Application Note

Guidelines for real-time RT-PCR assay optimization

Real-time RT-PCR has emerged as the preferred method for quantitating changes in gene expression. The reverse transcription (RT) step of real-time RT-PCR, converting RNA to cDNA is critical for accuracy in quantification and for detecting low copy messages. When performing real-time RT-PCR, the primers and enzymes for reverse transcription must be carefully chosen. The primers should allow reverse transcription of all targets of interest, and the reverse transcriptase should yield cDNA amounts that accurately represent the original RNA amounts to ensure accurate quantification. In addition, the effects of the components of the RT reaction on subsequent real-time PCR must be minimized.

Two-step and one-step RT-PCR

Real-time RT-PCR can take place as a two-step or one-step reaction. In one-step RT-PCR, both reverse transcription and real-time PCR take place in the same tube, with reverse transcription preceding PCR. With two-step RT-PCR, the RNA is first reverse transcribed into cDNA using oligo-dT primers, random oligomers, or gene-specific primers. An aliquot of the reverse-transcription reaction is then added to the real-time PCR (Figure1).

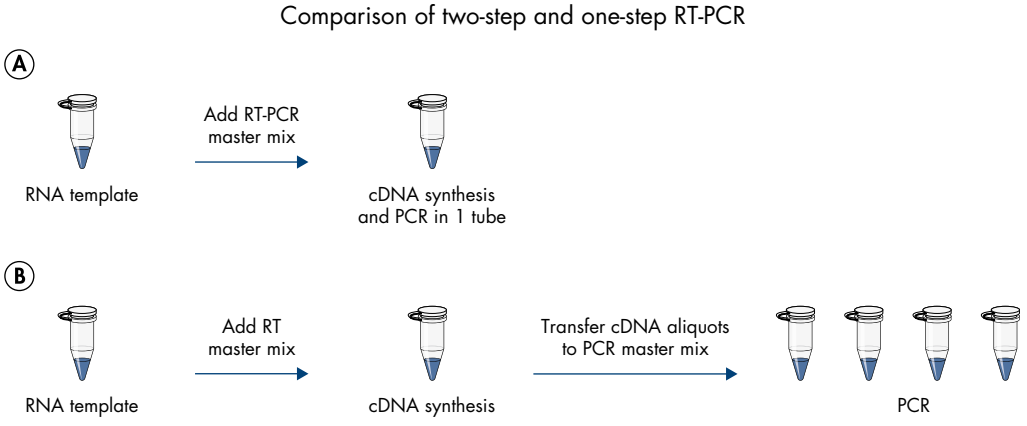


Figure 1. Comparison of two-step and one-step RT-PCR. A In one-step RT-PCR, reverse transcription and PCR take place sequentially in the same tube. **B** In two-step RT-PCR, cDNA is synthesized in 1 tube, and aliquots of the cDNA are transferred to other tubes for PCR.

Two-step RT-PCR uncouples cDNA synthesis and the subsequent real-time PCR reaction, making it possible to optimize the two reactions separately and choose between different types of RT primers, depending on experimental need. The ability to use oligo-dT primers or random oligomers for reverse transcription means that all different transcripts can be transcribed in a single RT reaction. In addition, precious RNA samples can be immediately transcribed into more stable cDNA for later use and long-term storage.

With fewer handling steps, one-step RT-PCR is easy to automate, reduces the chance of pipetting errors and contamination. One-step RT-PCR is a strong choice for quantitating the same gene(s)' expression repeatedly, for example, in high-throughput screening and diagnostic settings. (Table 1).

Table 1. Advantages of different RT-PCR procedures

Procedure	Advantages
Two-step RT-PCR	• Multiple PCRs from a single RT reaction
	• Flexibility with RT primer choice
	• Enables long-term storage of cDNA
One-step RT-PCR	• Easy handling
	• Fast procedure
	• High reproducibility
	• Low contamination risk

Choice of RT primers

There are three RT primer options - oligo-dT primers (typically 13–18mers), random oligomers (such as hexamers, octamers, or nonamers), and gene-specific primers. One-step RT-PCR is always performed with gene-specific primers as the downstream PCR primer is also the primer for reverse transcription. In two-step RT-PCR, the three types of primers, or combinations thereof, can be used for reverse transcription.

If oligo-dT primers are used, only mRNAs will be reverse transcribed starting from the poly-A tail at the 3' end. Oligo-dT primers are a popular choice for constructing cDNA libraries from eukaryotic mRNA as they bring the possibility of full length copies of mRNA.

Random oligomers enable reverse transcription from the entire RNA population (I.e. rRNA, tRNA, miRNA or non-Eukaryotic RNA). Random oligomers initiate reverse transcription from several positions within the RNA molecule. When used alone, random oligomers can lead to relatively short cDNA, but when used in combination with Oligo-dT primers, the random nature of the reverse transcription initiation can compensate for Oligo-dT primer, 3' end bias. A mix of random and oligo-dT primers, can produce a cDNA library covering all transcript sequences, including both 5' and 3' regions, improving cDNA synthesis for detection.

