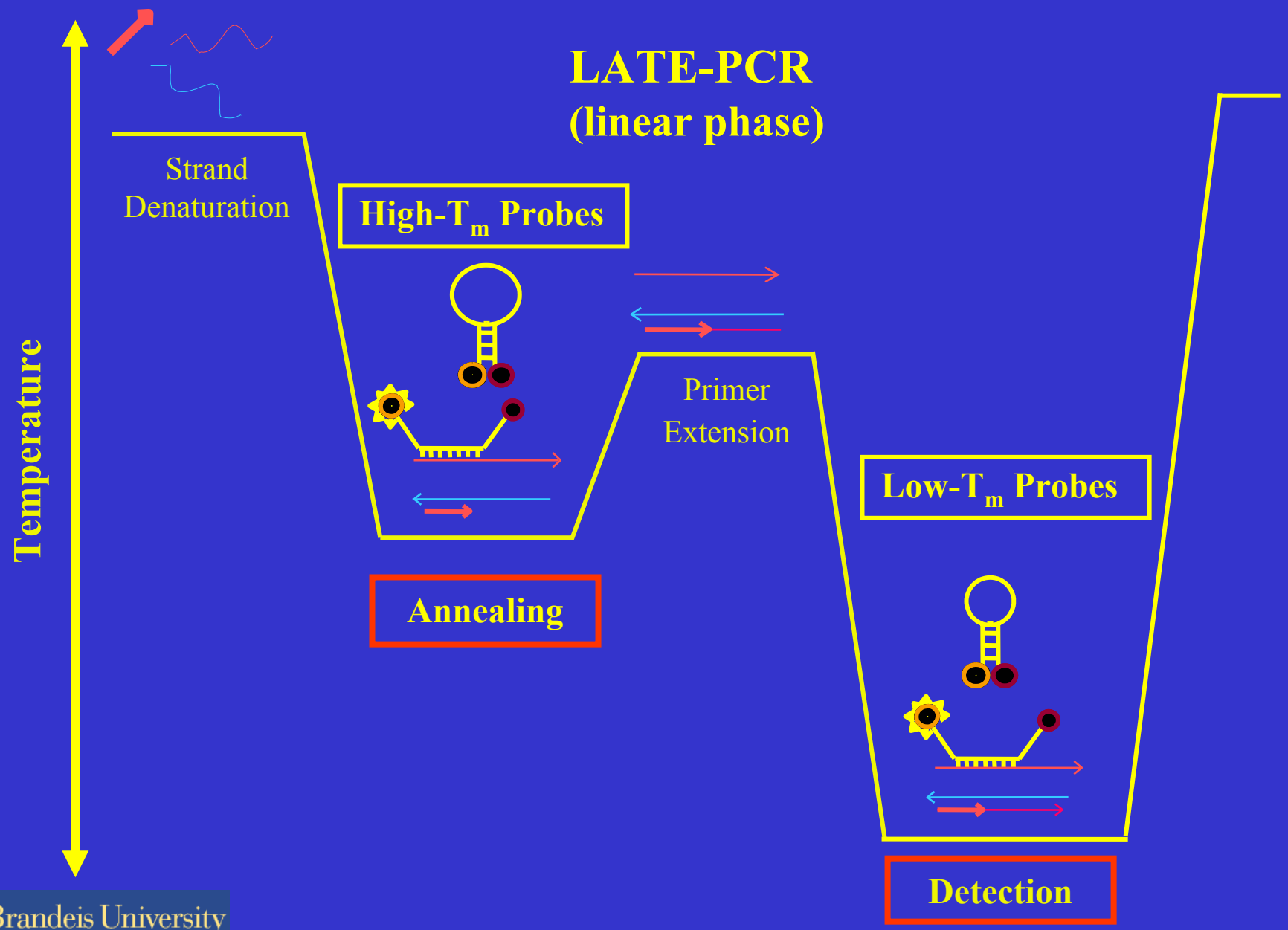


LATE-PCR Uncouples Annealing and Detection

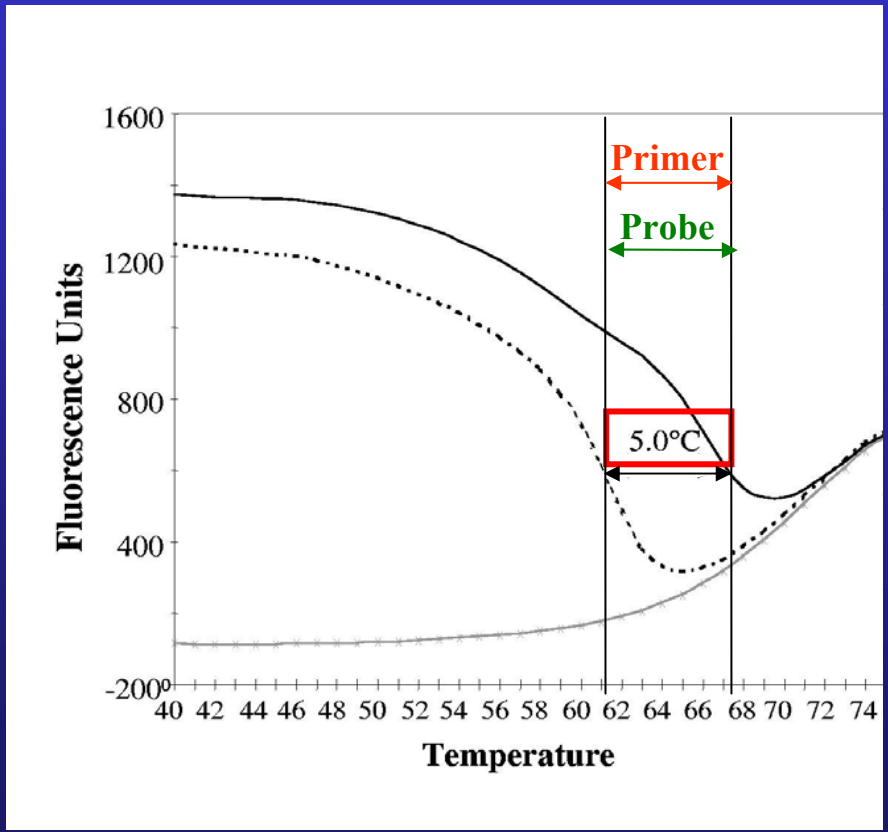


LATE-PCR Uncouples Annealing and Detection

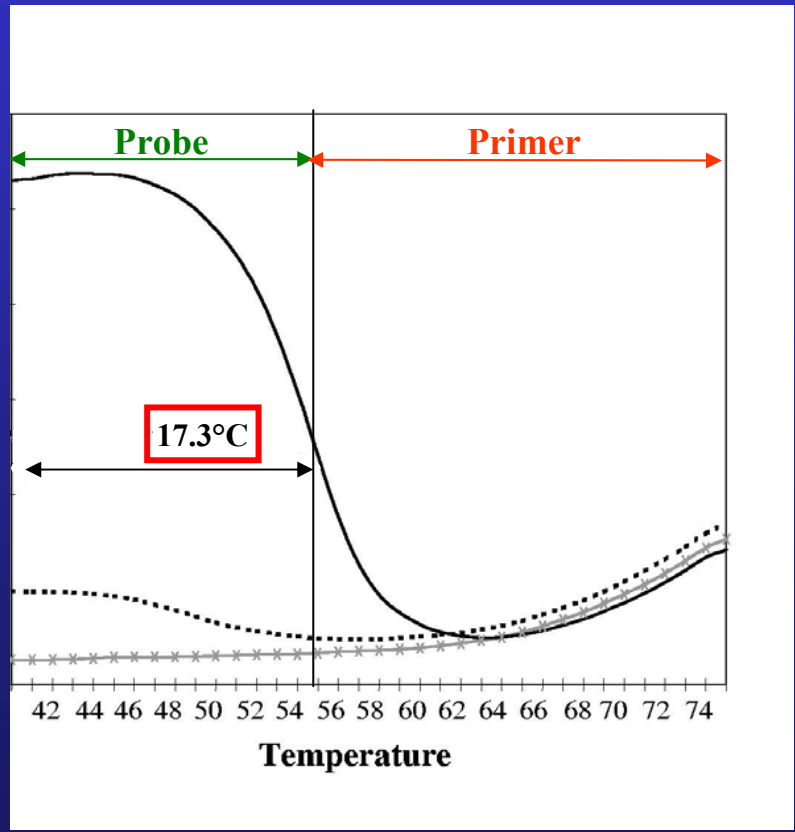


Advantages of Low-T_m Probes: Separate Temperature Window for Primer and Probe Design

High-T_m Molecular Beacon Melting Curve



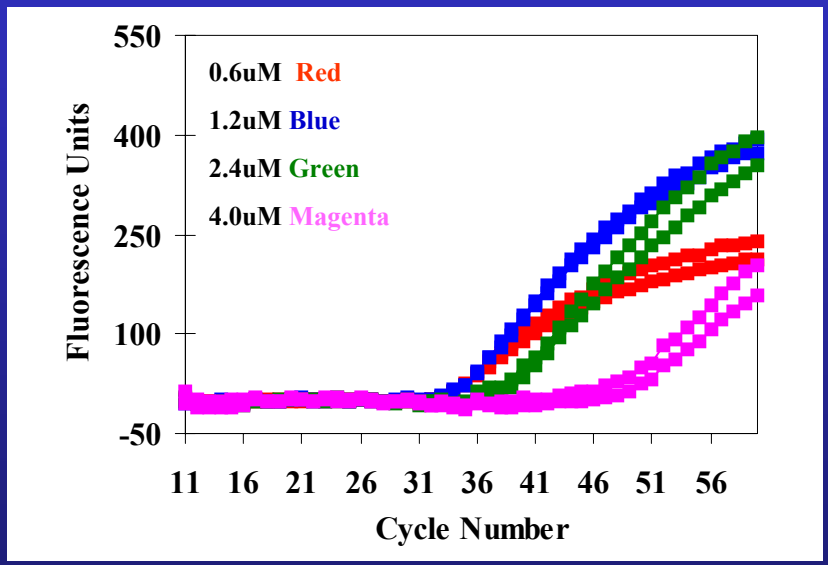
Low-T_m Molecular Beacon Melting Curve



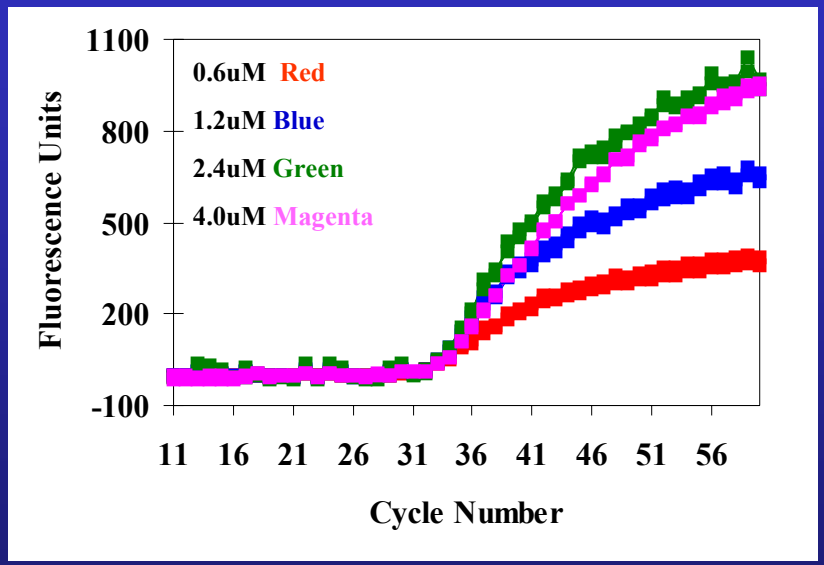
Sanchez *et al.* (2003) In Preparation for Nature Biotechnology

