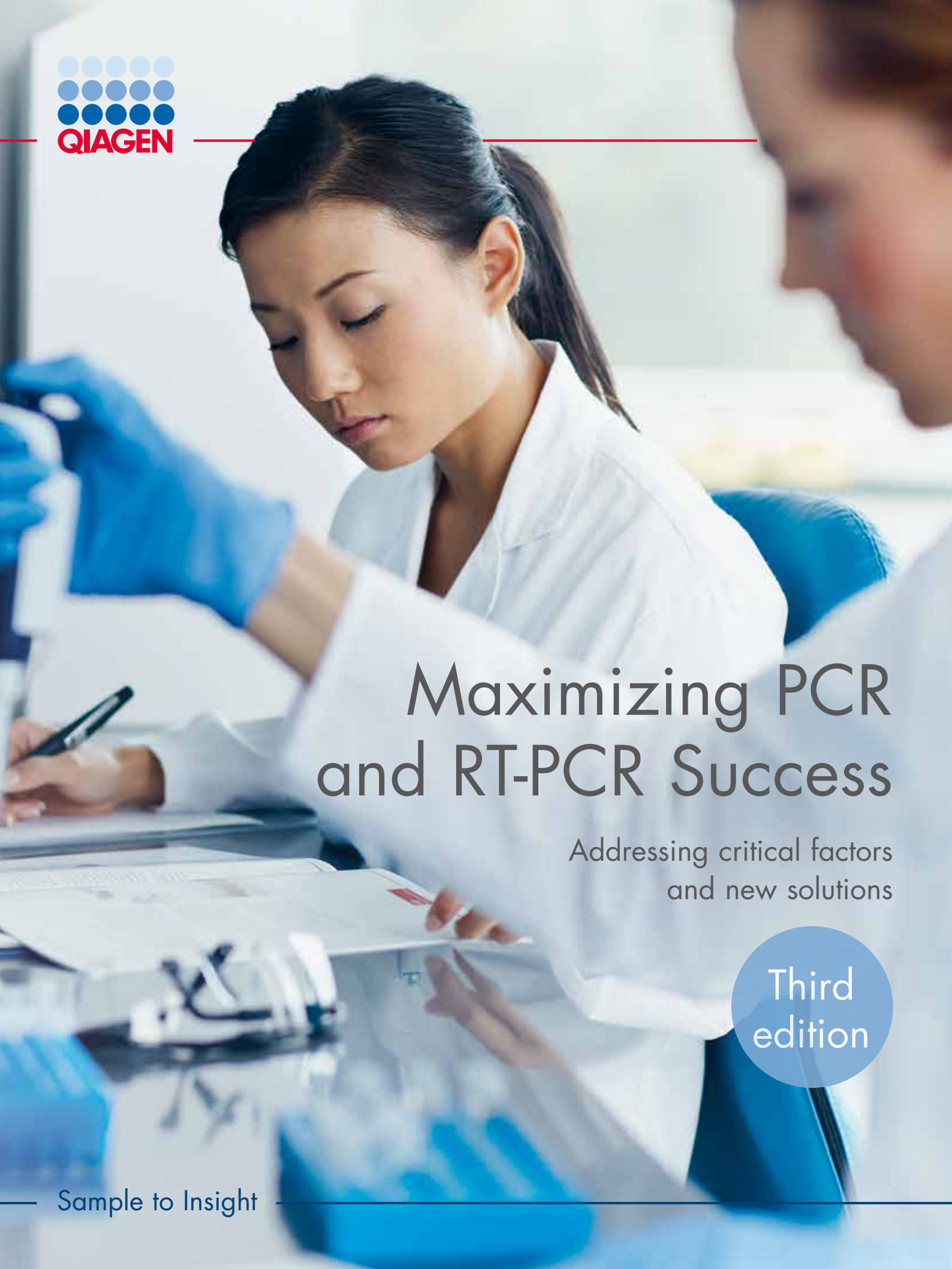

Maximizing PCR and RT-PCR Success

Addressing critical factors and new solutions

Third edition

Sample to Insight

Introduction

The invention of the polymerase chain reaction (PCR) by K. Mullis and coworkers in 1985 revolutionized molecular biology and molecular medicine. Major research areas, such as biomarker discovery, gene regulation and cancer research (see Figure 1) are challenging today's PCR technologies with more demanding requirements. These include the need for increased throughput while reducing costs, higher assay sensitivity and reliable data normalization. Assay development and evaluation, reproducibility of data and time to result are still major problems encountered by researchers.

Meeting today's challenges in PCR requires advances in all methods of the workflow that starts with sample collection, sample stabilization, and nucleic acid purification, and ends with amplification and detection. The following pages focus on the importance of amplification in meeting these challenges.

<table>
<tr><td></td><td colspan="3">Research areas</td><td colspan="3">Applications</td></tr>
<tr><td></td><td colspan="3">Biomarker discovery
Pathogen identification
Gene regulation research
Cell development research
Cancer research
Drug validation/applied biomarkers</td><td colspan="3">Gene silencing/siRNA/miRNA)
Epigenetics
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Food testing
Blood testing
Vaccination</td></tr>
<tr><td>Challenges</td><td>Sample quality and amount</td><td>Costs</td><td>Normalization</td><td>Sensitivity</td><td>Reliability of results</td><td>Time to result</td></tr>
<tr><td>Methods</td><td colspan="2">Sample collection and stabilization</td><td>Nucleic acid purification</td><td colspan="2">Amplification</td><td>Detection</td></tr>
</table>

Figure 1. Overview of research areas and associated challenges.

Critical factors influencing PCR success

Despite the fact that PCR amplification is performed routinely and that thousands of PCR protocols have been developed, researchers still encounter technical difficulties with PCR experiments and often fail to obtain specific amplification products. Although there are several different challenges (e.g., smearing, low yield, and nonspecific amplification), there are only two main reasons for PCR failure or poor results. These are the specificity of the reaction and template secondary structure.

This brochure presents a discussion of the factors that influence PCR and RT-PCR specificity and template denaturation – enabling successful results.

The factors discussed are:

- Choice of enzyme (page 3)
- Reaction conditions (i.e., choice of buffer, annealing temperature, etc) (page 7)
- Automatability of PCR (page 9)
- Ease of use (page 12)
- Primer design (page 14)
- Template quality (page 15)
- Challenging applications and new technologies (page 15)

Enzymes

Taq DNA polymerase

Several types of thermostable DNA polymerases are available for use in PCR, providing a choice of enzymatic properties (Table 1).

Taq DNA polymerase, isolated from the eubacterium *Thermus aquaticus*, is the most commonly used enzyme for standard end-point PCR. The robustness of this enzyme allows its use in many different PCR assays. However, as this enzyme is active at room temperature, it is necessary to perform reaction setup on ice to avoid nonspecific amplification. QIAGEN has overcome this limitation with the introduction of the novel TopTaq® DNA Polymerase. This innovative non-hot-start enzyme has limited access to primer and template at room temperature, allowing immediate reaction setup without the use of ice.

A number of modifications of the original "PCR polymerase" – *Taq* DNA polymerase – are now available for different downstream application needs, such as hot-start, single-cell, or multiplex PCR (see page 16). With an average error rate of 1 in 10,000 nucleotides, *Taq* DNA polymerase and its variants are less accurate than the thermostable enzymes of DNA polymerase family B. However, due to its versatility, *Taq* DNA polymerase is still the enzyme of choice for most routine applications and when used with a stringent hot-start, is suitable for several challenging PCR applications (Table 4, page 16).

Table 1. DNA polymerases used in PCR*

	DNA polymerase family A	DNA polymerase family B
Enzymes available	*Taq* DNA Polymerases,† Hot-start DNA polymerases‡	Proofreading enzymes§
5'–3' exonuclease activity	+	–
3'–5' exonuclease activity	–	+
Extension rate (nucleotides/second)	~ 150	~ 25
Error rate (per bp/per cycle)	1 in 10^3/10^4	1 in 10^5/10^6
PCR applications	Standard, hot-start, reverse transcription, real-time	High fidelity, cloning, site-directed mutagenesis
A-addition	+	Sometimes§

* Review article: Ishino, S. and Ishino, Y. (2014) DNA polymerases as useful reagents for biotechnology – the history of developmental research in the field. Front Microbiol. **5**, 465.

† For example, TopTaq DNA Polymerase.

‡ For example, HotStarTaq *Plus* DNA Polymerase.

§ HotStar HiFidelity DNA Polymerase provides A-addition action for easy TA/UA-cloning

Hot-start PCR polymerase

When amplification reaction setup is performed at room temperature, primers can bind nonspecifically to each other, forming primer–dimers. During amplification cycles, primer–dimers can be extended to produce nonspecific products, which reduces specific product yield. For more challenging PCR applications, the use of hot-start PCR is crucial for successful specific results. To produce hot-start DNA polymerases, *Taq* DNA polymerase activity can be inhibited at lower temperatures with antibodies or, even more effectively, with chemical modifiers that form covalent bonds with amino acids in the polymerase. The chemical modification leads to complete inactivation of the polymerase until the covalent bonds are broken during the initial heat activation step.

The unique hot-start procedure, based on chemical modification, provided with QIAGEN® hot-start enzymes is easily incorporated into any PCR program using a simple 5-minute (HotStarTaq® *Plus* DNA Polymerase) or 15-minute (HotStarTaq DNA Polymerase) initial denaturation step (Figure 2, next page).

High-fidelity DNA polymerase

Unlike standard DNA polymerases (such as *Taq* DNA polymerase), high-fidelity PCR enzymes generally provide a 3' –5' exonuclease activity for removing incorrectly incorporated bases. High-fidelity PCR enzymes are ideally suited to applications requiring a low error rate, such as cloning, sequencing, and site-directed mutagenesis. However, the 3' –5' exonuclease activity can degrade primers during PCR setup and the early stages of PCR. Nonspecific priming caused by shortened primers can result in smearing or amplification failure — especially when using low amounts of template (Figures 3 and 4). It should be noted that the proofreading function often causes high-fidelity enzymes to work more slowly than other DNA polymerases. In addition, the A-addition function required for direct UA- or TA-cloning is strongly reduced, resulting in the need for blunt-end cloning with lower ligation and transformation efficiency. These limitations have been overcome with HotStar HiFidelity DNA Polymerase, which incorporates a hot-start activation to its exonuclease activity, providing reliable and sensitive results, in contrast to enzymes from other suppliers (Figures 3 and 4). In addition, this enzyme also adds an A overhang during the final extension step allowing direct UA- or TA-cloning.

