Critical factors for synthesis of cDNA for real-time PCR analysis

A commonly used method for gene expression analysis is real-time two-step RT-PCR, where reverse transcription takes place in one tube, and subsequent real-time PCR quantification takes place in another tube. An important issue to consider is the choice of primers for the synthesis of cDNA from RNA. In addition, eliminating genomic DNA contamination in the RNA sample is critical, as it is often not possible to design PCR primers to specifically amplify a cDNA sequence without also amplifying the corresponding genomic DNA sequence. Both these issues are addressed by the optimized chemistries of the QuantiTect® Reverse Transcription Kit, which removes genomic DNA and synthesizes cDNA in just 20 minutes.

Correct choice of primers for reverse transcription

The most appropriate primers to use in cDNA synthesis depends on the real-time two-step RT-PCR application (Table 1). To allow reverse transcription of all regions of each transcript in an RNA sample, the QuantiTect Reverse Transcription Kit is supplied with an optimized RT primer mix that consists of a unique blend of oligo-dT and random primers. Use of this RT primer mix, in combination with Quantiscript® Reverse Transcriptase and Quantiscript RT Buffer, allows highly sensitive real-time two-step RT-PCR analysis. The sensitivity is greater than that achieved when either oligo-dT or random primers are used for reverse transcription (Figure 1).

Table 1. Recommended RT primers for various RT-PCR applications

Application	Recommended primers
RT-PCR of full-length transcripts or of long amplicons that start from the 3' end of transcripts	Oligo-dT primers
RT-PCR of short amplicons, with an even representation of cDNA sequences from the 5' to 3' end of transcripts	Random oligomers, or a mixture of oligo-dT primers and random oligomers. We recommend the RT primer mix supplied with the QuantiTect Reverse Transcription Kit to ensure that cDNA sequences covering the entire transcript are produced.

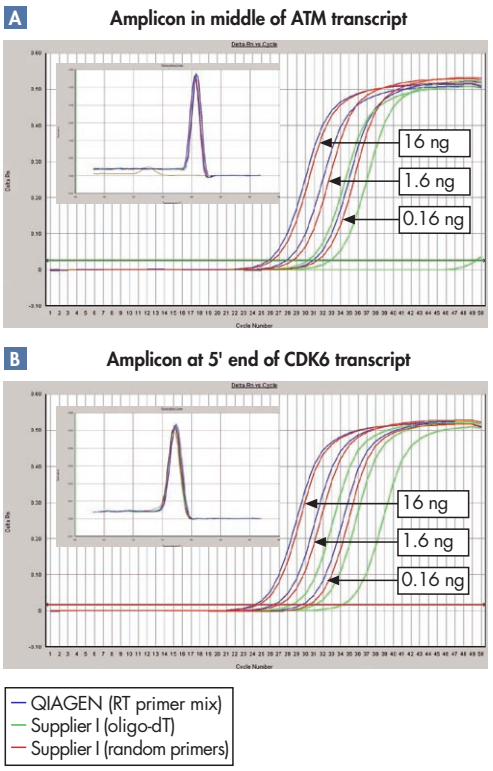

Figure 1. Greater sensitivity in real-time two-step RT-PCR using an optimized RT primer mix. cDNA was synthesized from tenfold serial dilutions of HeLa cell RNA (1000 ng, 100 ng, or 10 ng). Reactions were carried out using either the QuantiTect Reverse Transcription Kit (with supplied RT primer mix) or a kit from Supplier I (with either oligo-dT or random primers). Synthesized cDNA (16 ng, 1.6 ng, or 0.16 ng) was analyzed on the Applied Biosystems® 7500 Real-Time PCR System using the QuantiFast® SYBR® Green PCR Kit and the QuantiTect Primer Assay for A ATM (ataxia telangiectasia mutated) or B CDK6 (cyclin-dependent kinase 6). Melting curve analysis provided a single peak, demonstrating specific amplification (see insets).

