RT-qPCR Optimization Guide

INTRODUCTION

Reverse transcription quantitative PCR (RT-qPCR) combines reverse transcription and quantitative PCR (qPCR) for precisely detecting and measuring relative levels of RNA molecules in a sample. It can be employed in a two-step or one-step protocol.

Two-step RT-qPCR involves first strand cDNA synthesis from the RNA sample (total RNA or poly(A) enriched mRNA) using a reverse transcriptase (RT) enzyme. After reverse transcription, a small proportion (around 10%) of the cDNA is then transferred to separate qPCR reactions. This process allows multiple targets to be interrogated from the same initial RNA sample and can be useful for limiting samples or if material needs to be accessed for further testing.

One-step RT-qPCR combines the first-strand cDNA synthesis and the qPCR reaction into the same tube, simplifying the reaction set-up and reducing the possibility of contamination.

OPTIMIZATION

Priming method

Depending on the sample type quantity and the analysis strategy, the choice of primers used to initiate the reverse transcription can greatly impact the RT-qPCR results. Two-step RT-qPCR reactions offer the greatest flexibility as random primers, oligo(dT) or gene specific primers can be used individually or in strategies combining priming methods. The qScript® Flex cDNA Synthesis Kit is compatible with each of these priming strategies. In contrast, one-step RT-qPCR reactions always employ gene-specific primers for reverse transcription which are then also used for cDNA amplification in the qPCR reaction.

Random primers anneal throughout the RNA molecule and can be used for non-polyadenylated RNA, degraded RNA or RNA with extensive secondary structure. Random primers are ideal for generating large pools of cDNA with high yield. However, because they hybridize at multiple points throughout an RNA molecule, cDNA lengths will not be full sized and coverage may be reduced at the 3' ends.

Oligo(dT) primers are selective for mRNA as they hybridize to the poly(A)-tail at the 3' ends and allow for synthesis of longer cDNA molecules. However, oligo(dT) primers may result in loss of representation of degraded RNA or incomplete cDNA synthesis of RNA with internal secondary structure.

A combination of random and oligo(dT) primers can be used to combine the benefits of each priming method, such as is incorporated in the qScript XLT cDNA SuperMix.

Gene specific primers offer the greatest specificity but require a separate RT reaction for each target. Gene specific primers are required for one-step RT-qPCR because oligo(dT) or random primers will not allow for specific amplification of the cDNA target in the qPCR step.

Reverse transcription temperature

The temperature of the reverse transcription reaction can affect specificity, especially if gene specific primers are used. High temperature reverse transcription helps to prevent primer dimerization, melt RNA secondary structure and improve specificity of binding for gene specific primers. One drawback to high reverse transcription temperatures is that this can exacerbate metal-ion dependent RNA hydrolysis by free magnesium. Benefits of lower reverse transcription temperatures include improved RT stability and processivity for some enzymes, and increased primer hybridization to the RNA for primers that can form a strong 3' duplex.

Quantabio one-step RT-qPCR master mixes give optimal reverse transcription at 48–50°C but this temperature can be optimized between 42–55°C for qScript XLT 1-Step RT-qPCR ToughMix® and qScript 1-Step Virus ToughMix. UltraPlex® 1-Step ToughMix has been formulated for extended RT stability, allowing reverse transcription reactions up to 60°C for increased primer binding specificity.

Temperature range	General observations	Recommended for
48–50°C	▪ Compromise between relaxing secondary structure and maintaining enzyme stability	▪ Most applications ▪ Starting temperature range for protocol optimization
Lower (42–48°C)	▪ Maintain enzyme stability ▪ Ensure enzyme activity for full reaction time ▪ Maintain enzyme processivity	▪ Templates with low secondary structure ▪ Oligo(dT) priming
Higher (50–60°C)	▪ Relax secondary structure of template ▪ Increase specificity of gene specific primers ▪ Increase access for gene specific primers ▪ Increased risk of Mg^{2+} dependent RNA hydrolysis	▪ Templates with high secondary structure ▪ Gene specific priming method ▪ Methods using a thermostable RT such as qScript Ultra

Table 1 General guidelines for reverse transcription temperature optimization for RT-qPCR reactions.

