QIAGEN News

Optimization of multiplex PCR

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Multiplex PCR is a demanding PCR technique used for genetic screening, microsatellite analysis, and other applications where it is necessary to amplify several products in a single reaction. This technique often requires extensive optimization because primer–dimers and other nonspecific products may interfere with the amplification of specific products. Although primer–dimer formation can usually be avoided using hot-start PCR, amplification specificity is also influenced by other factors such as the PCR buffer and primer concentration. Here we describe an optimization strategy for simultaneous amplification of multiple PCR products from as few as 10 cells.

Materials and methods

Genomic DNA was isolated from mouse tails using the DNeasy™ Tissue Kit. Amplification reactions were prepared using 100 ng DNA, 1x QIAGEN® PCR Buffer (providing a final concentration of 1.5 mM $MgCl_2$), 200 µM each dNTP, primers at concentrations indicated in the text, and 2.5 units HotStarTaq™ DNA Polymerase in a total volume of 50 µl. Reaction mixtures were incubated at 95°C for 15 min to activate HotStarTaq DNA Polymerase, and then subjected to 35 cycles of amplification (94°C, 30 sec; 58°C, 1 min; 72°C, 2 min). PCR products were analyzed on a 2% agarose gel.

Single-cell suspensions were prepared from mouse spleen, and B lymphocytes were isolated by flow cytometry. Ten cells were sorted directly into 20 µl water in a PCR tube and immediately frozen on dry ice. Recommendations for single-cell PCR presented in the *HotStarTaq PCR Handbook* (February 1999) were followed. PCR products were analyzed on a 2% agarose gel.

Results and discussion

Optimization of cycling conditions for individual PCR products

The murine *p53* gene (Figure 1) was chosen as a model system for multiplex PCR optimization. Following recommendations published earlier (1), primer pairs were selected to amplify fragments easily resolved on an agarose gel.

The first step in optimizing amplification conditions for multiplex PCR (Table 1) was to determine one set of cycling conditions that produced comparable yields from each individual primer pair. Reactions were prepared using 100 ng genomic DNA, 0.2 µM each primer, and one of the following combinations of enzyme and buffer: *Taq* DNA polymerase and buffer from Supplier C, a hot-start enzyme and buffer from Supplier A, or HotStarTaq DNA Polymerase and buffer from QIAGEN. The yields of the expected PCR products, corresponding to exons 1 (384 bp), 4 (260 bp), 5 (186 bp), 10 (108 bp), and 11(544 bp), were comparable for each of the different enzymes (Figure 2). These optimized cycling conditions were used in all subsequent experiments.

Multiplex PCR using the Murine *p53* Gene

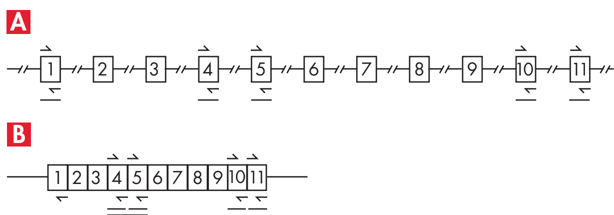

Figure 1 *Schematic of murine* p53 *gene structure and location of primers. Arrows indicate complementary primer binding sites; bars indicate expected PCR products.* **A** p53 *gene.* **B** p53 *pseudogene.*

Effect of hot-start PCR

Once conditions for amplifying individual products were determined, multiplex PCR was attempted using equimolar concentrations of each primer (Figure 3). In reactions where a hot start was not performed, primer–dimer formation was observed and only 2 specific products were detected.

Amplification of all 5 specific PCR products without primer–dimer formation was possible only when using hot-start PCR. The hot-start multiplex reactions also amplified a sixth specific product (446 bp) from a region spanning exons 4 and 5 of a *p53* pseudogene lacking the intervening intron (reference 2 and Figure 1B). In contrast to the poor amplification seen using the hot-start enzyme from Supplier A, the combination of HotStarTaq DNA Polymerase and QIAGEN PCR Buffer enabled specific amplification of all products at higher yields without further optimization.

Effect of primer concentration

In order to amplify all of the specific PCR products with equal efficiency, the concentrations of individual primer pairs were optimized (Figure 4). Concentrations were altered in increments of 0.1 µM for one primer pair at a time. Primer concentration was increased when the intensity of the corresponding band was too low, and decreased when the intensity of the band was too high. With multiplex PCR performed using HotStarTaq DNA Polymerase (QIAGEN), optimization resulted in similar high efficiencies for all specific products. In contrast, optimizing primer concentration did not substantially improve PCR performance using enzymes from other suppliers.