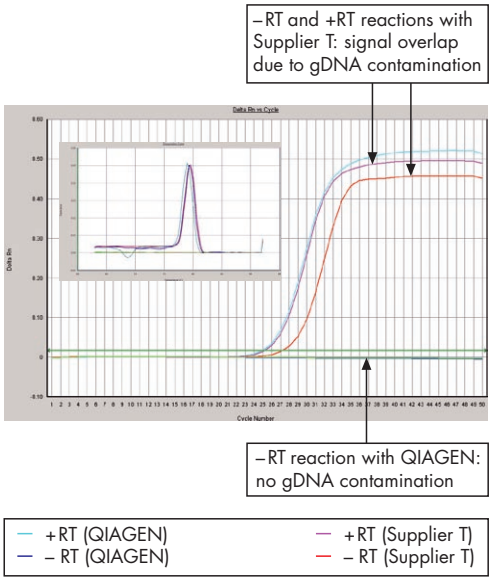

Figure 2. Effective removal of genomic DNA using gDNA Wipeout Buffer. Total RNA was purified from HeLa cells using the RNeasy® Mini Kit. cDNA was then synthesized from 1 µg RNA using either the QuantiTect Reverse Transcription Kit (with supplied RT primer mix) or a kit from Supplier T (with mix of oligo-dT and random primers). Reactions were carried out either with (+RT) or without (–RT) reverse transcriptase. Samples equivalent to 16 ng cDNA were analyzed on the Applied Biosystems 7500 Real-Time PCR System using the QuantiFast SYBR Green PCR Kit and a primer pair specific for the cDNA and genomic DNA sequences of E2F3 (E2F transcription factor 3). Melting curve analysis was also carried out (see inset): all reactions provided a single peak, demonstrating specific amplification of the intended amplicon.

Effective removal of genomic DNA contamination

Contamination of RNA samples with trace amounts of genomic DNA can interfere with real-time RT-PCR quantification if the PCR primers used are also able to amplify genomic DNA sequences. To overcome this problem, the QuantiTect Reverse Transcription Kit integrates cDNA synthesis with removal of genomic DNA (Table 2). RNA samples are briefly treated with gDNA Wipeout Buffer to eliminate genomic DNA contamination prior to efficient reverse transcription with the optimized RT primer mix, Quantiscript Reverse Transcriptase, and Quantiscript RT Buffer.

Table 2. Components of the QuantiTect Reverse Transcription Kit

Component	Advantages
gDNA Wipeout Buffer	Detection of cDNA sequences only in real-time RT-PCR
Quantiscript Reverse Transcriptase	Use of a wide range of RNA amounts (10 pg to 1 µg RNA) High sensitivity
Quantiscript RT Buffer	Read-through of difficult templates
RT Primer Mix	cDNA synthesis from all regions of transcripts, even from 5' regions

To demonstrate the effectiveness of gDNA Wipeout Buffer, the QuantiTect Reverse Transcription Kit was compared with a reverse-transcription kit from Supplier T that does not remove genomic DNA contamination. Using either kit in combination with the QuantiFast SYBR Green PCR Kit, real-time two-step RT-PCR was carried out to analyze the expression of E2F3 in HeLa cells. The primer pair used was designed to amplify both the cDNA and genomic DNA sequences of E2F3.

When the kit from Supplier T was used for the RT step, a sigmoidal amplification plot (Figure 2, red) was observed with the –RT control (i.e., control reaction without reverse transcriptase). This indicates that there was amplification of genomic DNA. The small difference of 2 cycles between the amplification plots for the –RT control and the +RT reaction (i.e., reaction with reverse transcriptase; see Figure 2, pink) suggests that 25% of the signal for the +RT reaction was due to amplification of contaminating genomic DNA.

When the RT step was performed using the QuantiTect Reverse Transcription Kit, the +RT reaction gave a sigmoidal amplification plot (Figure 2, light blue) as expected, demonstrating detection of cDNA sequence. The –RT control produced a flat amplification plot (Figure 2, blue), demonstrating that there was no amplification of genomic DNA. Therefore, gDNA Wipeout Buffer had effectively eliminated genomic DNA contamination.

High stability of gDNA Wipeout Buffer

As effective elimination of genomic DNA contamination is critical, it is important to use a robust reagent for DNA removal. gDNA Wipeout Buffer can be stored without risk of significant degradation and without the need for aliquoting. To demonstrate that the performance of the buffer is not impaired after long-term storage or after multiple freeze–thaw cycles, real-time two-step RT-PCR was carried out using gDNA Wipeout Buffer that had been stored under different conditions. C_T values for the –RT control were similar regardless of whether the gDNA Wipeout Buffer used was fresh, over 18 months old, or freeze-thawed 45 times (Figures 3 and 4).

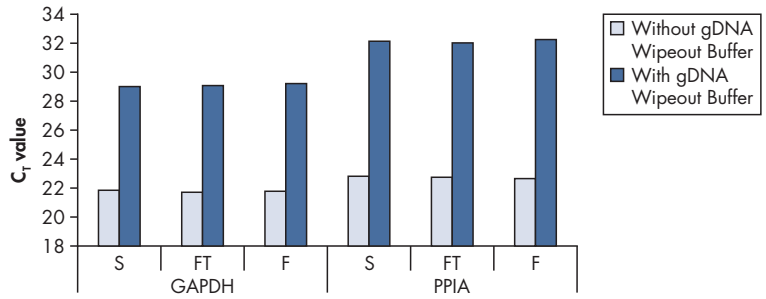

Figure 3. Stability of gDNA Wipeout Buffer after long-term storage and after multiple freeze–thaw cycles. Total RNA was purified from HeLa cells using the RNeasy Mini Kit and spiked with an equal amount of genomic DNA. Reverse-transcription reactions without reverse transcriptase were then carried out using the QuantiTect Reverse Transcription Kit. Genomic DNA was removed using gDNA Wipeout Buffer that was stored for over 18 months (**S**), frozen and thawed 45 times (**FT**), or fresh (**F**). Reactions were analyzed on the Applied Biosystems 7900 Real-Time PCR System using the QuantiFast SYBR Green PCR Kit and a primer pair specific for the cDNA and genomic DNA sequences of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or PPIA (peptidylprolyl isomerase A).