Reverse transcription time

Standard reverse transcription reactions can take up to 80 minutes to synthesize cDNA. Coupled with a further 1–2 h for PCR cycling, this can make RT-qPCR a time-consuming technique. Reduction in reverse transcription time can vastly improve protocol usability, particularly for RT-qPCR reactions used for diagnostics. However, with shorter reverse transcription time, complete cDNA synthesis from large quantities of RNA may not be achieved or full length cDNA might not be synthesized, particularly for long templates >1 kb. The Quantabio qScript XLT cDNA SuperMix kit allows flexibility of reverse transcription time between 30–70 minutes, allowing optimization for individual assays. The highly progressive reverse transcriptase in the qScript Ultra Flex Kit is able to transcribe targets in excess of 20 kb in just 10 minutes.

Quantabio one-step RT-qPCR master mixes contain an engineered MMLV reverse transcriptase enzyme to allow faster processivity, reducing first strand cDNA synthesis time to just 10 minutes (5 minutes for qScript 1-Step Virus ToughMix). These times also allow flexibility; the reverse transcription reaction can be extended to 20 min for longer templates.

Reaction time	General observations	Recommended for
Increased	▪ Higher yield ▪ Full length cDNA synthesis ▪ Greater sensitivity	▪ Long cDNA synthesis ▪ High input RNA quantity
Decreased	▪ Choose an RT engineered for high processivity	▪ RT engineered for high processivity ▪ Short targets

Table 2 General recommendations for optimization of reverse transcription time for RT-qPCR reactions.

RNase H Activity

The RNase H domain in native reverse transcriptases functions *in vivo* to cleave the RNA strand of RNA-DNA heteroduplexes during replication. For RT-qPCR, RNase H+ reverse transcriptases can improve qPCR amplification as they cleave the RNA template and enhance melting of the RNA-cDNA duplex during the first cycles of PCR. This prevents suppression of qPCR amplification by the RNA template ("template suppression"), resulting in higher sensitivity. However, RNase H activity can be a disadvantage for long RNA templates with complex secondary structure where RNA may be degraded prematurely while the RT is stalled, resulting in truncated cDNA (Figure 1).

Generally, two-step RT-qPCR protocols benefit from RNase H+ activity when long cDNA strands are synthesized. In one-step RT-qPCR reactions, where more often short cDNA strands are synthesized, template suppression is unusual. Quantabio offers both one and two-step RT-(qPCR) options with RNase H+ activity (e.g. qScript cDNA SuperMix) or reduced RNase activity (e.g. qScript XLT cDNA SuperMix and qScript XLT 1-Step RT-qPCR ToughMix). In addition, Quantabio one-step RT-qPCR master mixes benefit from accessory proteins which further prevent any suppression of cDNA synthesis by the RNA template.

RNase H activity	General observations	Recommended for
RNase H+	▪ Reduces RNA template suppression of PCR	▪ Applications where strong RNA:cDNA duplex is an issue ▪ Long cDNA synthesis from RNA templates with low secondary structure ▪ Increased sensitivity
RNase H–/ reduced RNase H activity	▪ No cleavage of long RNA templates	▪ Long cDNA synthesis from RNA templates with high secondary structure

Table 3 General recommendations for use of reverse transcriptase enzymes with RNase H+ or RNase H-/ reduced RNase H activity for RT-qPCR.

