Journal of Clinical Laboratory Analysis 16:47–51 (2002)

Multiplex Polymerase Chain Reaction: A Practical Approach

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Considerable time and effort can be saved by simultaneously amplifying multiple sequences in a single reaction, a process referred to as multiplex polymerase chain reaction (PCR). Multiplex PCR requires that primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions. In addition, methods must be available for the analysis of each individual amplification product from the mixture of all the products. Multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions. For a successful multiplex PCR assay, the relative concentration of the primers, concentration of the PCR buffer, balance between the magnesium chloride and deoxynucleotide concentrations, cycling temperatures, and amount of template DNA and Taq DNA polymerase are important. An optimal combination of annealing temperature and buffer concentration is essential in multiplex PCR to obtain highly specific amplification products. Magnesium chloride concentration needs only to be proportional to the amount of dNTP, while adjusting primer concentration for each target sequence is also essential. The list of various factors that can influence the reaction is by no means complete. Optimization of the parameters discussed in the present review should provide a practical approach toward resolving the common problems encountered in multiplex PCR (such as spurious amplification products, uneven or no amplification of some target sequences, and difficulties in reproducing some results). Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized purified nucleic acids. Where available, full use should be made of external and internal quality controls, which must be rigorously applied. As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical clinical syndromes and/or share similar epidemiological features. J. Clin. Lab. Anal. 16:47–51, 2002.

Key words: multiplex PCR; primer-to-template ratio; dNTP/$MgCl_2$ balance; PCR buffer concentration

INTRODUCTION

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more target sequences can be amplified by including more than one pair of primers in the same reaction. Multiplex PCR has the potential to produce considerable savings of time and effort in the laboratory. Since it was first described in 1988 (1), this method has been successfully applied in many areas of DNA testing, including gene deletion analysis (1), mutation and polymorphism analysis (2,3), quantitative analysis (4), and reverse-transcription (RT)-PCR (5). In the field of infectious diseases, multiplex PCR has been shown to be a valuable tool for identification of viruses (6–10), bacteria (11), and parasites (12).

The optimization of multiplex PCR can pose several difficulties, including poor sensitivity and specificity, and/or preferential amplification of certain specific targets. The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. One of the most important concepts in PCR is that of the optimal primer-to-template ratio. If the ratio is too high, primer-dimers are formed, as also occurs in conditions of very dilute template or excess primer. Primers must usually be in a 10^7 molar excess with respect to template. For most applica-

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Received 19 April 2001; Accepted 16 October 2001

DOI 10.1002/jcla.2058

tions, regardless of template concentration, the primer concentration cannot be raised much higher than 0.5 μM because of primer-dimer formation. Therefore, what determines the primer-to-template ratio is the amount and complexity of the template provided to the reaction. If the primer-to-template ratio is too low, product will not accumulate exponentially, since newly synthesized target strands will renature after denaturation (which reduces the yield considerably), or inhibit the formation of PCR product. Thus, primer dimers may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension (13). When more than a single target is to be amplified from the template, as is the case for multiplex PCR where multiple targets are amplified simultaneously with multiple primer pairs, it is necessary to adjust the primer-to-template ratio to avoid the formation of primer dimers. The optimization of multiplex PCR should aim to minimize such nonspecific interactions. Special attention to primer design parameters, such as homology of primers with their target nucleic acid sequences, length, GC content, and concentration, have to be considered (14). Hot start PCR often eliminates nonspecific reactions (production of primer dimers) caused by primer annealing at low temperature (4–25°C) before commencement of thermocycling (15). Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (primer length of 18–28 bp and a GC content of 45–60%) and should not display significant homology either internally or to one another (8,16).

Preferential amplification of one target sequence over another is a known phenomenon in multiplex PCRs (17). Two major classes of processes that induce this bias have been identified: PCR drift and PCR selection (18). PCR drift is a bias assumed to be due to fluctuation in the interactions of PCR reagents, particularly in the early cycles, which could arise in the presence of very low template concentration (17). PCR selection, on the other hand, is defined as a mechanism which favors the amplification of certain templates due to the properties of the target, the target's flanking sequences, or the entire target genome. These properties include interregion differences in GC content, leading to preferential accessibility of targets within genomes due to secondary structures and the gene copy number within a genome.