Advantages of Low-T_m Probes: Saturating Amounts for Increased Sensitivity

High-T_m Probes



Low-T_m Probes



Sanchez *et al.* (2003), In Preparation for Nature Biotechnology

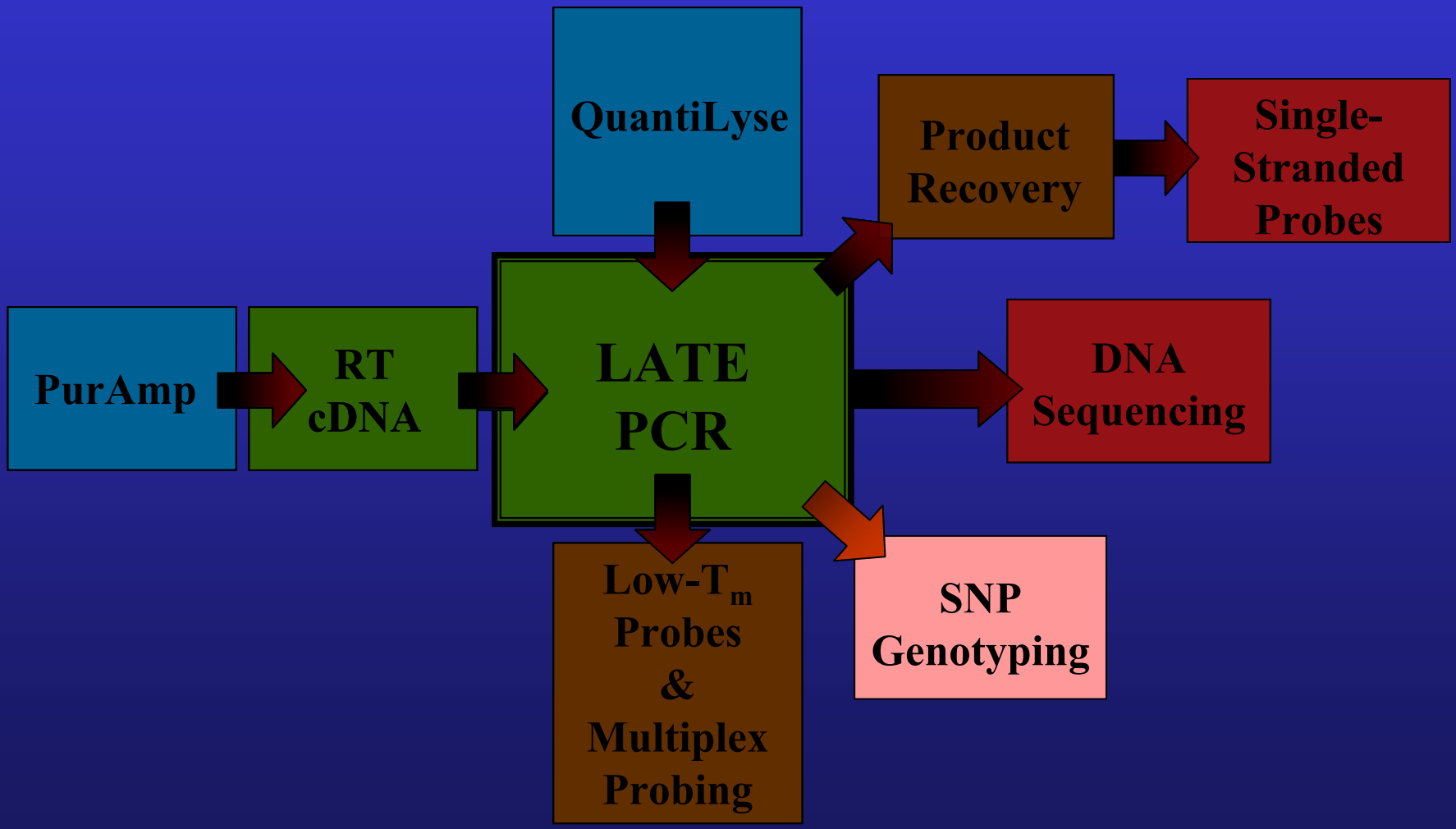
Benefits of Low-T_m Molecular Beacons

- Easier to Design – Not Constrained by Primer T_m
- Improved Sensitivity
- Do not Affect Amplification Efficiency
- Increased Allele Discrimination
- Expands Multiplexed Amplicon Detection

Low-T_m Molecular Beacons Provide Versatile and Sensitive Amplicon Detection in LATE-PCR