Multiplex PCR using just 10 cells

Previous reports suggest that multiplex PCR is unreliable when using low-concentration template DNA (3). To evaluate the robustness of amplification with HotStarTaq DNA Polymerase, multiplex PCR was attempted using just 10 cells. As the template concentration in these reactions is very low, we reduced the primer concentration to 1/5 of the concentration used in previous experiments. Although primer concentration was still high enough to cause primer–dimer formation, all of the specific products were successfully amplified from as few as 10 cells without the need for further optimization (Figure 5).

Optimized Cycling Conditions

	No hot start (Supplier C)					Hot-start enzyme (Supplier A)					HotStarTaq (QIAGEN)				
M	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5

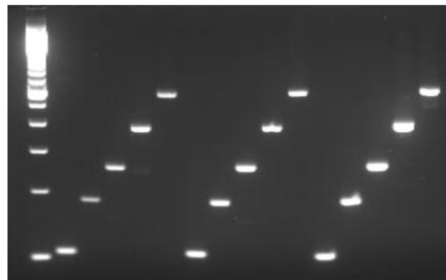

Figure 2 Fragments from the murine p53 gene were amplified from genomic DNA using reactions containing one of 5 different primer pairs. Reactions were prepared in parallel using either a Taq DNA polymerase from Supplier C (**No hot start**), a hot-start enzyme from Supplier A (**Hot-start enzyme**), or HotStarTaq DNA Polymerase (**HotStarTaq**) from QIAGEN. PCR products correspond to **1**: exon 10 (108 bp); **2**: exon 5 (186 bp); **3**: exon 4 (260 bp); **4**: exon 1 (384 bp); and **5**: exon 11 (544 bp). **M**: markers.

Effect of Hot Start PCR

	No hot start (Supplier C)	Hot-start enzyme (Supplier A)	HotStarTaq (QIAGEN)
M			

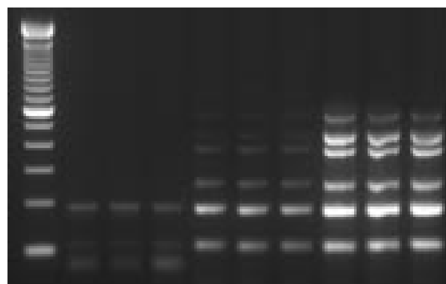

Figure 3 Fragments from the murine p53 gene were amplified from genomic DNA in multiplex PCR. Reactions were prepared in parallel using either a Taq DNA polymerase from Supplier C (**No hot start**), a hot-start enzyme from Supplier A (**Hot-start enzyme**), or HotStarTaq DNA Polymerase (**HotStarTaq**) from QIAGEN. **M**: markers.

Optimized Primer Concentration

	No hot start (Supplier C)	Hot-start enzyme (Supplier A)	HotStarTaq (QIAGEN)
M			

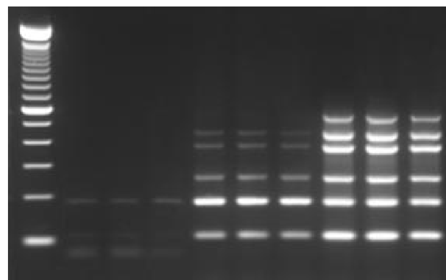

Figure 4 Fragments from the murine p53 gene were amplified from genomic DNA in multiplex PCR using optimized primer concentrations. Primer concentrations were: 0.2 µM (exon 1), 0.2 µM (exon 4), 1.0 µM (exon 5), 0.4 µM (exon 10), and 1.0 µM (exon 11). Reactions were prepared in parallel using either a Taq DNA polymerase from Supplier C (**No hot start**), a hot-start enzyme from Supplier A (**Hot-start enzyme**), or HotStarTaq DNA Polymerase (**HotStarTaq**) from QIAGEN. **M**: markers.

