Multiplex PCR: Rapid DNA Cycling in a Conventional Thermal Cycler

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Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more target sequences are simultaneously amplified in the same reaction. In the present study we investigated the limits to which the duration of multiplex PCR steps can be shortened using the thermal cycler Gene Amp PCR system 9600 (Perkin Elmer, Oak Brook, IL). The present multiplex PCR assay simultaneously detects five different herpes viruses (HSV-1, HSV-2, VZV, CMV, and EBV) and assesses sample suitability in a single amplification round of 40 cycles. It appears that when six target

sequences are simultaneously amplified in multiplex PCR, extension time is a critical parameter. Using a PCR protocol of 0 sec at 95°C, 0 sec at 60°C, and 0 sec at 74°C with Platinum Taq DNA polymerase (Life Technologies, Gaithersburg, MD), we were able to reduce the total cycling time of the multiplex PCR assay to as little as 55 min, without affecting the yield of PCR products or the specificity of the assay. It may be necessary to optimize each specific apparatus and template, but any such optimization would be trivial. *J. Clin. Lab. Anal.* 17:108–112, 2003. © 2003 Wiley-Liss, Inc.

Key words: multiplex PCR; rapid DNA cycling

INTRODUCTION

Automated in vitro DNA amplification with a heat-stable DNA polymerase requires temperature cycling of the sample (1). Temperature transition rates in commercial instruments are usually 1–3°C/sec when metal blocks or water are used for thermal equilibration and samples are contained in 0.2-mL polypropylene tubes (2). Conventional three-temperature PCR protocols require a sample to be heated to 94–96°C to denature double-stranded DNA into two single strands. The reaction sample is then cooled down to 40–60°C to anneal target-specific primers onto the single-stranded DNA. Finally, the reaction is heated again to the optimum reaction temperature (72–78°C) for Taq DNA polymerase to add complementary nucleotides and thus extend the primers to produce two copies of the original target strands. Any temperature cycling protocol for DNA amplification can be divided into six segments: three endpoint temperatures and three temperature transitions. Time spent in transition is usually wasted. The transition times after elongation and denaturation have no function. Rapid cooling after denaturation favors the kinetics process (primer

annealing to template/product) over the equilibrium process (product dimerization) (3). A significant portion of the cycling time is spent on heating and cooling the sample, as opposed to time spent at optimal denaturation, annealing, and elongation temperatures. Primer extension by a thermostable polymerase at the high temperatures at which polymerase chain reaction (PCR) is carried out proceeds at the rate of 35–100 bp/sec (4). Primer annealing, driven by a large primer excess, also requires <1 sec (5). The only purpose for prolonging the PCR steps for more than a few seconds would be to allow thermal equilibration to occur from the heat source to the heating block, the tube wall, and the reaction solution. For rapid temperature cycling, the

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sample container is just as important as the thermal cycler. However, no matter what container is used, temperature equilibration will always be achieved faster if the sample volume is small, the container wall is thin, and the surface-to-volume ratio of the sample exposed to the container wall is high. Extended amplification times of 2 or more hours are common in convenient block-type thermal cyclers. Time is required both to bring the sample block to temperature and to transfer heat to the sample through a microcentrifuge tube (1). In an alternative thermal cycling approach, air is used for the heat transfer and the samples are contained in thin glass capillary tubes (5,6). The rapid temperature response produced by this method allows transition times to be reduced to a minimum. Amplified product from genomic DNA can be obtained within 1 hr or less; however, these techniques require specialized equipment and thermal cyclers.

Multiplex PCR is a variant of PCR in which two or more target sequences are simultaneously amplified in the same reaction. Since it was first described in 1988 (7), this method has been successfully applied in many areas of DNA testing (8–15). Multiplex PCR requires that primers lead to amplifications of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions. The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize the reaction conditions. The primer pairs could enable equally efficient amplifications to be obtained for their target sequence in a multiplex reaction. Primers with nearly identical optimum annealing temperatures should work under similar conditions if they anneal with single copy sequences. Primer concentration is a critical parameter for successful multiplex PCR. If all primers in a reaction anneal with equal efficiency, they can be used at the same concentrations (12). Important factors in performing a successful multiplex PCR assay include 1) the relative concentration of the primers while the primer concentration is adjusted for each target sequence, 2) the concentration of the PCR buffer, 3) the balance between the magnesium chloride and deoxynucleotide concentrations, 4) the cycling temperatures, and 5) the amount of template DNA and Taq DNA polymerase used (16).

Because of specific problems associated with multiplex PCR, including no or uneven amplification of some target sequences, and difficulties in reproducing some results, we investigated the limits to which the duration of PCR steps could be shortened using the thermal cycler Gene Amp PCR system 9600 (Perkin Elmer, Oak Brook, IL).

MATERIALS AND METHODS

Multiplex PCR Primers

The PCR primers were synthesized with an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). The primer sequences H1P32/H1M32, H2M40/H2P4, VP22/VM20, CP15/CM3, and EP5/EM3 were chosen from the DNA sequence of HSV-1, HSV-2, VZV, CMV, and EBV viruses, respectively, and have already been reported (12,14,15). The primers for the α -tubulin coding sequence have also been previously reported (13).

