Board of Trustees of the Leland Stanford Junior University v. Roche Molecular Systems, Inc. et al Doc. 187 Att. 3

EXHIBIT C

Dockets.Justia.com

1

OPTIMIZATION OF PCRs

Michael A. Innis and David H. Gelfand

Polymerase Chain Reaction (PCR) is an ingenious new tool for molecular biology that has had an effect on research similar to that of the discovery of restriction enzymes and the Southern blot. PCR is so sensitive that a single DNA molecule has been amplified, and single-copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels. PCR can also be utilized for rapid screening and/or sequencing of inserts directly from aliquots of individual phage plaques or bacterial colonies. Enhancements, such as the use of thermostable DNA polymerases and automation, of the method invented by Kary Mullis (K. B. Mullis, U.S. patent 4,683,195, July 1987; U.S. patent 4,683,202, July 1987) (Saiki *et al.* 1985; Mullis *et al.* 1986; Mullis and Faloona 1987) have fostered the development of numerous and diverse PCR applications throughout the research community. Unquestionably, no single protocol will be appropriate to all situations. Consequently, each new PCR application is likely to require optimization. Some often encountered problems include: no detectable product or a low yield of the desired product; the presence of nonspecific background bands due to mispriming or misextension of the primers; the formation of "primer–dimers" that compete for amplification with

PCR Protocols: A Guide to Methods and Applications
Copyright © 1990 by Academic Press, Inc. All rights of reproduction in any form reserved.

Case 3:05-cv-04158-MHP Document 187-4 Filed 08/29/2007 Page 3 of 11

the desired product; and mutations or heterogeneity due to misincorporation. The objective of this chapter is to expedite the optimization process by discussing parameters that influence the specificity, fidelity, and yield of the desired product. These recommendations derive from our practical experience using native or recombinant *Taq* DNA polymerases obtained from Perkin-Elmer Cetus Instruments.

Standard PCR Amplification Protocol

While the standard conditions will amplify most target sequences, they are presented here principally to provide starting conditions for designing new PCR applications. It can be highly advantageous to optimize the PCR for a given application, especially repetitive diagnostic or analytical procedures in which optimal performance is necessary.

---

1. Set up a 100-μl reaction in a 0.5-ml microfuge tube, mix, and overlay with 75 μl of mineral oil:
   - Template DNA (10^5 to 10^6 target molecules*)
   - 20 pmol each primer ($T_m > 55$°C preferred)
   - 20 m*M* Tris–HCl (pH 8.3) (20°C)
   - 1.5 m*M* $MgCl_2$
   - 25 m*M* KCl
   - 0.05% Tween 20
   - 100 μg/ml of autoclaved gelatin or nuclease-free bovine serum albumin
   - 50 μ*M* each dNTP
   - 2 units of *Taq* DNA polymerase

   * 1 μg of human single-copy genomic DNA equals 3×10^5 targets; 10 ng of yeast DNA equals 3×10^5 targets; 1 ng of *Escherichia coli* DNA equals 3×10^5 targets; 1% of an M13 plaque equals 10^6 targets.
2. Perform 25 to 35 cycles of PCR using the following temperature profile:

Denaturation	96°C, 15 seconds (a longer initial time is usually desirable)
Primer Annealing	55°C, 30 seconds
Primer Extension	72°C, 1.5 minutes

3. Cycling should conclude with a final extension at 72°C for 5 minutes. Reactions are stopped by chilling to 4°C and/or by addition of EDTA to 10 m*M*.

Enzyme Concentration

A recommended concentration range for *Taq* DNA polymerase (Perkin-Elmer Cetus) is between 1 and 2.5 units (SA = 20 units/pmol) (Lawyer *et al.* 1989) per 100-μl reaction when other parameters are optimum. However, enzyme requirements may vary with respect to individual target templates or primers. When optimizing a PCR, we recommend testing enzyme concentrations ranging from 0.5 to 5 units/100 μl and assaying the results by gel electrophoresis. If the enzyme concentration is too high, nonspecific background products may accumulate, and if too low, an insufficient amount of desired product is made.