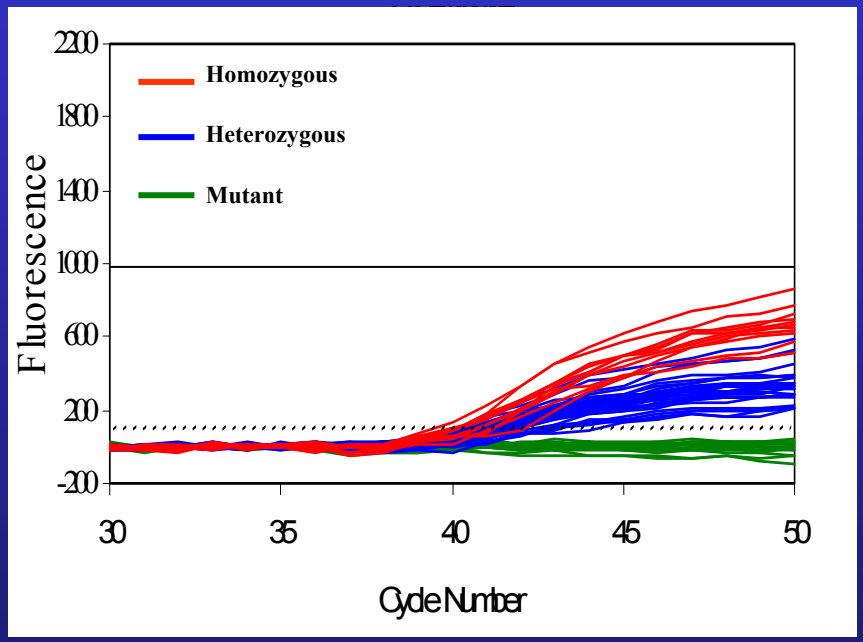
The LATE-PCR Platform Technologies

Sample Preparation Reaction Methods Product Analysis Applications

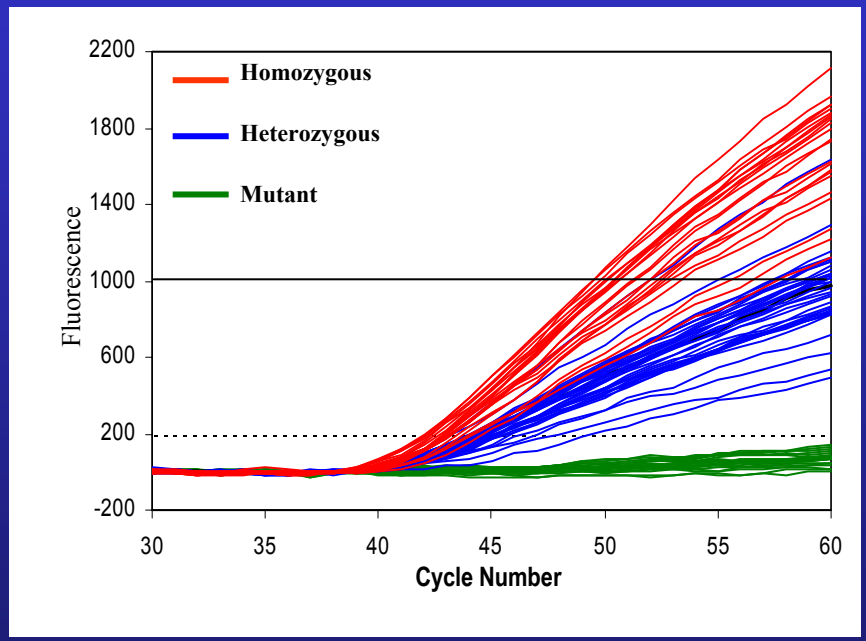


An Example of LATE-PCR Assay: Cystic Fibrosis

Symmetric PCR



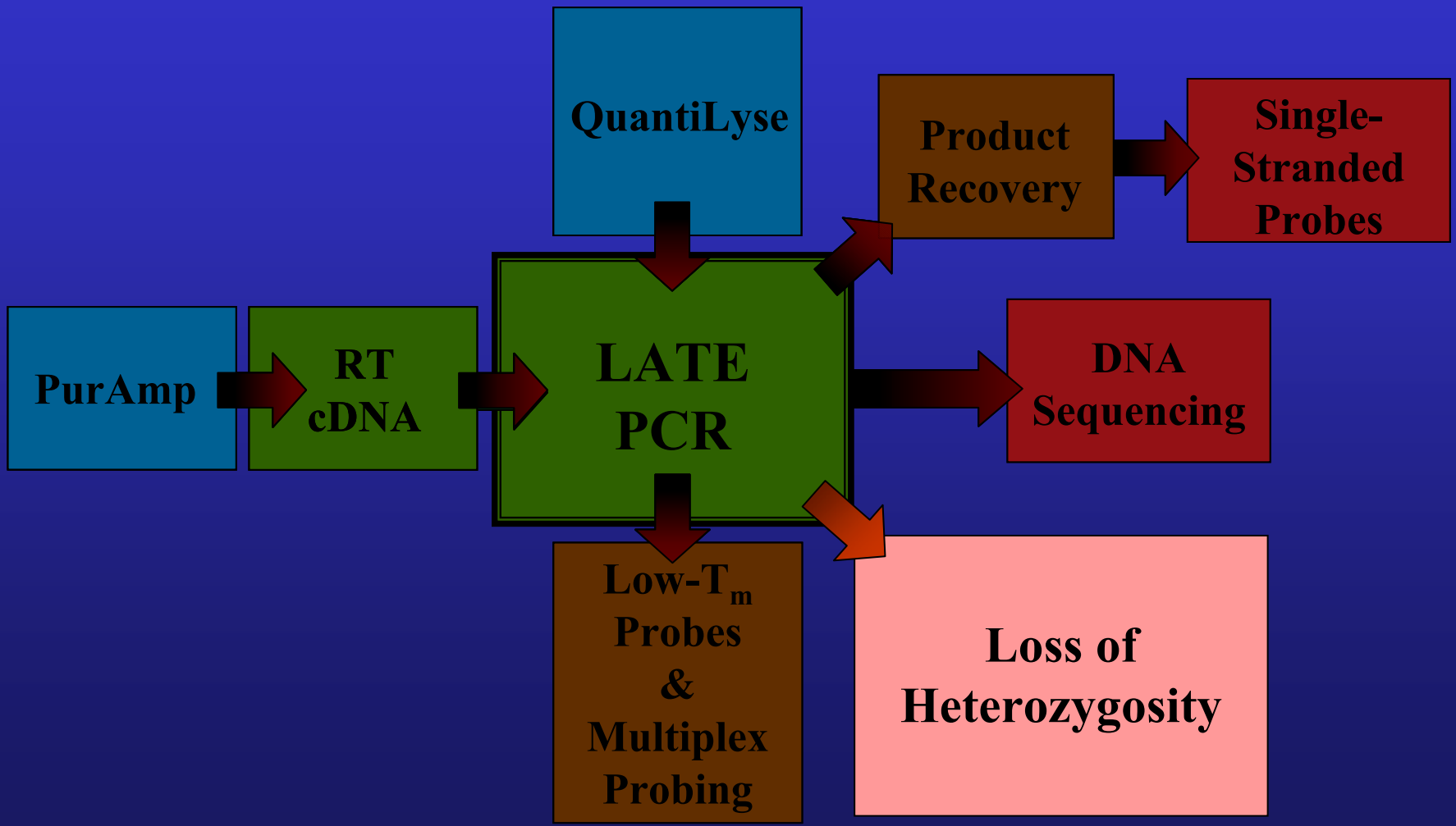
LATE-PCR



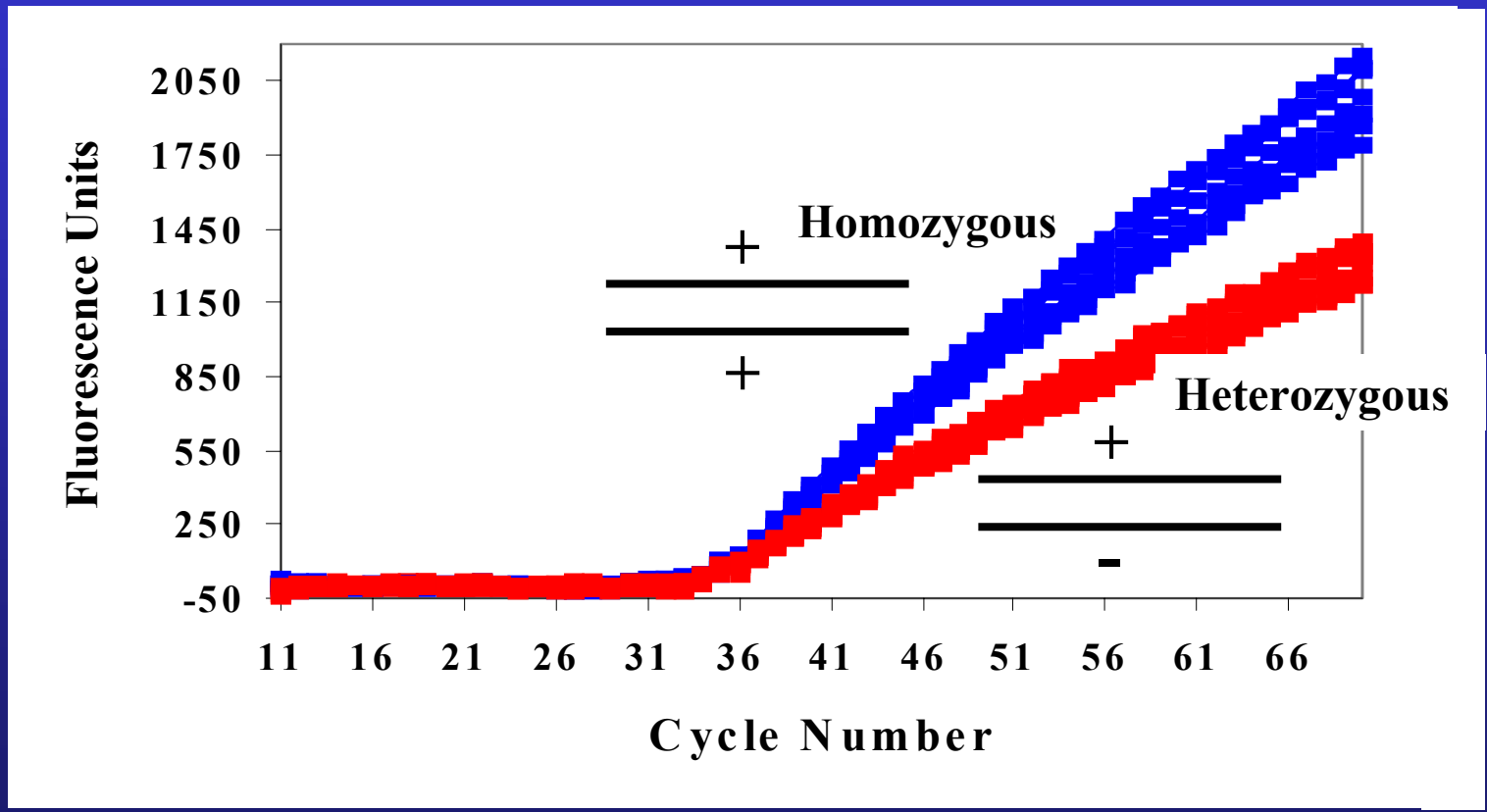
Pierce *et al.* (2003), In Preparation for Molecular Human Reproduction

The LATE-PCR Platform Technologies

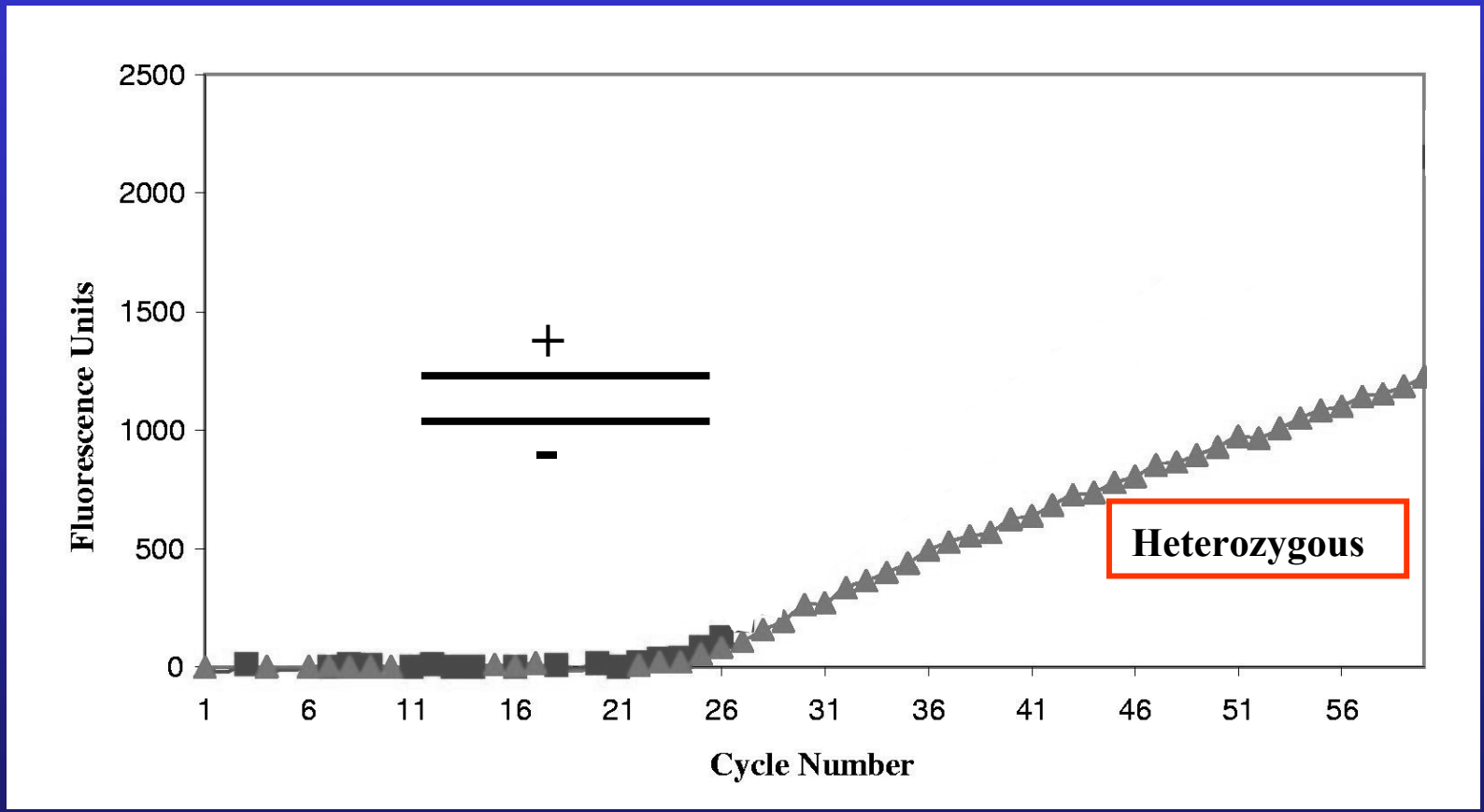
Sample Preparation Reaction Methods Product Analysis Applications