Viruses–DNA Extraction

HSV-1 strain F, HSV-2 strain G, VZV strain Ellen, CMV strain AD169, and EBV strain P-3 (American Type Culture Collection, Manassas, VA) were used in the multiplex PCR assay. All viral strains except EBV were propagated in tubes in MRC-5 cells (American Type Culture Collection) grown in minimal essential medium (Dulbecco's modified enriched medium/MEM-D) supplemented with 2% fetal calf serum, and examined daily until a typical cytopathic effect (CPE) was observed. When 75–90% CPE was observed, the tubes were frozen at -70°C . EBV strain P-3 was subjected to DNA extraction directly from the stock solution.

DNA extraction from infected cell cultures, as well as from uninfected MRC-5 cells, was performed using the Xtreme Genomic Purification Kit II (Pierce, Rockford, IL) according to the manufacturer's instructions. In addition, whole HSV-1 (strain McIntyre), HSV-2 (strain G), VZV (strain Rod), CMV (strain AD169), and EBV (strain B95-8) virus particles, counted by negative-stain electron microscopy (Advanced Biotechnologies Inc., Columbia, MA) prior to DNA extraction, were used in the present multiplex PCR assay in order to normalize the initial target concentration of each virus preparation (15).

DNA Amplification–Multiplex PCR

To optimize the multiplex PCR, titration of the primer concentration, primer-to-template ratio, dNTP/MgCl₂ balance, and Taq DNA concentration was performed (15).

The amplification was performed in 0.2-mL, thin-walled tubes with the Gene Amp PCR system 9600. The tubes were incubated at 95°C for 4 min to denature the templates and activate the DNA polymerases. Forty initial amplification cycles of 30 sec at 95°C , 40 sec at 60°C , and 50 sec at 74°C were compared to 0 sec at 95°C , 0 sec at 60°C , and 0 sec at 74°C . Amplification was carried out in a 50- μL final volume containing either

5 μ L, or 7.5 μ L 10 $\times$ reaction buffer (Stratagene, La Jolla, CA; or Invitrogen, Life Technologies, Gaithersburg, MD), 0.25 mM from deoxynucleotide triphosphate (dNTP), 50 pmol from each of the 12 primers, and 2.5 U of either cloned Pfu DNA polymerase (Stratagene) or Platinum Taq DNA polymerase (Invitrogen, Life Technologies). An optimal free Mg^{2+} concentration of 0.5 mM was necessary for the Platinum Taq DNA polymerase, yielding a final concentration of $MgCl_2$ of 1.5 mM. For the Pfu DNA polymerase, the Mg^{2+} concentration was 2 mM, and it was present in the final 1 $\times$ dilution of the cloned Pfu DNA polymerase 10 $\times$ reaction buffer. The normalized initial target concentration from each virus added in the multiplex PCR assay was adjusted to be 10³ active virus particles per reaction volume (50 μ L) counted by negative-stain electron microscopy (Advanced Biotechnologies Inc., Columbia, MA). No ramp times were programmed. After the last cycle, the samples were incubated for 10 min at 78°C to complete the extension of primers. Then 10 μ L of each amplified product were analyzed by agarose gel electrophoresis on 3.5% agarose (Nusieve 3:1; FMC, Rockland, ME) containing 1 μ g/mL of ethidium bromide in 1 $\times$ TBE buffer, and visualized in a UV transilluminator (Foto/Phoresis; Fotodyne, Hartland, WI).

RESULTS

Optimal results (maximum band intensity and minimal background nonspecific staining) were obtained with 0.8 and 1 mM dNTPs, while lower levels resulted in a decrease in the efficiency of amplification. PCR amplification reaction mixtures should contain free Mg^{2+} in excess of the total dNTP concentration. An optimal free Mg^{2+} concentration of 0.5 mM is necessary for Platinum Taq DNA polymerase. For Pfu DNA polymerase-based PCR, fidelity is optimal when the total Mg^{2+} concentration is 2 mM in a standard reaction mixture. This total Mg^{2+} concentration is present in the final 1 $\times$ dilution of cloned Pfu DNA polymerase 10 $\times$ reaction buffer, according to the manufacturer's instruction manual.

Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces the misextension of incorrect nucleotides at the 3' end of the primers. The annealing temperature (60°C) was chosen to be as high as possible, and care was taken to avoid reducing the sensitivity of the assay. The choice of a stringent hybridization temperature of 60°C avoided parasite bands while maintaining a normal amplification rate (Fig. 1, lanes 1–12).

Forty amplification cycles of 30 sec at 95°C, 40 sec at 60°C, and 50 sec at 74°C were compared to 0 sec at

95°C, 0 sec at 60°C, and 0 sec at 74°C (Fig. 1, lanes 1–4 and 5–12, respectively).

