5 Essential Tips for Real-Time PCR Success: From Beginner to Expert

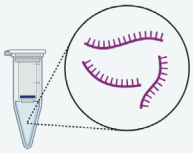
Whether preparing for your first Real-Time PCR experiment or a seasoned expert, when it comes to perfecting Real-Time PCR assays, everyone has room to maximize their success. Test your RT-PCR knowledge and consider these important tips based on your experience.

● Beginner ● Intermediate ● Advanced ● Expert

1. DESIGN GREAT PRIMERS AND PROBES

- Determine your reference gene; great primers may already exist
- Target amplicon size = 70–150 bp
- Span an exon-exon junction
- GC content = 40–60%
- T_m = 50–65°C
- Restrict the number of identical nucleotide runs
- Verify specificity with Primer-BLAST
- Ensure minimal secondary structures and prevent primer dimer formation with an online tool like IDT's OligoAnalyzer™
- Optimize T_a with a temperature gradient run on a qPCR device
- Use an online tool like IDT's PrimerQuest® to design specific primer/probe combinations
- Store stock oligos (100µM) and diluted aliquots (10–20µM) in nuclease-free H_2O at -20°C

2. ISOLATE HIGH-QUALITY RNA



- RNA is very sensitive to degradation; use PPE, ensure all tools and working area are sterilized with H_2O_2, and keep samples on ice
- Ensure high quality RNA (260/280 ratio of 1.8–2.0; ideally 2.0) and quantity (varies) before proceeding to cDNA synthesis
- If concerned about quality, run RNA out on a gel; smearing as opposed to two clean (2:1) bands indicates poor quality
- Treat with DNase to remove contamination from genomic DNA (gDNA) prior to cDNA synthesis

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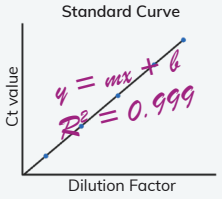
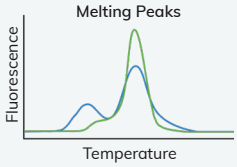
3. REFINE TEMPLATE QUANTITY & REACTION EFFICIENCY



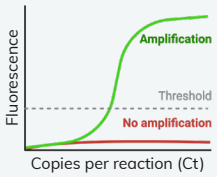
- Vortex and quick-spin down all reagents before use, making sure to use a low volume (e.g. P10) pipette when needed
- Serially dilute template cDNA to identify a dilution that yields ideal cycle threshold (Ct) values of 20–30
- Verify reaction efficiency $[10^{(-1/slope)}-1]*100\%$ and $R^2 > 0.95$ from a standard curve for all primer pairs; always use fresh dilutions
- Validate multiplexed assays together

4. INCLUDE THE PROPER CONTROLS



- A no template control (NTC) will determine if contamination is present in your master mix
- A positive control ensures the reaction conditions are met and helps determine if your new primers/probes work
- A no reverse transcriptase (RT) control will determine if you have DNA contaminants (e.g. from gDNA)
- For SYBR® based assays, use a melt curve at the end of cycling to determine that only one product is amplified
- An internal positive control will determine if PCR inhibitors are present

5. SELECT THE APPROPRIATE METHOD FOR Ct ANALYSIS: THRESHOLD VS REGRESSION



- Verify the cycling conditions are correct (especially if using a shared device)
- The regression mode of analysis is default on many qPCR devices
- For threshold analysis, set the baseline to be two cycles earlier than the Ct value for the most abundant sample; the threshold should be set to the product's exponential growth phase

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