Multiplex PCR: Critical Parameters and Step-by-Step Protocol

BioTechniques 23:504-511 (September 1997)

O. Henegariu, N.A. Heerema, S.R. Dlouhy, G.H. Vance and P.H. Vogt[1]
Indiana University, Indianapolis, IN, USA and [1]Heidelberg University, Heidelberg, Germany

ABSTRACT

By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. While numerous papers and manuals discuss in detail conditions influencing the quality of PCR in general, relatively little has been published about the important experimental factors and the common difficulties frequently encountered with multiplex PCR. We have examined various conditions of the multiplex PCR, using a large number of primer pairs. Especially important for a successful multiplex PCR assay are the relative concentrations of the primers at the various loci, the concentration of the PCR buffer, the cycling temperatures and the balance between the magnesium chloride and deoxynucleotide concentrations. Based on our experience, we propose a protocol for developing a multiplex PCR assay and suggest ways to overcome commonly encountered problems.

INTRODUCTION

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 (6), this method has been successfully applied in many areas of DNA testing, including analyses of deletions (2,8), mutations (14) and polymorphisms (11), or quantitative assays (10) and reverse-transcription PCR (7).

The role of various reagents in PCR has been discussed (3,9,12,13), and protocols for multiplex PCR have been described by a number of groups. However, few studies (5,15) have presented an extensive discussion of some of the factors (e.g., primer concentration, cycling profile) that can influence the results of multiplex analysis. In this study, over 50 loci were amplified in various combinations in multiplex PCRs using a common, KCl-containing PCR buffer. Because of specific problems associated with multiplex PCR, including uneven or lack of amplification of some loci and difficulties in reproducing some results, a study of the parameters influencing the amplification was initiated. Based on this experience, a step-by-step multiplex PCR protocol was designed (Figure 1), with practical solutions to many of the problems encountered. This protocol should be useful to those using PCR technology in both the research and the clinical laboratories.

MATERIALS AND METHODS

Standard Solutions and Reagents for the PCR

Nucleotides (dNTP) (Pharmacia Biotech [Piscataway, NJ, USA] or Boehringer Mannheim [Indianapolis, IN, USA]) were stored as a 100 mM stock solution (25 mM each dATP, dCTP, dGTP and dTTP). The standard 10× PCR buffer was made as described (Perkin-Elmer, Norwalk, CT, USA) and contained: 500 mM KCl, 100 mM Tris-HCl, pH 8.3 (at 24°C) and 15 mM $MgCl_2$. *Taq* DNA Polymerase was purchased from Life Technologies (Gaithersburg, MD, USA) or from Perkin-Elmer. Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) and glycerol were purchased from Sigma Chemical (St. Louis, MO, USA). Primers were either commercially obtained (Genosys [The Woodlands, TX, USA] or Research Genetics [Huntsville, AL, USA]) or synthesized locally and were used in a final concentration of 10–25 pmol/μL each. One set of primer pairs (sY) was used to map deletions on the human Y chromosome (8,16). Another 10–15 primer pairs were for the Duchenne muscular dystrophy (DMD) gene on human chromosome X (4). Other primers represent various polymorphic loci (microsatellites) on human chromosome 12 (Research Genetics). Primers were combined in multiplex mixtures as described in Table 1 and Figures 2b, 3b

and 5e. Genomic DNA was prepared using a standard sodium dodecyl sulfate (SDS)/proteinase K protocol (Boehringer Mannheim).

Basic PCR Protocol

The basic PCR (25 µL vol) included: autoclaved ultra-filtered water; PCR buffer (1×); dNTP mixture (200 µM each); primer(s) (0.04–0.6 µM each); DMSO, glycerol or BSA (5% - if used); *Taq* DNA polymerase (1–2 U/25 µL) and genomic DNA template (150 ng/25 µL). The components of the reaction can be added in any order, provided that water is added first. Pipetting was done on ice, and the vials were placed from ice directly into the preheated metal block or water bath (94°C) of the thermal cycler. For radioactive labeling, 1 µCi [^{32}P]dCTP (Amersham, Arlington Heights, IL, USA) was added to a 100 µL master mixture immediately before setting up the reaction. Results of PCR were the same when 100- or 25- or 6.2-µL reaction volumes were used. With smaller volumes, pipetting is critical, especially for dNTP. Various thermal cyclers were used during these studies and, with minor cycling adjustments, all performed well.

Gel Analysis of PCR Products

The PCR products of non-polymorphic loci (chromosomes X and Y) were separated by electrophoresis on 3% SeaKem® LE or NuSieve® (3:1) Agarose Gels (FMC BioProducts, Rockland, ME, USA) in 1× TAE [0.04 M Tris-acetate; 0.001 M EDTA (pH 8.0)] or 1× TBE [0.09 M Tris-borate; 0.002 M EDTA (pH 8.0)] buffer, respectively, at room temperature using voltage gradients of 7–10 V/cm. For any given gel analysis, the same volume of PCR products was loaded in each gel slot. Results were visualized after staining the gels in 0.5–1 µg/mL ethidium bromide. Sequencing gels (6% polyacrylamide [PAA]/7 M urea) were used for separation of the PCR products when the loci tested were polymorphic or a higher resolution was required. The equivalent of about 0.2 µL radioactively labeled PCR product was loaded in each gel lane, after mixing it in loading buffer. These gels were run in 0.6× TBE at 1800–2000 V (60 A) for about 2 h. Autoradiographs were obtained after overnight exposure.