Note: Taq DNA polymerase from different suppliers may behave differently because of different formulations, assay conditions, and/or unit definitions.

Deoxynucleotide Triphosphates

Stock dNTP solutions should be neutralized to pH 7.0, and their concentrations should be determined spectrophotometrically. Primary stocks are diluted to 10 m*M*, aliquoted, and stored at −20°C. A working stock containing 1 m*M* each dNTP is recommended. The stability of the dNTPs during repeated cycles of PCR is such that approximately 50% remains as dNTP after 50 cycles (Corey Levenson, personal communication).

Deoxynucleotide concentrations between 20 and 200 μ*M* each result in the optimal balance among yield, specificity, and fidelity. The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Both the specificity and the fidelity of PCR are increased by using lower dNTP concentrations than those originally recommended for Klenow-mediated PCR (1.5 m*M* each).

Low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides (Innis *et al.* 1988). One should decide on the lowest dNTP concentration appropriate for the length and composition of the target sequence; e.g., 20 μM each dNTP in a 100-μl reaction is theoretically sufficient to synthesize 2.6 μg of DNA or 10 pmol of a 400-bp sequence. Recently, the use of low, uniform dNTP concentrations (2 μM each) enabled highly sensitive ($1/10^7$), allele-specific amplification of *ras* point mutations (Ehlen and Dubeau 1989).

Magnesium Concentration

It is beneficial to optimize the magnesium ion concentration. The magnesium concentration may affect all of the following: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer–dimer artifacts, and enzyme activity and fidelity. *Taq* DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and dNTPs. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total dNTP concentration. Note that the presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium optimum.

Other Reaction Components

A recommended buffer for PCR is 10 to 50 mM Tris–HCl (between pH 8.3 and 8.8) when measured at 20°C; however, an extensive survey of other buffers has not been performed. Tris is a dipolar ionic buffer having a pK_a of 8.3 at 20°C, and a Δ pK_a of −0.021/°C. Thus, the true pH of 20 mM Tris (pH 8.3) at 20°C varies between 7.8 and 6.8 during typical thermal cycling conditions.

Up to 50 mM KCl can be included in the reaction mixture to facilitate primer annealing. NaCl at 50 mM, or KCl above 50 mM, inhibits *Taq* DNA polymerase activity (Innis *et al.* 1988).

While DMSO is useful in PCRs performed with the Klenow fragment of *E. coli* DNA polymerase I, 10% DMSO inhibits the activity of *Taq* DNA polymerase by 50% (see Chapter 16) and its use is not recommended for most applications [one exception is the protocol of

Chamberlain *et al.* (1988) that describes amplifying multiple sequences in the same reaction; also see Chapter 33].

Gelatin or bovine serum albumin (100 μg/ml) and nonionic detergents such as Tween 20 or Laureth 12 (0.05 to 0.1%; Mazer Chemicals, Gurnee, Illinois) are included to help stabilize the enzyme, although many protocols work well without added protein.

Primer Annealing

The temperature and length of time required for primer annealing depend upon the base composition, length, and concentration of the amplification primers. An applicable annealing temperature is 5°C below the true T_m of the amplification primers. Because *Taq* DNA polymerase is active over a broad range of temperatures, primer extension will occur at low temperatures, including the annealing step (Innis *et al.* 1988). The range of enzyme activity varies by two orders of magnitude between 20 and 85°C. Annealing temperatures in the range of 55 to 72°C generally yield the best results. At typical primer concentrations (0.2 μ*M*), annealing will require only a few seconds.

Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at the 3′ end of primers. Therefore, stringent annealing temperatures, especially during the first several cycles, will help to increase specificity. For maximum specificity in the initial cycle, *Taq* DNA polymerase can be added after the first denaturation step during primer annealing. Low extension temperature together with high dNTP concentrations favors misextension of primers and extension of misincorporated nucleotides. For these reasons, some investigators have argued that PCRs should perform better using longer primers and only two temperatures; e.g., from 55 to 75°C for annealing and extension, and 94 to 97°C for denaturation and strand separation (Kim and Smithies 1988; Will Bloch, personal communication).