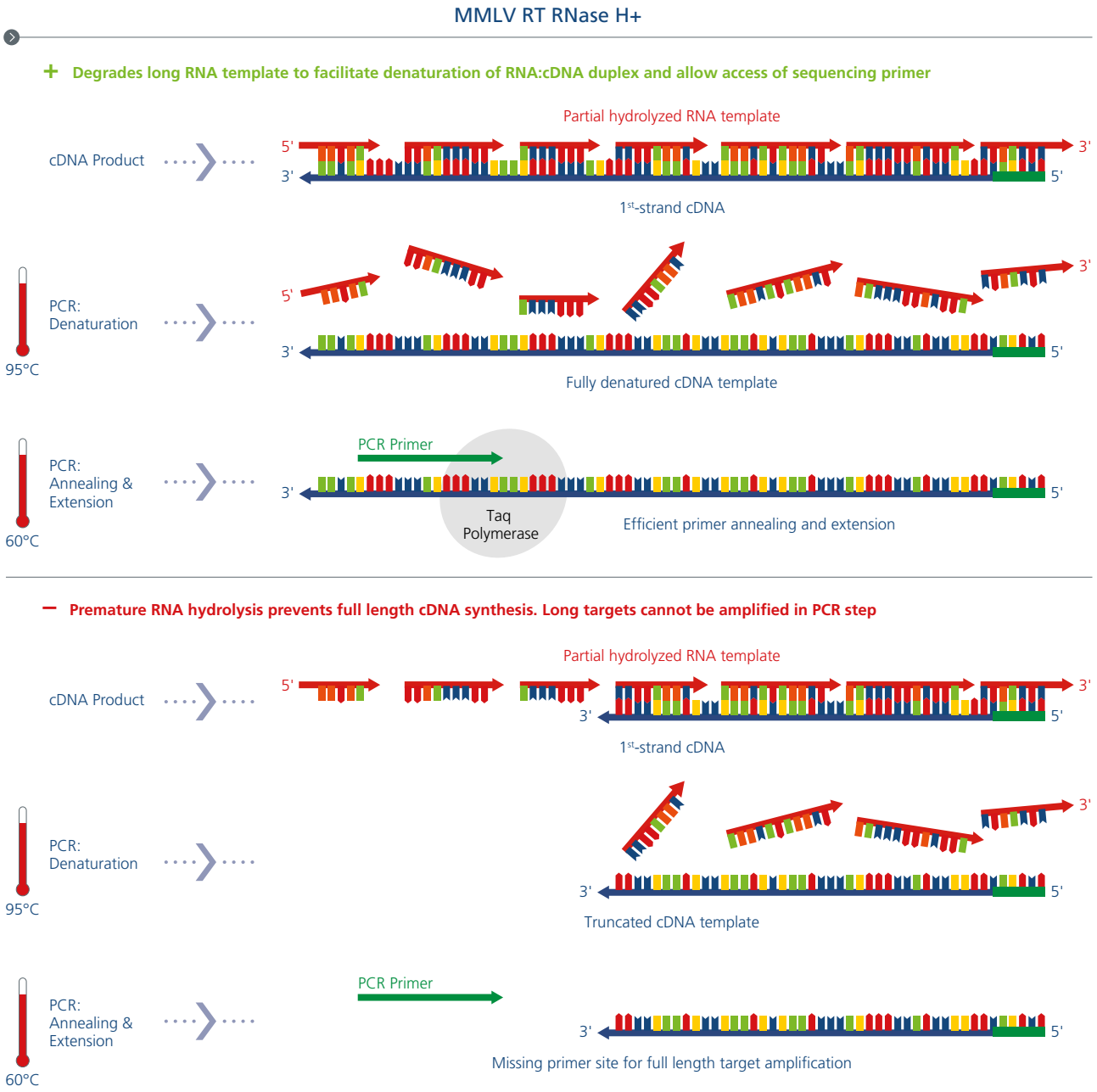

Figure 1 **Advantages and disadvantages of MMLV RT RNase H+ activity.** RNase H+ activity degrades RNA template, facilitating melting of the RNA:cDNA duplex during PCR and preventing RNA template suppression of PCR. However for long templates, RNase H+ activity can relsult in premature RNA hydrolysis and prevents full length cDNA synthesis.

Mg^{2+} concentration

Magnesium is an important cofactor for both the RT and DNA polymerase. For qPCR, magnesium chloride or magnesium sulfate is typically used at a final concentration of 3 mM. Higher concentrations can further increase enzyme activity and consistency. Raising the magnesium concentration as high as 6 mM can improve reaction efficiency for single product amplification. However, lower magnesium concentrations can reduce competitive binding and decrease primer-dimer formation. In addition, at high temperatures, free magnesium can cause metal-ion dependent hydrolysis of the RNA template. The Quantabio one-step RT-qPCR master mixes have optimized Mg^{2+} concentration to favor both reverse transcription and qPCR reactions.