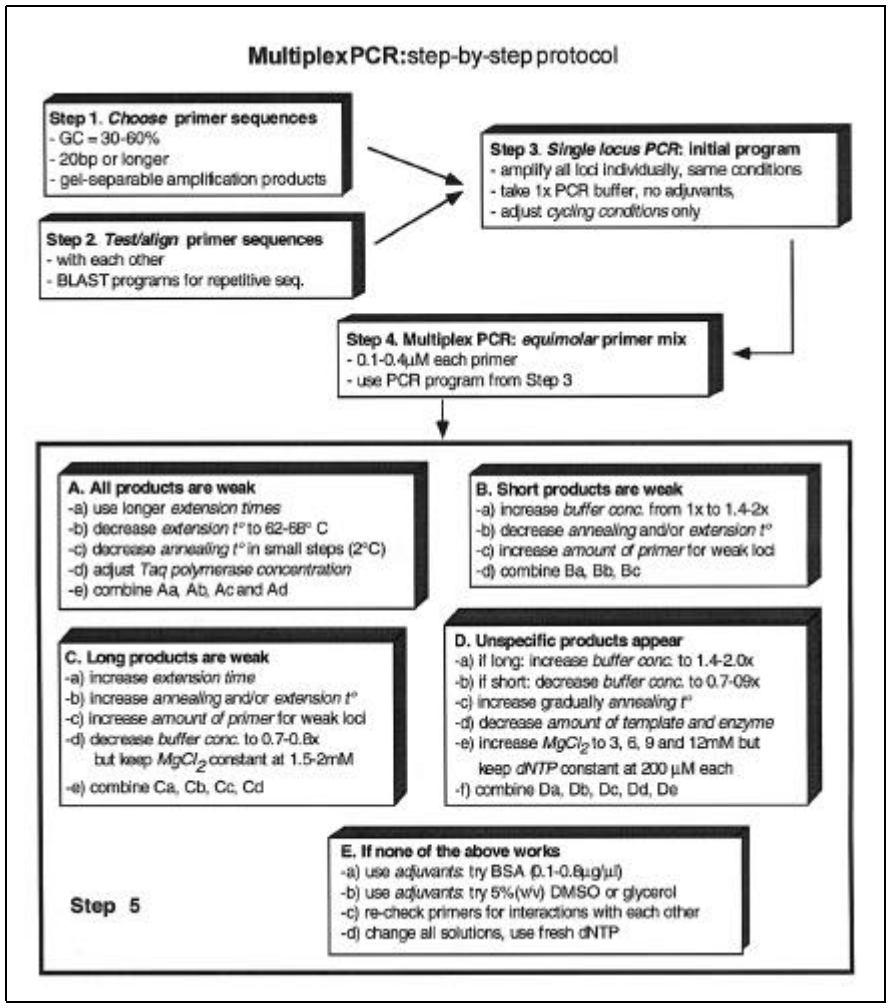

Figure 1. Step-by-step protocol for the multiplex PCR.

RESULTS AND DISCUSSION

Based on many experiments, a protocol for establishing a multiplex PCR has been designed (Figure 1), including a number of practical solutions to some of the most commonly encountered problems. For convenience and ease of use, the words in italic characters link the scheme with various points presented in Materials and Methods and the following subsections.

Basic Principles of the Multiplex PCR

DNA primers (Steps 1 and 2). Primer selection followed simple rules: primer length of 18–24 bp or higher and a GC content of 35%–60%, thus having an annealing temperature of 55°-58°C or higher. Longer primers (DMD primers, 28-30 bp) allowed the reaction to be performed at a higher annealing temperature and yielded less unspecific products. To calculate the melting point and test for possible primer-primer interactions, "Primers 1.2" (a freeware that can be downloaded from **ftp.bio.indiana.edu**) was used. To test for possible repetitive sequences, many of the primers used were aligned with the sequence databases at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) family of programs.

Single locus PCR (Step 3). A PCR

program to amplify all loci individually was designed. Reaction mixture included 1× PCR buffer, 0.4 µM each primer, 5% DMSO and 1 U *Taq* DNA polymerase/25 µL reaction volume. Results of PCR were compared when the reactions were done consecutively in the same thermal cycler, or in parallel, in machines of the same model and in machines of different models or manufacturers. Results were very reproducible when the same machine or same machine model was used but could markedly differ when the same exact PCR program was used on thermal cyclers from different manufacturers. However, with adjustments in only the cycling conditions, results became reproducible even in different types of machines. We have observed that for the loci tested (100–300-bp long), yield of some products was increased by decreasing the extension temperature. For individually amplified loci, the annealing time (from 30–120 s) and the extension time (from 30–150 s) did not visibly influence the results, but the specificity and yield of PCR product were increased or decreased by changes in annealing temperature. To amplify the 22 Y-specific loci (Figure 2a), PCR program A gave best results (Table 2).

Multiplex PCR: equimolar primer mixture (Step 4). Combining the primers in various mixtures and amplifying many loci simultaneously (Table 1 and Figure 2b), required alteration/optimization of some of the parameters of the reaction. When the multiplex reaction is performed for the first time, it is useful to add the primers in equimolar amounts. The results will suggest how the individual primer concentration and other parameters need to be changed. Examples of some useful changes are illustrated and discussed below; however, these examples do not necessarily follow the exact order as listed in the protocol (Figure 1) since a number of parameters (e.g., extension temperature) are referred to more than once.