Primer Extension

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are tradi-

tionally performed at 72°C because this temperature was near optimal for extending primers on an M13-based model template (D. Gelfand, unpublished). Estimates for the rate of nucleotide incorporation at 72°C vary from 35 to 100 nucleotides second^{-1} depending upon the buffer, pH, salt concentration, and the nature of the DNA template (Innis *et al.* 1988; Saiki and Gelfand 1989). An extension time of one minute at 72°C is considered sufficient for products up to 2 kb in length. However, longer extension times may be helpful in early cycles if the substrate concentration is very low, and at late cycles when product concentration exceeds enzyme concentration (approximately 1 n*M*) (Will Bloch, personal communication).

Denaturation Time and Temperature

The most likely cause for failure of a PCR is incomplete denaturation of the target template and/or the PCR product. Typical denaturation conditions are 95°C for 30 seconds, or 97°C for 15 seconds; however, higher temperatures may be appropriate, especially for G+C-rich targets. It only takes a few seconds to denature DNA at its strand-separation temperature (T_{ss}); however, there may be lag time involved in reaching T_{ss} inside the reaction tube. It is a good idea to monitor the temperature inside one reaction tube with a low-mass thermocouple probe (see Chapter 51). Incomplete denaturation allows the DNA strands to "snap back" and, thus, reduces product yield. In contrast, denaturation steps that are too high and/or too long lead to unnecessary loss of enzyme activity. The half-life of *Taq* DNA polymerase activity is >2 hours, 40 minutes, and 5 minutes at 92.5, 95, and 97.5°C, respectively (See Chapter 16).

Cycle Number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized. A common mistake is to execute too many cycles. To quote Kary Mullis, "If you have to go more than 40 cycles to amplify a

single-copy gene, there is something seriously wrong with your PCR." Too many cycles can increase the amount and complexity of nonspecific background products (see Plateau Effect). Of course, too few cycles give low product yield. Some guidelines for number of cycles versus starting target concentration are provided:

Number of target molecules	Number of cycles
3×10^5	25 to 30
1.5×10^4	30 to 35
1×10^3	35 to 40
50	40 to 45

Primers

Primer concentrations between 0.1 and 0.5 μM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent artifact termed a primer–dimer. Nonspecific products and primer–dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs, and primers, resulting in a lower yield of the desired product.

Some simple rules aid in the design of efficient primers. Typical primers are 18 to 28 nucleotides in length having 50 to 60% G+C composition. The calculated T_ms for a given primer pair should be balanced. For this purpose, one can use the rule-of-thumb calculation of 2°C for A or T and 4°C for G or C (Thein and Wallace 1986). Depending on the application, T_ms between 55°C and 80°C are desired. One should avoid complementarity at the 3′ ends of primer pairs as this promotes the formation of primer–dimer artifacts and reduces the yield of the desired product. Also, runs (three or more) of C′s or G′s at the 3′ ends of primers may promote mispriming at G+C-rich sequences and should be avoided, when possible, as should palindromic sequences within primers. If all else fails, it usually helps to try a different primer pair. A less obvious reason for some primers failing to work is the presence of secondary structure in the template DNA. In this case, substitution of 7-deaza-2′-deoxyGTP for dGTP has been very useful (see Chapter 7).

The design of special-purpose primers is discussed in other chap-

ters. Briefly, primers may contain 5′ extensions or mismatches for incorporating restriction enzyme sites, an ATG start codon, or promoter sequences into the target sequence (see Chapter 11). Mismatched bases can be placed internally for mutagenesis (see Chapter 22). Degenerate primers can be used to isolate novel genes on the basis of similarity and/or amino acid sequence (see Chapters 5 and 6). Some authors have suggested using inosine in primers instead of using degenerate primers (Knoth *et al.* 1988). When using degenerate primers, it helps to avoid degeneracy at the 3′ ends, because mismatched bases are inefficiently extended.