Enzyme inhibitors

Enzyme inhibitors are an important consideration for both one- and two-step RT-qPCR where such inhibitors will be present during or carried over into reverse transcription and qPCR reactions. The use of organic solvents (e.g. ethanol) and chaotropic salts (e.g. guanidine) are necessary for RNA extraction and purification, but these can inhibit enzymatic reactions either by direct interaction with the enzymes or with co-factors. In addition, where samples contain different levels of inhibitors, sample comparison may not be accurate. The use of an RNA isolation method that minimizes inhibitor carryover can alleviate these issues, but this is not always possible. Moreover, in clinical settings there is increasing attention toward using unprocessed patient samples for reaction input. In such cases, choosing an inhibitor resistant enzyme formulation is vital.

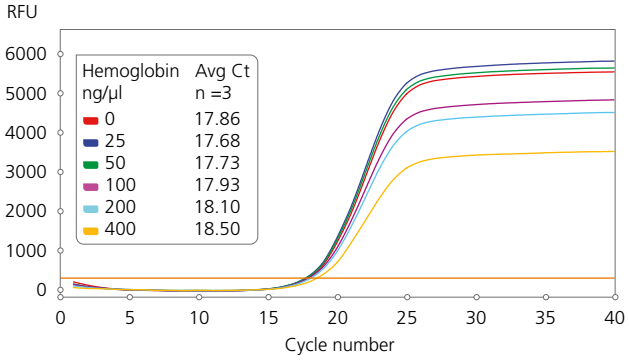

Figure 2 **Inhibitor tolerant RT-qPCR.** The ToughMix formulation ensures high RT-qPCR performance in the presence of increasing concentrations of hemoglobin.

Quantabio's engineered, application-specific enzymes combined with optimized ToughMix buffer formulations allow superior performance in the presence of inhibitors. Particularly for one-step RT-qPCR, UltraPlex 1-step ToughMix allows bypass of time consuming and expensive sample purification, resulting in accurate and reliable quantification of RNA directly from crude lysates.

DNase treatment

Carryover of gDNA into RT-qPCR reactions can result in non-specific primer binding, unintended gDNA amplification and erroneous results for gene expression studies. One method to avoid gDNA amplification in RT-qPCR is to design primers to amplify only cDNA, for example by flanking an intron or spanning exon-exon junctions. Where this is not possible, the input RNA sample can be treated with DNase I enzyme to degrade any contaminating DNA.

Following DNase treatment, inactivation and/or removal of the enzyme is important as DNase can degrade newly synthesized cDNA in the reverse transcription reaction. DNase treatment can be carried out either on column or in solution. In-solution treatment requires inactivation of the enzyme, typically at 65°C. At this temperature, magnesium (required for DNase I activity) can cause magnesium-dependent RNA hydrolysis. Therefore, a magnesium ion chelating agent (e.g. EDTA) is often used to sequester Mg^{2+} during heat treatment. However, if EDTA is carried over into the reverse transcription reaction it can sequester the Mg^{2+} required for RT activity, inhibiting the RT enzyme.

Compared to in-solution DNase treatment, on-column DNase treatment typically requires more enzyme but does not require enzyme heat-inactivation in the presence of EDTA, as the enzyme

is removed by salt washes of the silica matrix. Therefore, either on-column DNase treatment, or in solution DNase treatment with a reaction stop solution that does not contain EDTA, is preferred.

Alternatively, gDNA contamination can be removed during total RNA extraction with a spin column method such as in the Extracta Plus RNA kit. The kit includes a DNA removal column that binds gDNA and elutes RNA, resulting in efficient DNA removal without DNase treatment.

Controls

For accurate analysis of RT-qPCR results, experiments should include appropriate replicates and controls. At least three technical replicates are necessary for each experimental and control sample to minimize errors due to pipetting. For two-step RT-qPCR, the use of a "SuperMix" formulation for cDNA synthesis, such as qScript cDNA SuperMix or qScript XLT cDNA SuperMix, where the enzyme, buffer components and primers are pre-mixed can improve accuracy by reducing pipetting steps.

The expression of an endogenous "internal control" should also be measured to control for differences in RNA extraction, quantification and reaction set up. For gene expression experiments, multiple reference genes should be analyzed for stable gene expression. A reference gene with an expression pattern that is unaltered by the treatment or different time points to be tested should be selected.