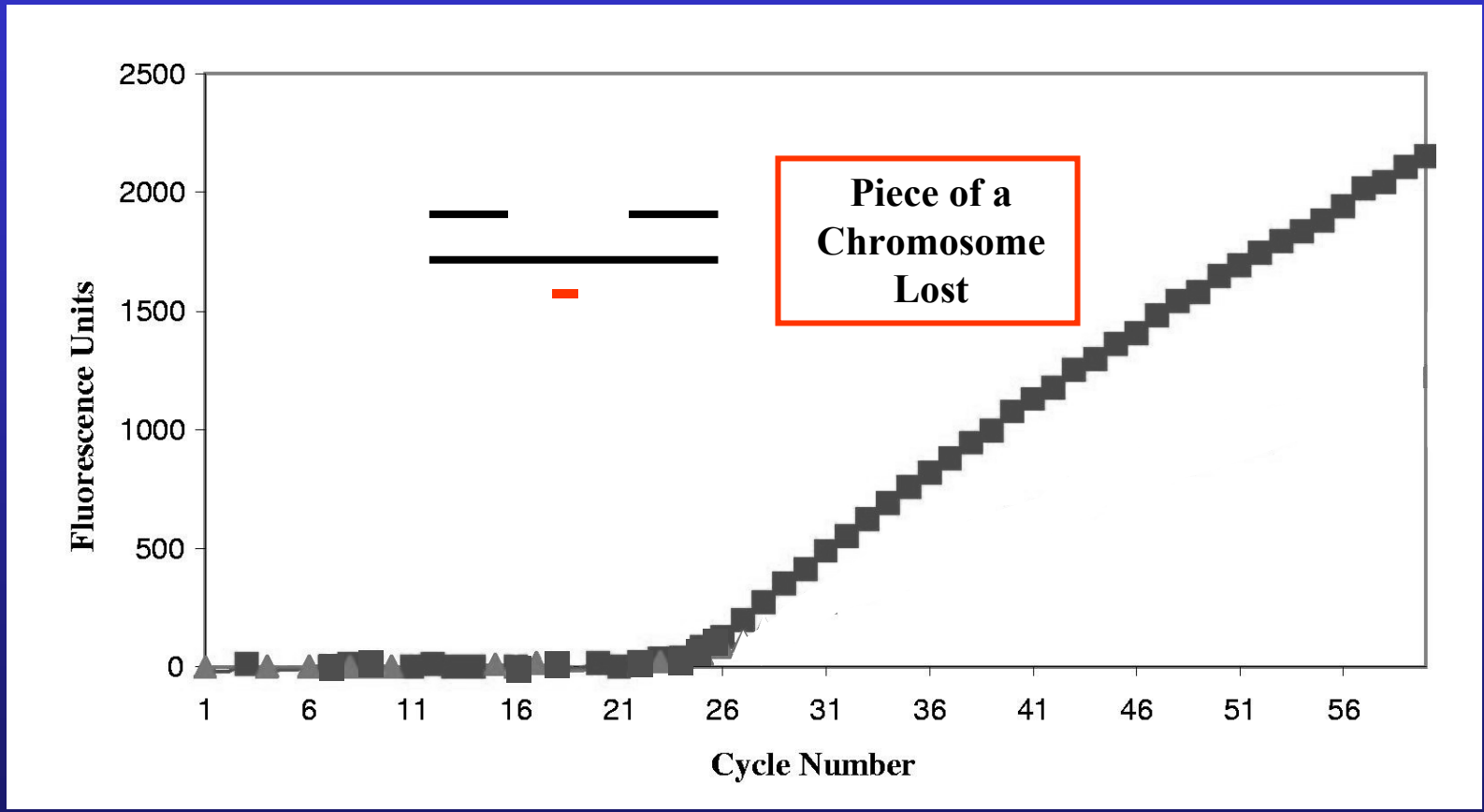
The Linear Kinetics of LATE-PCR



Loss of Heterozygous and Oncogenesis

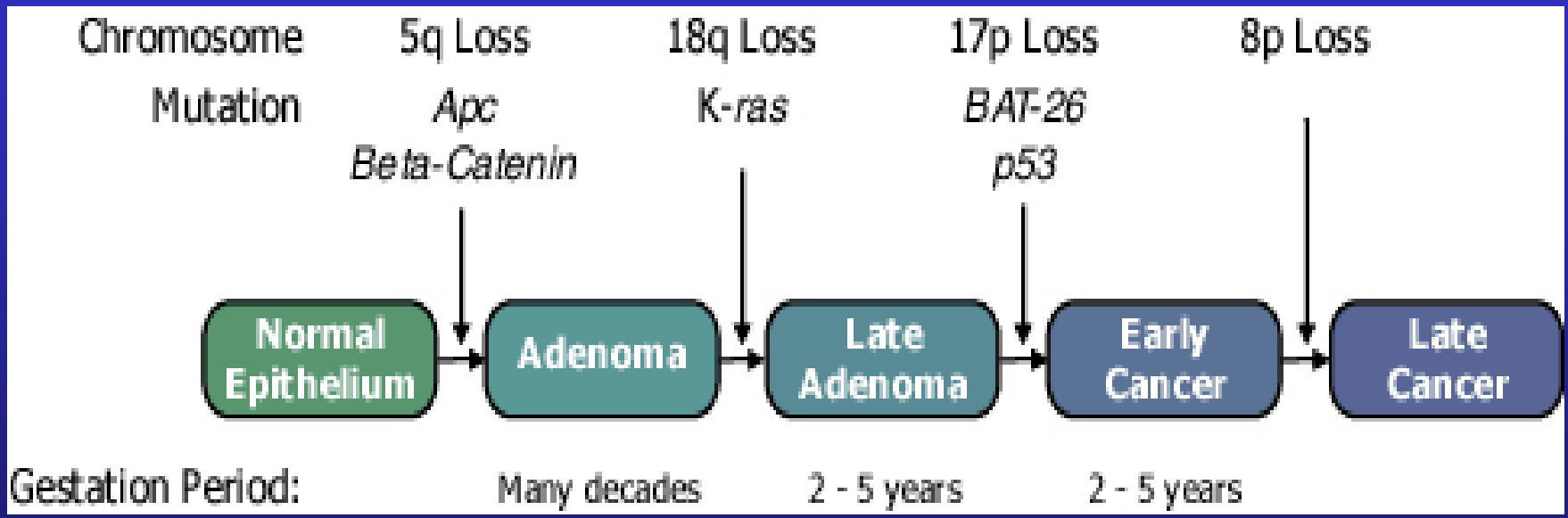


Simple Assay for Loss of Heterozygosity



The American Cancer Society and National Cancer Institute recommend annual colorectal cancer screening for the more than 74 million Americans over the age of 50. In reality, only a fraction of this population is being screened routinely for this disease.

Colorectal Cancer Is a Disease That Is Well Understood From a Genomics Point of View

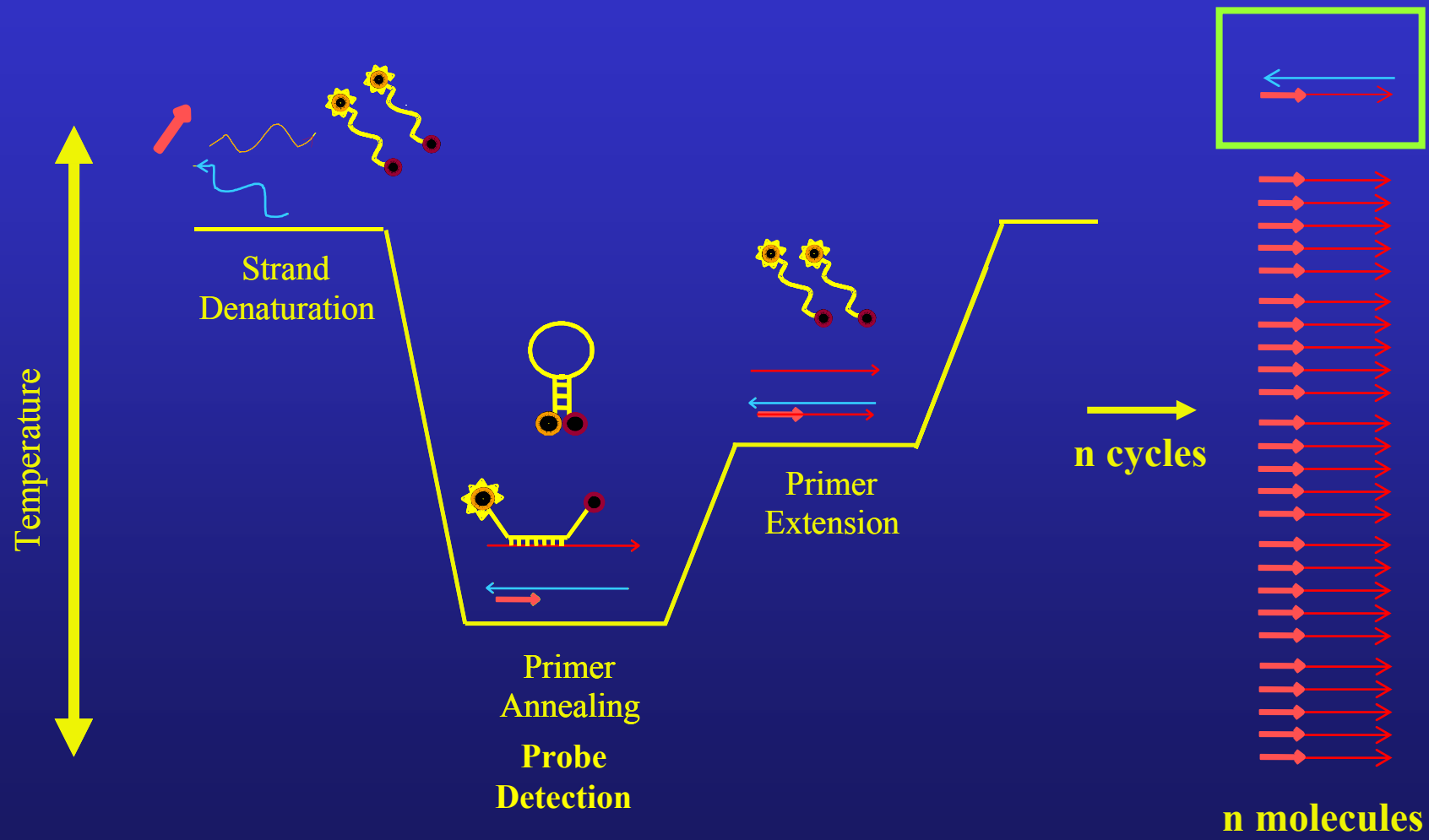


Sequencing Mutant Amplicons Would Be Informative

But Before Sequencing it is Critical to Suppress Amplification Errors

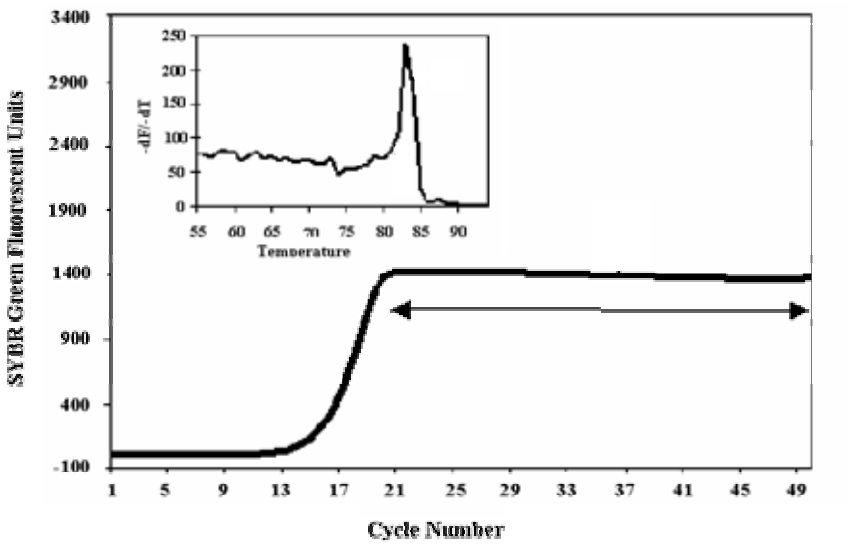
Product Evolution

In LATE-PCR: Double-Stranded DNA Molecules Should Remain Constant During Linear Amplification



