Fundamentals of Real-Time PCR

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Senior Product Specialist
Polymerase Chain Reaction (PCR)

- Unamplified DNA
- Strand Denaturation
- Primer annealing
- Primer extension

Cycling:
Exponential amplification of PCR products

Denaturation
Annealing
Extension
Limitations of Traditional End-Point PCR

- Low sensitivity
- Poor precision
- Results are not expressed as numbers
- Ethidium bromide staining is not quantitative
- Post-PCR processing required
- Narrow dynamic range (<2 logs)
Alternative Quantitative Methods

- Northern Blots
- RNase protection assays
- In Situ hybridization
- Competitive PCR
- cDNA arrays
Problems Associated With These Alternative Methods

- Difficulty achieving high throughput
- Using large RNA/DNA quantities
- Limited dynamic range
- Threat of contamination
- Difficulty designing controls
- Difficulty creating and optimizing quantitative assays
Goals For Improvement of Quantitative PCR

- Eliminate use of gel electrophoresis
- Increase reproducibility
- Enable use of internal controls/standards
- Reduce turnaround time
- Increase throughput
- Reduce sample amount usage
Quantitative Real-Time PCR

Detection of PCR product growth throughout the amplification process

• No post-PCR processing required
• Collects data during high-precision exponential phase
3 Phases of PCR

**Exponential:**
- Exact doubling of product
- Reaction is very precise and specific

**Linear:**
- The reaction components are becoming limited
- The reaction efficiency is dropping

**Plateau:**
- The reaction has stopped
- No more products are being made
Large Dynamic Range

Amplification of serial dilutions of 18S rRNA target in 16 replicates

Standard curve showing 9 logs of linear dynamic range
Real-Time PCR Chemistries

**SYBR® Green I dye**

Binds double stranded DNA

**Fluorogenic 5' Nuclease Assay**

Uses a TaqMan® probe
Fluorogenic 5' Nuclease Assay

*FRET* = Fluorescence Resonance Energy Transfer
Fluorogenic 5' Nuclease Assay

Displacement during Polymerization

Forward Primer

Cleavage

Reverse Primer
TaqMan® MGB Probes

- Minor Groove Binder (MGB) enhances the melting temperature ($T_m$) of the probe resulting in shorter probes
  - shorter probes provide better discrimination

[R NFQ MGB] : Reporter Dye

NFQ : Non-Fluorescent Quencher

MGB : Minor Groove Binder ($T_m$ Enhancer)
SYBR® Green I Dye Assay Chemistry

Denaturation

Polymerization

Polymerization Complete
Terminology

Baseline:
The initial cycles prior to any detectable amplification, in which there is little change in fluorescent signal.
Threshold:

Level at which fluorescence is detected in reactions during the exponential phase of PCR.
Cycle Threshold ($C_T$): The cycle (point in time) at which the PCR product crosses the threshold of detection.
$R_n$: Reporter signal divided by the passive reference ROX™ Dye signal. Normalized to account for pipetting variation.
\( \Delta R_n \): Normalized reporter signal minus background (baseline level).
Types of Quantitation Assays

Absolute quantitation
Provides absolute measurement of starting copy number
- Requires standards of known quantity
- e.g. Detecting DNA copy number for forensics purposes

Relative quantitation
Forensic Applications

Is there any (amplifiable) DNA?

How much is there?
From Fluorescence to Copy Number

$C_T$ is directly proportional to log of amount of input template
High (100%) Amplification Efficiency

1 cycle = 2 fold expression difference
Types of Quantitation Assays

- Absolute quantitation
- Relative quantitation

Provides accurate discrimination between relative amounts of starting material:
- e.g. Comparing expression levels of wildtype vs. mutated alleles
- e.g. Comparing expression levels of a gene across different tissues or between different biological conditions
- e.g. Validating array results
Relative Quantitation

Calibrator = The sample used as the basis for comparative results
Relative Quantitation

**Endogenous Control**

= Target used to normalize for sample handling
(e.g. 18S rRNA, GAPDH, β-actin)

- **t =0**
  - total RNA
  - cDNA

- **t=12**
  - total RNA
  - cDNA

- **t=24**
  - total RNA
  - cDNA

- **t=48**
  - total RNA
  - cDNA
Comparative $C_T$ Method

- **$t = 0$**
  - Endogenous control: $C_t=10$
  - Target gene: $C_t=24$

- **$t = 12$ h**
  - Endogenous control: $C_t=9$
  - Target gene: $C_t=25$

- **$t = 24$ h**
  - Endogenous control: $C_t=9$
  - Target gene: $C_t=24$

- **$t = 48$ h**
  - Endogenous control: $C_t=11$
  - Target gene: $C_t=23$

**Legend:**
- Red: Endogenous control
- Green: Target gene

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Comparative $C_T$ Method Calculation:

**Normalized to endogenous control:**

\[
C_T^{48hrs} - C_T^{Endo.\ Control} = \Delta C_T^{48hrs}
\]

\[
C_T^{0hrs, Calibrator} - C_T^{Endo.\ Control} = \Delta C_T^{0hrs}
\]

**Normalized to calibrator sample:**

\[
\Delta C_T^{48hrs} - \Delta C_T^{0hrs, Calibrator} = \Delta \Delta C_T
\]

**Relative fold change:**

\[
2^{(-\Delta \Delta C_T)} = 2^{2} = 4
\]

There is a 4-fold overexpression of my gene at $T=48h$ compared $T=0h...48hrs$ after drug treatment!
Applications

♦ Real-Time Detection
  – Absolute Quantitation
  – Relative Quantitation

♦ End Point Detection
  – Allelic Discrimination (SNP)
  – +/- Assay (IPC)
  • Pathogen Detection
Allelic Discrimination (SNP)

♦ Determines the genotype of samples
  • Possible to differentiate a single nucleotide polymorphism (SNP)
Allelic Discrimination Assay

Allele C - only VIC<sup>R</sup> dye signal is generated

Allele T - only FAM™ dye signal is generated
# Allelic Discrimination (SNP)

<table>
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<tr>
<th>Allele X</th>
<th>Allele Y</th>
<th>Positive Ref</th>
<th>Task</th>
<th>Call</th>
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<td>AL-1</td>
<td>99.98</td>
<td>Automatic</td>
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</table>

**Allelic Discrimination Plot**

- **Legend:**
  - Undetermined
  - Allele X
  - Allele Y
  - Allele X&Y
  - Undetermined
  - NTC

**Setup**

- Marker: C_2484870_10
- Col: Undeter

**Results**

- **Allelic Disc Plot:**
  - TT
  - CT
  - CC

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Applications

♦ Real Time Detection
  – Absolute Quantitation
  – Relative Quantitation

♦ End Point Detection
  – Allele Detection (SNP)
  – +/- Assay (IPC)
    • Pathogen Detection
Internal Positive Control (IPC)

♦ Distinguish true target negative from PCR inhibition

♦ Co-amplified with target DNA without compromising amplification of the target sequence
# Plus/Minus assay with IPC

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<td>U (+)</td>
<td>U (+)</td>
<td>U (+)</td>
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**Disconnected Colors:**
- **Red Circle:** Indicates a non-specific reaction.
- **Green Circle:** Indicates a specific reaction.
Important Considerations

- Reagents
- Chemistry
- Assay
- Instrument
- Software
RNA to Amplified cDNA: 1-Step vs. 2-Step

**1-STEP**
- Closed tube (no contamination)
- Easy-to-use

**2-STEP**
- Archive-ready sample
Formats: Master Mix vs. Core Reagent

Core reagents allow flexibility and optimization

- **AmpliTaq Gold® DNA Polymerase**
- **AmpErase® UNG**
- **10X Buffer**
- **dNTPs**
- **MgCl₂**

Master mixes are easy-to-use and convenient

*All components in one tube!*
Advantage of Using a ROX™ Dye Normalizer

Improves precision

Compensates for small fluorescent fluctuations that can occur from well-to-well

Reporter / Reference
Precision with ROX™ Dye

As the concentration of passive reference decreases, the st. dev. increases; thus decreasing precision.

With only 20% of the Passive Reference Dye I, amplification becomes noisy with broad CT spread.

At 100% of the Passive Reference Dye I, CT replicates are tight and precise.
Not all ROX Dyes are Rock Solid!