Control reactions that lack reverse transcriptase, "no RT controls", are useful to identify signal from gDNA contamination. When using a cDNA SuperMix kit formulation, it is not feasible to construct a control reaction that includes all components except the RT. In this case, the most direct method to test for presence of gDNA is to by-pass the reverse transcription step and use an equivalent amount of the RNA preparation directly for PCR amplification.

A "no template control" should also be included to identify possible contamination during preparation of samples. For one-step RT-qPCR, a no template control should be included for each different gene analyzed. For more detailed guidance on the relevant controls and methods of analysis for relative expression using RT-qPCR, see Bustin et al. 2009.[1]

Reference

[1] Stephen A Bustin, et al., The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clinical Chemistry, Volume 55, Issue 4, 1 April 2009, Pages 611–622, "**https://doi.org/10.1373/clinchem.2008.112797**"

TROUBLESHOOTING

Issue	Causes	Troubleshooting
Primer-dimer formation	Since RT enzymes can extend from a DNA primer on a DNA template, primer-dimer formation may start during the RT step	▪ Increase temperature of reverse transcription reaction ▪ Re-design primers with lower T_m ▪ Decrease Mg^{2+} concentration ▪ Use two-step RT-PCR
Signal in no-RT control	DNA contamination in the RNA sample can result in erroneously high, non-specific signal or signal in no-RT control	▪ Use primers that flank an intron or that span an exon-exon junction ▪ Treat input RNA sample with DNase I (note, this can result in excess Mg^{2+} carryover into the reverse transcription)
Poor cDNA yield	RNA is degraded/ low quality	▪ Use two-step RT-qPCR with random priming method where polyA signal could be degraded ▪ Avoid oligo(dT) priming ▪ Improve RNA extraction method ▪ Use reverse transcriptase with reduced RNase H activity
	Inhibition of RT (e.g. by organic solvents or chaotropic salts)	▪ Use RT enzyme engineered to overcome inhibitors ▪ Use master mix with inhibitor resistant formulation ▪ Dilute input RNA
	RNA input is too low	▪ Use more input RNA ▪ Use gene specific priming and/or one-step RT-qPCR to detect low abundance RNA
	Presence of RNases which degrade RNA template	▪ Use RNase contamination control procedures ▪ Use one-tube RT reaction master mixes to reduce risk of contamination during set up
Poor amplification efficiency or no amplification	Carry over of DNase I from DNase treatment of RNA sample can degrade cDNA or amplified DNA during PCR step	▪ If treating RNA sample with DNase, ensure full deactivation of DNase or sufficient washing with column based methods before reverse transcription
	Inactivation of DNase step with chelating agent (e.g. EDTA) can lead to sequestration of Mg^{2+} ions and inhibition of RT/ polymerase activity	▪ Use on-column DNase treatment method ▪ Use DNase treatment with a 'reaction stop' solution that does not include EDTA
	Inhibition of qPCR	▪ Use enzyme engineered to overcome inhibitors ▪ Use master mix with inhibitor resistant formulation ▪ Dilute input RNA (one-step RT-qPCR) or cDNA (two-step RT-qPCR)
Multiple peaks in melt curve	Non-specific amplification of contaminating DNA or non-specific primer binding	▪ Check for DNA contamination in RNA sample ▪ Increase temperature of reverse transcription reaction ▪ Use gene specific priming for reverse transcription ▪ Change primer design to prevent off-target priming ▪ Reduce extension time during PCR ▪ Optimize annealing temperature with gradient PCR
qPCR Reaction efficiency >110%	Inhibition of reaction	▪ Use enzyme engineered to overcome inhibitors ▪ Use master mix with inhibitor resistant formulation ▪ Dilute input RNA (one-step RT-qPCR) or cDNA (two-step RT-qPCR)
qPCR Reaction efficiency <90%	Suboptimal reagent concentrations	▪ Vary concentration of primers, Mg^{2+} or polymerase
	Suboptimal PCR cycling	▪ Check primer design, Tm of primers should be within 5°C of each other ▪ Optimize annealing temperature with gradient PCR ▪ Optimize extension time according to polymerase processivity rate ▪ Increase initial denaturation and final extension times for PCR

SUMMARY OF OPTIMIZATION PROCESS

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