Table 1. List of Primers Used in the Multiplex Mixtures

Name (locus)	Size (bp)	Name (locus)	Size (bp)	Name (locus)	Size (bp)	Name (locus)	Size (bp)
Y-1		**Y-2**		**Y-3**		**Y-4**	
sY84 DYS273	326	sY143 DYS231	311	sY86 DYS148	320	sY14 SRY	472
sY134 DYS224	301	sY157 DYS240	285	sY105 DYS201	301	sY95 DYS280	303
sY117 DS209	262	sY81 DYS271	209	sY82 DYS272	264	sY127 DYS218	274
sY102 DYS198	218	sY182 KALY	125	Y6HP35 DYS274	226	sY109 DYF43S1	233
sY151 KALY	183	sY147 DYS232	100	Y6PHc54 n.a.	166	sY149 DYS1	132
sY94 DYS279	150			sY153 DYS237	139		
sY88 DYS276	123			sY97 DYS281	104		

DMD exon No.	Size (bp)	DMD exon No.	Size (bp)	Name (locus)	Size (bp)
X-1		**X-3**		**12-1**	
No. 45	547	PM	535	AFM263zd1 D12S332	317-341
PM	535	No. 3	410	AFM205ve5 D12S93	271-291
No. 19	459	No. 50	271	AFM205xg3 D12S310	243-253
No. 17	416	No. 6	202	AFM211wb6 D12S98	228-238
No. 51	388	No. 60	139	AFM206ze5 D12S94	183-201
No. 8	360			AFM299zd5 D12S349	165-181
No. 12	331			AFM135xe3 D12S87	142-168
No. 44	268			AFM122xf6 D12S85	105-125
No. 4	196				

n.a. = locus not assigned.
PM = promoter region

Optimization of Multiplex PCR Cycling Conditions

Extension temperature (Step 5, A–C). Figure 2c illustrates the results obtained when four different amplification mixtures containing equal amounts (0.4 µM each) of different Y-chromosome primers were subjected to multiplex PCR with program A and program B (Table 2); the latter program had a higher extension temperature (72°C) and longer annealing and extension times. In general, there was a visibly higher yield of PCR products for mixtures Y-1, Y-3* and Y-4 with program A. In addition, with program B, some products are missing (in Y-1 and Y-2) and some unspecific products appear (in Y-1 and Y-3*). The results with program B were considered less desirable overall and suggested that the higher extension temperature in program B decreased the amplification of

Table 2. Cycling Conditions/PCR Programs

	Program A	Program B	Program C
First Denaturing	94°C, 4 min	94°C, 4 min	94°C, 4 min
Denature	94°C, 30 s	94°C, 30 s	94°C, 30 s
Anneal	54°-56°C, **30 s***	54°C, **1 min**	54°C, **45 s**
Extend	**65°C, 1 min** 32 cycles	**72°C, 1 min, 20 s** 32 cycles	**65°C, 2 min** 32 cycles
Final Extension	65°C, 3 min	72°C, 3 min	65°C, 3 min
	Program D	**Program E**	**Program F**
First Denaturing	94°C, 4 min	94°C, 4 min	none
Denature	94°C, 30 s	94°C, 30 s	94°C, 30-45 s
Anneal	55°C, 30 s	54°C, 45 s	**56°-58°C, 45 s**
Extend	65°C, **4 min** 32 cycles	65°C, **2 min** **45 cycles**	**68°C, 2 min, 30 s** 35 cycles
Final Extension	65°C, 3 min	65°C, 5 min	none

Bold characters show most important modifications when programs are compared.
*Program A was used with two different annealing temperatures, according to the type of PCR amplification (see Results and Discussion).