We used 74°C as the standard elongation temperature. For medium-length targets in the present multiplex PCR assay (147–527 bp), the sum of which was 1,614 bp, an elongation duration of 0 sec was sufficient for Platinum Taq DNA polymerase (Fig. 1, lanes 9–12), whereas 50 sec was necessary for cloned Pfu DNA polymerase to achieve similar target synthesis (Fig. 1, lanes 1 and 2). Raising the buffer concentration to 1.5 $\times$ improves the efficiency of the multiplex reaction when the amplification procedures are short (17). This beneficial effect was seen clearly on both DNA polymerases (Fig. 1, lanes 7, 8, 11, and 12) when 0 sec was used in the three-temperature PCR protocol, but cloned Pfu DNA polymerase produced only a subset of the expected target amplifications (Fig. 1, lane 8).

DISCUSSION

Extended amplification times of 2 or more hours are common in convenient block-type thermal cyclers. Time is required both to bring the sample block to temperature and to transfer heat to the sample through a microcentrifuge tube (1). Extended amplification times of 2–3 hr are common, and long transition times make it difficult to determine optimal temperatures and times for each stage.

Annealing and denaturation are claimed to occur almost instantaneously once the sample has reached the appropriate temperature. Rapid cooling after denaturation favors the kinetics process (primer annealing to template/product) over the equilibrium process (product dimerization). Classical kinetic studies on DNA renaturation also predict rapid annealing because of the high primer concentration used in DNA amplification (3).

Our results suggest that denaturation and annealing do in fact occur very quickly in DNA amplification. The time required for denaturation and annealing apparently are minimal. With the Gene Amp PCR system 9600, the calculated sample temperature reaches 1° from the set temperature (94°C for denaturation and 59°C for annealing).

The initial target concentration in PCR is typically 10²–10⁴ copies per reaction volume. As the concentration of the DNA target increases, the melting temperature (T_m) of that duplex also increases. If the denaturation temperatures are too low for complete denaturation (88–90°C), the T_m of that duplex also increases with increasing DNA concentration, and the denaturation becomes less efficient. If this is the case, and incomplete denaturation occurs, there will be no exponential increase of the target DNA for these cycles. It is important, therefore, to denature templates at 95°C.

Fig. 1. Multiplex PCR: conventional time cycling (lanes 1–4, 30 sec at 95°C, 40 sec at 60°C, 50 sec at 74°C) and rapid time cycling (lanes 5–12, 0 sec at 95°C, 60°C, and 74°C). Lanes M: molecular weight marker $\phi\chi 174$ RF DNA/HaeIII fragments. Lanes 1, 3, 5, 7, 9, and 11: negative control (uninfected cells). Lanes 2, 4, 6, 8, 10, 12: initial target concentration adjusted to be 10^3 active virus particles from each virus type (HSV-1, EBV, HSV2, CMV, and VZV) added in the multiplex PCR assay. Lanes 1, 2, and 5–8: cloned Pfu DNA polymerase. Lanes 3, 4, and 9–12: Platinum Taq DNA polymerase. Lanes 1–6, 9, and 10: $1 \times$ reaction buffer. Lanes 7, 8, 11, and 12: $1.5 \times$ reaction buffer. The predicted size in bp of each specific for each virus amplicon is indicated on the right.

With the Gene Amp PCR system 9600, using thin-walled reaction tubes, a denaturation time of 0 sec was sufficient with a 50- μ L reaction volume. The amount of time spent on annealing has a direct effect on the specificity of the amplification reaction. As more time is spent at the annealing temperature, there is a greater chance of nonspecific priming and extension of undesirable product. The 50- μ L reaction volume tubes required 0 sec. An annealing time of 0 sec also maximizes specificity. Primer extension is not instantaneous and the elongation time required depends on the length of the amplified product. Taq DNA polymerase is highly processive, with an extension rate of more than 100 nucleotides/sec at 70°C.

Since the transition to the optimum extension temperature is more rapid, Taq DNA polymerase has more time to extend the specific product, as well as any nonspecific products. The recommended procedure is to reduce the incubation time, so that only a specific

product is extended and the extension of nonspecific product is minimized.

It appears that extension time in multiplex PCR when six target sequences are amplified simultaneously is a critical parameter that affects the yield of PCR products. For Platinum Taq DNA polymerase, an extension time of 0 sec/1.6 Kb of amplified templates was sufficient for maximum synthesis of six PCR targets. In contrast, cloned Pfu DNA polymerase-based multiplex PCR amplifications required an extension time of 50 sec/1.6 Kb of amplified templates to achieved similar target synthesis. Extension time is the most critical parameter affecting the yield of PCR products obtained when Pfu DNA polymerase, a proofreading DNA polymerase, is used.

In multiplex PCR, thermal cycling parameters should be chosen carefully to ensure that the shortest denaturation times to avoid enzyme inactivation and/or template damage, adequate extension times (depending on the

Taq or Pfu polymerases used) to achieve full-length target synthesis, and annealing temperatures near the primer melting temperature to improve specificity of the desired PCR products are used.

Using these protocols with Platinum Taq DNA polymerase, we were able to reduce the total cycling time of the multiplex PCR assay from 2 hr 40 min to as little as 55 min, without affecting the yield of PCR products or the specificity of the assay. It may be necessary to optimize each specific apparatus and template; however, our experience with the Gene Amp PCR system 9600 and the reported experimental protocol indicates that any such optimization would be trivial.

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