Technical Information

Seven challenges to successful one-step RT-PCR

One-step RT-PCR is highly suited to gene expression studies because it enables the rapid analysis of messenger RNA (mRNA) in small amounts. At its core, the technique involves three phases. RNA is isolated from cell or tissue samples. Then, the target RNA is reverse transcribed to complementary DNA (cDNA) using the enzyme reverse transcriptase and specific primers. The resulting cDNA is then amplified via conventional PCR techniques using a heat-stable DNA polymerase. Reverse transcription and PCR take place in the same tube, which reduces handling, minimizes contamination risk and facilitates automation.

However, one-step RT-PCR is not without its challenges. To help researchers overcome these challenges, we developed the QIAGEN OneStep *Ahead* RT-PCR Kit. It has the fastest cycling protocol on the market, enabling the full reaction to be run in just one hour, and it addresses the seven common challenges to successful one-step RT-PCR.

Challenge one: RNA with secondary structures or high GC contents

If the RNA in the sample material has complex secondary structures, the reverse transcriptase may stop or dissociate from the RNA template. The resulting truncated cDNA is then not amplified during PCR as it is missing the downstream primer-binding site. The reverse transcriptase can also skip over looped-out regions of RNA, which are then excluded from the synthesized cDNA.

If the RNA has a high GC content, the tight association of RNA–DNA hybrids can interfere with primer binding and prevent DNA polymerases from progressing. In the PCR step, the resulting cDNA with internal deletions is amplified, appearing as shortened PCR products.

The QIAGEN OneStep *Ahead* RT-PCR Kit combines Omniscript® and Sensiscript® in the unique Q-Solution reverse transcriptases to ensure high affinity for any RNA template, even one with secondary structures and high GC contents. Q-Solution facilitates reverse transcription and amplification of such templates to generate complete cDNA (Figure 1).

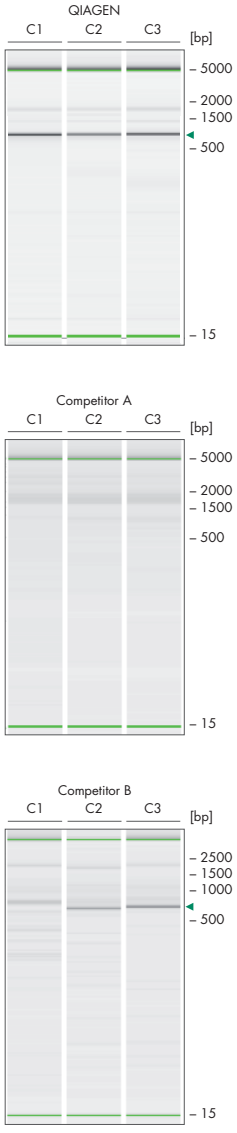

Figure 1. The QIAGEN OneStep *Ahead* RT-PCR Kit reliably transcribes RNA to cDNA. HeLa total RNA (100 ng) was used as the template for amplification of TNFRI (581 bp) with a GC ratio of 67.1%. Reactions were performed in triplicate, according to the suppliers' instructions. The green arrows indicate the specific product.

Challenge two: Nonspecific amplification during the reaction setup

Selecting appropriate primers is essential in RT-PCR. Nonspecific priming reduces the sensitivity of the process, leading to reduced yields of specific products or failure of the RT-PCR. It is common for nonspecific binding to occur when the amplification reaction is set up at room temperature. Primers can bind to each other, forming primer–dimers. During amplification cycles, primer–dimers can be extended to produce nonspecific products. The strict setup temperature requirement complicates protocols and hinders the possibility of high-throughput setup for automation.

The QIAGEN OneStep *Ahead* RT-PCR kit uses hot-start RT-enzymes that are completely inactive at ambient temperatures. This enables convenient room temperature setup and even allows the reactions to be kept at room temperature for a certain time before cycling (Figure 2). Not only does this reduce nonspecific priming, it also facilitates high-throughput automated workflows.