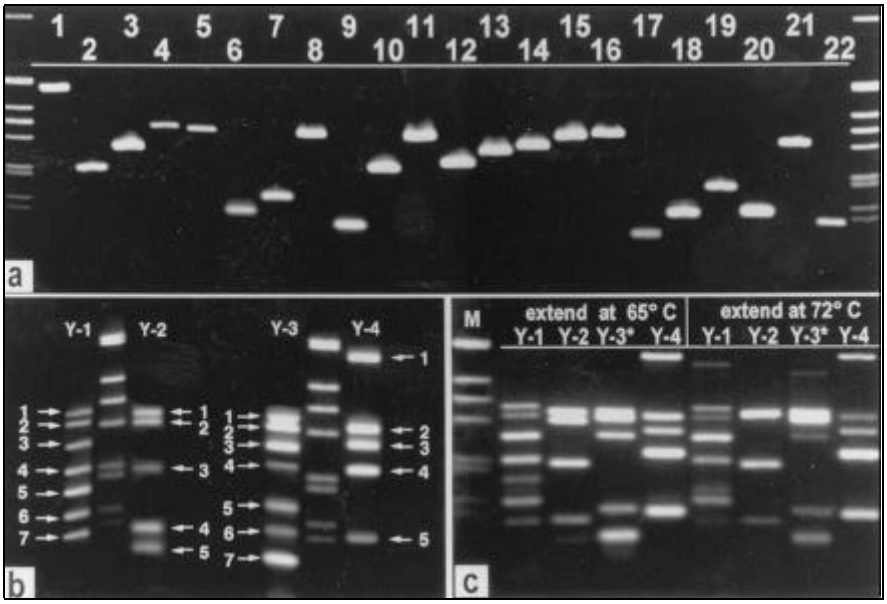

Figure 2. (a) Single-locus PCR. Amplification of the sY loci using 1× PCR buffer and program A. On the gel, the products are arranged in increasing order of sY number (1=sY14, 2=sY81, 3=sY82, 4=sY84, 5=sY86, 6=sY88, 7=sY94, 8=sY95, 9=sY97, 10=sY102, 11=sY105, 12=sY109, 13=sY117, 14=sY127, 15=sY134, 16=sY143, 17=sY147, 18=sY149, 19=sY151, 20=sY153, 21=sY157 and 22=sY182). All products had the expected length, and there was no visible unspecific amplification. In all gels, lanes without a label show the size marker (1-kb ladder; Life Technologies). (b) Optimized multiplex reactions. Multiplex PCR with primer mixtures Y-1 (sY84, sY134, sY117, sY102, sY151, sY94 and sY88), Y-2 (sY143, sY157, sY81, sY182 and sY147), Y-3 (sY86, sY105, sY82, Y6HP35, Y6Phc54, sY153 and sY97) and Y-4 (sY14, sY95, sY127, sY109 and sY149) in 1.6× PCR buffer (PCR program E). Mix Y-3* is mixture Y-3 without primers Y6HP35 and Y6Phc54. Arrows indicate the expected amplification products. (c) Extension temperature. Multiplex PCR with mixtures Y-1 to Y-4 with PCR programs A and B (Table 2). All amplification products are visible in the first four lanes (extension at 65°C). In the last four lanes (extension at 72°C), bands are missing in Y-1 and Y-2, and unspecific products appear in Y-1 and Y-3*. Length marker in all figures = 1-kb ladder. In all images, electrophoresis was conducted from top to bottom.

some loci, even though we tried to compensate using a longer annealing time and slightly longer extension time.

Extension time (Step 5, A, B and D). In multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products. Two experiments illustrated the influence of the extension time. In one experiment, a Y-chromosome primer pair (Y6BaH34pr, 910bp) was added to a X-chromosome primer mixture (X-3). The results (Figure 3b) showed that increasing the extension time in the multiplex PCR (program A vs. program D) increased the amount of longer products. In another experiment, four Y multiplex mixtures were amplified using PCR programs C and A (Figure 3a and Table 2). Visibly higher yields of PCR products were obtained for all Y mixtures when a longer extension time was used.

Annealing time and temperature (Step 5, A–D; Figure 1). Modification of the annealing time from 20 s to 2 min did not alter the amplification efficiency (not shown), but the annealing temperature was one of the most important parameters. Although many individual loci could be specifically amplified at 56°–60°C, our experience showed that lowering the annealing temperature by 4°–6°C was required for the same loci to be co-amplified in multiplex mixtures. This is demonstrated in Figure 3, d–f, which depict an optimal multiplex annealing temperature of 54°C for primers individually usable at 60°C. At 54°C, although unspecific amplification probably occurs (e.g., Figure 3c), it is overcome by the concurrent amplification of an increased number of specific loci in the multiplex reaction and thus remains invisible. Similarly, when many specific loci are simultaneously amplified, the more efficiently amplified loci will negatively influence the yield of product from the less efficient loci. This is due to the fact that PCR has a limited supply of enzyme and nucleotides, and all products compete for the same pool of supplies.

Number of PCR cycles. Primer mixture Y-3* was used to amplify two different genomic DNA samples, stop-

ping the reaction after increasing numbers of cycles (Figure 4a). One of the two genomic DNAs was a better template, possibly due to the higher quality and/or amount of DNA. Both of them, however, show a gradual increase in the yield of all bands with the number of cycles. The most obvious variation in the amount of products was around 25 cycles (for ethidium bromide-stained gels). Twenty-eight to thirty cycles are usually sufficient for a reaction; little is gained by increasing cycle number up to 60.

Optimization of Multiplex Reaction Components

Initially, there was some variation from test to test when the same PCR program was used (e.g., Figures 2c and 3a). Solving this reproducibility problem required adjustments of PCR components.

Amount of primer (Step 5, B and C). Initially, equimolar primer concentrations of 0.2–0.4 µM each were used in the multiplex PCR (Figure 3c), but there was uneven amplification, with some of the products barely visible even after the reaction was optimized for the cycling conditions. Overcoming this problem required changing the proportions of various primers in the reaction, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong" loci. The final concentration of the primers (0.04–0.6 µM) varied considerably among the loci and was established empirically.

dNTP and $MgCl_2$ concentrations (Step 5D).

dNTP. The significance of the dNTP concentration was tested in a multiplex PCR test with primer mixture Y-4. Magnesium chloride concentration was kept constant (3 mM), while the dNTP concentration was increased stepwise from 50–1200 µM each (Figure 4b). The best results were at 200 and 400 µM each dNTP, values above which the amplification was rapidly inhibited. Lower dNTP concentration (50 µM) allowed PCR amplification but with visibly lower amounts of products. dNTP stocks are sensitive to thawing/freezing cycles. After 3–5 such cycles, multiplex PCRs often did not work well; products became almost completely invisible. To avoid such problems, small aliquots (2–4 µL, 10–20 reactions) of dNTP (25 mM each) can be made and kept frozen at -20°C and centrifuged before use. This "low stability" of dNTP is not so obvious when single loci are amplified.

