

1. **Poor primer and probe design.** For the most efficient design of PCR primer and probe sets for real-time qRT-PCR, we strongly recommend using primer design software. Most primer design programs include adjustable parameters for optimal primer and probe design. These parameters consider primer/probe Tm complementarity, and secondary structure as well as amplicon size and other important factors. Restricting the number of identical nucleotide runs is also recommended. When designing amplicons in eukaryotic targets, choose PCR primers that span at least one exon-exon junction in the target mRNA to prevent amplification of the target from contaminating genomic DNA.

2. **Using poor quality RNA.** Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield. RNA should either be prepared from fresh tissue, or from tissue treated with an RNA stabilization solution such as RNAlater™ (see www.ambion.com/techlib/resources/ralater for more information). The importance of using full length RNA for reverse transcription depends on the application. Amplicons for real-time qRT-PCR are typically short (70-250 bp). As a result, some degradation of the RNA can be tolerated. If it is not possible to use completely intact RNA, design primers to anneal to an internal region of the gene of interest. Note that for truly quantitative RT-PCR, partially degraded RNA may not give an accurate representation of gene expression.

3. **Not using “master mixes”.** qRT-PCR is a highly sensitive tool for analyzing RNA. As the PCR amplifies the target, errors are simultaneously amplified. Therefore, variability should be kept to a minimum whenever possible. A “master mix”, or mixture of the reaction reagents, should be used when setting up multiple reactions to minimize sample-to-sample and well-to-well variation and improve reproducibility. To further reduce wear-to-wear variation, a reference dye such as ROX can be added to the master mix.

4. **Introducing cross-contamination.** All surfaces in the PCR area should be routinely decontaminated to prevent cross-contamination – use of a DNA decontamination solution, such as DNAase, that destroys DNA is recommended (see www.ambion.com/techlib/realtime for more information). A "No Template Control" (NTC) should be run to rule out cross contamination of reagents and surfaces. The NTC includes all of the RT-PCR reagents except the RNA template. Typically the RNA is simply substituted with nuclease-free water. No product should be synthesized in the NTC; if a product is amplified, it indicates that one or more of the RT-PCR reagents is contaminated with the amplicon.

5. **Not using a "- RT" control.** It is virtually impossible to completely eliminate genomic DNA from RNA preparations. Therefore, it is important to include a minus-reverse transcriptase control (No Amplification Control® or NAC) in qRT-PCR experiments. Typically, the NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. If a product is seen in the NAC, it probably indicates that contaminating DNA is present in the sample. For information about DNase treatment, see Getting Rid of Contaminating DNA at www.ambion.com/techlib/tn/81/817.html. Also note the article on page 7 in this issue about a new hyperactive DNase that can further eliminate contaminating genomic DNA compared to standard DNase I.

6. **Using an inappropriate normalization control.** The reliability of any qRT-PCR experiment can be improved by including an invariant endogenous control in the assay to correct for sample to sample variations in qRT-PCR efficiency and errors in sample quantitation. The expression level of a good control should not vary across the samples being analyzed. 18S rRNA is often used as a control because it is less variant in expression level than traditional internal controls such as β-actin or GAPDH. For more information, see Using 18S rRNA as an Internal Control for Relative RT-PCR at www.ambion.com/techlib/tn/83/839.html.

7. **Not setting the baseline and threshold properly.** To obtain accurate Cq values the baseline needs to be set two cycles earlier than the Cq value for the most abundant sample. For real-time qRT-PCR data to be meaningful, the threshold should be set when the product is in exponential phase. Typically this is set at least 10 standard deviations from the baseline.

8. **Dissociation (melting) curves are not performed when using SYBR® Green.** Ideally, the experimental samples should yield a sharp peak (first derivative plot) at the melt temperature of the amplicon, whereas the NAC and NTC will not generate significant fluorescent signal. This result indicates that the products are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest. If the dissociation curve reveals a series of peaks, it indicates that there is not enough discrimination between specific and non-specific reaction products. To obtain meaningful data, optimization of the qRT-PCR protocol would be necessary.

9. **The efficiency of the reaction is poor.** The efficiency (E) of the reaction can be calculated by the following equation: 

\[ E = 10^{\frac{-1}{Slope}} - 1 \]

The efficiency of the PCR should be 90-110% (−3.62 s.d. slope = 3.1). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure, and primer design, to name a few. Although valid data can be obtained that fall outside of the efficiency range, the qRT-PCR should be further optimized or alternative amplifications designed.

10. **Using an inappropriate range for standard curves.** Standard curves should be prepared for each gene under study for RNA quantitation (absolute or relative quantitation), or for verification of the efficiencies of the reactions for comparative quantitation (ΔΔCt). The standard curve should extend above and below the expected abundance of your target. Additional input quantities can be included such as the minimum and maximum RNA amounts above and below the limit of detection to help differentiate between specific and non-specific products.

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