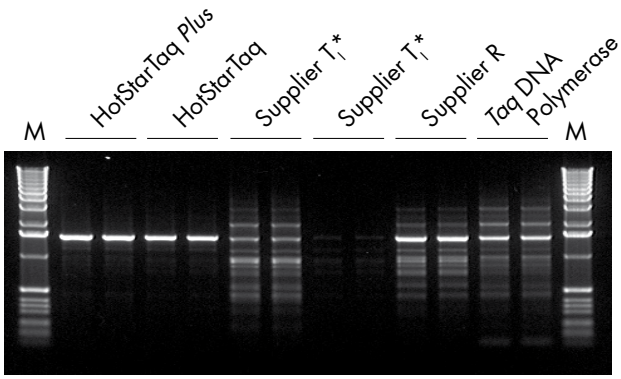

Figure 2. Highest specificity with HotStarTaq *Plus* Polymerase. PCR was carried out using QIAGEN HotStarTaq *Plus*, HotStarTaq and *Taq* DNA Polymerases and three hot-start PCR enzymes from the indicated suppliers. Parallel reactions were performed following the suppliers' recommendations, using 50 ng human genomic DNA. A 1.5 kb fragment of the human CFTR gene was amplified in 35 PCR cycles. **M**: markers.

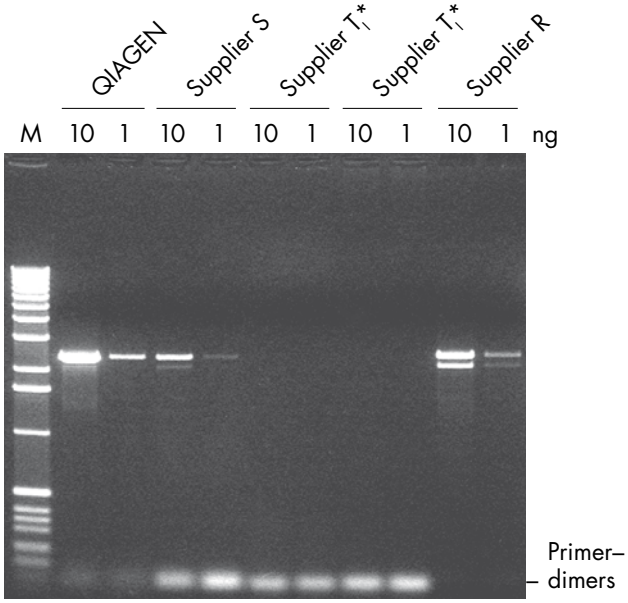

Figure 3. The importance of optimized high-fidelity systems for sensitive and the reliable PCR. High-fidelity PCR was performed using HotStar HiFidelity DNA Polymerase Kit (QIAGEN) and the four high-fidelity PCR enzymes from the indicated suppliers. A 2.3 kb fragment of the human IL9R gene was amplified from the indicated amounts of genomic DNA in 40 PCR cycles. **M**: marker. The HotStar HiFidelity DNA Polymerase Kit provided highly sensitive results using 1 ng template.

* Two different enzymes from Supplier T_i.

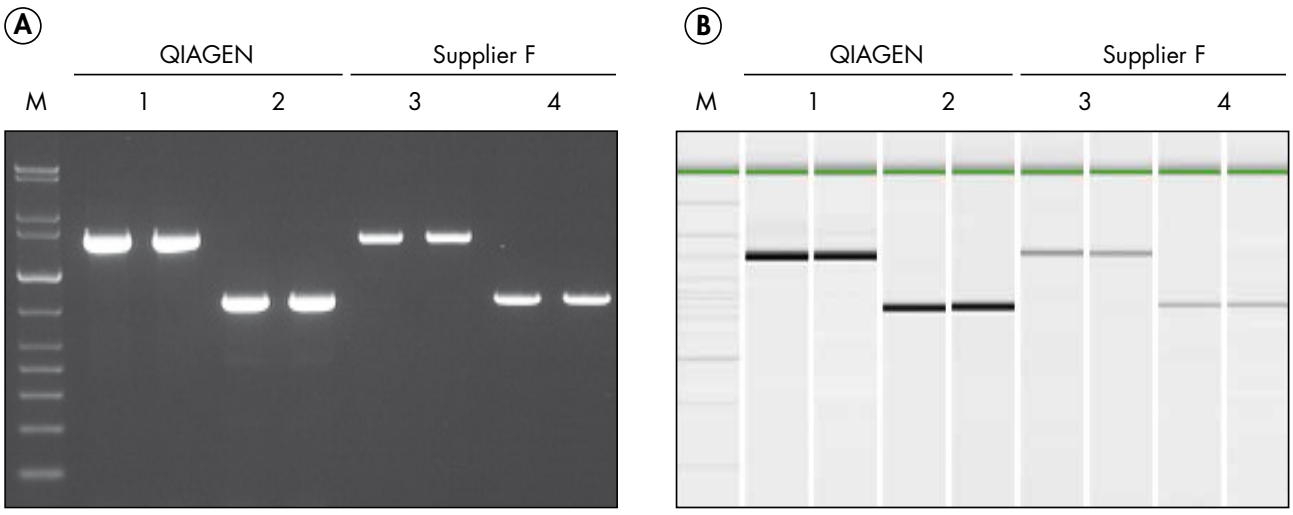

Figure 4. Highly reliable and sensitive PCR with QIAGEN's HotStar HiFidelity DNA Polymerase Kit. PCR was performed using the HotStar HiFidelity DNA Polymerase Kit and a high-fidelity DNA polymerase from Supplier F and analyzed by **A** agarose gel electrophoresis and by **B** the QIAxcel® Advanced System. Amplicons of 1.5 kb (lanes 1 and 3) and 750 bp (lanes 2 and 4) were generated using 100 ng human genomic DNA as a template. HotStar HiFidelity DNA Polymerase provided higher yields and more sensitive results compared with the polymerase from Supplier F.

Reverse transcriptases

RT-PCR allows the analysis of RNA using a combination of reverse transcription and PCR. cDNA is synthesized from RNA templates using reverse transcriptases — RNA-dependent DNA polymerases 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.

The single-stranded cDNA produced by reverse transcription is more susceptible to nonspecific primer annealing at lower temperatures than double-stranded DNA (e.g., genomic DNA). Nonspecific annealing can result in poor amplification specificity which, especially when combined with limiting cDNA quantity or low transcript abundance, leads to reduced sensitivity and poor reproducibility.

Amplification specificity is crucial for successful RT-PCR and is best achieved by combining innovative buffer solutions with specially modified reverse transcriptases and hot-start PCR.

The use of optimized reverse transcription buffers and specially developed reverse transcriptases (such as Omniscript® and Sensiscript® contained in the QIAGEN OneStep RT-PCR Kit and QIAGEN OneStep *Ahead* RT-PCR Kit) can resolve secondary structures that commonly occur with single-stranded RNA molecules.

To further increase specificity and enable room-temperature setup, the QIAGEN OneStep *Ahead* RT-PCR Kit includes an RT-blocker that keeps the reverse transcriptase inactive at ambient temperatures; therefore preventing it from nonspecific amplification of primer–dimers. When the reaction is heated to the catalytic optimum of 50–55°C, the blocker dissociates from the RT enzyme rendering it fully active (Figure 5).

