

Eight 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 eight common challenges to successful one-step RT-PCR.

Challenge one: The influence of annealing temperature on sensitivity

Nonspecific priming can 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 1). 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.

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. Reverse transcriptase has residual activity at room temperature, meaning that primers, which bind to each other during reaction setup, can be extended. The resulting primer–dimers are preferentially amplified in the subsequent PCR, reducing the specificity and sensitivity of the reaction. Therefore, most kits require that setup be performed on ice, which 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 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.

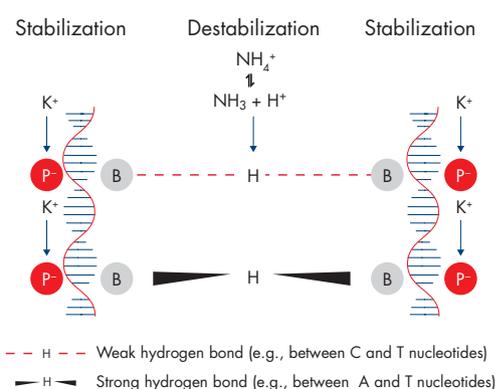


Figure 1. The QIAGEN OneStep *Ahead* RT-PCR Kit dual-cation PCR buffer. The buffer contains both K^+ and NH_4^+ to ensure high yields of specific PCR products over a wide range of annealing temperatures. It destabilizes nonspecifically bound primers, providing a more robust reaction environment.

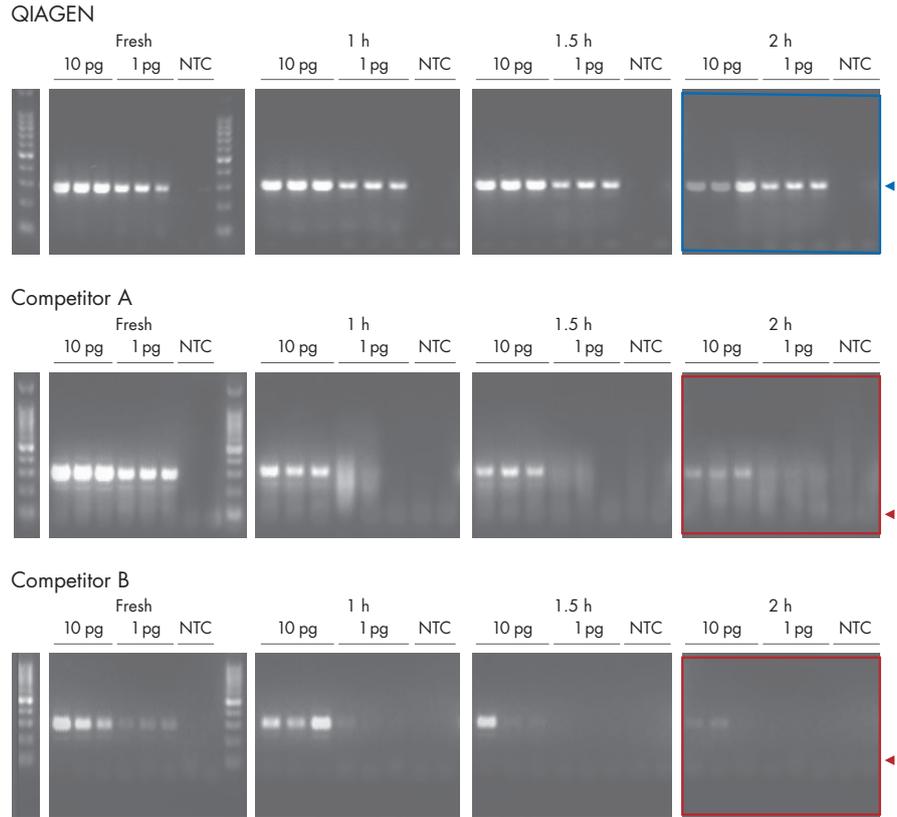


Figure 2. RT-PCR setup with the QIAGEN OneStep Ahead RT-PCR Kit is stable at room temperature. HeLa total RNA (10 and 1 pg) was used as a template for amplification of ACTB in triplicate. Reactions were either set up on ice or left at room temperature for the times indicated before analysis. With the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

Challenge three: 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 four: RNA with secondary structures or high GC content

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, resulting in the amplification of RT-PCR products with internal deletions.

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 reduced amount of cDNA results in lower sensitivity.

The QIAGEN OneStep Ahead RT-PCR Kit combines Omniscript® and Sensiscript® to ensure high affinity for any RNA template. In addition, a unique Q-Solution is provided as an optional additive that can be used for GC-rich amplicons (Figure 3).

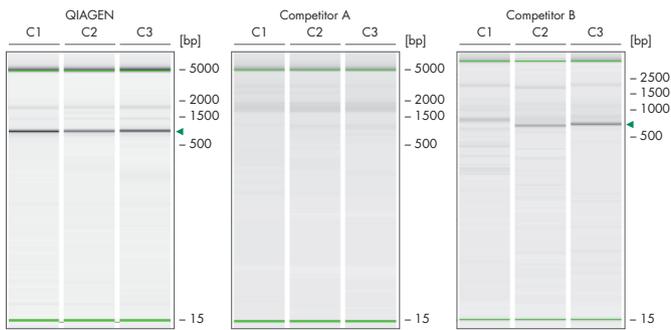


Figure 3. The QIAGEN OneStep Ahead RT-PCR Kit includes unique Q-Solution that reliably transcribes GC-rich RNA to cDNA. HeLa total RNA (100 ng) was used as the template for amplification of TNFRI (581 bp) with a GC ratio of 67.1%. Reactions were performed in triplicate, according to the suppliers' instructions. The green arrows indicate the specific product.

Challenge five: 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 six: 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 that can be added to the master mix and a blue one to add to the template. When the template is pipetted into the master mix, the solution turns green. The dyes also serve as gel-tracking dyes during electrophoresis, eliminating the need to add gel-loading or tracking dye after cycling.



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 certainty, a positive control would be needed in each single reaction. The QIAGEN OneStep Ahead RT-PCR Kit is optimized for duplex PCR and is able to co-amplify an internal positive control with every single reaction.

Challenge eight: Target RNA detection threshold

Low abundance mRNA can be difficult to detect. Some sample material may only contain minute amounts of the target RNA. In such cases, the chemistry of the RT-PCR reagents must be optimized for extremely high sensitivity – even to the picogram level. We have optimized the chemistry of the QIAGEN OneStep Ahead RT-PCR Kit solution to detect RNA contents as low as 1 pg. In addition, the 2.5x master mix allows for the input of more sample RNA.

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 offers excellent convenience to the user, with setup at room temperature 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 <i>Ahead</i> RT-PCR Kit (50)	6 vials for 50 reactions: 1 x 500 µl OneStep <i>Ahead</i> RT-PCR Master Mix, 1 x 50 µl OneStep <i>Ahead</i> 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 <i>Ahead</i> RT-PCR Kit (200)	8 vials for 200 reactions: 2 x 1 ml OneStep <i>Ahead</i> RT-PCR Master Mix, 1 x 200 µl OneStep <i>Ahead</i> 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 <i>Ahead</i> RT-PCR Kit (2000)	75 vials for 2000 x 25 µl reactions: 20 x 1 ml OneStep <i>Ahead</i> RT-PCR Master Mix, 10 x 200 µl OneStep <i>Ahead</i> 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

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