Plateau Effect

The term "plateau effect" is used to describe the attenuation in the exponential rate of product accumulation that occurs during late PCR cycles concomitantly with the accumulation of 0.3 to 1 pmol of the intended product. Depending on reaction conditions and thermal cycling, one or more of the following may influence plateau: (1) utilization of substrates (dNPTs or primers); (2) stability of reactants (dNTPs or enzyme); (3) end-product inhibition (pyrophosphate, duplex DNA); (4) competition for reactants by nonspecific products or primer–dimer; (5) reannealing of specific product at concentrations above 10^{-8} M (may decrease the extension rate or processivity of *Taq* DNA polymerase or cause branch-migration of product strands and displacement of primers); and (6) incomplete denaturation/strand separation of product at high product concentration.

An important consequence of reaching plateau is that an initially low concentration of nonspecific products resulting from mispriming events may continue to amplify preferentially. Optimizing the number of PCR cycles is the best way to avoid amplifying background products.

Fidelity Considerations

Conditions that promote misincorporation include when deoxynucleotide concentrations are well below the K_m (i.e., <1 μM) or when the concentration of one dNTP is low relative to the other

three. We demonstrated that a respectable "chain-termination" sequencing ladder can be generated without using dideoxynucleotides by limiting one dNTP in each of four separate sequencing reactions (Innis *et al.* 1988). In contrast, we did not observe misincorporation bands (background on sequencing gels) when the concentration of the four dNTPs was >10 μM each and balanced. We recommend using balanced concentrations of dNTPs to diminish misincorporation errors. Because misincorporated bases cannot be proofread (*Taq* lacks a 3′ to 5′ exonuclease activity) and mismatched bases are inefficiently extended, misincorporation errors that do occur during PCR promote chain termination. Chain termination restricts the amplification of defective molecules and helps to maintain fidelity.

What determines whether a mismatch is extended? Petruska *et al.* (1988) showed (for *Drosophila* DNA polymerase α) that enzymatic discrimination against elongating mismatched termini is based mainly on K_m differences: a matched A–T terminus was found to be extended 200 times faster than a G–T mismatch was and 1400 and 2500 times faster than C–T and T–T mismatches were, respectively. The same is likely to be true for *Taq* DNA polymerase; therefore, the concentration of dNTPs in the reaction is predicted to have a substantial effect on the fidelity of PCR (at high dNTP concentrations, i.e., >1 mM, mismatches will be extended more efficiently). In combination, high-temperature annealing/extension (>55°C) and low dNTP concentrations (10 to 50 μM each) give the highest fidelity in the final PCR product.

Literature Cited

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.

Ehlen, T., and L. Dubeau. 1989. Detection of *ras* point mutations by polymerase chain reaction using mutation-specific, inosine-containing oligonucleotide primers. *Biochem. Biophys. Res. Commun.* **160**:441–447.

Innis, M. A., K. B. Myambo, D. H. Gelfand, and M. A. D. Brow. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. USA* **85**:9436–9440.

Kim, H.-S., and O. Smithies. 1988. Recombinant fragment assay for gene targeting based on the polymerase chain reaction. *Nucleic Acids Res.* **16**:8887–8903.

Knoth, K., S. Roberds, C. Poteet, and M. Tamkun. 1988. Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. *Nucleic Acids Res.* **16**:10932.

Lawyer, F. C., S. Stoffel, R. K. Saiki, K. Myambo, R. Drummond, and D. H. Gelfand. 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *J. Biol. Chem.* **264**:6427–6437.

Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155**:335–350.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51**:263–273.

Petruska, J., M. F. Goodman, M. S. Boosalis, L. C. Sowers, C. Chaejoon, and I. Tinoco, Jr. 1988. Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc. Natl. Acad. Sci. USA* **85**:6252–6256.

Saiki, R. K., and D. H. Gelfand. 1989. Introducing AmpliTaq DNA polymerase. *Amplifications* **1**:4–6.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350–1354.

Thein, S. L., and R. B. Wallace. 1986. The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In *Human genetic diseases: a practical approach* (ed. K. E. Davis), p. 33–50. IRL Press, Herndon, Virginia.