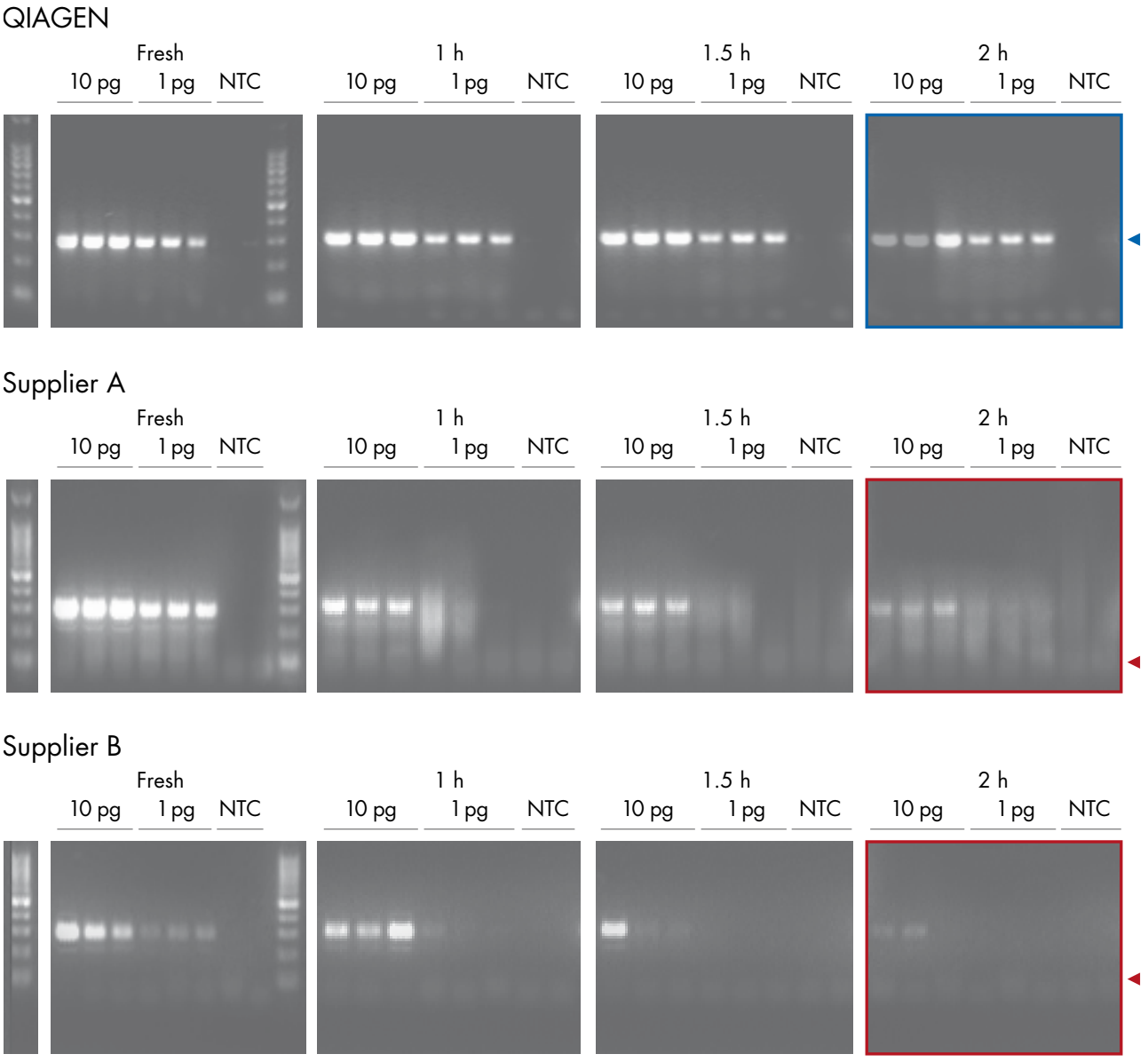

Figure 5. Superior stability after reaction setup at room temperature. HeLa total RNA (10 and 1 pg) was used as a template for amplification of ACTB in triplicates, according to suppliers' instructions. Reactions were either set up on ice or left at room temperature for the times indicated before analysis on a 2% agarose gel. Distinct, gene-specific bands are observed with the QIAGEN OneStep *Ahead* RT-PCR Kit even after a 2-hour incubation at room temperature before cycling (blue arrow), whereas reactions performed with kits from other suppliers deteriorate as time progresses. Gene-specific bands appear significantly weaker, if present at all, while primer–dimers (red arrows) become more prominent.

Reaction conditions

Primer annealing specificity and PCR buffers

In PCR, annealing occurs between the primers and complementary DNA sequences in the template. Primer annealing must be specific for successful amplification. Due to the high concentration of primers necessary for efficient hybridization during short annealing times, primers can anneal to non-complementary sequences. Amplification of products from nonspecific annealing competes with specific amplification and may drastically reduce the yield of the specific product (Figure 2, page 4).

The success of PCR largely depends on maintaining a high ratio of specific to nonspecific annealing of the primer molecules. Annealing is primarily influenced by the components of the PCR buffer (in particular the cations) and annealing temperature. Special cation combinations can maintain high primer annealing specificity over a broad range of annealing temperatures. This eliminates the need for optimization of annealing temperatures for each individual primer–template system and also allows the use of non-ideal PCR systems with different primer annealing temperatures.

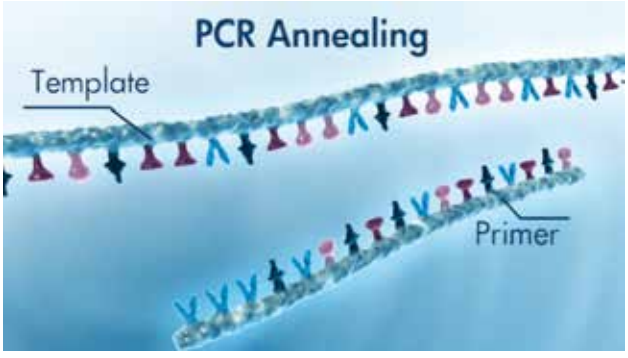

Figure 6. Animation on QIAGEN's unique PCR buffer system. Watch the video **here.**

A balanced combination of cations promotes specific primer annealing

Cations in commonly used PCR buffers bind to the negatively charged phosphate groups on the DNA backbone and thereby neutralize these negative charges. This weakens the electrorepulsive forces between the DNA template and primer molecule leading to more stable hybridization of the primer. Most commercially available PCR buffers contain only one monovalent cation, K^+, which stabilizes both specific and nonspecific primer annealing. This often results in smearing and nonspecific DNA amplification, which leads to lower product yields. QIAGEN has found that the balanced combination of K^+ and NH_4^+ used in all QIAGEN PCR buffer formulations can strongly increase primer annealing specificity.

The improved specificity is caused by ammonium ions destabilizing the weak hydrogen bonds at mismatched bases (Figures 6 and 7).

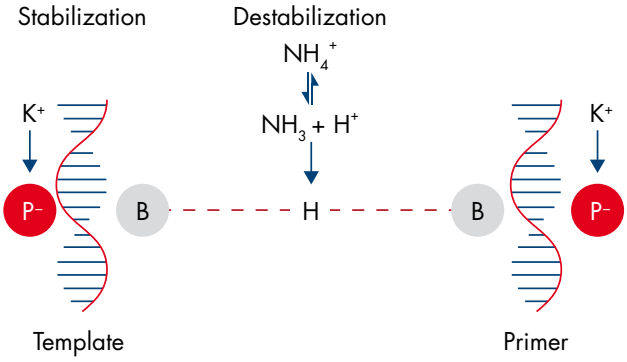

Figure 7. Effect of unique QIAGEN PCR Buffer. K^+ binds to the phosphate groups (P) on the DNA backbone, stabilizing the annealing of the primers to the template. NH_4^+, which exists both as the ammonium ion and as ammonia under thermal-cycling conditions, can interact with the hydrogen bonds between the bases (B), destabilizing principally the weak hydrogen bonds at mismatched bases. The combined effect of the two cations maintains the high ratio of specific to non-specific primer–template binding over a wide temperature range.

Annealing temperature

The optimal primer annealing temperature is dependent on the base composition (i.e., the proportion of A, T, G, and C nucleotides), primer concentration, and ionic reaction environment. Using QIAGEN PCR buffers, containing both K^+ and NH_4^+, delivers high yields of specific PCR product over a wide range of annealing temperatures. This specificity is achieved by destabilizing nonspecifically bound primers, providing a more robust reaction environment and eliminating the need for tedious annealing temperature optimization. In contrast, the range of optimal PCR annealing temperatures is smaller and less predictable when using a PCR or one-step RT-PCR buffer that only contains K^+, as illustrated in Figure 8.

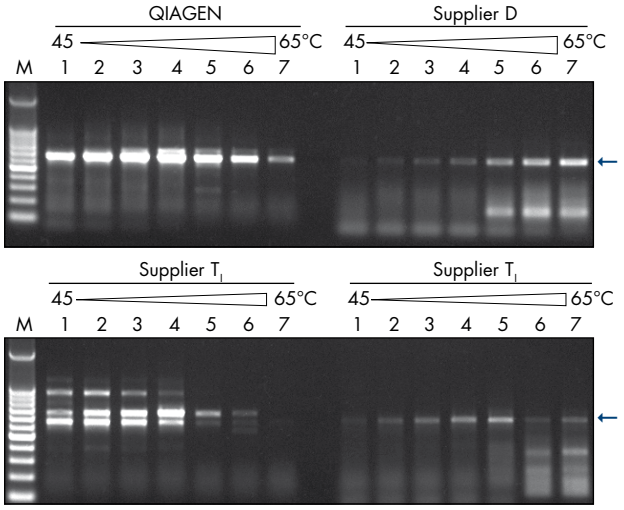

Figure 8. Influence of annealing temperature on one-step RT-PCR specificity. One-step RT-PCR was performed using kits from the indicated suppliers (two kits from supplier T_I) over a range of annealing temperatures. A 1289 bp fragment from the human RCC1 gene was reverse transcribed and amplified from Hela RNA (arrow). **M**: marker. High levels of specific amplification without optimization were observed only with the QIAGEN OneStep RT-PCR Kit.

Magnesium ion concentration

Magnesium ions are a critical DNA polymerase cofactor necessary for enzyme activity. In a manner similar to K^+ (see Figure 7), Mg^{2+} also binds to DNA, primers and nucleotides contained in the amplification reaction. The Mg^{2+} concentration is generally higher than that of dNTPs and primers, and some optimization may be necessary for different template and primer concentrations. Higher than optimal concentrations of Mg^{2+} can stabilize nonspecific binding and is often indicated by decreased yields of specific PCR products (Figure 9) and the appearance of background smear or other PCR artifacts. The destabilizing effect of NH_4^+ (provided in the QIAGEN PCR Buffer) on nonspecific primer annealing maintains the predominance of specific annealing over a range of Mg^{2+} concentrations and greatly reduces the need to optimize Mg^{2+} concentration.

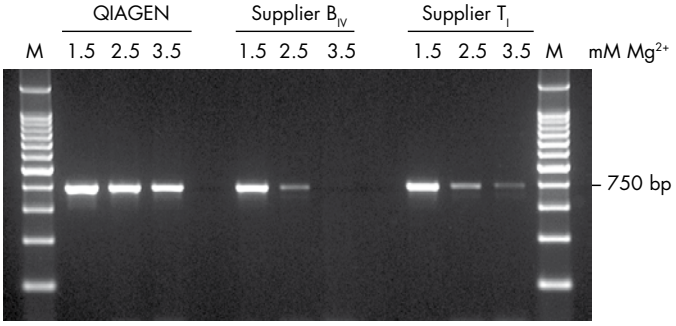

Figure 9. Influence of Mg^{2+} concentration on PCR success. A 750 bp product from the human PRP gene was amplified in 50 µl reactions using 20 ng genomic DNA from leukocytes and DNA polymerases from the indicated suppliers. PCR buffer contained 1.5, 2.5 or 3.5 mM Mg^{2+}. PCR products (5 µl) were subjected to electrophoresis on a 1.5% agarose gel. **M**: marker. High levels of specific amplification at all Mg^{2+} concentrations were observed only using QIAGEN's *Taq* DNA Polymerase and its innovative buffer system.

PCR additives

Various PCR additives or enhancers are available for improving PCR results. It is claimed that these reagents relieve secondary DNA structure (e.g., in GC-rich regions or in long amplification products), lower template melting temperature, enhance enzyme processivity, stabilize DNA polymerases, or prevent attachment of polymerases to plasticware. Commonly used PCR additives include dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and glycerol. Most QIAGEN PCR kits include the novel reagent Q-Solution® (see selection guides, pages 23–25), which changes the dynamics of the PCR reaction to increase sensitivity when amplifying GC-rich DNA and targets with strong secondary structures (Figure 10).