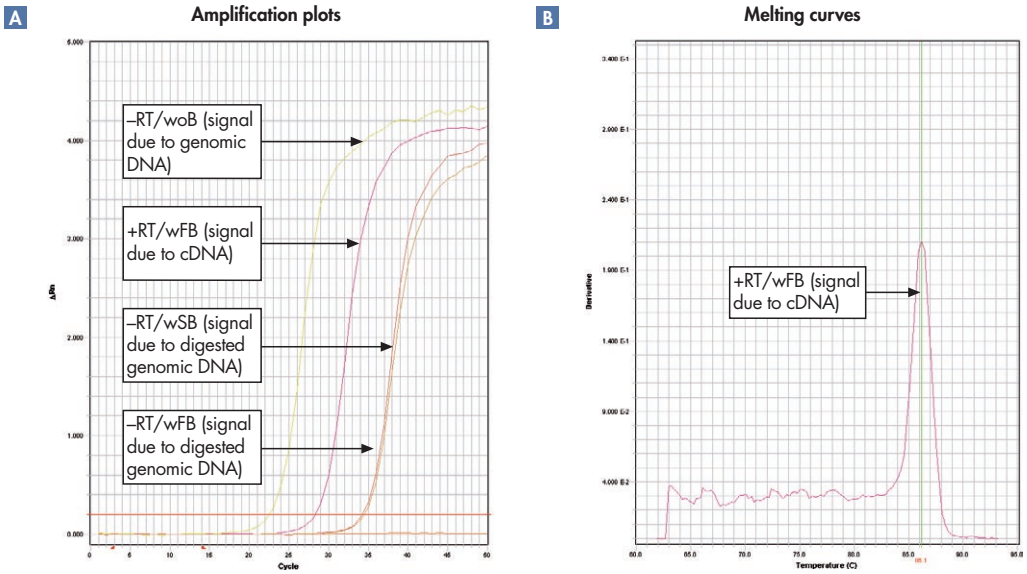

Figure 4. Stability of gDNA Wipeout Buffer after 18 months of storage. Total RNA was purified from HeLa cells using the RNeasy Mini Kit and spiked with an equal amount of genomic DNA. cDNA was then synthesized using the QuantiTect Reverse Transcription Kit. Synthesized cDNA was analyzed on the Applied Biosystems 7900 Real-Time PCR System using the QuantiFast SYBR Green PCR Kit and a primer pair specific for the cDNA and genomic DNA sequences of PPIA (peptidylprolyl isomerase A). **–RT/woB**: –RT control reaction without gDNA Wipeout Buffer; **+RT/wFB**: +RT reaction with fresh gDNA Wipeout Buffer; **–RT/wSB**: –RT control reaction with 18-month-old gDNA Wipeout Buffer; **–RT/wFB**: –RT control reaction with fresh gDNA Wipeout Buffer.

Conclusions

- The RT primer mix supplied with the QuantiTect Reverse Transcription Kit contains a unique blend of oligo-dT and random primers that delivers greater sensitivity in real-time two-step RT-PCR, regardless of the position of the amplicon within the transcript sequence.
- The QuantiTect Reverse Transcription Kit includes gDNA Wipeout Buffer, which provides effective removal of genomic DNA contamination in RNA samples.
- gDNA Wipeout Buffer remains stable after long-term storage and multiple freeze–thaw cycles, and does not need to be aliquoted prior to storage.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Reverse Transcription Kit (10)	Trial kit for 10 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205310
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311
QuantiTect Reverse Transcription Kit (200)	For 200 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205313
QuantiFast SYBR Green PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast SYBR Green PCR Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204054
QuantiTect Primer Assay (200)	For 200 x 50 µl reactions or 400 x 25 µl reactions: 10x QuantiTect Primer Assay (lyophilized) supplied in single tube	Varies†
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74104

* Larger kit size also available; please visit www.qiagen.com.

† Order assays at www.qiagen.com/GeneGlobe. Assays also available in 96- and 384-well plates.

Visit www.qiagen.com/goto/QuantiTectRT to find out more about the QuantiTect Reverse Transcription Kit

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