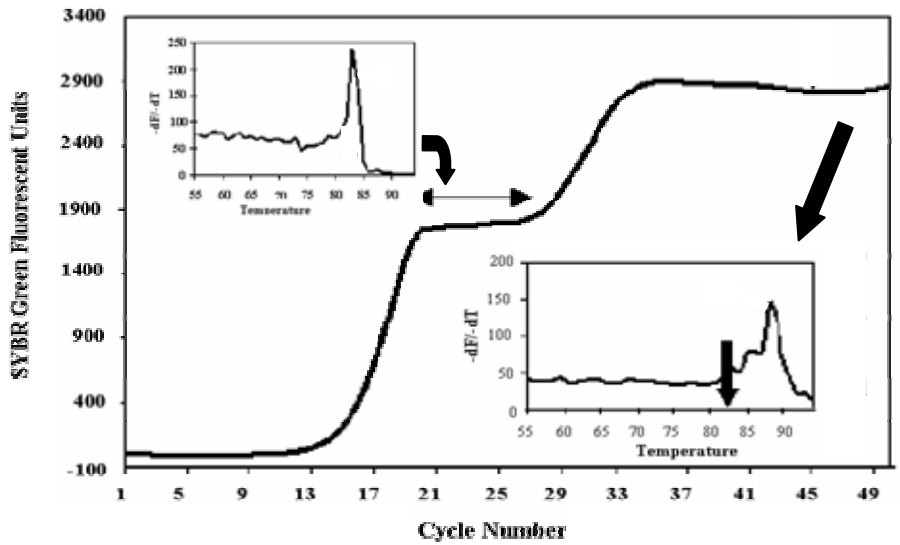
The Phenomenon of Product Evolution

Stringent Conditions



No Evolution: ds-Molecules Constant

Non-Stringent Conditions

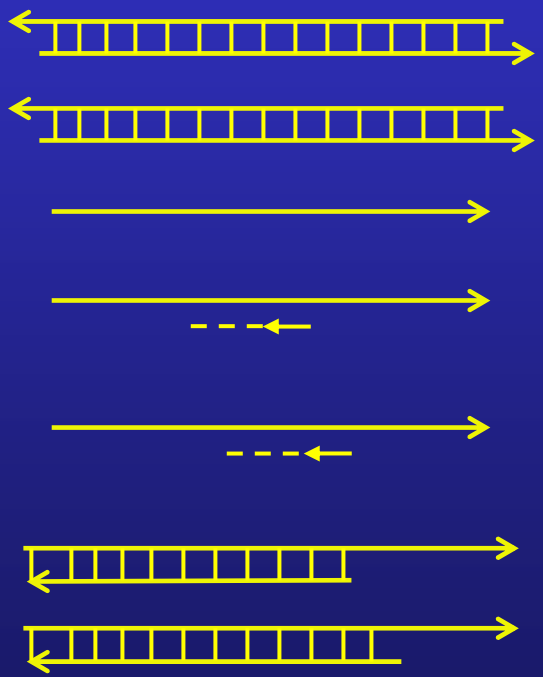


+ Evolution: ds-Molecules Increase

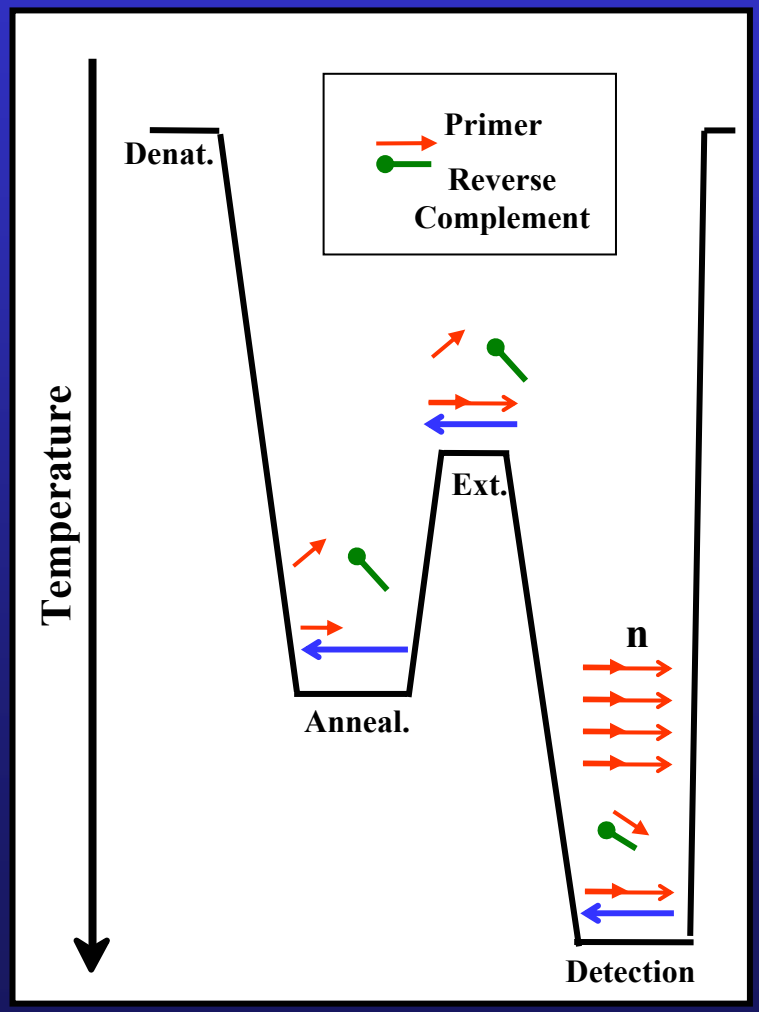
Hypotheses I to Explain Product Evolution

Primer Mis-Priming

Of the Single-Strands



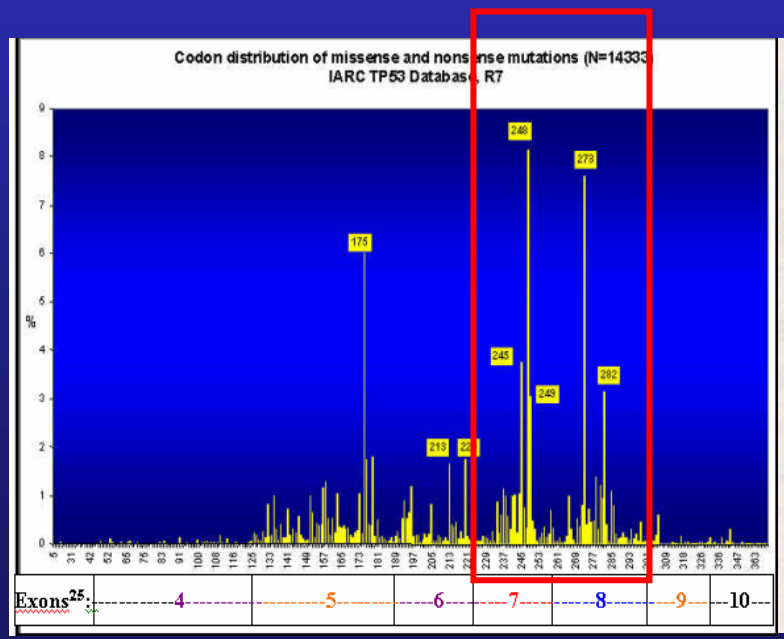
Solution:
Primer Reverse Complement



p53 Mutations

- found in the majority of Li-Fraumeni syndrome cases, an autosomal dominantly inherited disorder
- most frequently observed somatic genetic events, occurring in ~50% of all cancers

600 nucleotides



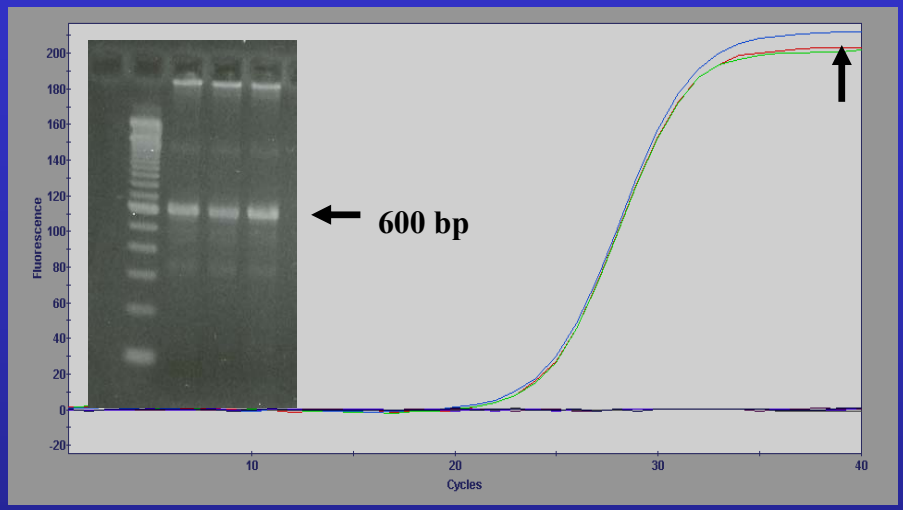
p53 Missense Mutations In Conserved Sites of DNA Binding Domain

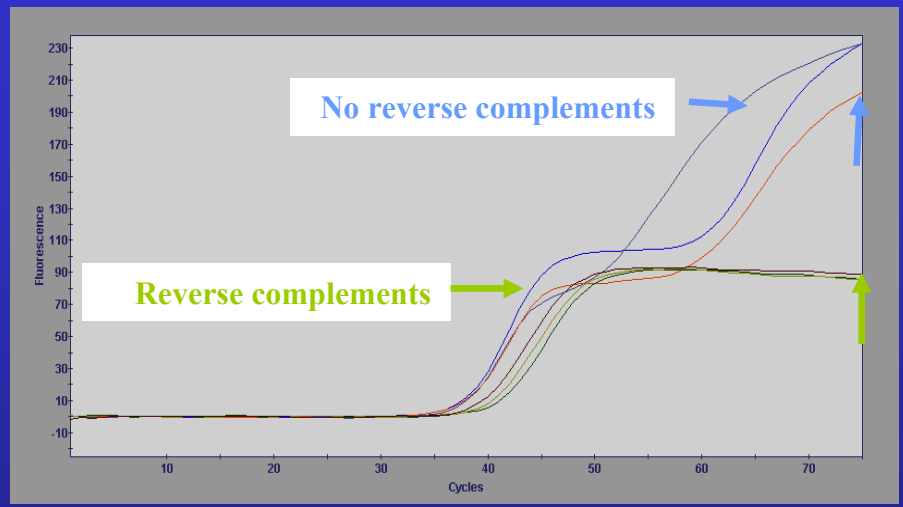
Missense mutations affect >90 amino acids in p53