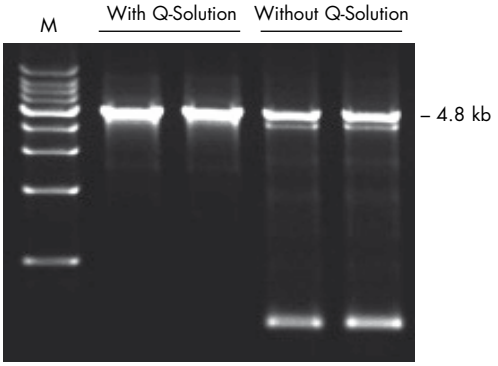

Figure 10. Influence of Q-Solution on PCR success. A 4.8 kb fragment was amplified in standard reactions using TopTaq DNA Polymerase with or without Q-Solution. **M**: marker. Specific amplification was achieved only in reactions containing Q-Solution.

Automatibility of PCR

Reproducibility and standardization in each step of a PCR experiment is the basis for successful results. Reliable tools for automated setup of reactions and sensitive platforms for fragment detection are required to achieve this.

PCR setup

Manual reaction setup is error prone due to pipetting variability, which can result from incorrect pipet calibration and/or human error. This leads to inconsistencies between different experiments and labs. Maintaining pipetting precision and ensuring reproducibility is even more difficult when pipetting into 96- or 384-well plates. The results obtained can vary from lab to lab and from researcher to researcher. Comparison of results and subsequent downstream analysis is therefore challenging. Manual pipetting of PCR reagents increases the risk of contamination with nucleases – and this is especially critical when using RNA as a template. Manual pipetting is also time consuming and tedious, and can result in repetitive strain injury.

QIAGEN's automated platform for high-precision PCR setup

To eliminate pipetting errors and to ensure consistency, speed, and reliability in your research, QIAGEN offers the QIAgility® – an automated platform for rapid, high-precision PCR setup in all formats. The innovative QIAgility can set up multiple master mixes and process multiple PCR setups in parallel, streamlining your research and increasing productivity. The risk of contamination is greatly reduced, errors associated with manual handling are minimized, and improved yields are achieved.

Analysis of PCR fragments

Post-PCR analysis and detection is commonly performed using agarose gel electrophoresis. Traditional agarose gel electrophoresis is time consuming and labor intensive, especially if there are large number of samples to be analyzed. Gel preparation also involves exposure to hazardous chemicals such as ethidium bromide. Thorough analysis of data in terms of fragment sizes and concentration is challenging, especially when data are to be compared with previously analyzed PCR products. Several factors such as the agarose quality and the percentage of agarose used affect the duration of electrophoresis and can influence results. Use of a high voltage during an electrophoretic run often results in smearing of nucleic acids, making analysis of results difficult. Standardization is of key importance when comparing data from different gel runs and this places a great emphasis on accurate electrophoresis conditions and record keeping.

QIAGEN's automated platform for effortless DNA fragment analysis

For automated, high-resolution capillary electrophoresis, QIAGEN offers the QIAxcel Advanced System. DNA fragment analysis of 12 samples can be performed in as little as 3 minutes (Figure 11). Ready-to-run gel cartridges allow 96 samples to be analyzed with a minimum of hands-on interaction, reducing manual handling errors and eliminating the need for tedious gel preparation. With a resolution of 3–5 bp for fragments smaller than 0.5 kb, the QIAxcel Advanced System ensures greater accuracy than slab-gel methods, as well as greater confidence in data interpretation. Hands-free sample loading and self-contained components minimize exposure to hazardous chemicals such as ethidium bromide.

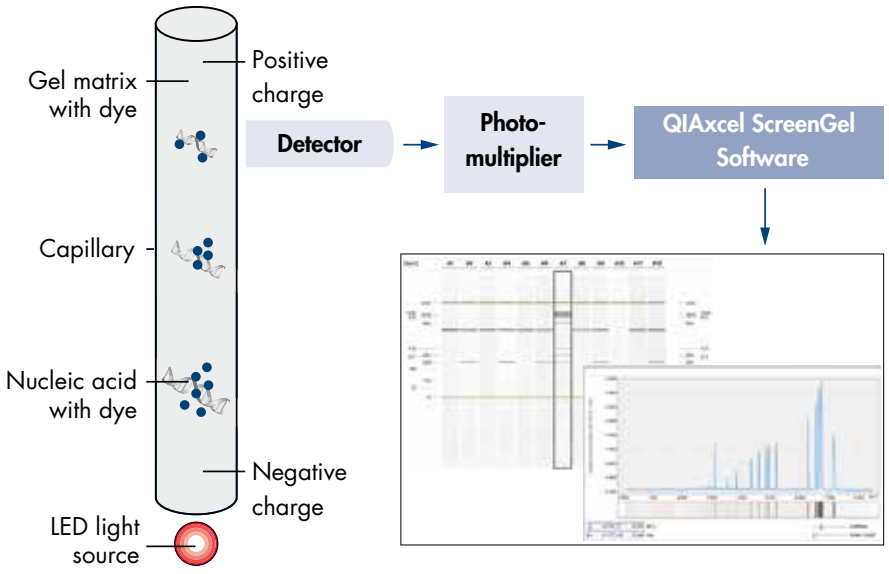

Figure 11. QIAxcel Advanced process. Nucleic acid molecules are size separated by applying a current to a gel-filled capillary, and detected as they migrate toward the positively charged terminus. The signal data pass through a photomultiplier and are converted to an electropherogram and gel image by the QIAxcel ScreenGel Software.

Automatic primary analysis

Using ScreenGel® software, the QIAxcel operating software, you can define electrophoretic patterns and their respective biological meaning within the assay. After a run, the software automatically analyses the results, searches for possible pattern matches and generates a comprehensive report to simplify data interpretation. This feature is particularly valuable when analyzing of large pools of samples.

A complete solution for your research

QIAGEN's end-point PCR kits, together with automated platforms for PCR setup and DNA fragment analysis, standardize the entire PCR workflow – from convenient PCR setup with the QIAgility (Figure 12) and specific amplification with QIAGEN end-point PCR kits to fast and precise DNA fragment detection with the QIAxcel Advanced System (Figure 13).

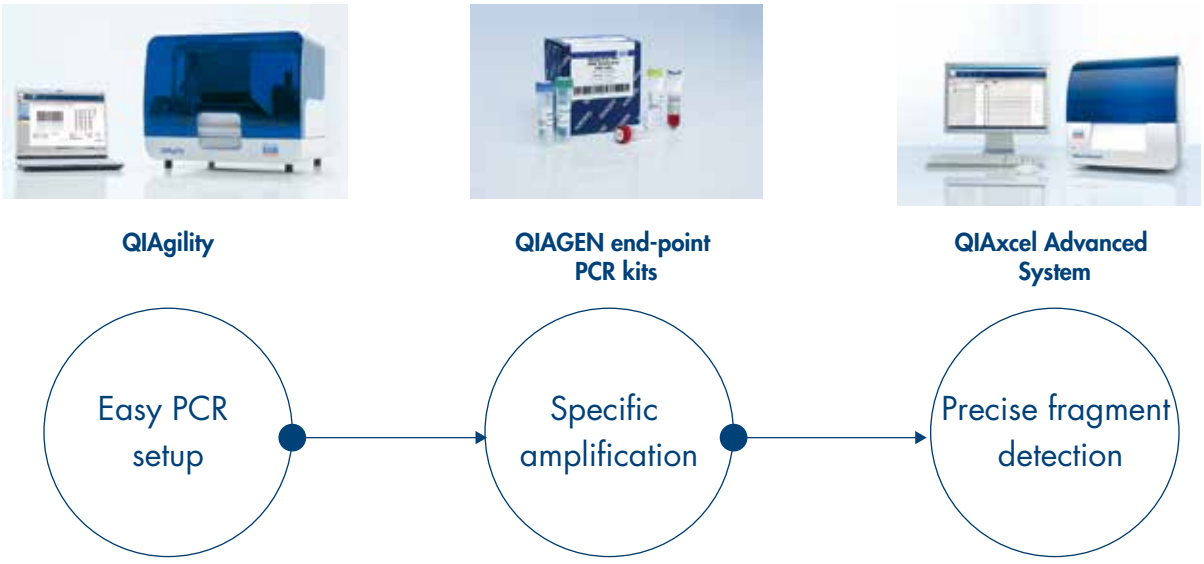

Figure 12. Standardized PCR workflow. To learn more about QIAGEN's complete solution for PCR automation and to view application data, visit **www.qiagen.com/pcr-literature**.

