Critical Factors For Successful Real-Time RT-PCR

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Annealing

Specific product
High yield
High sensitivity

Nonspecific product
Low yield
Low sensitivity
SYBR Green Detection

→ Detection of specific & non-specific PCR products
Specificity & SYBR Green Based Detection

Non-specific PCR products result in:

- Non-specific fluorescent signals (e.g. by primer-dimers)
- Reduced sensitivity
- Inaccurate quantification
Factors Influencing PCR Specificity

- Amount of template
- Primer design
- Cations
- Initial artifact generation by *Taq* DNA polymerase
Effects of Cations

Template

Primer

Stabilizing

Destabilizing

K^+

NH_3^+ + H^+ ⇌ NH_4^+

K^+

P

B

H

P

B
Variable Mg$^{2+}$ Concentration

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M 1.5 4.0 M

Supplier A

M 1.5 4.0 mM
Effect of Mg$^{2+}$ Concentration

A. QIAGEN, Mg$^{2+}$ concentration supplied

B. Supplier R, Mg$^{2+}$ concentration supplied

C. Supplier R, optimized Mg$^{2+}$ concentration
PCR Specificity: Initial PCR Cycle

Nonspecific amplification starts during

- Reaction setup at room temperature
- Initial heating phase of thermal cycler
## Different Hot Start Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA Polymerase</th>
<th>Hot Start (Supplier A)</th>
<th>Antibody-Mediated (Supplier I)</th>
<th>No Hot Start (Supplier R)</th>
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<tbody>
<tr>
<td>HotStar DNA Polymerase</td>
<td>M</td>
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<tr>
<td>HotStartTaq DNA Polymerase</td>
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<tr>
<td>Hot Start enzyme (Supplier A)</td>
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<tr>
<td>Antibody-mediated (Supplier I)</td>
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<tr>
<td>No hot start (Supplier R)</td>
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</tbody>
</table>

- **497 bp**
Effect of Specificity on PCR Sensitivity (SYBR Green)

- Supplier A
- QIAGEN

Copies
- $10^2$
- 10
- 5
- 0
Improved Sensitivity in Probe-based Assays

100 ng at $C_T$ 22.2
10 pg at $C_T$ 35.5
Efficiency: 98%

100 ng at $C_T$ 25.5
10 pg at $C_T$ 44.4
Efficiency: 89% (61%)

Gene Expression Assay Mm_Bcl2
QuantiTect PCR & RT-PCR Kits

- Balanced combination of KCl and (NH$_4$)$_2$SO$_4$
  → Specific primer annealing during each cycle

- Stringent hot start with HotStarTaq DNA Polymerase
  → High PCR specificity in initial PCR step

- Accurate & sensitive quantitation of transcripts

- Optimized for use with any real-time cycler

- Ready-to-use master mix format
RT-PCR: The Reverse Transcription Step

- Methods
- Efficiency, Sensitivity and Specificity
- Primers
- Template
One-Step and Two Step RT-PCR

- One-Step RT-PCR
  - Single tube reaction
  - Direct link of both steps
  - RT starts from reverse PCR primer
  - Fast & reproducible procedure

- Two-Step RT-PCR
  - Two reaction setups
  - Temporally and physically separated
  - Various types of RT primers
  - 1 RT for multiple transcripts
  - Long-term storage of cDNA
One Step RT-PCR: Problems and Solutions (I)

- Inhibition of PCR by RT enzyme
  - Water
  - 10% mock RT
  - 20% mock RT
  - 30% mock RT
  - No template control

- Inhibition relief by
  - Optimized RT/PCR enzyme ratio
  - Additives (proprietary, patented technology)
One Step RT-PCR: Problems and Solutions (II)

- Efficiency and cDNA yield
  - High-affinity RT enzymes ➔ No truncated cDNA
  - Buffer additives ➔ High RT temperature

- Specificity and Sensitivity
  - HotStarTaq ➔ No interference with RT step
  - Balanced ion composition ➔ High annealing specificity
Reliable Quantitation in One-Step Real-time RT-PCR

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Supplier A

Supplier I

Supplier S
Choice of RT Primer

- **Selectivity**
  - Gene-specific (Oligo-dT)

- **cDNA length**
  - Oligo-dT (Gene specific)

- **Amplicon position**
  - Gene-specific (Random oligomers)

- **Flexibility**
  - Oligo-dT + Random Oligomers
Effect of RT Primer Choice

Amplicon – 3‘-end: 2 kb

[Oligo-dT] 1µM
[(N)₉] 10µM
[Oligo-dT+(N)₉] 1µM/10µM

Amplicon – 3‘-end: 6 kb

Oligo-dT
Oligo-dT+(N)₉
(N)₉
Oligo-dT+(N)₉
Oligo-dT
Effect of RT Volume in Real-time PCR Reaction

Target A

Target B
Summary: Reverse Transcription & Real-Time PCR

- High-affinity RT Enzymes and Buffer Additives
  → Sensitive and linear one-step quantification

- Use of RT Primer Mixture
  → High flexibility in amplicon choice

- Limited RT template volume
  → Inhibition-free and reliable amplification
QIAGEN R&D Group Modification/Amplification