Gene specific primers are designed based on known sequences of the target RNA. Gene specific primers increase sensitivity, as all of the reverse transcription activity is directed to a specific message.

A universal priming method for the RT step of real-time two-step RT-PCR should allow amplification and detection of any PCR product regardless of transcript length and amplicon position, and achieve this with high sensitivity and reproducibility (Table 2).

Table 2. Suitability of primer types for RT-PCR

Application	Recommended type of primer
RT-PCR of specific transcript:	Gene-specific primer gives highest selectivity and only the RNA molecule of choice will be reverse transcribed
RT-PCR of long amplicon:	Oligo-dT or gene-specific primers
RT-PCR of an amplicon within long transcript:	Gene-specific primers, random oligomers, or a mixture of oligo-dT primers and random nonamers (see section 8.1.1) are recommended so that cDNA covering the complete transcript is produced

Effect of RT volume added to two-step RT-PCR

In two-step RT-PCR, the addition of the completed reverse-transcription reaction to the subsequent amplification reaction transfers not only cDNA template, but also salts, dNTPs, and RT enzyme. The RT reaction buffer, which has a different salt composition to that of the real-time PCR buffer, can adversely affect real-time PCR performance. However, if the RT reaction forms 10% or less of the final real-time PCR volume, performance will not be significantly affected. Use of 3 µl of RT reaction in a 20 µl PCR (i.e., 15% of the final volume) can lead to significant inhibition of real-time PCR (Figure 3). We recommend testing dilutions of the RT reaction in real-time PCR to check the linearity of the assay. This helps to eliminate any inhibitory effects of the RT reaction mix that might affect accurate transcript quantification.

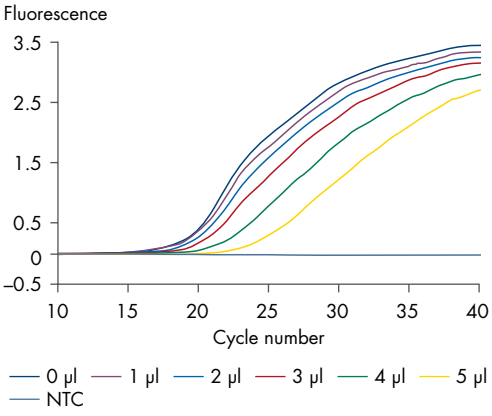
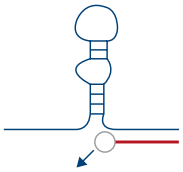
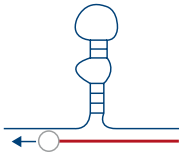


Figure 2. Inhibition of real-time PCR by addition of RT reaction. Real-time PCR (20 µl volume) was carried out using plasmid DNA as template. The volumes of RT reaction (without template RNA) indicated above were added to the PCR to determine their effect on amplification.

Effect of RNA secondary structure

RNA secondary structure can create a problem in transcribing full length RNA. Regions of RNA with complex secondary structure can cause the reverse transcriptase to stop or dissociate from the RNA template causing truncation or cDNA with internal deletions (Table 3).

Table 3. Effects of complex secondary structure on RT-PCR: RT effects

Cause	Effect on cDNA	RT-PCR result
(A) RT stops or dissociates cDNA at RNA region with complex secondary structure	 Truncated cDNA products	cDNA products missing primer-binding site not amplified
(B) RT skips RNA region with complex secondary structure	 Shortened cDNA products with internal deletions	Shortened PCR products with internal deletions
(C) RT reads through	 Full-length cDNA products	Full-length RT-PCR products

Truncated cDNA, missing the downstream primer-binding site, are not amplified during PCR. cDNAs with internal deletions are amplified and appear as shortened PCR products giving misleading results.

Problematic secondary structure in your RNA can be hard to predict, but high GC content in a target gene can indicate that RNA secondary structure will be difficult to melt apart. For this reason, an initial 5 minute, 65°C denaturation step to relax the RNA is common. Alternatively, using a specialized RT enzyme that moves through secondary structure with higher efficiency, even at standard RT incubation temperatures (37–42°C) would be advisable.

RT-PCR primer design

General primer-design rules for PCR are also applicable in RT-PCR to avoid mispriming and primer–dimer formation. These effects are even more pronounced in RT-PCR, where cDNAs produced during reverse transcription are more susceptible to nonspecific priming due to their single-stranded nature. Nonspecific priming in RT-PCR reduces the sensitivity of the process, leading to reduced yields of specific products or failure of the RT-PCR altogether.

Genomic DNA contamination in the RNA can cause a signal increase in the RT-PCR, if primers bind to the genomic sequence that initially codes for the transcript of interest. This leads to over-quantification of the respective message. To avoid amplification of contaminating genomic DNA, primers for RT-PCR should be designed so that one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon. Such primers will anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA (Figure 3).

Alternatively, removing gDNA contamination is highly recommended, either by DNAse I treatment, or preferably by an integrated gDNA removal. This is done either during sample preparation, or cDNA synthesis, as provided by various commercially available kits. This is particularly useful, if efficient intron-exon spanning primers cannot be designed.