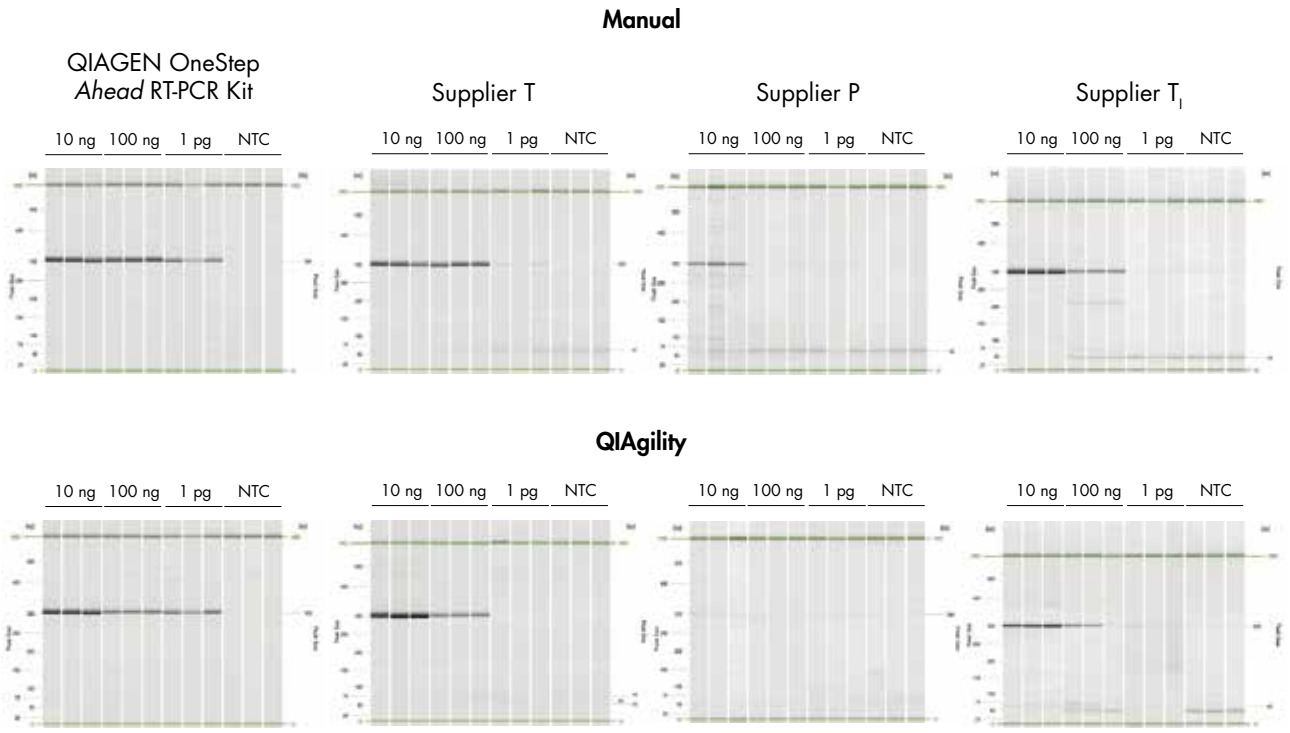

Figure 13. Comparison of manual reaction setup with automated setup using QIAgility. QIAxcel readout of one-step RT-PCR–amplified 295 bp actin beta (ACTB) mRNA target. The QIAGEN OneStep *Ahead* RT-PCR Kit provides superior sensitivity and specificity compared with other suppliers. Manual versus automated setup with the QIAGEN kit yields comparable results, while kits from other suppliers yield poorer results with automated setup, due to the longer exposure to room temperature during the automated setup process.

Ease of use

In addition to factors such as the choice of enzyme, reaction conditions, template quality and primer design, researchers must also consider of use and convenience during reaction setup. The robustness of a particular PCR reaction under various conditions – such as different salt concentrations, annealing temperatures, and cycle numbers – must be evaluated.

Ease of handling

Many PCR reagents include a number of additional features (e.g., master mixes, visualization dyes, or convenient room-temperature setup) for streamlining the PCR procedure and reducing the risk of handling errors. QIAGEN offers a range of kits providing these features as well as dNTPs and integrated gel loading dye for further time savings (Figure 14). Hot-start PCR enzymes that exhibit no activity at room temperature are available for convenient PCR and RT-PCR setup. In addition, a novel enzyme-stabilizing additive in TopTaq DNA Polymerase Kits improves the ease of setup when compared with standard PCR enzymes and allows the enzyme to be stored in a refrigerator – eliminating the need to freeze and thaw the reagent. For a comprehensive overview of the exceptional handling features of QIAGEN PCR and RT-PCR products, see pages 23–25.

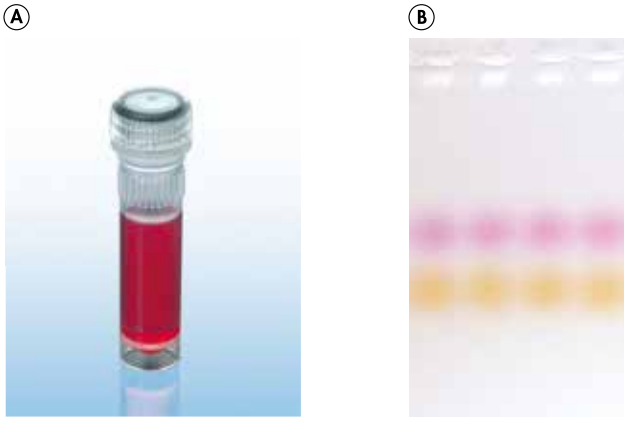

Figure 14. Integrated loading dye for streamlined handling. A CoralLoad® PCR Buffer, provided with many QIAGEN PCR kits, contains gel-tracking dyes for easier pipetting, **B** enabling immediate gel loading of PCR samples and easy visualization of DNA migration.

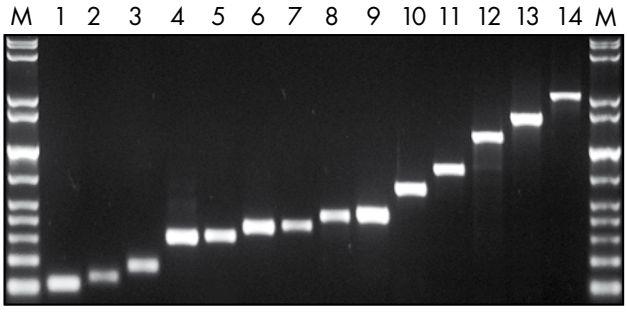

Figure 15. High PCR product yields without the need for optimization. TopTaq DNA Polymerase was used to amplify 14 different PCR products from mammalian genomic DNA ranging in size from 100 bp to 2 kb according to the standard preoptimized protocol and using identical cycling conditions.

One protocol for all assays

QIAGEN's unique dual-cation buffer system – provided with every QIAGEN PCR and RT-PCR kit – ensures highly specific amplification, resulting in significant time and cost savings. No time-consuming optimization or gradient PCR is required for the development and verification of new assays; successful results can be easily obtained using just a single, optimized PCR protocol that can be programmed into the thermocycler (Figure 15).

Significant time and cost savings

The simple reaction setup, fast procedure, consistent results, and ease of use ensured by QIAGEN PCR kits result in substantial savings in time and costs, as well as reagents, because optimization of PCR parameters is no longer necessary. The need for repeated experiments is eliminated and PCR success is achieved at the first attempt. PCR assay development is straightforward and easy, using a single, optimized protocol (Figure 16).

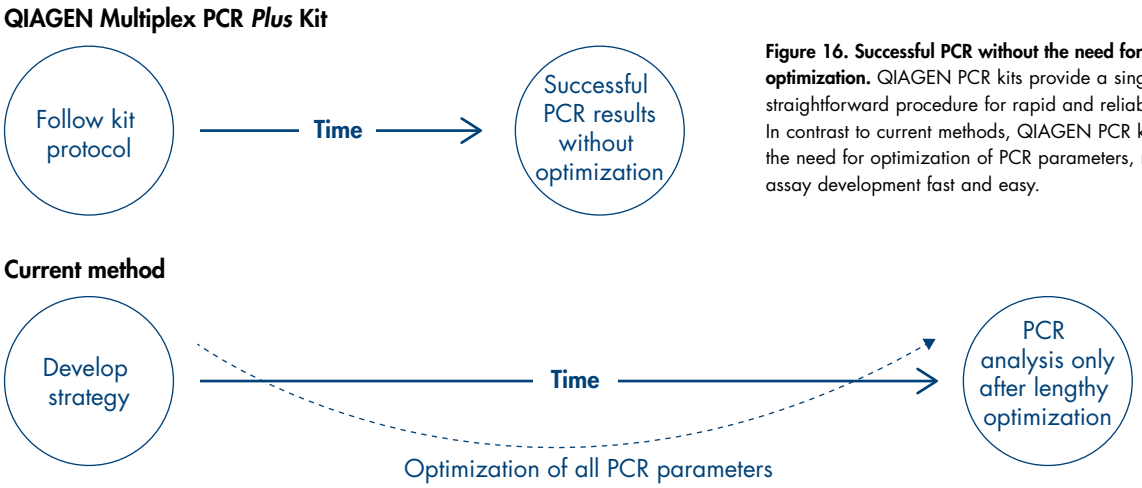

Figure 16. Successful PCR without the need for optimization. QIAGEN PCR kits provide a single, straightforward procedure for rapid and reliable results. In contrast to current methods, QIAGEN PCR kits eliminate the need for optimization of PCR parameters, making assay development fast and easy.

Primer design

Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR. Table 2 provides an overview of primer design and use for standard and multiplex PCR, as well as one-step RT-PCR.

Table 2. Guidelines for the design and use of primers

	Standard PCR	Multiplex PCR	One-step RT-PCR
Length	18–30 nt	21–30 nt	18–30 nt
GC content	40–60%	40–60%	40–60%
T_m calculation	2°C x (A+T) + 4°C x (G+C) The T_m of all primer pairs should be similar.	2°C x (A+T) + 4°C x (G+C) The T_m of all primer pairs should be similar. For optimal results, the T_m should be between 60 and 88°C.	2°C x (A+T) + 4°C x (G+C) The T_m of all primer pairs should be similar. The T_m should not be lower than the temperature of the reverse transcription (e.g., 50°C).
Estimating optimal annealing temperature	5°C below the calculated T_m	5–8°C below the calculated T_m (when greater than 68°C) 3–6°C below the calculated T_m (when 60–67°C)	5°C below the calculated T_m
Location	–	–	**To prevent detection of gDNA:** Primer hybridizes to the 3' end of one exon and the 5' end of the adjacent exon. Alternatively, the primer hybridized to a flanking region that contains at least one intron. If only the mRNA sequence is known, choose primer annealing sites that are 300–400 bp apart.
Sequence	Avoid complimentarity in the 2–3 bases at the 3' end of the primer pairs. Avoid mismatches between the 3' end of the primer and the template. Avoid runs of three or more C at the 3' end of the primer. Avoid complimentarity within primers and between the primer pair. Avoid a T at the 3' end. Ensure primer sequence is unique for your template sequence.		
Concentration, A_{260} unit equivalence	20–30 µg	20–30 µg	20–30 µg
	Input 0.1–0.5 µM of each primer (0.2 µM recommended)	Input 0.2 µM of each primer	Input 0.5–1 µM of each primer (0.6 µM recommended)
Storage	Dissolved in TE, store at –20°C	Dissolved in TE, store at –20°C	Dissolved in TE, store at –20°C

Template quality

Because PCR consists of multiple cycles of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol, chloroform, salts, ethanol, EDTA and other chemical solvents than single-step, enzyme-catalyzed processes. These impurities, which are commonplace in home-made template preparations, can reduce the sensitivity and efficiency of PCR amplification. Template preparation based on phenol extraction, ethanol precipitation, or salting-out procedures may not remove all contaminants and, thus, adversely affect PCR reliability (Table 3). We recommend QIAGEN sample prep technologies using silica membrane-based kits (e.g., QIAamp® and QIAprep® Kits) for reliable PCR results. Additionally, whole genome amplification kits, which amplify very small amounts of genomic DNA while introducing no sequence bias, can be used if the starting amount of DNA is limiting (e.g., REPLI-g® Kits).