$MgCl_2$. A recommended magnesium chloride concentration in a standard PCR is 1.5 mM at dNTP concentrations of around 200 µM each. To test the influence of magnesium chloride, a multiplex PCR (mixture Y-3) was performed, keeping dNTP concentration at 200 µM and gradually increasing magnesium chloride from 1.8–10.8 mM (Figure 4c). Amplification became more specific (unspecific bands disappeared), and the products acquired comparable intensities (at 10.8 mM). In PCRs with up to 20 mM $MgCl_2$, products became barely visible, as if the reactions were inhibited (not shown).

dNTP/$MgCl_2$ balance. To work properly, *Taq* DNA polymerase requires free magnesium (besides the

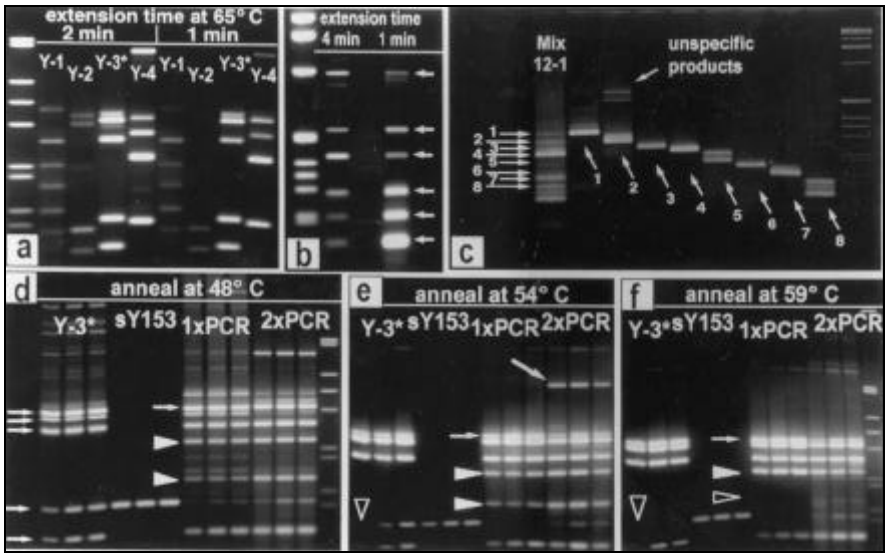

Figure 3. (a) Extension time. Multiplex PCR of mixtures Y-1 to Y-4, comparing PCR programs C (2-min extension time) and A (1-min extension time, 54°C annealing temperature). Comparison of equivalent lanes shows an improvement in yield when extension time is 2 min. Some faint unspecific bands appear, possibly due to the low buffer concentration (1×). (b) Extension time. Multiplex PCR with mixture X-3 (primers for DMD gene exons Nos. PM, 3, 50, 6, 60) and primer pair Y6BaH34 (910-bp product, upper arrow). Primers giving shorter amplification products are preferentially amplified with short extension times (1-min, program A). (c) Equimolar primer mixture. PCR with individual primer pairs of mixture 12–1 (separate and multiplex), using program F. Products are arranged on the gel according to their decreasing length. Individual products have comparable intensities. When equimolar amounts of primers were mixed for the multiplex reaction (first lane), some products were not efficiently amplified but unspecific products disappeared. (d–f) Annealing temperature, buffer concentration and number of primers. Multiplex amplification of mixture Y-3* (first three lanes in each gel), primer pair sY 153 (lanes 4–6) and mixture Y-3 (lanes 7–12 in 1× or 2× PCR buffer) on three different template DNAs using three PCR programs differing in annealing temperature (48°, 54° or 59°C). Lanes 1–9 on each gel show reactions in 1× PCR buffer. Lanes 10–12 on each gel show reactions in 2× PCR buffer. Lanes 7–12 on each gel (under 1× PCR and 2× PCR) were with primer set Y-3. The very last lane in Figure 3, d and f is the marker (1-kb ladder). Small horizontal arrows indicate the expected products of mixture Y-3* (five products) including the longest specific product on the gel. Oblique arrow (3e) indicates a strong unspecific product. Solid arrowheads indicate the two extra products expected in mixture Y-3 (total of seven products) compared with Y-3*. Arrowhead outlines show positions of some missing products (e.g., 3e, first lane). With multiplex amplification at 48°C, many unspecific bands appear. In 1× PCR buffer, the sY153 product is stronger when amplified in mixture Y-3* (5 primer pairs) than in mixture Y-3 (7 primer pairs), which shows that at least for some products, an increased number of simultaneously amplified loci can influence the yield at some specific loci. Raising the PCR buffer concentration from 1× to 2× allows a more even amplification of all specific products and helps to decrease the intensity of many longer unspecific products (compare lanes 7–9 vs. 10–12). The strong 470–480-bp unspecific band (oblique arrow) seen with 2× buffer was eliminated by varying the proportion of different primers in the reaction (compare with Y-3, Figure 2b). At 59°C the sY153 product can be seen only when 2× buffer is used or when the locus is amplified alone.

magnesium bound by the dNTP and the DNA) (9). This is probably why increases in the dNTP concentrations (Figure 4b) can rapidly inhibit the PCR, whereas increases in magnesium concentration often have positive effects (Figure 4c). By combining various amounts of dNTP and $MgCl_2$, it was found that 200 µM each dNTP work well in 1.5–2 mM $MgCl_2$, whereas 800 µM dNTP require at least 6–7 mM $MgCl_2$. The threshold for the reaction was roughly 1 mM $MgCl_2$ when 200 µM dNTP was used, with reduced PCR amplification below this $MgCl_2$ concentration.

PCR buffer (KCl) concentration. Comparison of PCR buffers (Step 5, B–D).