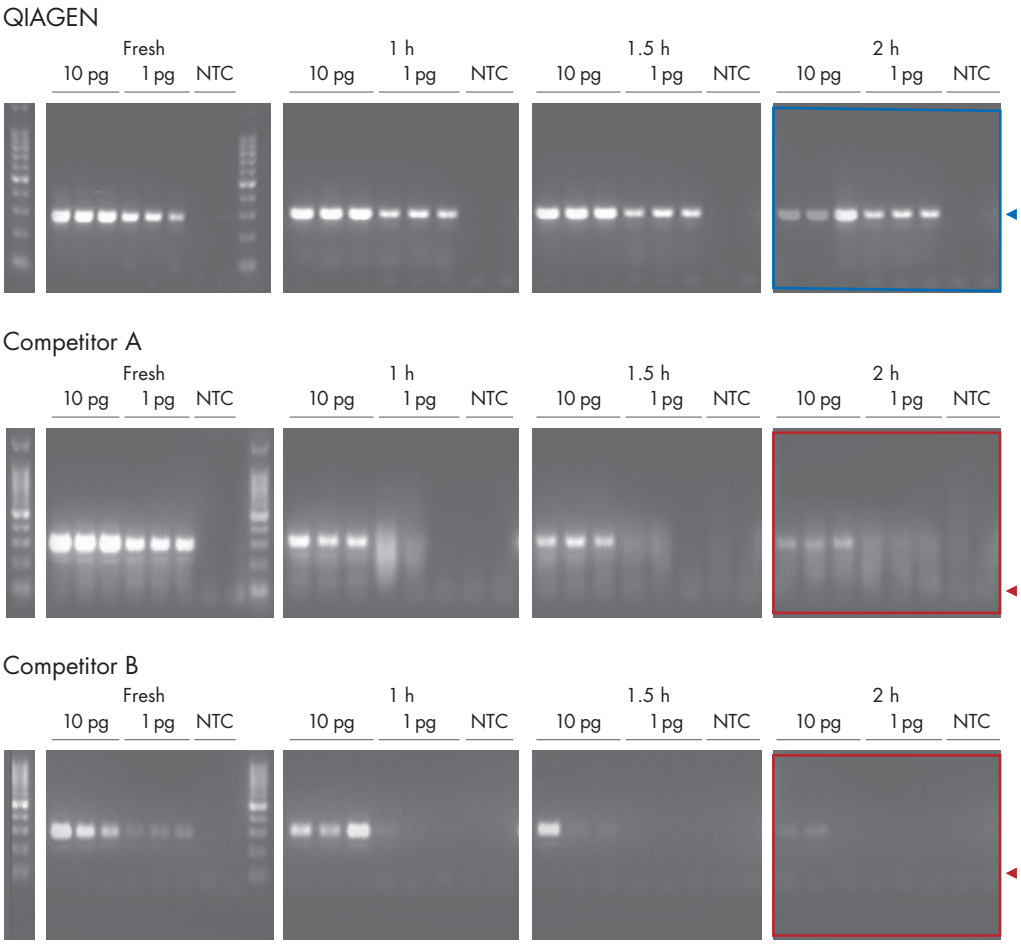

Figure 2. RT-PCR setup with the QIAGEN OneStep *Ahead* RT-PCR Kit is stable at room temperature. HeLa total RNA (10 and 1 pg) was used as a template for amplification of ACTB in triplicate. Reactions were either set up on ice or left at room temperature for the times indicated before analysis. With the QIAGEN kit, distinct, gene-specific bands are observed even after 2 h incubation at room temperature (blue arrow).

Challenge three: The influence of annealing temperature on sensitivity

Nonspecific priming can also occur when the annealing temperature is not optimized. The single-stranded cDNA produced by reverse transcription is more susceptible to nonspecific primer annealing at lower temperatures than double-stranded genomic DNA. Nonspecific amplification competes with specific amplification and may drastically reduce yields.

The QIAGEN OneStep *Ahead* RT-PCR Kit has a master mix that includes a dual-cation PCR buffer (Figure 3). This helps to maintain high primer annealing specificity over a broad range of temperatures, eliminating the need for optimization for each individual primer–template system. It also allows the use of assays with different primer annealing temperatures.