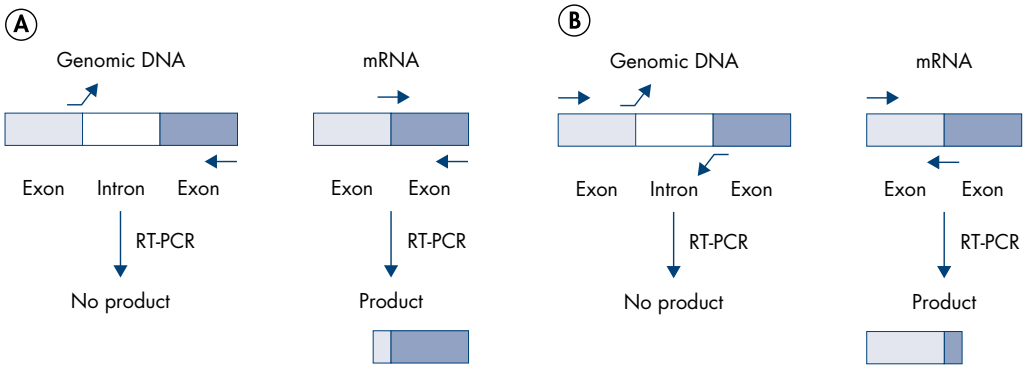


Figure 3. No coamplification of genomic DNA. Primer design to eliminate signals from contaminating genomic DNA. **A** Forward primer crosses an intron/exon boundary. **B** Reverse primer crosses an intron/exon boundary.

To detect amplification of contaminating DNA, RT-PCR primers should be designed to flank a region that contains at least one intron. Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). Size difference in products is used to detect the presence of contaminating DNA.

If only the mRNA sequence is known, choose primer annealing sites that are at least 300–400 bp apart. It is likely that fragments of this size from eukaryotic DNA contain splice junctions. As explained in the previous point, such primers may be used to detect DNA contamination. ▷

If a choice must be made, primer efficiency and specificity are paramount. With access to very efficient and specific primers designed in just one exon, it would be ill advised to use less efficient/specific primers to cover exon-intron junctions.

In summary, the following factors should be considered when designing primers for RT-PCR:

- Annealing temperature can affect RT-PCR efficiency and sensitivity
- High primer concentrations can cause mispriming and primer–dimer formation
- A stringent hot start is essential for optimal RT-PCR performance
- Primer design in RT-PCR can prevent signals from contaminating DNA. For best results, DNA-free RNA should be used in order to avoid misquantification caused by DNA in RT-PCR.

Enzymes used in RT-PCR

cDNA is synthesized from RNA templates using reverse transcriptases — RNA-dependent DNA polymerases are normally isolated from a variety of retroviral sources (e.g., from Avian Myeloblastosis Virus [AMV] or Moloney murine leukemia virus [MMLV]). Although thermostable DNA polymerases such as Tth DNA polymerase also exhibit reverse transcriptase activity under specific conditions, these enzymes are not as efficient for reverse transcription as mesophilic reverse transcriptases.

Multiplex RT-PCR

Multiplex, real-time RT-PCR allows simultaneous quantification of several RNA targets in the same reaction. Multiplexing is possible with either one- or two-step RT-PCR. Multiplex RT-PCR offers many advantages for applications such as gene expression analysis, viral load monitoring, and genotyping. The target gene(s) as well as an internal control are co-amplified in the same reaction, eliminating the well-to-well variability that would occur if separate amplification reactions were carried out. The internal control can be either an endogenous gene that does not vary in expression between different samples or an exogenous nucleic acid. For viral load monitoring, the use of an exogenous nucleic acid as internal control allows the following parameters to be checked: the success of sample preparation, the absence of inhibitors, and the success of PCR. Multiplex analysis ensures high precision in relative gene quantification, where the amount of a target gene is normalized to the amount of a control reference gene. Quantification of multiple genes in a single reaction also reduces reagent costs, conserves precious sample material, and allows increased throughput.

Multiplex RT-PCR is made possible by the use of sequence-specific probes that are each labeled with a distinct fluorescent dye and an appropriate quencher moiety. This means that the emission maxima of the dyes must be clearly separated and must not overlap with each other. In addition, reactions must be carried out on an appropriate real-time cycler that supports multiplex analysis (i.e. the excitation and detection of several non-overlapping dyes in the same well or tube).

Real-time RT-PCR is a sensitive and accurate method of quantitating gene expression, if the crucial RT step of real-time RT-PCR is performed with care. Attention must be given to common RT pitfall factors such as choosing the appropriate assay type, RT enzyme and probe type and accounting for secondary structure and gDNA presence during experimental design. Properly optimized, real-time RT-PCR can be utilized with great efficiency.

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References

1. Tacke, E. et al (1995) Transposon tagging of the maize Glossy2 locus with the transposable element En/Spm. Plant J. **8**, 907.

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