Highly efficient multiplex PCR using novel reaction chemistry

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Multiplex PCR is a powerful technique enabling amplification of two or more products in a single reaction. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required, such as typing of normal and genetically modified animals and plants, detection of pathogens or genetically modified organisms (GMOs), or for microsatellite analyses. Multiplex PCR assays can be tedious and time-consuming to establish and lengthy optimization procedures, such as adjusting primer concentrations, Mg^{2+} concentration, and amount of enzyme are often necessary. In many cases, the results are still disappointing and further extensive optimization may be required. However, due to novel developments in the reaction chemistry, multiplex PCR assays are now simple and straightforward to establish.

The combined use of a highly stringent hot start with a unique PCR buffer specifically developed for multiplex reactions, makes the QIAGEN® Multiplex PCR Kit highly suited for multiplex PCR applications. The newly developed reaction buffer, containing Factor MP, a special multiplex PCR-enhancing synthetic factor, eliminates the need for optimization — even when using equimolar primer concentrations.

In this study, we describe the effects of different reaction parameters and how the QIAGEN Multiplex PCR Kit minimizes the need for optimization of multiplex PCR assays. The results were visualized either by gel electrophoresis or using the Agilent 2100 Bioanalyzer.

Materials and methods

Genomic DNA was isolated from human K562 cells using the DNeasy® Tissue Kit and 20 ng was used as template for multiplex PCR. PCR was performed using either QIAGEN Multiplex PCR Master Mix (containing HotStarTaq® DNA Polymerase), or using standard *Taq* DNA polymerase in an otherwise identical reaction mixture, or using a *Taq* DNA polymerase with antibody-mediated hot start and KCl reaction buffer from Supplier I. Equal volumes of each multiplex PCR were analyzed on the Agilent 2100 Bioanalyzer using the DNA 1000 LabChip® Kit or by agarose gel electrophoresis.

Results and discussion

Effect of novel multiplex PCR buffer chemistry on primer annealing

With multiplex PCR, the annealing efficiencies of the different primers in the reaction are usually dissimilar. Although it is possible to design primers that have a similar annealing temperature, the T_m of each primer does not always provide a good indication of its annealing efficiency. In addition, other factors such as the 3'-end sequence of primers may affect the efficiency of primer extension by *Taq* DNA polymerase. Efficient primer annealing and extension, irrespective of primer sequence, is achieved by Factor MP in QIAGEN Multiplex PCR Master Mix. Factor MP increases the local concentration of primers at the template DNA and stabilizes specifically bound primers (Figure 1). In addition, nonspecific primer binding is avoided by a balanced combination of salts and the specially optimized NH_4^+ concentration in the QIAGEN Multiplex PCR Master Mix. NH_4^+, which exists predominantly as ammonia (NH_3) under thermal-cycling conditions, interacts with the relatively weak hydrogen bonds formed when primers bind nonspecifically to the template DNA and destabilizes these nonspecifically bound primers. Figure 2 shows that in contrast to conventional PCR reagents, the formulation of the new QIAGEN Multiplex PCR Master Mix ensures comparable efficiencies for annealing and extension of all primers in the reaction without further optimization. ▶

Effect of Factor MP and NH_4^+ Ions on Primer Annealing

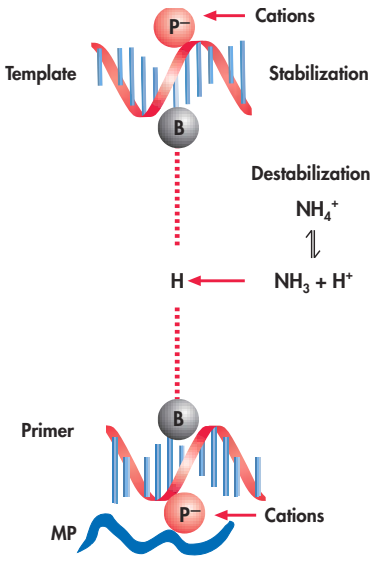

Figure 1 *Mg^{2+} and other salts bind to phosphate groups (**P⁻**) on the DNA backbone, which stabilizes the annealing of the primers to the template. NH_4^+, which exists both as the ammonium ion and as ammonia under thermal-cycling conditions, can interact with the hydrogen bonds between the bases, destabilizing principally the weak hydrogen bonds at mismatched bases (**B**) of nonspecifically bound primers. Factor MP (**MP**) increases the concentration of the primers at the template and stabilizes specifically bound primers, enabling efficient extension of all primers in the reaction.*