Visit **www.qiagen.com** to see our complete range of DNA purification products, all of which provide pure DNA from a wide variety of sample types and ensure accurate PCR results.

In addition to DNA quality, the amount of template used for PCR may also influence PCR success – especially too much template, which often results in smearing or nonspecific amplification products.

Table 3. Impurities exhibiting inhibitory effects on PCR

Impurity	Inhibitory concentration
SDS	>0.005% (w/v)
Phenol	>0.2% (v/v)
Ethanol	>1% (v/v)
Isopropanol	>1% (v/v)
Sodium acetate	≥5 mM
Sodium chloride	≥25 mM
EDTA	≥0.5 mM
Hemoglobin	≥1 mg/ml
Heparin	≥0.15 i.U./ml
Urea	>20 mM
RT reaction mixture	≥15% (v/v)

Challenging PCR methods and new technologies

Standard PCR applications are routinely used in numerous research areas, such as biomarker discovery, genotyping, gene regulation and cancer research. New PCR applications have been developed to address the need for increased sophistication and accuracy. These developments have been driven by demands for increased throughput while reducing costs, increasing assay sensitivity and ensuring reliable data normalization (Table 4, next page). Specialized PCR buffer systems and associated additives are often necessary to meet these challenges and ensure successful results.

Table 4. Increasing success with challenging PCR applications

Application	Challenge	Amplification method
Genotyping	Parallel amplification of multiple products	Multiplex PCR
High-throughput/fast PCR	Reduce time to result	Multiplex PCR/fast-cycling, hot-start PCR
Single-cell PCR	Increased sensitivity	Highly specific hot-start PCR
Cloning	Sequence accuracy Amplification of long PCR products	High-fidelity PCR Long-range PCR
Detection of methylated DNA	PCR specificity	Methylation-specific PCR
Viral load monitoring and gene expression analysis	Increased sensitivity	One-step RT-PCR

Multiplex PCR

Multiplex PCR employs different primer pairs in the same reaction for simultaneous amplification of multiple targets. This type of PCR often requires extensive optimization of annealing conditions for maximum amplification efficiency of the different primer–template systems and is often compromised by nonspecific PCR artifacts. A stringent hot-start procedure and specially optimized buffer systems are absolutely crucial for successful multiplex PCR. In our **Multiplex PCR User Guide** we provide tips and tricks to overcome challenges in multiplex PCR (Figure 17).

Compared with standard PCR systems using only two primers, an additional challenge of multiplex PCR is the varying hybridization kinetics of different primer pairs. Primers that bind with high efficiency could utilize more of the PCR reaction components, thereby reducing the yield of other PCR products. This often results in unamplified DNA sequences and absence of expected PCR products. QIAGEN has overcome this problem with a specially adapted multiplex PCR buffer (provided with QIAGEN Multiplex PCR Kit and the QIAGEN Multiplex PCR *Plus* Kit). This buffer contains a unique synthetic additive, Factor MP, which further promotes stable and efficient annealing of different primers to the nucleic acid template. The increased hybridization efficiency and primer stability provides excellent product yields – even for primer pairs that normally bind suboptimally to their target sequence under the chosen conditions (Figure 18). This principle has been extended with the Type-it® Microsatellite PCR Kit, where each step of the procedure has been preoptimized – from template amount to precise cycle number. With this unique kit format, development of microsatellite assays with a high number of targets is easier than ever before (Figure 19).

Figure 17. Multiplex PCR Webapp.

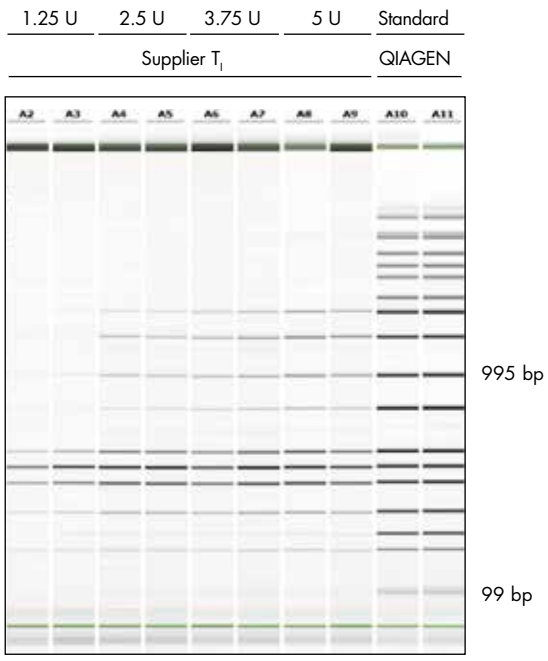

Figure 18. Efficient 19-plex PCR using the QIAGEN Multiplex PCR *Plus* Kit. Multiplex PCR of 19 targets (99–955 bp) was performed using standard conditions for the QIAGEN Multiplex PCR *Plus* Kit, without further optimization (QIAGEN) or using a variety of concentrations of a hot-start DNA polymerase from Supplier AII. Analysis was performed using the QIAxcel Advanced System. The QIAGEN Multiplex PCR *Plus* Kit resulted in specific amplification of all targets without the need for optimization. Despite lengthy optimization using different enzyme concentrations, multiplex PCR using the kit from Supplier AII resulted in missing fragments, even when using higher concentrations.

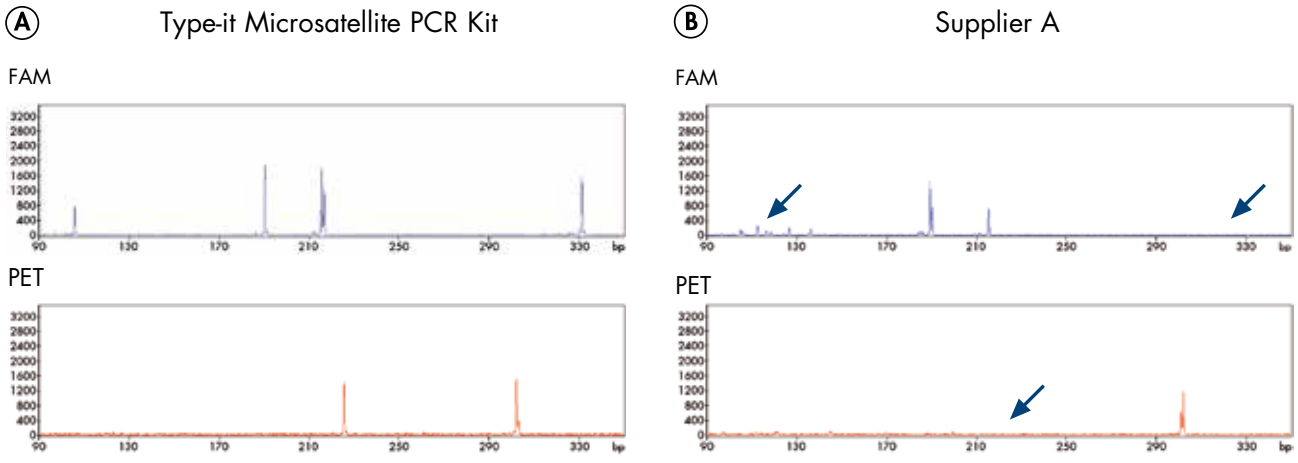

Figure 19. Optimization-free and reliable 13-plex STR analysis using the Type-it Microsatellite PCR Kit. Only two out of four channels (FAM™ and PET®) of a 3730*xl* DNA Analyzer (Thermo Fisher Scientific Inc.), representing 6 of 13 analyzed STR loci are shown. Using the Type-it Microsatellite PCR Kit under standard conditions, all 6 STR loci are reliably amplified. Despite the increased number of cycles and preoptimized PCR conditions for the hot-start method of Supplier A – compared with 25 cycles and standard conditions for the Type-it Microsatellite Kit – several specific peaks are missing and nonspecific signals are observed (arrows).

Fast-cycling PCR

Faster PCR amplification enables increased PCR throughput and allows researchers to spend more time on downstream analysis.

The demand for reducing time-to-result (see Figure 1, page 2) is met by the recent development of faster PCR techniques. Fast PCR can be achieved using new thermal cyclers with faster ramping times or through innovative PCR chemistries that allow reduced cycling times due to significantly shortened DNA denaturation, primer annealing, and DNA extension times. Fast-cycling PCR reagents must be highly optimized to ensure amplification specificity and sensitivity.

The QIAGEN Fast Cycling PCR Kit enables successful fast-cycling, hot-start PCR even on standard thermal cyclers, through the use of the novel Q-Bond® Molecule. This molecule dramatically increases the binding affinity of DNA polymerase to single-stranded DNA, allowing the annealing time to be reduced to just 5 seconds. The unique buffer formulation and optimized DNA polymerase concentration also enables a significant reduction in denaturation and extension times. Visit **www.qiagen.com/PCR-literature** to access literature on fast-cycling PCR.

> " Your kit allowed me to consistently and reproducibly amplify forensic DNA samples from 5000 year old bone samples in a short amount of time. This is a great tool for obtaining clean results in the shortest amount of time possible "
>
> Dr. Alex Nikitin. Assistant Professor, Grand Valley State University, Minnesota.

