Building Tools for the Analysis of Cellular Heterogeneity

LATE-PCR and Allied Technologies for Analysis of Genetic Heterogeneity in Single Cells & Single DNA Molecules

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The LATE-PCR Technology Platform

Sample Preparation

Quantitative recovery of nucleic acids from single cells

PurAmp (DNA & RNA)
The LATE-PCR Technology Platform

Sample Preparation

Amplification Method

Efficient amplification of single-stranded DNA

PurAmp (DNA & RNA) → LATE PCR
The LATE-PCR Technology Platform

Sample Preparation

Amplification Method

PurAmp (DNA & RNA) → LATE PCR → PrimeSafe

Suppresses non-specific amplification products
The LATE-PCR Technology Platform

Sample Preparation

Amplification Method

Product Analysis

PurAmp (DNA & RNA)

LATE PCR

Increased Detection Sensitivity

Multiplexing

PrimeSafe

Direct DNA Sequencing
Genetic Heterogeneity at the Single Cell Level: The Hypoxia Transcriptional Response

Low O$_2$ concentration
(Hypoxia)

HIF$\alpha$, HIF1$\beta$

Hypoxia-response element

Target genes

Glycolytic enzymes

Anaerobic Metabolism

HIF$\alpha$ activates hypoxia response
TP53 programmed cell death
p14 ARF regulates hypoxia response
HSP70 copes with hypoxic stress
EPO erythropoiesis
VEGF angiogenesis

Low O$_2$ concentration (Hypoxia) activates the hypoxia response, leading to the activation of HIF$\alpha$, HIF1$\beta$, p14 ARF, HSP70, EPO, and VEGF. This process involves the regulation of glycolytic enzymes and anaerobic metabolism to cope with hypoxic stress.
Limitations of Conventional PCR

Symmetric RT-PCR
Equal concentration of amplification primers

real-time

detection background
Amplification stops because double-stranded DNA inhibits Taq DNA polymerase.

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Symmetric RT-PCR
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Amplification stops because double-stranded DNA inhibits Taq DNA polymerase

Co-Amplification of Abundant & Rare Targets

Abundant targets prevent amplification of rare targets

Fluorescence
Exponential Phase
Plateau Phase

Detection background

Cycle Number

0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00

0 5 10 15 20 25 30
Linear-After-The-Exponential (LATE) RT-PCR

Unequal concentration of amplification primers

Limited accumulation of double-stranded DNA products does not stop PCR

detection background

real-time

end-point

The LATE-PCR Solution
The LATE-PCR Solution

Linear-After-The-Exponential (LATE) RT-PCR
Unequal concentration of amplification primers
Limited accumulation of double-stranded DNA products does not stop PCR

Co-Amplification of Abundant & Rare Targets

Symmetric PCR

Double-stranded DNA competes with binding of detection probes
LATE-PCR Provides Increased Detection Sensitivity

**Symmetric PCR**

Double-stranded DNA competes with binding of detection probes

**LATE-PCR**

Single-stranded DNA products are saturated with detection probes for maximum sensitivity
Multiplex LATE-PCR Assays for Analysis of the Hypoxia Response in Single Cells

Hypoxia-response element

Target genes

HIFα, TP53, p14 ARF, HSP70, EPO, VEGF

HIF1α, HIF1β

HSP70, EPO, VEGF

HIF1α, TP53, p14 ARF

Graphs showing expression levels for HIF1α, TP53, p14 ARF, HSP70, EPO, and VEGF over time.
Optimized Multiplexed LATE-PCR Assays Using PrimeSafe

- HIF1α
- TP53
- p14ARF
- HSP70
- EPO
- VEGF

Hypoxia-response element → Target genes

Graphs showing expression levels of HIF1α, TP53, p14ARF, HSP70, EPO, and VEGF.
Quantitative Endpoint Analysis

Quantitative Endpoint Analysis

Quantitative Real-Time Analysis

DNA + RNA copy number

Heat shock time (mins)

obtained from Ct

obtained from end point

Activation of Gene Expression (min)

Fluorescence

Cycle Number

DNA + RNA copy number

Obtained from Ct

Obtained from end point

Cycle Number

Activation of Gene Expression (min)
Multiplex LATE-PCR Assays for Analysis of the Hypoxia Response in Single Cells
Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes
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- Neoplasia is driven by the accumulation of genetic changes.
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Does mitochondria undergoes random genetic changes during neoplastic progression?
Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes

- Neoplasia is driven by the accumulation of genetic changes
- Does mitochondria undergoes random genetic changes during neoplastic progression?
- A single cell has ~1000 mitochondrial DNAs
Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes

- Neoplasia is driven by the accumulation of genetic changes

- Does mitochondria undergoes random genetic changes during neoplastic progression?

- A single cell has ~ 1000 mitochondrial DNAs

- Detection of random, low frequency mutations requires analysis of single mitochondrial DNAs
A 10-plex LATE-PCR Assay for Analysis of Single Mitochondrial DNA

- 10 primers sets for 8 genes/regions: HV1, HV2, 12srRNA, ND1, ND4, CO1, CO2, and CytB
- Covering 30% of the mitochondrial genome

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>Amplicon Length (bp)</th>
<th>% Gene Covered</th>
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<tbody>
<tr>
<td>HV1</td>
<td>420</td>
<td>96-590 (495)</td>
<td>100</td>
</tr>
<tr>
<td>HV2</td>
<td>300</td>
<td>33-440 (408)</td>
<td>100</td>
</tr>
<tr>
<td>12srRNA</td>
<td>954</td>
<td>106-899 (794)</td>
<td>83</td>
</tr>
<tr>
<td>ND1</td>
<td>957</td>
<td>197-780 (584)</td>
<td>61</td>
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<tr>
<td>ND4</td>
<td>1378</td>
<td>105-539 (434)</td>
<td>63</td>
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<tr>
<td></td>
<td></td>
<td>804-1240 (436)</td>
<td></td>
</tr>
<tr>
<td>CO1</td>
<td>1542</td>
<td>36-608 (573)</td>
<td>37</td>
</tr>
<tr>
<td>CO2</td>
<td>684</td>
<td>123-644 (522)</td>
<td>76</td>
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<tr>
<td>Cytb</td>
<td>1135</td>
<td>105-498 (394)</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>606-983 (378)</td>
<td></td>
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</tbody>
</table>
Amplification Products from the 10-Plex Assay

**Cal Orange**
- Cyb-1
- HV1
- 12s

**Quasar**
- ND4-1
- CO2

**Fam**
- ND4-2
- HV2

**Cal Red**
- Cyb-2
- ND1
- CO1-1
Sequencing of Individual Products from the 10-Plex

HV1

HV2

12s

CO1-1

CO2

Cyb1

Cyb2

ND1

ND4-1

ND4-2
Analysis of Single Mitochondrial DNAs Identifies Random Mutations Not Seen in Bulk DNA