KCl or PCR buffer concentration. Raising the buffer concentration to 2× (or only the KCl concentration to 100 mM) improved the efficiency of the multiplex reaction (Figure 4d and also Figure 3, d–f), this effect being more important than using any of the adjuvants tested (DMSO, glycerol or BSA). Generally, primer pairs with longer amplification products worked better at lower salt concentrations, whereas primer pairs with short amplification products worked better at higher salt concentrations, where longer products become harder to denature (compare 0.4× with 2.8× in Figure 4d). For example, pair sY94 (melting point ca. 58°C) is favored over both sY88 (melting point ca. 58°C) and sY151 (melting point ca. 52°C) at 0.8× buffer but not at higher salt concentrations. The proper buffer concentration may help overcome other factors (product size, GC

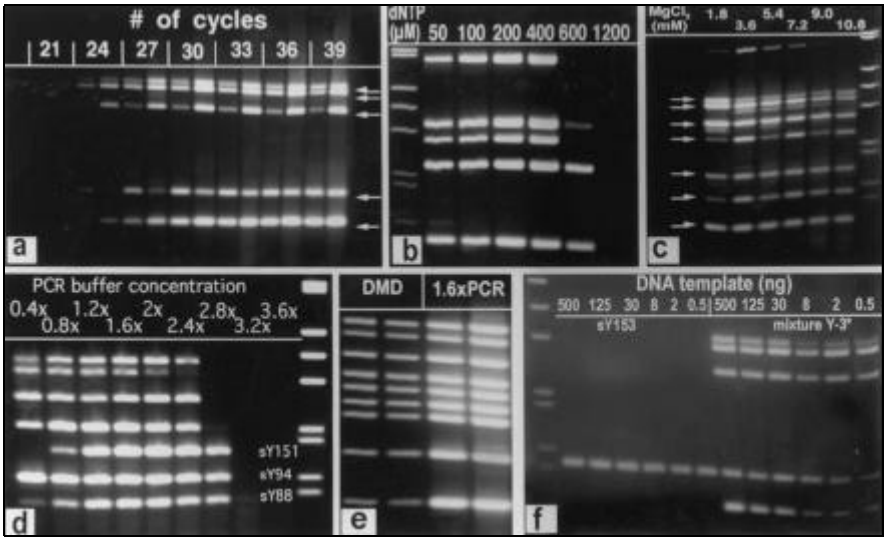

Figure 4. (a) Number of cycles. Amplification with two different DNA templates using primer mixture Y-3* in 1.4× PCR buffer, with increasing numbers of cycles by units of three. (b) dNTP concentration. PCR amplification using mixture Y-4 in 2× PCR buffer (3 mM $MgCl_2$) and increasing concentrations of dNTP (50, 100, 200, 400, 600 and 1200 µM). Most efficient amplification is seen at concentrations of 200–400 µM dNTP. Further increase in the dNTP concentration inhibits the reaction when $MgCl_2$ concentration is kept constant. (c) $MgCl_2$ concentration. Multiplex PCR was performed with mixture Y-3 in 1.4× PCR buffer, using PCR program E and gradually raising the concentration of $MgCl_2$. (d) PCR buffer concentration. Amplification products of mixture X-1 (DMD gene exons Nos. 45, PM, 19, 17, 51, 8, 12, 44 and 4) using increasing concentrations of PCR buffer and program E. As the stringency in the reaction mixture decreases, shorter products are amplified more efficiently, whereas the intensity of longer products gradually decreases. For this particular primer mixture, the optimal buffer concentration was 1.2×–1.6×. (e) Comparison of PCR buffers. Comparison of multiplex PCR of mixture X-1 in the DMD buffer and the 1.6× KCl-based PCR buffer, using the same proportion of ingredients (DNA, *Taq* DNA polymerase, primer amount) and PCR program E. For every DNA sample tested, the amounts of products were increased when 1.6× PCR buffer was used. Only four lanes are shown, although the gel had more samples loaded, and identical results were observed. (f) Amount of template DNA. Various amounts of template DNA were amplified with primer sY153 and mixture Y-3* in 2× PCR buffer with program E. Reaction volumes were 25 µL. There were no major differences using 500 or 30 ng DNA; however, some bands became weaker as the DNA amount was further decreased to 0.5 ng/25 µL reaction. No major differences due to the DNA template concentration were seen when primer pair sY153 was used alone.

content, etc).

Comparison of PCR buffers. We have compared a previously described multiplex PCR buffer (6), called "DMD" for the purpose of this paper, with the less complex, KCl-based buffer in the multiplex reaction. The 5× "DMD" buffer contains 83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl (pH 8.8), 33.5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 850 μg/mL BSA, and it is used at 1× final concentration together with 10% DMSO and 1.5 mM each dNTP (1,2,4). When tested with the DMD gene primers (mixture X-1) the regular KCl-based PCR buffer at 1.6× worked better than the "DMD" buffer (visibly higher yield of products) (Figure 4e). Results were reproducible in dozens of patient DNA samples tested. The KCl-based buffer is less complex and easier to adjust and optimize. Also, since the fidelity of the *Taq* DNA polymerase is higher at lower dNTP concentrations (9), using the KCl-based buffer (which requires much less dNTP) can be beneficial when the PCR products need to be further analyzed for mutations.

Amount of template DNA and *Taq* DNA polymerase (Step 5, A and D). At DNA template quantities between 30 and 500 ng/25 μL reaction, mixture Y-3* showed no significant differences (Figure 4f); however, below 30 ng the amount of some of the products decreased. When the amount of template DNA is very low (pg of DNA), efficient and specific amplification can be obtained by further lowering the annealing temperature, sometimes by as much as 10°–12°C (data not shown).