The alteration of other PCR components, such as PCR buffer constituents, dNTPs, $MgCl_2$, and enzyme concentrations in multiplex PCR greater than those reported for most uniplex PCRs usually results in considerable improvement in the sensitivity and/or specificity of the test (8). Increasing the concentration of these factors may increase the likelihood of mispriming, with subsequent production of nonspecific amplification products. However, optimization of those components in multiplex PCRs that are designed for simultaneous amplification of multiple targets is usually beneficial. Variation in concentrations of reaction components greater than those used in uniplex PCR reflects the competitive nature of the PCR process.

Prior to application in a clinical setting, multiplex PCRs must be evaluated for their sensitivity and specificity as compared with their corresponding uniplex PCRs (8–10).

Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. The latter is a major contribution to false-positive results due to carryover contamination, although protocols against contamination, including PCR controls (reaction and specimens extraction controls), must be implemented in all PCR protocols. Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered. Multiplex PCRs that amplify target sequences along with the presence of external or internal control target nucleic acids to indicate reaction failure have been developed (9,10).

Annealing temperature is 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 coamplified in multiplex mixtures. 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.

OPTIMIZATION OF MULTIPLEX REACTION COMPONENTS

Amount of Primer

Initially, equimolar primer concentrations of 0.1–0.5 μM each are used in the multiplex PCR. When there is uneven amplification, with some of the products barely visible even after the reaction was optimized for the cycling conditions, changing the proportions of various primers in the reaction is required, 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 (0.04–0.5 μM) may vary considerably among the loci and is established empirically (8). For low copy number or high-complexity DNA, the primer concentration should be 0.3–0.5 μM. For high copy number or low-complexity DNA, the primer concentration should be 0.04–0.4 μM.

dNTP and $MgCl_2$ Concentrations

dNTP

$MgCl_2$ concentration is kept constant (2 mM), while the dNTP concentration is increased stepwise from 0.5–1.6 mM. The best results are between 200 and 400 μM each dNTP values, above which the amplification is rapidly inhibited. Lower dNTP concentration (100 μM each dNTP) allowed

PCR amplification but with visibly lower amounts of products (8). dNTP stocks are sensitive to thawing/freezing cycles. After three to five such cycles, multiplex PCRs often did not work well. To avoid such problems, small aliquots of dNTP can be made and kept frozen at –20°C. This "low stability" of dNTP is not so obvious when single loci are amplified.

$MgCl_2$

Optimization of Mg^{2+} is critical since Taq DNA polymerase is a magnesium-dependent enzyme. In addition to Taq DNA polymerase, the template DNA primers and dNTPs bind Mg^{2+}. Therefore, the optimal Mg^{2+} concentration will depend on the dNTP concentration, specific template DNA, and sample buffer composition. If primers and/or template DNA contain chelators such as EDTA or EGTA, the apparent Mg^{2+} optimum may be shifted. Excessive Mg^{2+} concentration stabilizes the DNA double strand and prevents complete denaturation of DNA, which reduces yield. Excessive Mg^{2+} can also stabilize spurious annealing of primer to incorrect template sites, decreasing specificity. On the other hand, inadequate Mg^{2+} concentration reduces the amount of product.

dNTP/$MgCl_2$ Balance

To work properly, Taq DNA polymerase requires free magnesium (besides the magnesium bound by the dNTP and the DNA). This is probably why increases in the dNTP concentrations can rapidly inhibit the PCR, whereas increases in magnesium concentration often have positive effects. By combining various amounts of dNTP and $MgCl_2$, it was found that 200 µM of each dNTP work well in 1.5–2 mM $MgCl_2$. The threshold for the reaction was roughly 0.5–1 mM $MgCl_2$ over the total dNTP concentration, with reduced PCR amplification below this $MgCl_2$ concentration (8–10).

PCR Buffer Concentration

Raising the buffer concentration to 2X improves the efficiency of the multiplex reaction. This was more effective than any of the adjuvants tested [dimethyl sulfoxide (DMSO), glycerol, and bovine serum albumin (BSA)]. Primer pairs with longer amplification products work better at lower salt concentrations, whereas primer pairs with short amplification products work better at higher salt concentrations (16).

