LATE-PCR

Linear-After-The-Exponential

A Patented Invention of the Laboratory of

Human Genetics and Reproductive Biology

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Laboratory Co-Inventors

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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

- QuantiLyse
- Product Recovery
- Single-Stranded Probes
- DNA Sequencing
- Genotyping
- Analysis of Multiple Targets
- LATE PCR
- RT cDNA
- PurAmp
Hybridization & Melting of DNA Strands is Temperature-Dependent

Tm, the Melting Temperature, Hybridization 50% of the Time
Symmetric (conventional) PCR

Temperature

Strand Denaturation

Primer

Extension

Primer

Annealing

Probe

Detection

$n$ cycles

$2^n$ molecules
Limitations of Real-Time Symmetric PCR

Lower Sensitivity for Smaller Number of DNA Targets

Lack of Reliability Among Replicate Samples

Symmetric PCR Generates High Concentrations of Both Strands

- Primer Extension
- Primer Annealing
- Probe Detection
- Strand Denaturation

Temperature

n cycles

$2^n$ molecules
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

2^n molecules
Asymmetric PCR As a Solution to Amplicon Strand Reannealing

Phase II – Linear Amplification

Temperature

n cycles

n molecules

Strand Denaturation

Primer Annealing

Probe Detection

Primer Extension
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…”

The LATE-PCR Platform Technologies

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- Low-Tₘ Probes & Multiplex Probing

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Symmetric PCR

\[ C_T = 39.6 \pm 1.2 \]

Asymmetric PCR

\[ C_T = 45.2 \pm 1.2 \]

LATE-PCR

\[ C_T = 39.0 \pm 0.8 \]

- 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 $T_m^L$ Is Above Excess Primer $T_m^X$

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

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

<table>
<thead>
<tr>
<th>Sequence</th>
<th>nM</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symmetric PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’-CCTTCTCTCTGCCCCCTGGT-3’</td>
<td>1000</td>
<td>64.8</td>
<td>+ 0</td>
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<tr>
<td>5’-GCCAGGGGTTCCACTACGTAGA-3’</td>
<td>1000</td>
<td>64.3</td>
<td></td>
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<tr>
<td><strong>Conventional Asymmetric PCR</strong></td>
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<td></td>
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<tr>
<td>5’-CCTTCTCTCTGCCCCCTGGT-3’</td>
<td>25</td>
<td>58.9</td>
<td>- 5</td>
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<tr>
<td>5’-GCCAGGGGTTCCACTACGTAGA-3’</td>
<td>1000</td>
<td>64.3</td>
<td></td>
</tr>
<tr>
<td><strong>LATE-PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’-GCCCTCTCTCTGCCCCCTGGT -3’</td>
<td>25</td>
<td>64.0</td>
<td>+ 0</td>
</tr>
<tr>
<td>5’-GCCAGGGGTTCCACTACGTAGA-3’</td>
<td>1000</td>
<td>64.3</td>
<td></td>
</tr>
</tbody>
</table>

Sanchez *et al.* (2003), In Preparation for Nature Biotechnology
Validation of LATE-PCR Primer Design

\[(T_m^L - T_m^X) < 0\]  \quad \text{Inefficient}

\[(T_m^L - T_m^X) \geq 0\]  \quad \text{Efficient}

Sanchez et al. (2003), In Preparation for Nature Biotechnology
LATE-PCR: Rational Design Allows A Broad Range of Primer Ratios

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

Rule 1: \((T_m^L - T_m^X) \geq 0\)
Increasing the Value of ($T_{mL} - T_{mX}$) also Increases Amplification Specificity

100 genomes, Annealing Temp. 2°C Below $T_{mL}$

Pierce et al. (2003), In Preparation for Nucleic Acid Res.
The Problem of Product Amplicon Strand Competition

Rule 2: \((Tm^A - Tm^X) \leq 18^\circ C\)
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

- PurAmp
- RT cDNA
- LATE PCR
- QuantiLyse
- Product Recovery
- DNA Sequencing
- Single-Stranded Probes
- SNP Genotyping
- Low-T_m Probes
LATE-PCR Uncouples Annealing and Detection

Symmetric PCR

Annealing And Detection

Strand Denaturation

Primer Extension

Temperature
LATE-PCR Uncouples Annealing and Detection

LATE-PCR (linear phase)

Strand Denaturation

Annealing And Detection

 Primer Extension

Primer
Extension

Symmetric PCR

LATE-PCR

LATE-PCR Uncouples Annealing and Detection

LATE-PCR (linear phase)

Annealing

Detection

Strand Denaturation

Primer Extension

Temperature
LATE-PCR Uncouples Annealing and Detection

LATE-PCR (linear phase)

Primer Extension

 Strand Denaturation

Annealing

Low-Tm Probes

Detection
LATE-PCR Uncouples Annealing and Detection

LATE-PCR (linear phase)

Strand Denaturation

High-T<sub>m</sub> Probes

Annealing

Primer Extension

Low-T<sub>m</sub> Probes

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

QuantiLyse

Product Recovery

Single-Stranded Probes

PurAmp  RT cDNA

LATE PCR

Low-T_m Probes & Multiplex Probing

DNA Sequencing

SNP Genotyping

Applications

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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

PurAmp  
RT  
cDNA

LATE PCR

QuantiLyse

Product Recovery

Single-Stranded Probes

DNA Sequencing

Low-T_m Probes & Multiplex Probing

Loss of Heterozygosity
The Linear Kinetics of LATE-PCR
Loss of Heterozygous and Oncogenesis

![Graph showing fluorescence units over cycle number with + and - symbols and a red box indicating Heterozygous.]
Simple Assay for Loss of Heterozygosity

Piece of a Chromosome Lost
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

Diagram:
- Chromosome Mutations: 5q Loss, 18q Loss, 17p Loss, 8p Loss
- Pathway:
  - Normal Epithelium → Adenoma → Late Adenoma → Early Cancer → Late Cancer
- Gestation Period:
  - Many decades → 2-5 years → 2-5 years
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

Non-Stringent Conditions

No Evolution: ds-Molecules Constant

+ Evolution: ds-Molecules Increase
Hypotheses I to Explain Product Evolution

**Primer Mis-Priming**
**Of the Single-Strands**

**Solution:**
**Primer Reverse Complement**

Diagram illustrating the process of primer mis-priming and the proposed solution of using 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

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

- **600 bp**
- **88°C**

No reverse complements

Reverse complements
LATE PCR Analysis of p53 gene from Single Cell Double Stranded Probe and Two Reverse Complements
The LATE-PCR Platform Technologies

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- LATE PCR
- RT cDNA
- PurAmp
The Problem of Substrate Preparation for Pyrosequencing

**The Problem**

- PCR in the presence of biotin-modified primer
- Binding of PCR products to streptavidin-coated beads
- Washing, discharge of strand with unmodified primer, and neutralization
- Annealing of bead-attached template to sequencing primer

**The Solution**

- LATE-PCR
  - Add sequencing primer

**Pyrosequencing Analysis**
Pyrosequencing
The LATE-PCR Platform Technologies

Sample Preparation  Reaction Methods  Product Analysis  Applications

PurAmp  RT cDNA  LATE PCR  Low-T<sub>m</sub> Probes & Multiplex Probing  QuantiLyse  Product Recovery  DNA Sequencing  SNP Genotyping  Single-Stranded Probes
Primer Extension

Primer Annealing

Primer

Annealing

Strand Denaturation

Temperature

n cycles

n molecules

Automated Product Recovery

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

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- SNP Genotyping

CHIP FORMAT

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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