Effect of PCR Buffer on Multiplex PCR

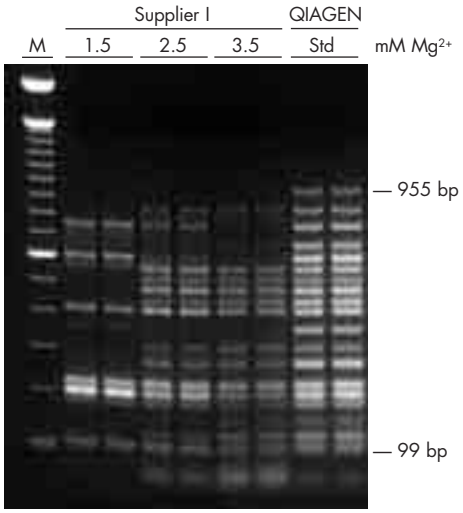

Figure 2 *Multiplex PCR of 19 targets (99–955 bp) was carried out for 35 cycles using standard conditions (**Std**) for the QIAGEN Multiplex PCR Kit (**QIAGEN**) without optimization, or using the indicated Mg^{2+} concentrations with a hot-start enzyme and supplied KCl-based buffer from Supplier I (**Supplier I**). Equal volumes of each multiplex PCR were analyzed on a 1.7% agarose gel stained with ethidium bromide. **M**: markers.*

Effect of hot start on multiplex PCR specificity

Typically, a primer concentration of 0.2–0.5 µM is used in conventional PCR. In contrast, the total primer concentration in multiplex PCR can be as high as 2–4 µM, depending on the number of different primer pairs in the reaction. The large number of primers often results in the generation of nonspecific PCR products and primer-dimers, reducing the specificity and sensitivity of the multiplex PCR. Using a stringent hot start to increase PCR specificity can prevent the generation of these nonspecific products. Figure 3 shows a comparison of hot-start multiplex PCR using the QIAGEN Multiplex PCR Kit, and multiplex PCR using a standard *Taq* DNA polymerase without a hot start. Use of standard *Taq* DNA polymerase results in the generation of large amounts of primer-dimers and low efficiency of amplification. In contrast, the stringent hot start provided by HotStarTaq DNA Polymerase in the QIAGEN Multiplex PCR Kit leads to reproducible and efficient amplification of all 13 PCR products, without generating nonspecific artifacts.

Conclusions

- Establishing multiplex PCR assays is easy and fast using the QIAGEN Multiplex PCR Kit. Tedious optimization procedures are almost eliminated.
- The kit is highly suited for multiplex applications, including genotyping of transgenic organisms, detection of pathogens or GMOs, and microsatellite genotyping, for example, short tandem repeat (STR) and variable number tandem repeat (VNTR) analyses.
- Factor MP in the multiplex PCR buffer allows efficient annealing of multiple primers under identical cycling conditions.
- The stringent hot start provided by HotStarTaq DNA Polymerase eliminates nonspecific PCR products and primer-dimer formation in multiplex PCR. ■