Single-cell PCR

Single-cell PCR provides a valuable tool for genetic characterization using a limited amount of starting material. By flow cytometry or micromanipulation, individual cells of interest can be isolated based on cell-surface markers or physical appearance. Amplification of low-abundance template molecules – as low as one or two gene copies – requires a PCR system that is highly efficient, specific, and sensitive, such as HotStarTaq *Plus* DNA Polymerase (Figure 20).

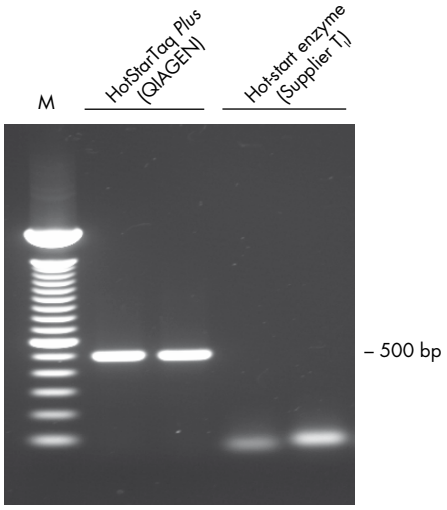

Figure 20. Successful single-cell PCR. A 500 bp fragment of the murine p53 gene was amplified from single cells isolated by flow cytometry and directly sorted into individual PCR tubes. Reactions were prepared in parallel using HotStarTaq *Plus* DNA Polymerase (**QIAGEN**) and a hot-start enzyme and buffer from Supplier T_1 (**Hot-start enzyme**). **M**: marker. A single specific fragment was only attained using HotStarTaq *Plus* DNA Polymerase.

Long-range PCR

PCR products of up to 4 kb can be routinely amplified using standard PCR protocols. However, amplification of PCR products longer than 4 kb often fails without lengthy optimization. Reasons for failure include nonspecific primer annealing, secondary structures in the DNA template, and suboptimal cycling conditions – all factors which have a greater effect on the amplification of longer PCR products than on shorter ones.

Preventing DNA damage, such as DNA depurination, is of particular importance for amplification of long PCR products, as a single DNA lesion within the template is sufficient to stall the PCR enzyme. DNA damage during PCR cycling can be minimized with specific buffering substances that stabilize the pH of the reaction. The QIAGEN LongRange PCR Kit is optimized for the amplification of PCR products up to 40 kb in size. DNA pre-incubated in QIAGEN LongRange PCR Buffer shows similar PCR product yield compared to non-damaged control DNA, demonstrating that the protecting function of the buffer system provides an optimal reaction environment for the amplification of long PCR products (Figure 21). Unlike kits from other suppliers, the QIAGEN LongRange PCR Kit overcomes long-range PCR challenges, owing to its unique features (Figure 22).

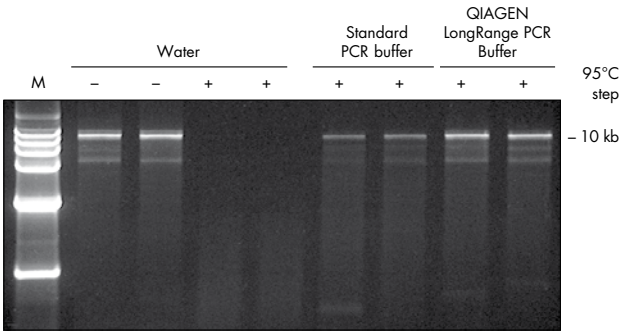

Figure 21. LongRange PCR Buffer protects genomic DNA from excessive damage. Genomic DNA from leukocytes in water (**Water**), long-range PCR buffer from Supplier A (**Standard PCR buffer**) or QIAGEN LongRange PCR Buffer (**QIAGEN LongRange PCR Buffer**) were used directly for PCR (**–**) or incubated at 95°C (**+**) before PCR. Amplification reactions (50 µl) were performed using the QIAGEN LongRange PCR Kit. **M**: marker. Greater DNA damage, as indicted by reduced product yield, is observed with pre-treatment of DNA in water or standard long-range PCR buffers than with pre-treatment in QIAGEN LongRange PCR Buffer.

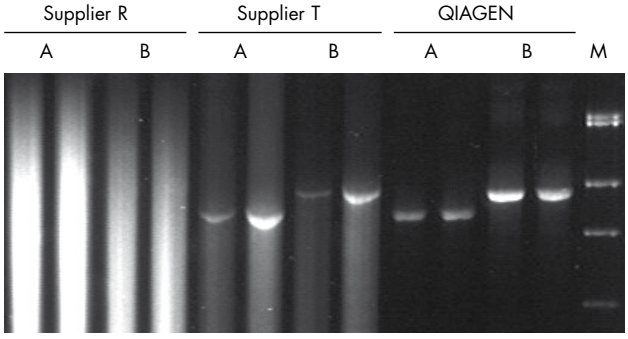

Figure 22. Successful long-range PCR using the QIAGEN LongRange PCR Kit. PCR was performed with the indicated kits, using 40 ng human genomic DNA as a template. In contrast to kits from Supplier R and Supplier T, the QIAGEN LongRange PCR Kit provided successful amplification, resulting in amplicons of 7.6 kb and 8.9 kb, using a simplified, time-saving protocol. **M**: marker.

Methylation-specific PCR (MSP)

MSP enables the methylation status of target DNA to be determined after sodium bisulfite treatment (e.g., using the EpiTect® Fast Bisulfite Kit). The method requires two sets of primers to be designed: one set that anneals to unchanged cytosines (i.e., methylated in the genomic DNA) and one set that anneals to uracil resulting from bisulfite treatment of cytosines not methlyated in the genomic DNA. Amplification products derived from the primer set for unchanged sequences indicates the cytosines were methylated and thus protected from alteration.*

Stringent and highly specific PCR conditions must be used to avoid nonspecific primer binding and the amplification of PCR artifacts. This is particularly important as the conversion of unmethylated cytosines to uracils reduces the complexity of the DNA and increases the likelihood of nonspecific primer–template binding. HotStarTaq *Plus* DNA Polymerase, designed for amplification of templates with high GC-content DNA, has been successfully used for MSP (Figure 23). In addition, QIAGEN's dedicated EpiTect MSP Kit is specially optimized for highly reliable MSP in epigenetics applications.

* Review article: Hernández H. G., et al. (2013) Optimizing methodologies for PCR-based DNA methylation analysis. Biotechniques **55**, 181.

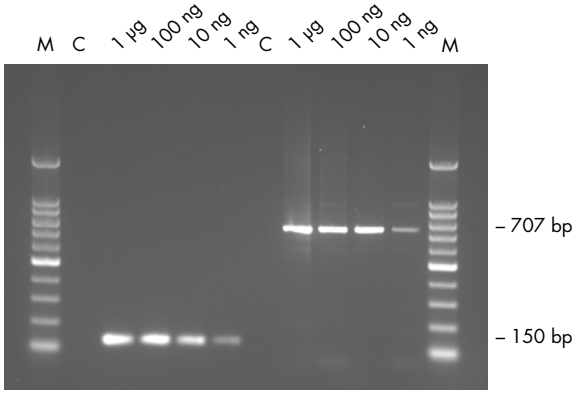

Figure 23. Highly sensitive MSP amplification. Human genomic DNA was purified from blood using the QIAamp DNA Blood Mini Kit, and various amounts (1 ng – 1 µg) were converted using the EpiTect Bisulfite Kit. PCR was performed using the HotStarTaq *Plus* Master Mix Kit and two sets of primers designed to amplify converted DNA. 5 µl of each product was loaded onto a 1.3% agarose gel. The HotStarTaq *Plus* Master Mix Kit allowed specific amplification from all DNA concentrations. **M**: marker; **C**: negative control.

Viral research with one-step RT-PCR

RNA secondary structure can affect RT-PCR results in a number of ways. During reverse transcription, regions of RNA with complex secondary structure can cause the reverse transcriptase to stop or dissociate from the RNA template. Truncated cDNAs that do not include the downstream primer-binding site are not amplified during PCR. In some cases, the reverse transcriptase skips looped structures, resulting in deletions in the cDNA which lead to truncated PCR products. When amplifying a low-abundance transcript or viral sequences, these problems are even more critical. A well-balanced system, consisting of reverse transcriptase, a stringent hot-start enzyme, and an optimized buffer system is crucial for applications such as viral detection or gene expression analysis, where maximum sensitivity is often required (Figure 24). Download our **specialized protocol** for one-step RT-PCR for viral samples. The buffer provided with the QIAGEN OneStep RT-PCR Kit and QIAGEN OneStep *Ahead* RT-PCR Kit allows reverse transcription to be performed at an elevated temperature (50°C). This high reaction temperature improves the efficiency of the reverse transcriptase reaction by disrupting secondary structures and is particularly important for one-step RT-PCR performed with limiting template amounts.

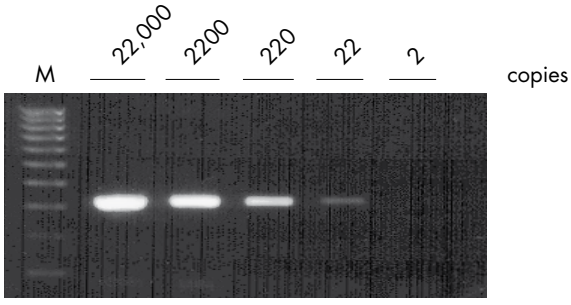

Figure 24. Efficient detection of viral RNA. A 336 bp fragment of F-gene mRNA was reverse-transcribed and amplified from Sendai virus RNA isolated from persistently infected Vero cells. Reactions were prepared using the QIAGEN OneStep RT-PCR Kit and the indicated number of viral genome copies.* **M**: markers.

* Data kindly provided by H. Rausch, Max Planck Institute for Biochemistry, Martinsried, Germany as part of the project "Experimental control of virological work at safety levels 2 and 3 in Bavaria," supported by the Bavarian Ministry of the Environment.