Amount of Template DNA and Taq DNA Polymerase

When the amount of template DNA is very low, efficient and specific amplification can be obtained by further lowering the annealing temperature. Different concentrations of Taq DNA polymerase were tested. The most efficient enzyme concentration seems to be around 2.5 U/50 µl reaction volume. Excessive enzyme quantity, possibly due to the high glycerol concentration in the stock solution, resulted in an unbalanced amplification of various loci and a slight increase in the background. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as $MgCl_2$ and dNTPs, and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance must be directed to the factors affecting annealing and/or extension rates.

Use of Adjuvants: DMSO, Glycerol, BSA

The most difficult multiplex PCR reactions can be significantly improved by using a PCR additive, such as DMSO, glycerol, formamide, and betaine, which relax DNA, thus making template denaturation easier.

In the multiplex PCR, DMSO and glycerol gave conflicting results. Therefore, the usefulness of these adjuvants needs to be tested in each case. BSA, in concentrations up to 0.8 µg/µl increased the efficiency of the PCR much more than did DMSO or glycerol (16).

DISCUSSION

The optimal combination of annealing temperature and salt (buffer) 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 products. In multiplex PCR, adjusting primer amount for each locus is also essential. The list of factors that can influence the reaction is by no means complete. Nevertheless, optimization of the parameters should provide a basic approach to some of the common problems of multiplex PCR (such as spurious amplification products, uneven or no amplification of some target sequences, and difficulties in reproducing some results). While there is no clear theoretical limit to the number of sequences that can be amplified simultaneously, the constraints on establishing conditions for specific and interpretable reactions generally limit the useful number of target sequences. A multiplex PCR reaction to amplify as much as nine segments of the human dystrophin gene has been reported (1,16).

Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized, purified nucleic acids. Where available, full use should be made of external and internal quality controls, which must be rigorously applied (9,10).

As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical clinical syndromes and/or share similar epidemiological features (19,20).

PCR has revolutionized the field of infectious disease diagnosis. To overcome the inherent disadvantage of cost and to improve the diagnostic capacity of the test, multiplex PCRs for the detection of viral, bacterial, and/or other infectious agents in one reaction tube have been developed. Efforts to enhance sensitivity and specificity, and to facilitate automation have resulted in numerous publications regarding the application of multiplex PCR in the diagnosis of infectious agents, especially those which target viral nucleic acids (21).

Multiple techniques, including RT-PCR (20), PCR (1,9–12), real-time PCR (22), ligase chain reaction (23,24), and transcription-mediated amplification (24), have been developed to amplify and detect genes in a manner similar to multiplex assays.

Specific gene fusions observed in solid tumors are extremely useful diagnostic markers. An assay based on multiplexing of primers and probes using real-time PCR, which enables the detection (under identical PCR conditions) of the different fusions, was reported for the routine genetic diagnosis of small round cell tumors (22). The advantage of this multiplex fluorescent analysis of chromosome translocations is that this method diminishes the manipulation of PCR products and lowers considerably the risk of cross-contamination linked to carryover of RT-PCR products. It also constitutes an important step towards the automation of cancer-specific gene fusion detection. The ligase chain reaction (LCR) involves repetitive cycles of ligation of two adjacent pairs of oligonucleotides to form longer ligated products in a template-dependent manner. Multiplex LCR was reported for the simultaneous determination of the normal and mutant alleles causing cystic fibrosis. Discrimination of mutations was achieved using competitive LCR with six oligonucleotides and with multiplex-competitive LCR using 12 oligonucleotides to detect both alleles for two mutations in a single tube (23). Although equipment providing various degrees of automation has been adapted to these techniques, full automation is not yet available. As the processes become more refined, automated, and standardized, nucleic acid detection methods will displace current methods for rapid clinical laboratory diagnosis. Analogies between immunoassay development and progress in nucleic acid detection methods are appropriate. The first immunoassays were research-laboratory methods that were subsequently transferred to clinical laboratories. The ultimate automated DNA analyzer would combine a multiplex diagnostic assay with DNA array technology, which would offer a fundamentally new approach to routine DNA diagnostics (25). Microarrays require specialized robotics and imaging equipment that are currently only commercially available from a few companies. However, miniaturization and automation are the keys to high-throughput applications. Genomic microarray technology allows the assessment of multiple gene targets that bind amplified products; this has many applications, including cancer, microbiology diagnostics, genotyping, gene expression, pharmacogenomics, and environmental control (25–27).