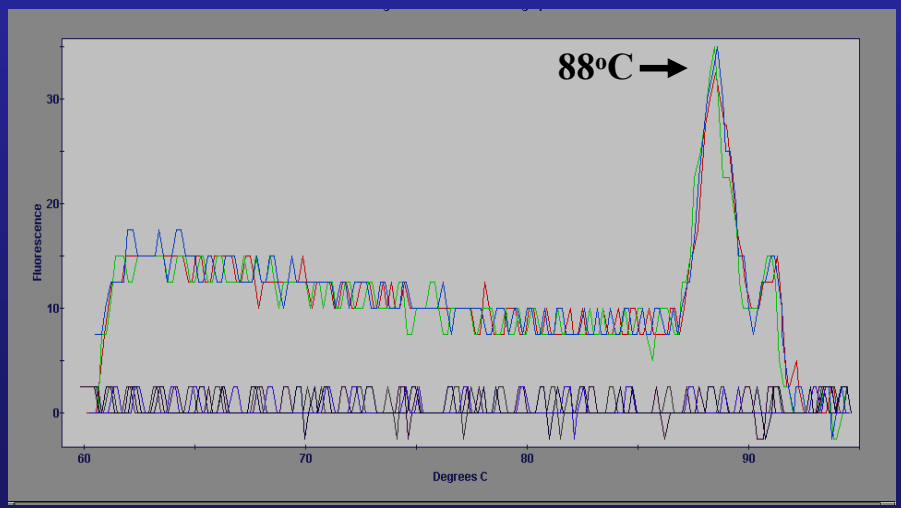
Three "Hotspots" account for 30%

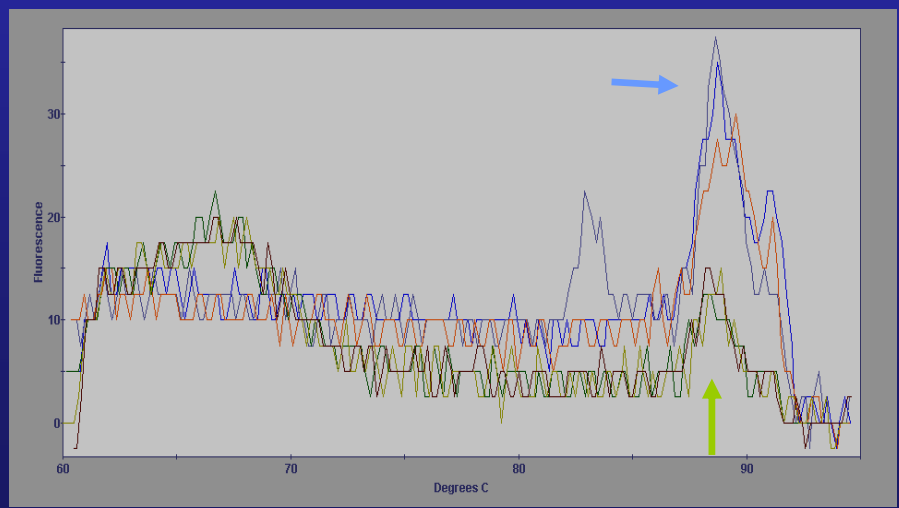
Alter capacity of p53 to bind to DNA

Amplification of the p53 Exon 7-8 Amplicon

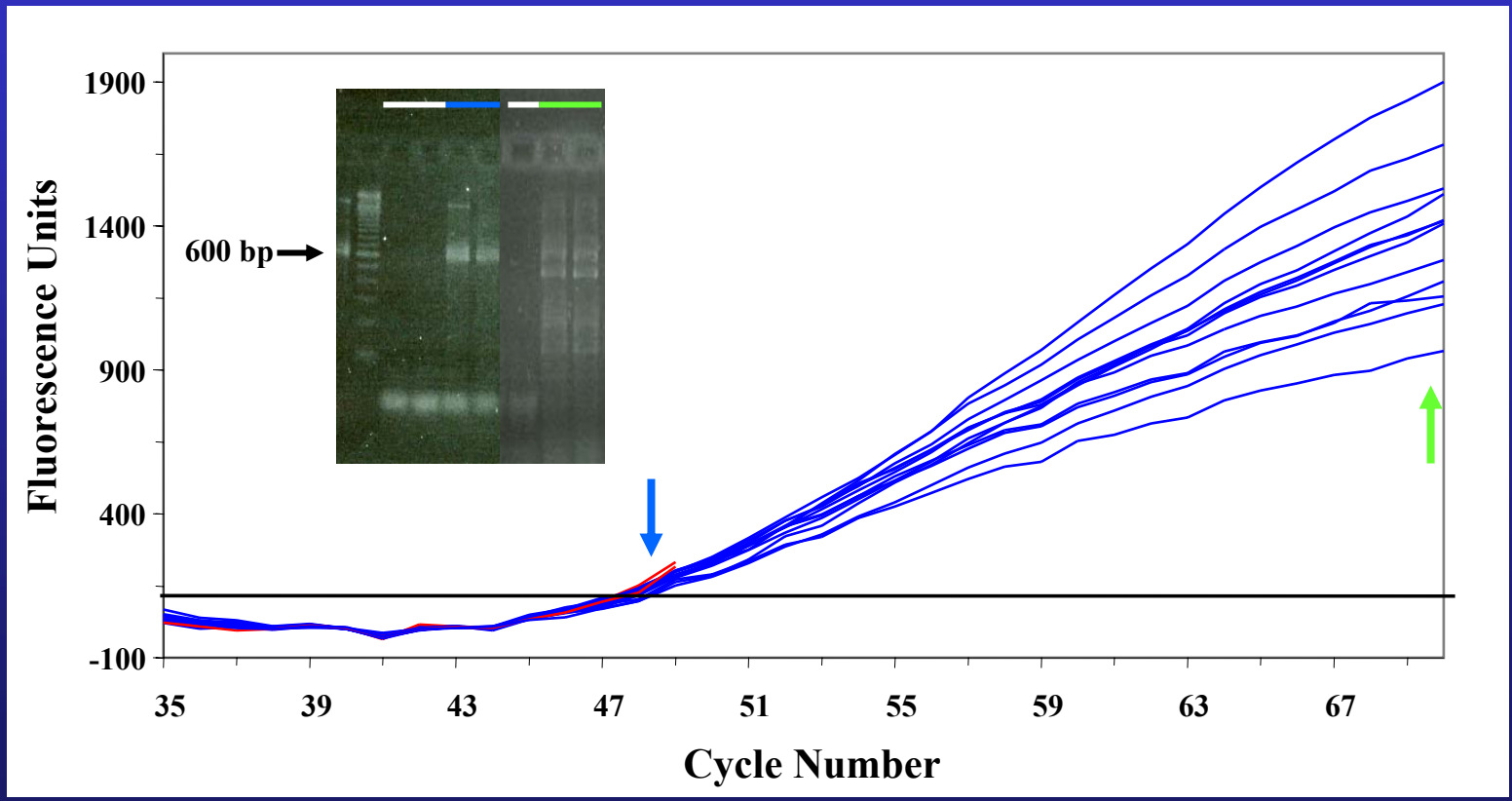






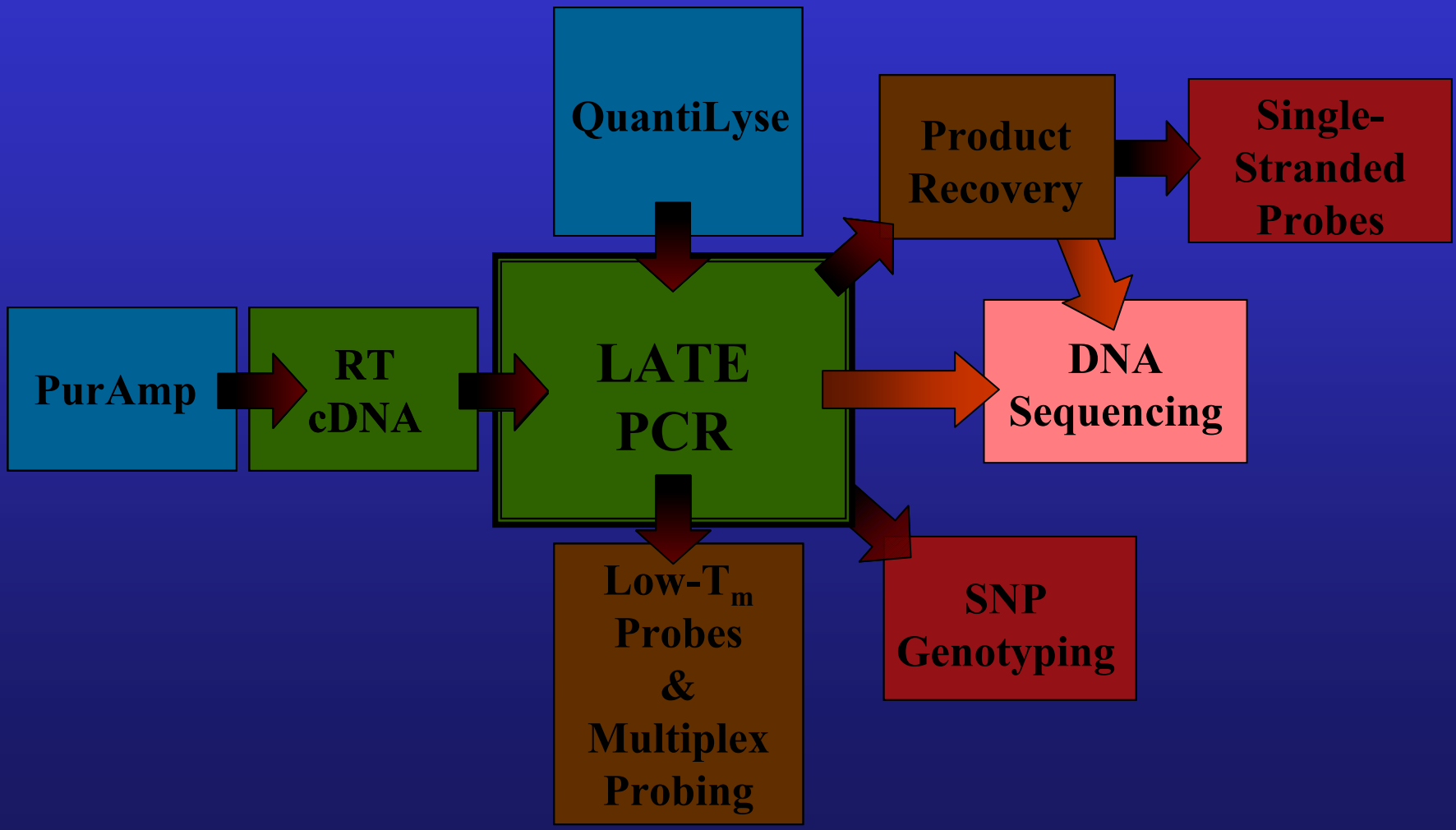


LATE PCR Analysis of p53 gene from Single Cell Double Stranded Probe and Two Reverse Complements