In addition, the new QIAGEN OneStep *Ahead* RT-PCR Kit adds increased process safety and convenience to viral detection. The RT-blocker keeps the reverse transcription enzyme inactivated at ambient temperatures and enables room-temperature reaction setup, as well as high-throughput use in automated workflows. Its inbuilt RNase inhibitor protects the sample from RNase contamination and the optional use of dual-color pipetting control (Figure 25) reduces the risk of human pipetting error. The kit comes in a convenient master mix format with an ultrafast cycling protocol that takes just 1 hour. For improved sequence accuracy and amplification of long fragments up to 4 kb, the mix also includes a high fidelity/proofreading polymerase, which makes the kit particularly useful for protocols including subsequent sequencing of targets.

Figure 25. Dual-color pipetting control in the QIAGEN OneStep *Ahead* RT-PCR Kit. See how the control works **here.**

Summary

PCR is a powerful and still-developing tool for modern molecular biology. Recent advances in PCR have enabled increased sensitivity and specificity coupled with even faster results. Following the simple guidelines presented in this brochure will allow you to achieve optimal PCR results – even at your first attempt. QIAGEN PCR and RT-PCR products have been specifically developed to provide superior results without the need for optimization. In addition, our range of products offer convenient features, such as master mix formats, gel-loading PCR buffers and streamlined protocols, as well as automation for every step of the workflow – from setup to detection. We also provide a range of reliable quantitative PCR and RT-PCR kits, as well as whole genome amplification kits. Visit **www.qiagen.com/PCR-literature** and find out more about overcoming challenges in end-point PCR with QIAGEN's PCR solutions.

Whatever your PCR application, QIAGEN has a kit to suit your needs (see selection guides, page 23–25).

Ordering Information

Product	Features	Cat. no.
Standard PCR – for standard and specialized PCR applications		
TopTaq DNA Polymerase (250)*	Fridge storage and room temperature setup	200203
TopTaq Master Mix Kit (250)	Fridge storage and room temperature setup	200403
Taq DNA Polymerase (250)*	With ready-to-load PCR buffer	201203
Taq PCR Master Mix Kit (250)*	Ready-to-use master mix format	201443
Taq PCR Core Kit (250 U)*	Complete kit format with dNTP mix	201223
Hot-start PCR – for fast and highly specific amplification in all applications		
HotStarTaq *Plus* DNA Polymerase (250)*	With ready-to-load PCR buffer	203603
HotStarTaq *Plus* Master Mix Kit (250)*	Ready-to-use master mix format	203643
HotStarTaq DNA Polymerase (250)*	With PCR enhancer — Q-Solution	203203
HotStarTaq Master Mix Kit (250)*	Ready-to-use master mix format	203443
Fast-cycling, hot-start PCR – for ultrafast and specific amplification on any thermal cycler		
QIAGEN Fast Cycling PCR Kit (200)*	Ready-to-use master mix format	203743
High-fidelity PCR – for highly sensitive and reliable high-fidelity hot-start PCR		
HotStar HiFidelity Polymerase Kit (100)*	Complete kit format with dNTP mix	202602
Long-range PCR – for sensitive and accurate amplification of long fragments		
QIAGEN LongRange PCR Kit (20)*	With dNTP mix	206401
Multiplex PCR – for highly specific and sensitive amplification of multiple targets		
QIAGEN Multiplex PCR Kit (100)*	Ready-to-use master mix format	206143
QIAGEN Multiplex PCR *Plus* Kit (30)*	Ready-to-use and ready-to-load master mix format	206151
Type-it Microsatellite PCR Kit (70)*	Microsatellite assay development without optimization	206241
Type-it Mutation Detect PCR Kit (200)	Multiplex PCR assay development without optimization	206343
One-step RT-PCR – for fast, highly sensitive, and successful one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	Complete kit format with dNTP mix	210210
QIAGEN OneStep *Ahead* RT-PCR Kit (50)	Complete kit in master mix format	220211
Methylation-specific PCR – for highly accurate methylation-specific PCR (MSP)		
EpiTect MSP Kit (100)	Hot-start PCR with bisulfite-converted DNA	59305
dNTPs – for sensitive and reproducible PCR and RT-PCR		
dNTP Set (100 µl)*	100 mM each dATP, dCTP, dGTP, dTTP	201912

* Other kit sizes/formats available, please inquire or visit **www.qiagen.com**. Also visit our website to see our complete range of quantitative PCR and whole genome amplification products.

Discover our complete range of PCR products at **www.qiagen.com/PCR**

Selection Guide

QIAGEN multiplex end-point PCR solutions

		Type-it Microsatellite PCR Kit	Type-it Mutation Detect PCR Kit	QIAGEN Multiplex PCR Kit	QIAGEN Multiplex PCR *Plus* Kit
Application		**Genotyping**		**Any multiplex PCR application**	
Starting material		**gDNA**		**gDNA/cDNA**	
Application	STR and microsatellite analysis	●		●	●
	Mutation detection		●	●	●
	SNP loci amplification		●	●	●
	Single-cell PCR			●	●
	Preamplification			●	●
PCR performance	Hot-start (15 min activation)			●	
	Hot-start (5 min activation)	●	●		●
	Q-Solution (PCR enhancer for difficult templates)	●	●	●	●
	Maximum sensitivity and specificity	●	●	●	●
	Amplification-product size	≤0.5 kb	≤3.5 kb	≤3.5 kb	≤3.5 kb
Ease of use and convenience	PCR buffer with tracking dyes (CoralLoad)		●		●
	Room-temperature setup	●	●	●	●
	Fridge storage	●*	●*	●†	●*
	Master mix format, including nucleotides	●	●	●	●

●: Recommended kit. * Up to 2 months. † Up to 6 months.

Selection Guide

QIAGEN PCR application

Sample type	Application	QIAGEN Multiplex PCR Kit	QIAGEN Multiplex PCR Plus Kit	Type-it Microsatellite PCR Kit*	Type-it Mutation Detect PCR Kit*	QIAGEN Fast Cycling PCR Kit	QIAGEN LongRange PCR Kit	HotStar HiFidelity Polymerase Kit	HotStarTaq DNA Polymerase	HotStarTaq Master Mix Kit	HotStarTaq Plus DNA Polymerase	HotStarTaq Plus Master	Taq DNA Pol
		PCR kits											
DNA/ cDNA	Standard PCR						●					●	●
	Standard PCR, hot-start					●		●	●	●	●	●	
	High-fidelity PCR						●	●					
	Fast-cycling PCR					●							
	Long-range PCR						●						
	Multiplex PCR	●	●	●	●								
	STR and microsatellite analysis	●	●	●									
	Detection of mutations	●	●		●				●	●	●	●	
	Amplification of SNP loci	●	●		●				●	●	●	●	
	Single-cell PCR	●	●			●			●	●	●	●	
	Methylation-specific PCR (MSP)												
	Nested PCR	●	●			●			●	●	●	●	
	DNA virus detection	●	●			●			●	●	●	●	
RNA	Reverse transcription												
	One-step RT-PCR												
	Single-cell, one-step RT-PCR												
	RNA virus detection												

* Dedicated Type-it PCR Kits for genotyping using HRM® Technology or using TaqMan® probes are also available. Visit **www.qiagen.com/PC** PCR-based genotyping solutions.

Mix Kit	ymerase	q PCR Core Kit	Taq PCR Master Mix Kit	TopTaq DNA Polymerase	TopTaq Master Mix Kit	EpiTect MSP Kit	QIAGEN OneStep RT-PCR Kit	QIAGEN OneStep Ahead RT-PCR Kit	Sensiscript	Omniscript
						RT-PCR kits				
●		●	●	●						
							●			
							●			
					●					
									●	●
						●	●			
						●	●			
						●	●			

R-literature and download our brochure on

Selection Guide

QIAGEN PCR application

Your benefit	Advanteges	QIAGEN Multiplex PCR Kit	QIAGEN Multiplex PCR *Plus* Kit	Type-it Microsatellite PCR Kit*	Type-it Mutation Detect PCR Kit*	QIAGEN Fast Cycling PCR Kit	QIAGEN LongRange PCR Kit	HotStar HiFidelity Polymerase Kit	HotStarTaq DNA Polymerase	HotStarTaq Master Mix Kit	HotStarTaq *Plus* DNA Po	HotStarTaq Pl	Ta
		PCR kits											
Reliable results	No need to optimize	**All QIAGEN PCR and RT-PCR Kits**											
Speed	Ultrafast PCR					●							
	Multiplex PCR	●	●	●	●								
PCR performance	Hot-start (15 min activation)	●							●	●			
	Hot-start (5 min activation)		●	●	●	●		●			●	●	
	Maximal specificity	●	●	●	●	●		●	●	●	●	●	
	Maximal sensitivity	●	●	●	●	●		●	●	●	●	●	
	Q-Solution (PCR enhancer for difficult templates)	●	●	●	●	●	●	●	●		●		●
	Fidelity	1x	1x	1x	1x	1x	3x	>10x	1x	1x	1x	1x	1x
	Amplification-product size (kb)	≤3.5	≤3.5	≤0.5	≤3.5	≤3.5	≤40	≤5	≤5	≤5	≤5	≤5	≤5
Ease of use and convenience	Direct UA/TA cloning	**All QIAGEN PCR and RT-PCR Kits**											
	PCR buffer with gel tracking dyes (CoralLoad)		●		●	●					●	●	●
	Room-temperature setup	●	●	●	●	●		●	●	●	●	●	
	Fridge storage	●*	●†	●†	●†				●*	●*	●†	●†	
	Complete kit format including nucleotides						●	●					
	Master mix format including nucleotides	●	●	●	●	●				●		●	

* Up to 2 months
† Up to 6 months

...ymerase	...us Master Mix Kit	...q DNA Polymerase	Taq PCR Core Kit	Taq PCR Master Mix Kit	TopTaq DNA Polymerase	TopTaq Master Mix Kit	EpiTect MSP Kit	QIAGEN OneStep RT-PCR Kit	QIAGEN OneStep Ahead RT-PCR Kit	Sensiscript	Omniscript
							RT-PCR kits				
							●				
				●	●						
						●					
				●	●	●	●	●			
				●	●	●	●	●			
●		●			●	●					
1x	1x	1x	1x	1x	1x	3x	1x	1x			
≤5	≤5	≤5	≤5	≤0.2	≤5	≤4	≤4	≤4			
●		●	●								
		●	●			●					
		●	●								
●					●		●	●			
	●		●	●		●					

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.

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