The future of automation of DNA diagnostic methods holds much exciting promise.

REFERENCES

1. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 1988;16:11141–11156.
2. Rithidech KN, Dunn JJ, Gordon CR. Combining multiplex and touch down PCR to screen murine microsatellite polymorphisms. BioTechniques 1997;23:36–45.
3. Shuber AP, Skoletsky J, Stern R, Handelin BL. Efficient 12-mutation testing in the CFTR gene: a general model for complex mutation analysis. Hum Mol Genet 1993;2:153–158.
4. Zimmermann KD, Schogl B, Plaimauer B, Manhalter JW. Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. BioTechniques 1996;21:480–484.
5. Crisan D. Molecular diagnostic testing for determination of myeloid lineage in acute leukemias. Ann Clin Lab Sci 1994;24:355–363.
6. Heredia A, Soriano V, Weiss SH, et al. Development of a multiplex PCR assay for the simultaneous detection and discrimination of HIV-1, HIV-2, HTLV-I and HTLV-II. Clin Diagn Virol 1996;7:85–92.
7. Casa I, Pozo F, Trallero G, Echevarria JM, Tenorio A. Viral diagnosis of neurological infections by RT multiplex PCR: a search for entero- and herpes viruses in a prospective study. J Med Virol 1999; 57:145–151.
8. Markoulatos P, Samara V, Siafakas N, Plakokefalos E, Spyrou N, Moncany M. Development of a quadriplex polymerase chain reaction for human cytomegalovirus. J Clin Lab Anal 1999;13:99–105.
9. Markoulatos P, Mangana-Vougiouka O, Koptopoulos G, Nomikou K, Papadopoulos O. Detection of sheep poxvirus in skin biopsy samples by a multiplex polymerase chain reaction. J Virol Methods 2000;14: 214–219.
10. Markoulatos P, Georgopoulou A, Kotsovassilis C, Karabogia-Karaphillides P, Spyrou N. Detection and typing of HSV-1, HSV-2 and VZV by a multiplex polymerase chain reaction. J Clin Lab Anal 2000;14:214–219.
11. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J Clin Microbiol 1997;35:2854–2858.
12. Harris E, Kropp G, Belli A, Rodriguez B, Agabian N. Single step multiplex PCR assay for characterization of New World Leishmania complexes. J Clin Microbiol 1998;36:1989–1995.
13. Ruano G, Fenton W, Kidd KK. Biphasic amplification of very dilute DNA samples via "booster" PCR. Nucleic Acids Res 1989;17: 5407.
14. Brownie J, Shawcross S, Theaker J, et al. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res 1997;25:3235–3241.
15. Chou Q, Russel M, Birch DE, Raymond J, Bloch W. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy number amplifications. Nucleic Acids Res 1992;11:1717–1723.
16. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques 1997;23:504–511.
17. Mutter GL, Boyton KA. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res 1995;23:1411–1418.
18. Wagner A, Blackstone N, Cartwright P, et al. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst Biol 1994;43:250–261.
19. Jackson R, Morris DJ, Cooper RJ, Bailey AS, Klapper PE, Cleator

GM. Multiplex polymerase chain reaction for adenovirus and herpes simplex virus in eye swabs. J Virol Methods 1996;56:41–48.
20. Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J Clin Microbiol 1998;36:2990–2995.
21. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE. Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 2000;13:559–570.
22. Peter M, Gilbert E, Delattre O. A multiplex real-time PCR assay for the detection of gene fusions observed in solid tumors. Lab Invest 2001;81:905–912.
23. Fang P, Bouma S, Jon C, Gordon J, Beaudet AL. Simultaneous analysis of mutant and normal alleles for multiple cystic fibrosis mutations by the ligase chain reaction. Hum Mutat 1995;6:144–151.
24. Allain JP. Genomic screening for blood-borne viruses in transfusion settings. Clin Lab Hematol 2000;22:1–10.
25. Cuzin M. DNA chips: a new tool for genetic analysis and diagnostics. Transfus Clin Biol 2001;8:291–296.
26. Ladner DP, Leamon JH, Hamann S, et al. Multiplex detection of hotspot mutations by rolling circle-enabled universal microarrays. Lab Invest 2001;81:1079–1086.
27. Li J, Chen S, Evans DH. Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR. J Clin Microbiol 2001;39:696–704.