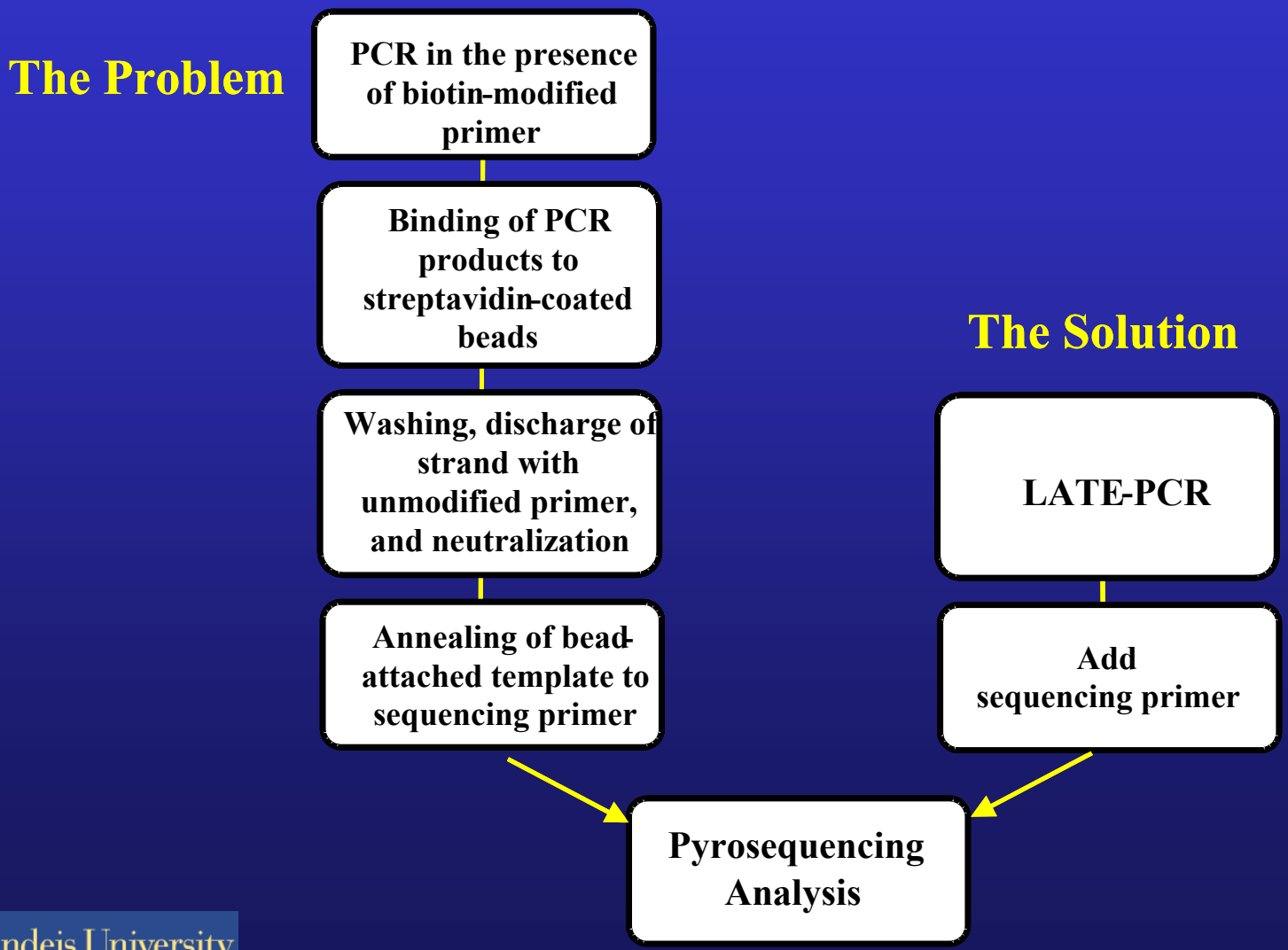


The LATE-PCR Platform Technologies

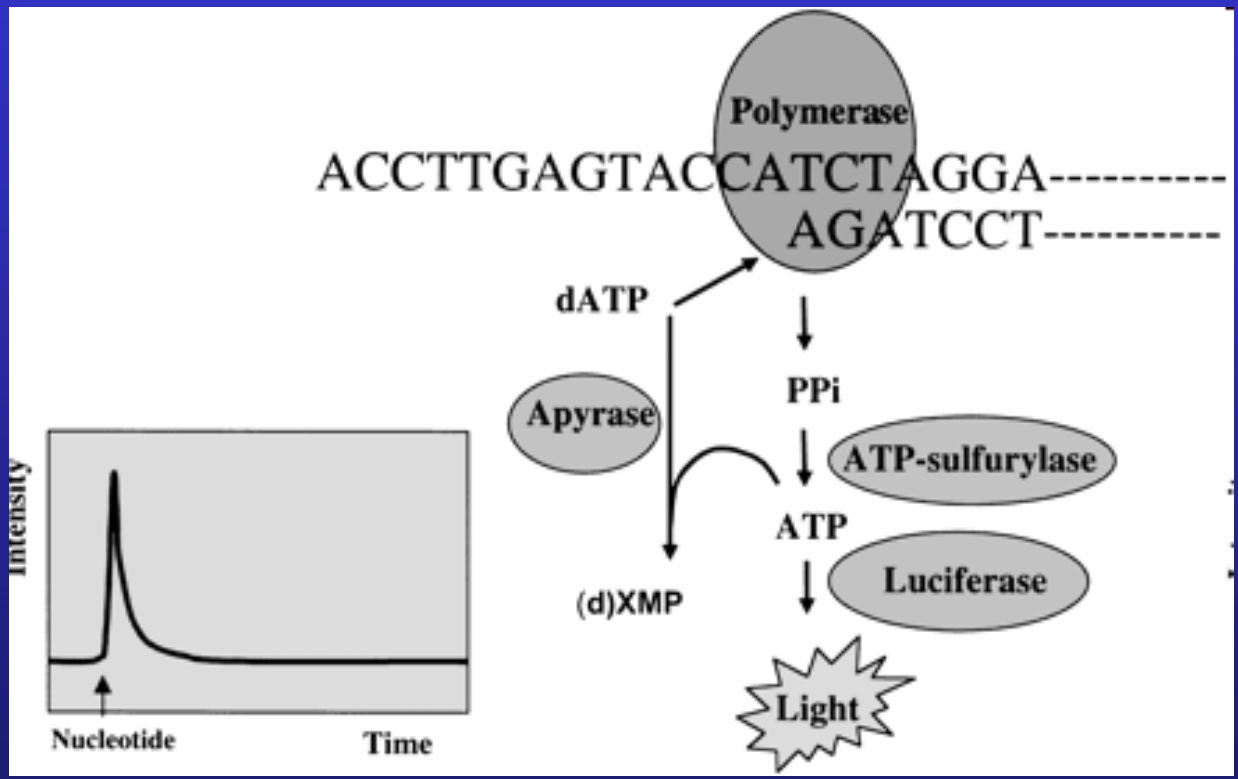
Sample Preparation Reaction Methods Product Analysis Applications