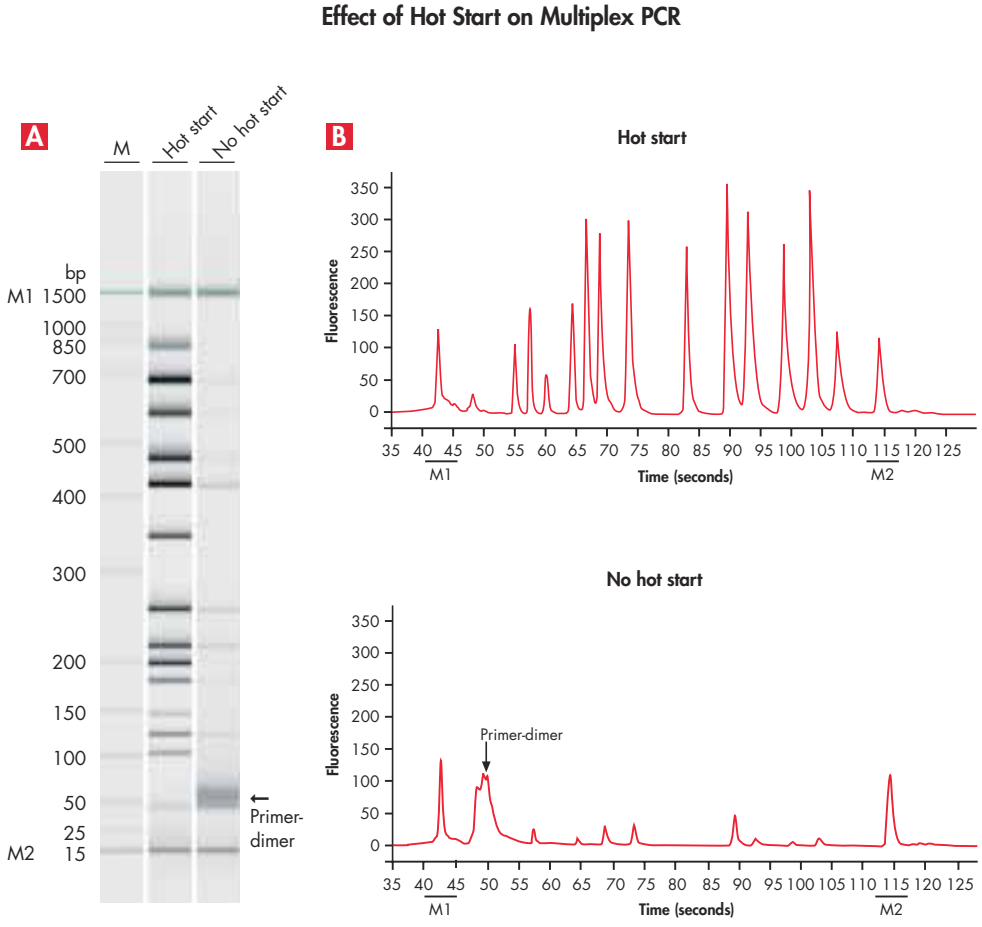

Figure 3 *Multiplex PCR of 13 targets (99–955 bp) was carried out for 35 cycles in a 50 µl reaction volume using the standard protocol in the* QIAGEN Multiplex PCR Handbook. *Multiplex reactions were carried out using QIAGEN Multiplex PCR Master Mix (containing HotStarTaq DNA Polymerase)* ***(Hot start)*** *or a standard* Taq *DNA polymerase* ***(No hot start)****. Equal volumes of each multiplex PCR were analyzed on the Agilent Bioanalyzer 2100 using the DNA 1000 LabChip Kit.* ***A*** *Gel-like image of collected data.* ***B*** *Agilent Bioanalyzer scan of multiplex PCR.* ***M****: markers;* ***M1****: upper marker;* ***M2****: lower marker.*

Ordering Information

Product	Contents	Cat. No.
QIAGEN Multiplex PCR Kit (100)	For 100 x 50 µl multiplex PCR reactions: 2x QIAGEN Multiplex PCR Master Mix (containing HotStarTaq DNA Polymerase and providing a final concentration of 3 mM $MgCl_2$, 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), distilled water (2 x 1.7 ml)	206143
Related products		
HotStarTaq DNA Polymerase (250 U)	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	203203
Taq DNA Polymerase (250 U)	250 units *Taq* DNA Polymerase, 10x PCR Buffer (containing 15 mM $MgCl_2$), 5x Q-Solution, 25 mM $MgCl_2$	201203
DNeasy Tissue Kit (50)	50 DNeasy Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504