Side-by-side comparison of four Master Mixes with comparable Passive Reference Dye I concentration

Applied Biosystems TaqMan® Universal PCR Master Mix produces the lowest standard deviation, therefore the most precise results!
Reaction Setup

TaqMan® Kit

- High specificity
- Multiplexing capability
- End-point assay detection
- Rare transcript and low level pathogen detection

SYBR® Green Kit

- Economical
- TaqMan® probe sensitivity not required
- Pre-screening targets
Dissociation Curve Analysis
- Displays melting temperature of the product generated in SYBR® Green assays
Gold Standard: AB Real-Time PCR reagent line

- TaqMan® Master Mix
  - Universal Master Mix
  - Fast TaqMan Master Mix
    - Improves time to result from 2 hours to about 35 minutes
- Power SYBR® Green Master Mix
  - Provide high sensitivity with less than 10 copies
  - High quality manufacturing ensure consistent lot-to-lot performance
- RT-Master Mix and core reagent
  - One-step or two-step RT reactions

Coming soon
- Reduced assay optimization time
- Reduced experimental validation

- Reduced running time
- Reduced dependency on accurate pipetting
- More extensive validation required
Your Choice of Assays

• **TaqMan® Gene Expression Assays**
  - An extensive list of pre-designed and qualified TaqMan® probes and primers ready for order
    - Inventoried (off-the-shelf)
      >40,000 gene expression assays for human, mouse, and rat
    - Non-inventoried (made-to-order)
      - >600,000 assays for human, mouse, rat, arabidopsis, and drosophila
  - Bioinformatics and information content
  - www.allgenes.com

• **Custom TaqMan® Gene Expression Assays**
  - Submit your sequence and Applied Biosystems will design and synthesize your assay
    - Custom made, single tube, ready-to-use
    - Same format as inventoried TaqMan Gene Expression Assays
    - For all species

• **Support for user designed assays**
  - Rapid Assay Development Guidelines
Rapid Assay Development Guidelines

• Primer and probe design using Primer Express® software
• The use of TaqMan® Universal PCR Master Mix or SYBR® Green PCR Master Mix
• Universal thermal cycling parameters
• Default primer and probe concentrations eliminate assay optimization
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<td>Fast 96-well</td>
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<td>Installation specification</td>
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<td>2-fold discrimination with 99.7% confidence level</td>
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<td>Plus/Minus Detection</td>
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<td>Real-Time throughput</td>
<td>Up to 5,000 wells per day (unattended operation) with Automation Accessory</td>
<td>Over 1000 wells per 8 hour work day</td>
<td>Up to 480 wells per 8 hour work day</td>
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Applied Biosystems 7900HT and 7500 Fast Real-Time PCR Systems

• Complete systems designed to run fast in a standard 96-well configuration

• Can perform absolute or relative quantitation assays in about 35 minutes

• Increase productivity by providing faster time to result

• Includes a complete Fast system: hardware, software, reagents and consumables

• Comparable data on both fast and standard
TaqMan® Low Density Array

– Convenient new consumable format
– Seamlessly integrates Applied Biosystems wide selection of assay products with the Applied Biosystems 7900HT Fast Real-Time PCR System

- Easy sample loading, 8 loading ports
  - No need for robotics
  - Standardization between experiments and labs

- 8 channels each with 48 reaction chambers
- 384 reaction chambers
What is Multicomponent?
- Contribution of individual dye component is displayed throughout the PCR cycle
Software Highlights

• Gene Expression
  • Fully automated data analysis (baseline and threshold for all assays)
  • Automated calculation of relative quantitation
  • Data from up to 10 plates integrated into a single study
Gene Expression 2002
Real-time PCR and its bottlenecks

\[ nM = ? \text{pmol} = ? \mu L \]
Gene Expression Today
Most bottlenecks of real-time PCR removed

TaqMan Gene Expression Assays
Custom TaqMan Gene Expression Assays
Online Ordering Catalog

Automated Gene Expression Analysis Software
Expectations in Gene Expression Studies

- Reproducibility ✓
- Accuracy ✓
- Flexibility (Scalability) ✓
- Standardization ✓
- High Throughput ✓
- Informative Data Sets ✓
- Convenience ✓
Complete Integrated Solution

• Complete line of REAGENTS and consumables
  +

• Your choice of ASSAYS
  +

• High-quality Real-time PCR INSTRUMENTS
  +

• Easy-to-use SOFTWARE for setup and complete data analysis
  =

Enabling scientific discovery!
Questions & Discussion…

Thank You!!!
Licensing and Trademarks

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For Research Use Only. Not for use in diagnostic procedures.
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Micro Fluidic Card developed in collaboration with 3M Company.

**SYBR Green Master Mix** -
The SYBR® Green dye is sold pursuant to a limited license from Molecular Probes, Inc.

**7300/7500 and 7900HT Fast Instruments** -
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