The Problem of Substrate Preparation for Pyrosequencing

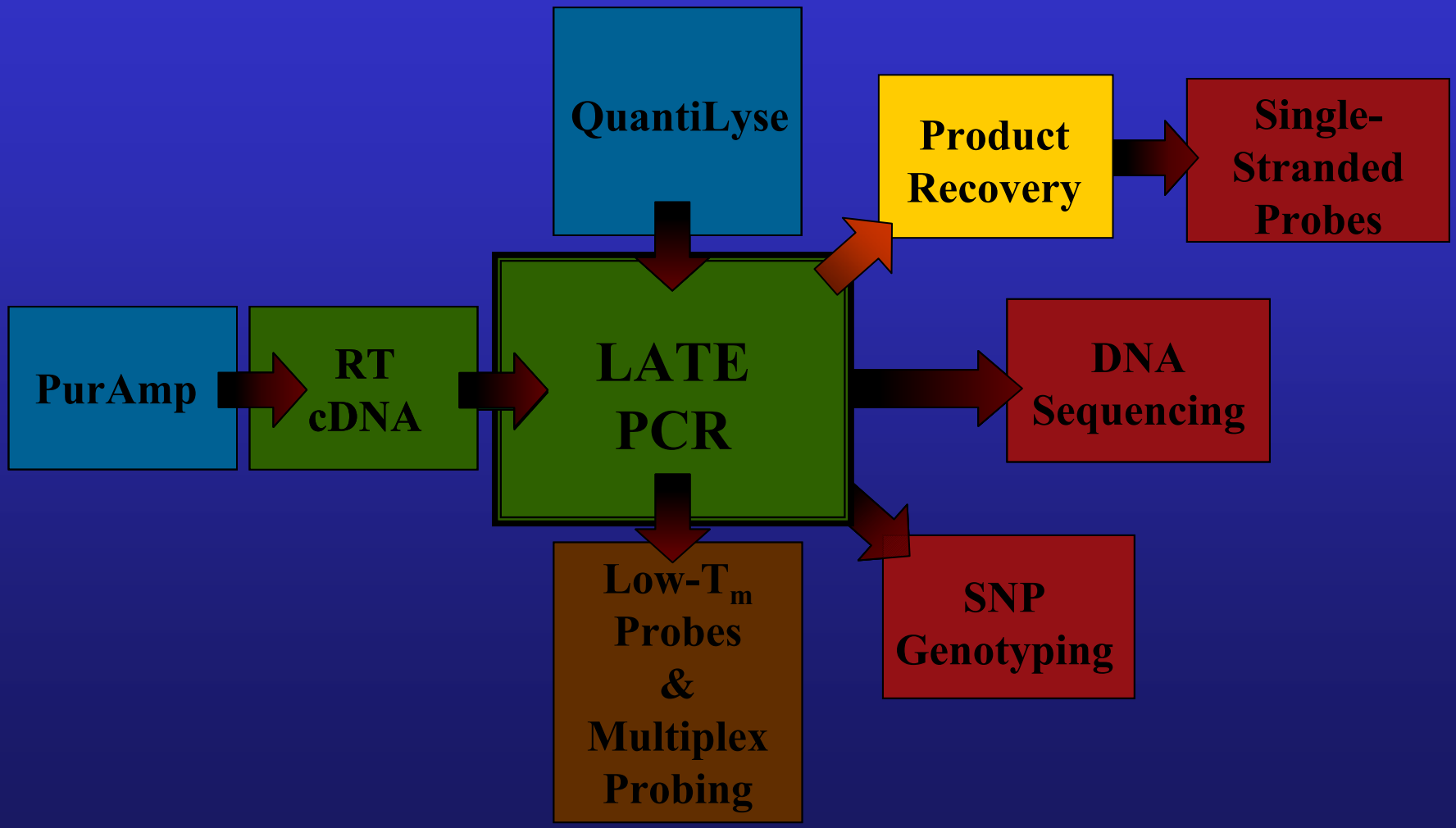


Pyrosequencing

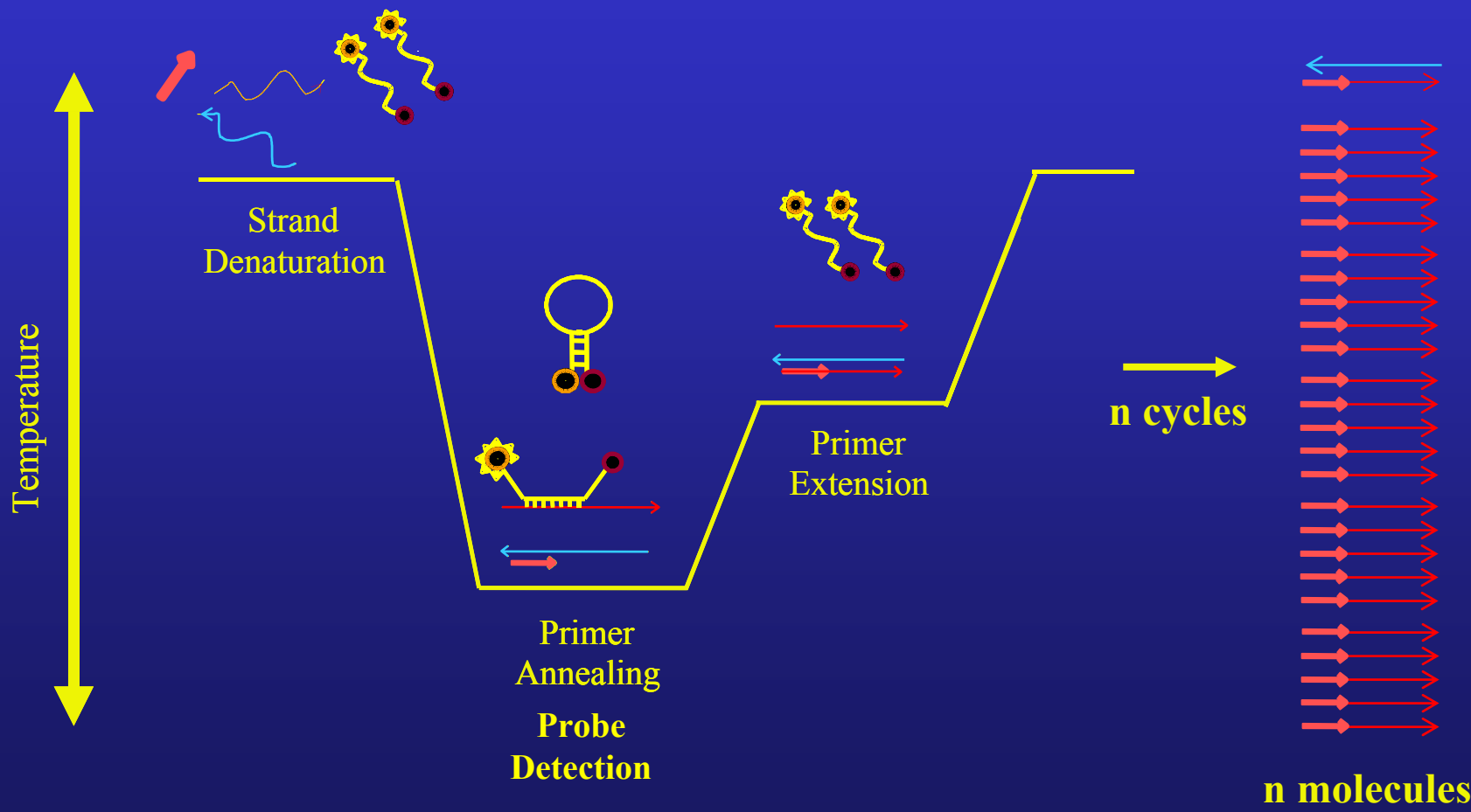


The LATE-PCR Platform Technologies

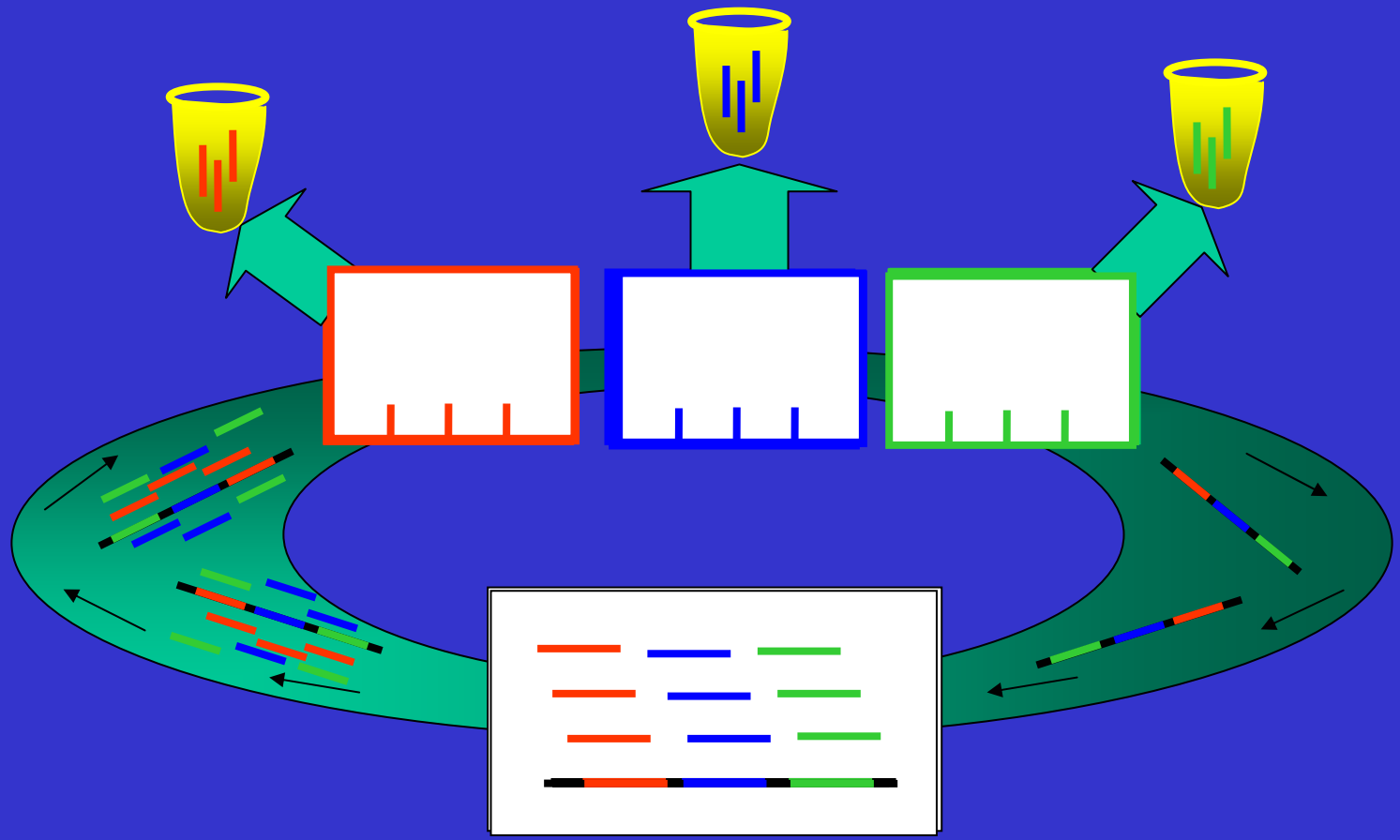
Sample Preparation | Reaction Methods | Product Analysis | Applications



Automated Product Recovery



The Race Track Reaction Chamber: Amplification & Product Recovery in a Closed System Format



PCR Reaction Chamber

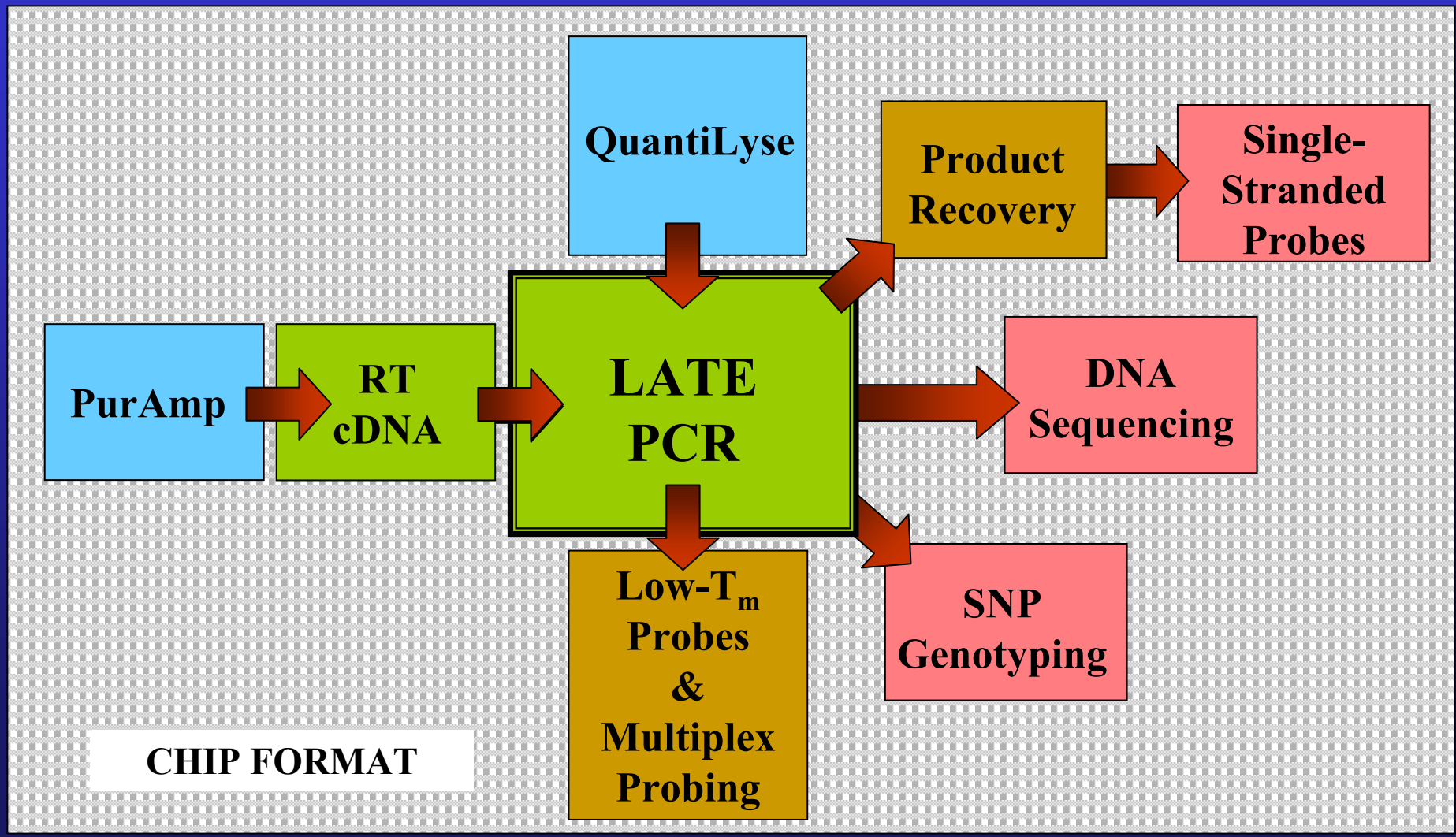
Benefits of the RaceTrack Reaction Chamber

- Unlimited Single-Strand DNA Yield
- Eliminates Product Evolution
- Enables Simultaneous Amplification of Multiple Targets
- Automatable – No Operator Input Required

The Race Track Reaction Chamber is a Closed Tube Solution for Multiplex LATE-PCR Applications

The LATE-PCR Platform Technologies

Sample Preparation Reaction Methods Product Analysis Applications



Why Will Users Adopt These Technologies ?

- Simple One-Tube Methods for Preparation of DNA and/or RNA
- Primer Design Made More Rational
- Thermal Cycle Profiles Made More Precise and Easier to Design
- Probe Design Made Easier and More Reliable
- Rational Approaches to Analysis and Recovery of Multiple Targets
- SLIO: Suppression of Amplification Errors in all Forms of PCR
- Quantitative End Point Assays
- Direct DNA Sequencing
- PurAmp: Quantitative RT-PCR Using Internal DNA as Controls

Additional Advantages of LATE-PCR

- Compatible With Existing PCR Equipment
- Lower Cost (Greater Sensitivity Means Less Reagents)
- Ideal for Lab-On-A-Chip Format

LATE-PCR and Several Allied Technologies

Are Available for Licensing

From Brandeis University

Brandeis University