Different concentrations of *Taq* DNA Polymerase (Perkin-Elmer) were tested using primer mixture Y-3 (Figure 5a). The most efficient enzyme concentration seemed to be around 0.4 μL or 2 U/25 μL reaction volume. Too much enzyme, possibly because of the high glycerol concentration in the stock solution, resulted in an unbalanced amplification of various loci and a slight increase in the background. Five native *Taq* DNA polymerases, from five different sources, performed similarly on mixture Y-4 in 1.6× PCR buffer using 2 U/25 μL (Figure 5b).

Use of adjuvants: DMSO, glycerol, BSA (Step 5E). Various authors recommend DMSO and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific products) of PCR, when used in concentrations varying between 5%–10% (vol/vol) (9). However, in the multiplex reaction, these adjuvants gave conflicting results. For example, 5% DMSO improved the amplification of some products and decreased the amount of others, whereas some loci were not influenced at all (Figure 5c). Similar results were obtained with 5% glycerol (data not shown). Therefore, the usefulness of these adjuvants needs to be tested in each case. BSA, in concentrations up to 0.8 μg/μL (higher than previously described) increased the efficiency of the PCR much more than either DMSO or glycerol. BSA did not have an inhibitory effect on any of the loci amplified (data not shown).

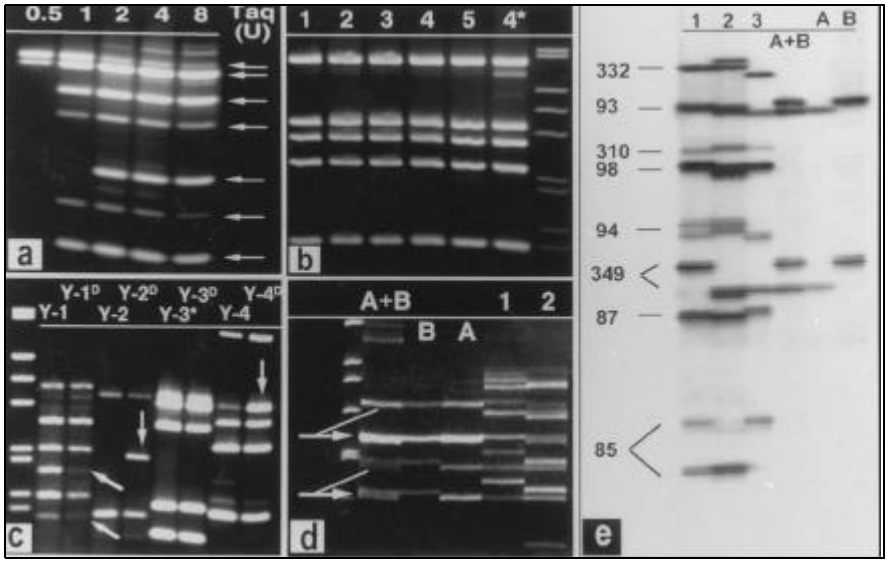

Figure 5. (a) Amount of enzyme. Amplification products of mixture Y-3, after using 0.5, 1, 2, 4 and 8 U/25 μL reaction volume are shown. Arrows indicate the expected positions of the amplification products. The most appropriate enzyme concentration was between 1–2 U/25 μL. (b) Source of enzyme. Multiplex PCR of mixture Y-4 in 1.6× PCR buffer uses *Taq* DNA polymerases from five sources. Lane 4* shows the products obtained when the enzyme from lane 4 was used in the buffer provided by the vendor. An unspecific product appeared. (c) Use of adjuvants. Comparative multiplex PCR using the Y-specific mixtures with 5% DMSO (superscript D) and without DMSO, in 1× buffer. Loci sY151 and sY88 from mixture $Y\text{-}1^D$ (oblique arrows) are stronger when no DMSO is used. However, DMSO helps amplify (vertical arrows) locus sY81 in mixture Y-2 and locus sY95 in mixture Y-4. (d) Nondenaturing PAA gel separation. Simultaneous PCR amplification of loci D12S93 and D12S349 performed on genomic DNA from two human-rodent cell lines, GM 10868 (A) and GM 12072 (B), each containing a different copy of human chromosome 12, and their combination (A+B). Although in lanes A and B each locus should have yielded only one allele (i.e., one band), on a nondenaturing polyacrylamide gel, each of the two expected products (arrows) was accompanied by another one running slower on the gel (oblique lines). A similar aspect persisted in lane A+B. Lanes labeled 1 and 2 show separation of amplification products of mixture 12-1 (including eight D12S polymorphic loci, the numbers of which are indicated to the left side of Panel e) on two different genomic template DNAs. (e) Denaturing PAA gels. Sequencing gel separation of the same multiplex products as in Figure 4e, after "hot" PCR. Lanes A and B show mono-allelic amplification of the respective polymorphic loci (D12S93 and D12S349). Lane A+B shows simultaneous amplification of both alleles at each locus. Lanes 1 and 2 show results using primer mixture 12-1 on two different human genomic DNAs, with polymorphisms detected at some loci. Lane 3 shows results after multiplex PCR with mixture 12-1 on DNA from hybridoma cell line GM 10868 yielding homozygous amplification of all loci tested. Numbers to the left of the figure indicate the D12S loci tested.

Agarose vs. Polyacrylamide Gels

Agarose. Multiplex PCR products, differing from each other by 30–40 bp in length could be conveniently separated on 3% gels of commonly used agaroses, such as SeaKem or NuSieve (FMC BioProducts). Overnight separation of products at lower voltage gradients notably decreased the sharpness of individual PCR bands, especially when the products were smaller than 400–500 bp.