Table 1. General guidelines for optimizing multiplex PCR

Step	Guideline
Step 1	**Optimize cycling conditions for individual primer pairs** Prepare 50-µl reactions containing only one primer pair: ◆ 100–200 ng genomic DNA ◆ 0.2 µM each primer ◆ 1x QIAGEN PCR Buffer ◆ 1.25–2.5 units HotStarTaq DNA Polymerase Adjust temperature and duration of annealing and extension steps until similar product yields are obtained with all primer pairs.
Step 2	**Perform multiplex PCR** Use equimolar concentrations of all primer pairs and the optimized cycling conditions from step 1.
Step 3	**Optimize multiplex conditions** Adjust the following parameters according to the guidelines below until the desired yield is obtained: ◆ Primer concentration in steps of 0.1–0.2 µM ◆ Annealing temperature in steps of 1°C ◆ Extension time in steps of 30 sec No product detected: ◆ Increase primer concentration ◆ Decrease annealing temperature Low product yield: ◆ Increase primer concentration ◆ For short products, decrease extension time ◆ For long products, increase extension time Excessive product yield: ◆ Decrease primer concentration ◆ For long products, decrease extension temperature to 68°C.
Step 4	**If necessary, perform further optimization** (to be used if steps 1–3 do not produce desired results) ◆ Use 1x Q-Solution* ◆ Use 1x Q-Solution* and decrease annealing temperature in steps of 1°C

**Q-Solution is a PCR additive provided with HotStarTaq DNA Polymerase.*

References

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Multiplex PCR using 10 Cells

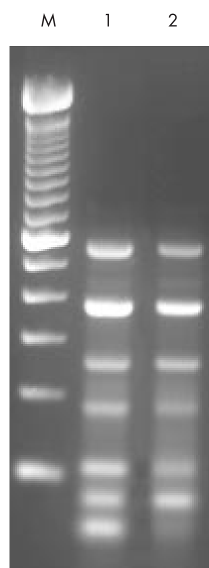

Figure 5 *Multiplex PCR amplification of fragments from the murine p53 gene using 10 cells. Reactions were prepared in parallel (**1, 2**) with 20% of the primer concentrations used in Figure 4. **M**: markers.*

Conclusion

Fast and efficient optimization of multiplex PCR was performed following the guidelines presented in Table 1. The combination of HotStarTaq DNA Polymerase and QIAGEN PCR Buffer produced successful results in a stringent test using template DNA from just 10 cells. When using standard concentrations of template DNA (100 ng per reaction), only PCR performed with HotStarTaq DNA Polymerase enabled detection of all specific products.

HotStarTaq DNA Polymerase from QIAGEN is inactive during reaction setup and is activated only by incubation for 15 minutes at 94°C. Since most nonspecific products are the result of primer annealing at low temperatures, this high-temperature activation step prevents the formation of primer–dimers and other nonspecific products that can interfere with the amplification of the desired products.

QIAGEN PCR Buffer significantly increases the specificity of amplification (4), and in these experiments permitted high-yield amplification of all multiplex products. Although special buffer formulations and the use of different buffer concentrations have been recommended for multiplex PCR (5, 6), QIAGEN PCR Buffer performed well without modification in multiplex PCR even using low-concentration template DNA. The balanced combination of NH_4^+ and K^+ in QIAGEN PCR Buffer allows specific amplification over a wide range of Mg^{2+} concentrations and annealing temperatures, which is necessary for robust performance in multiplex PCR (4, 7). ■

Ordering Information

Product	Contents	Cat. No.
HotStarTaq DNA Polymerase (250)	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer*, 5x Q-Solution, 25 mM $MgCl_2$	203203
HotStarTaq DNA Polymerase (1000)	1000 units HotStarTaq DNA Polymerase, 10x PCR Buffer*, 5x Q-Solution, 25 mM $MgCl_2$	203205
HotStarTaq Master Mix Kit (250)	3 x 0.85 ml HotStarTaq Master Mix† containing 250 units HotStarTaq DNA Polymerase total, 2 x 1.7 ml distilled water	203443
HotStarTaq Master Mix Kit (1000)	12 x 0.85 ml HotStarTaq Master Mix† containing 1000 units HotStarTaq DNA Polymerase total, 8 x 1.7 ml distilled water	203445

** Contains 15 mM $MgCl_2$*
† Provides a final concentration of 1.5 mM $MgCl_2$ and 200 µM each dNTP

Purchase of HotStarTaq DNA Polymerase or *Taq* DNA Polymerase products is accompanied by a limited license to use them in the Polymerase Chain Reaction (PCR) process for research and development activities in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e. an authorized thermal cycler.