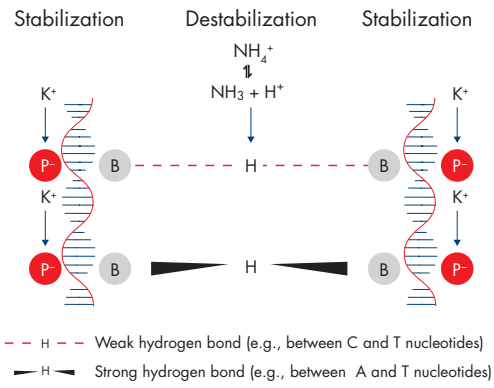

Figure 3. The QIAGEN OneStep *Ahead* RT-PCR Kit dual-cation PCR buffer. The buffer contains both K^+ and $NH4^+$ to ensure high yields of specific PCR products over a wide range of annealing temperatures. It destabilizes nonspecifically bound primers, providing a more robust reaction environment.

Challenge four: Pipetting errors

When pipetting colorless solutions on a large scale, e.g., into a 96-well plate, it can be very difficult to keep track of the solutions already added to each well. This kit offers a simple yet very effective solution: a visual pipetting control, consisting of an inert yellow dye in the master mix and a blue one to add to the template. When the template is pipetted into the master mix, the solution turns to green. The dyes also serve as gel tracking dyes during electrophoresis.

Challenge five: RNase contamination

RNA-cleaving RNase is widely dispersed in our environment. It is even present in large quantities on human skin. Therefore, even when considerable care is taken, RNase contamination of sample material can easily occur and potentially destroy the RNA in your sample. The QIAGEN OneStep *Ahead* RT-PCR Kit includes an RNase inhibitor that prevents the RNA decay caused by accidental RNase contamination.

Challenge six: PCR-borne mutations

PCR amplification using normal Taq DNA polymerase is prone to a certain error rate in the DNA replication process. The typical point mutation rate is 1 in 9,000. Sequence accuracy is essential to gain insight into the biology of the sample. By adding an extra high-fidelity enzyme with 3' to 5' exonuclease proofreading activity, we've elevated the overall fidelity of the PCR step and improved the processivity, thereby allowing for the amplification of longer targets (up to 4 kb).

Challenge seven: False negative results

The absence of a band on your gel could mean the absence of the RNA target sequence – but it could also mean that something failed during PCR. To know with confidence whether the target RNA is absent or the PCR needs to be repeated, a positive control is needed for each experiment. For absolute confidence, a positive control would be needed in each single reaction.

To ensure that you can interpret results with confidence, the QIAGEN OneStep *Ahead* RT-PCR Kit is optimized for duplex PCR and it co-amplifies an internal positive control with every single reaction.

Summary

The new QIAGEN OneStep *Ahead* RT-PCR Kit provides a convenient format for highly sensitive and specific RT-PCR. The kit contains optimized components that allow both reverse transcription and PCR amplification to take place in a single tube. The unique enzyme combination and specially developed reaction buffer ensure efficient, highly specific reactions without extra optimization. Additionally, the kit is offers excellent convenience to the user, with room temperature setup and a visual pipetting control. Finally, it has the fastest cycling protocol on the market – you can run the entire one step RT-PCR in just one hour.

Ordering Information

Product	Contents	Cat. no.
QIAGEN OneStep *Ahead* RT-PCR Kit (50)	6 vials for 50 reactions: 1 x 500 µl OneStep Ahead RT-PCR Master Mix, 1 x 50 µl OneStep Ahead RT Mix, 1 x 200 µl Template Tracer, 1 x 50 µl Master Mix Tracer, 1 x 1.9 ml water, 1 x 400 µl Q-Solution	220211
QIAGEN OneStep *Ahead* RT-PCR Kit (200)	8 vials for 200 reactions: 2 x 1 ml OneStep Ahead RT-PCR Master Mix, 1 x 200 µl OneStep Ahead RT Mix, 1 x 200 µl Template Tracer, 1 x 50 µl Master Mix Tracer, 2 x 1.9 ml water, 1 x 2 ml Q-Solution	220213
QIAGEN OneStep *Ahead* RT-PCR Kit (2000)	75 vials for 2000 x 25 µl reactions: 20 x 1 ml OneStep Ahead RT-PCR Master Mix, 10 x 200 µl OneStep Ahead RT Mix, 10 x 200 µl Template Tracer, 10 x 50 µl Master Mix Tracer, 20 x 1.9 ml water, 5 x 2 ml Q-Solution	220216

To find out more kits features and how it can help you with gene expression and virus detection studies, visit **qiagen.com/OneStepAhead**

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

www.qiagen.com