Polyacrylamide (PAA) gels. To separate PCR products differing in only

a few bp in length (for example, microsatellite markers), 6%–10% PAA gels are required. Whereas nondenaturing PAA gels work very well for nonpolymorphic loci, unusual bands appear when microsatellites are separated on this type of gel. For example, in an analysis of some chromosome 12-specific polymorphic loci from two hybridomas, each carrying one copy of human chromosome 12, for every locus tested, there were 2 bands on the nondenaturing PAA gel (e.g., Figure 5d). On a regular 6% PAA/7 M urea sequencing gel, radiolabeled PCR products showed one allele at each locus (compare products between Figure 5, d and e).

We have presented a series of examples of testing various parameters to optimize multiplex PCR. Optimal combination of two of these parameters, annealing temperature and KCl (salt) concentration, is essential in any PCR to obtain highly specific amplification products. Magnesium chloride concentration needs only to be proportional to the amount of dNTP, and these values can be constant for any reaction. Although gradually increasing magnesium chloride concentrations may further influence the reaction, the other two parameters mentioned seem to be much more important in obtaining specific, high yields of PCR product(s). In multiplex PCR, adjusting primer amount for each locus is also essential. Figure 1 illustrates a rational approach for developing efficient multiplex PCRs. The list of various factors that can influence the reaction is by no means complete. Nevertheless, optimization of the parameters as presented in this work should provide a basic way of approaching some of the common problems of multiplex PCR.

REFERENCES

1. **Abbs, S. and M. Bobrow.** 1992. Analysis of quantitative PCR for the diagnosis of deletion and duplication carriers in the dystrophin gene. J. Med. Genet. *29*:191-196.
2. **Anonymous.** 1992. Diagnosis of Duchenne and Becker muscular dystrophies by polymerase chain reaction. A multicenter study. JAMA *267*:2609-2615.
3. **Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl.** 1994. The polymerase chain reaction, p. 15.1.1.-15.1.4. *In* K. Janssen (Ed.), Current Protocols in Molecular Biology, Vol. 2. Greene Publishing Associates and John Wiley & Sons, New York.
4. **Beggs, A.H., M. Koenig, F.M. Boyce and L.M. Kunkel.** 1990. Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. Hum. Genet. *86*:45-48.
5. **Chamberlain, J.S., R.A. Gibbs, J.E. Ranier and C.T. Caskey.** 1990. Multiplex PCR for the diagnosis of Duchenne muscular dystrophy, p. 272-281. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.), PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego.
6. **Chamberlain, J.S., R.A. Gibbs, J.E. Ranier, P.N. Nguyen and C.T. Caskey.** 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. *16*:11141-11156.
7. **Crisan, D.** 1994. Molecular diagnostic testing for determination of myeloid lineage in acute leukemias. Ann. Clin. Lab. Sci. *24*:355-363.
8. **Henegariu, O., P. Hirschmann, K. Kilian, S. Kirsch, C. Lengauer, R. Maiwald, K. Mielke and P. Vogt.** 1994. Rapid screening of the Y chromosome in idiopathic sterile men, diagnostic for deletions in AZF, a genetic Y factor expressed during spermatogenesis. Andrologia *26*:97-106.
9. **Innis, M.A. and D.H. Gelfand.** 1990. Optimization of PCRs, p 3-13. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.), PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego.
10. **Mansfield, E.S., J.M. Robertson, R.V. Lebo, M.Y. Lucero, P.E. Mayrand, E. Rappaport, T. Parrella, M. Sartore, S. Surrey and P. Fortina.** 1993. Duchenne/Becker muscular dystrophy carrier detection using quantitative PCR and fluorescence-based strategies. Am. J. Med. Genet. *48*:200-208.
11. **Mutirangura, A., F. Greenberg, M.G. Butler, S. Malcolm, R.D. Nicholls, A. Chakravarti and D.H. Ledbetter.** 1993. Multiplex PCR of three dinucleotide repeats in the Prader-Willi/Angelman critical region (15q11-q13): molecular diagnosis and mechanism of uniparental disomy. Hum. Mol. Genet. *2*:143-151.
12. **Saiki, R.K.** 1989. Optimization of the polymerase chain reaction, p. 25-30. *In* H.A. Erlich, R. Gibbs and H.H. Kazazian Jr. (Eds.), Current Communications in Molecular Biology. CSH Laboratory Press, Cold Spring Harbor, NY.
13. **Saiki, R.K.** 1989. The design and optimization of the PCR, p. 7-16. *In* H.A. Erlich (Ed.), PCR Technology: Principles and Applications for DNA Amplification. Stockton Press, New York.
14. **Shuber, A.P., J. Skoletsky, R. Stern and B.L. Handelin.** 1993. Efficient 12-mutation testing in the CFTR gene: a general model for complex mutation analysis. Hum. Mol. Genet. *2*:153-158.
15. **Vandenvelde, C., M. Verstraete and D. Van Beers.** 1990. Fast multiplex polymerase chain reaction on boiled clinical samples for rapid viral diagnosis. J. Virol. Methods *30*:215-227.
16. **Vogt, P.H., A. Edelmann, S. Kirsch, O. Henegariu, P. Hirschmann, P. Kiesewetter, F.M. Köhn, W.B. Schill et al.** 1996. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. Hum. Mol. Genet. *5*:933-943.

Received 6 June 1996; accepted 25 March 1997.

Address correspondence to:

Octavian Henegariu
Department of Medical and Molecular Genetics
Indiana University School of Medicine
Medical Research and Library Building
975 W Walnut Street
Indianapolis, IN 46202-5251, USA
Internet: ohenega@indyunix.iupui.edu