Optimization of Polymerase Chain Reactions

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1. Introduction

The polymerase chain reaction (PCR) is a powerful method for fast in vitro enzymatic amplifications of specific DNA sequences. PCR amplifications can be grouped into three different categories: standard PCR, long PCR, and multiplex PCR. Standard PCR involves amplification of a single DNA sequence that is less than 5 kb in length and is useful for a variety of applications, such as cycle sequencing, cloning, mutation detection, etc. Long PCR is used for the amplification of a single sequence that is longer than 5 kb and up to 40 kb in length. Its applications include long-range sequencing; amplification of complete genes; PCR-based detection and diagnosis of medically important large-gene insertions or deletions; molecular cloning; and assembly and production of larger recombinant constructions for PCR-based mutagenesis (1,2). The third category, multiplex PCR, is used for the amplification of multiple sequences that are less than 5 kb in length. Its applications include forensic studies; pathogen identification; linkage analysis; template quantitation; genetic disease diagnosis; and population genetics (3–5). Unfortunately, there is no single set of conditions that is optimal for all PCR. Therefore, each PCR is likely to require specific optimization for the template/primer pairs chosen. Lack of optimization often results in problems, such as no detectable PCR product or low efficiency amplification of the chosen template; the presence of nonspecific bands or smeary background; the formation of “primer-dimers” that compete with the chosen template/primer set for amplification; or mutations caused by errors in nucleotide incorporation. It is particularly important to optimize PCR that will be used for repetitive diagnostic or analytical procedures where optimal amplification is required. The objective of this chapter is to discuss the parameters that may affect the specificity, fidelity, and efficiency of PCR, as well as approaches that can be taken to achieve optimal PCR amplifications.

Optimization of a particular PCR can be time consuming and complicated because of the various parameters that are involved. These parameters include the following: (1) quality and concentration of DNA template; (2) design and concentration of primers; (3) concentration of magnesium ions; (4) concentration of the four deoxynucleotides (dNTPs); (5) PCR buffer systems; (6) selection and concentration of DNA polymerase; (7) PCR thermal cycling conditions; (8) addition and concentrations of PCR additives/cosolvents; and (9) use of the “hot start” technique. Optimization of PCR
may be affected by each of these parameters individually, as well as the combined interdependent effects of any of these parameters.

2. Materials

1. Template DNA (e.g., plasmid DNA, genomic DNA).
2. Forward and reverse PCR primers.
3. MgCl$_2$ (25 mM).
4. dNTPs (a mixture of 2.5 mM dATP, dCTP, dGTP, and dTTP).
5. 10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 25°C.
6. Thermal stable DNA polymerase (e.g., Taq DNA polymerase).
7. PCR additives/cosolvents (optional; e.g., betaine, glycerol, DMSO, formamide, bovine serum albumin, ammonium sulfate, polyethylene glycol, gelatin, Tween-20, Triton X-100, β-mercaptoethanol, or tetramethylammonium chloride).

3. Methods

3.1. Setting Up PCR

The common volume of a PCR is 10, 25, 50, or 100 µL. Although larger volumes are easier to pipet, they also use up a larger amount of reagents, which is less economical. All of the reaction components can be mixed together in a 0.5-mL PCR tube in any sequence except for the DNA polymerase, which should be added last. It is recommended to mix all the components right before PCR cycling. Although it is not necessary to set up the PCR on ice, some published protocols recommend it.

For each PCR, the following components are mixed together:

1. Template DNA (1–500 ng).
2. Primers (0.05–1.0 µM).
3. Mg$^{2+}$ (0.5–5 mM).
4. dNTP (20–200 µM each).
5. 1× PCR buffer: 1 mM Tris-HCl and 5 mM KCl.
6. DNA polymerase (0.5–2.5 U for each 50 µL of PCR).

As a real-life example, the following PCR was set up to amplify the cII gene from bacteriophage lambda DNA (total volume = 50 µL):

1. 1 µL of 1 ng/µL lambda DNA (final amount = 1 ng).
2. 1 µL of 50 µM forward PCR primer (final concentration = 1 µM).
3. 1 µL of 50 µM reverse PCR primer (final concentration = 1 µM).
4. 5 µL of 25 mM MgCl$_2$ (final concentration = 2.5 mM).
5. 4 µL of 2.5 mM dNTPs (final concentration = 200 µM).
6. 5 µL of 10× PCR buffer (final concentration = 1×).
7. 0.25 µL of 5 U/µL Taq DNA polymerase (final amount = 1.25 U).

3.2. PCR Cycling

A common PCR cycling program usually starts with an initial dissociation step at 92 to 95°C for 2 to 5 min to ensure the complete separation of the DNA strands. Most PCR will reach sufficient amplification after 20 to 40 cycles of strand denaturation at 90 to 98°C for 10 s to 1 min, primer annealing at 55 to 70°C for 30 s to 1 min, and primer extension at 72 to 74°C for 1 min per kilobase of expected PCR product. It is suggested that a final extension step of 5 to 10 min at 72°C will ensure that
all amplicons are fully extended, although no solid evidence proves that this step is necessary. For example, the cycling program used to amplify the previously described lambda cII gene is as follows: initial denaturation for 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, then held at 4°C.

3.3. Verifying PCR Amplification

To measure the success of a PCR amplification, 5 to 10 µL of the final PCR product is run on a 1 or 2% agarose gel and visualized by staining with ethidium bromide. The critical questions are as follows: (1) Is there a band on the gel? (2) Is the band at the expected size? (3) Are there any nonspecific bands beside the expected PCR band on the gel? (4) Is there smear on the gel? A successful PCR amplification should display a single band with the expected size without nonspecific bands and smear.

4. Notes

1. The quality and concentration of DNA templates can directly affect the outcome of PCR amplifications. To achieve satisfactory amplification, certain baseline conditions may be used as a starting point for optimizing a PCR amplification. For a typical PCR, $10^4$ to $10^7$ molecules of template DNA is recommended. For long PCR (>5 kb), $10^7$ to $10^8$ molecules of a high copy number template DNA (e.g., 1–10 ng of lambda DNA) is recommended. For amplification from genomic DNA, use 100 to 500 ng of template DNA. In multiplex PCR, two- to fivefold more DNA template than what is needed for a typical PCR should be used.

   There are several methods for purifying DNA for PCR amplification, including commercially available kits, as well as standard methods (6). Long PCR amplification has the most stringent requirement for the quality of template DNA. Care should be taken to prevent template DNA damages from nicking, shearing, and depurination (7). Analysis by pulsed-field agarose gel electrophoresis is typically recommended for template DNA used in long PCR to assure its purity and integrity.

2. Appropriate primer design, as well as use of the proper primer concentration, is critical for successful PCR amplification. There are a variety of computer programs available for designing primers and they vary significantly in selection criteria, comprehensiveness, and user-friendliness (8–11). The purpose of primer design is to achieve a balance between the specificity and efficiency of an amplification. Specificity defines how frequently mispriming occurs, whereas efficiency represents the increase of the amount of PCR product over a given number of cycles. The following guidelines should be considered when designing primers. The optimal primer size is usually between 18 and 28 bases. Shorter primers are generally less specific but may result in more efficient PCR, whereas longer primers improve specificity yet can be less efficient. Primers from both directions should have melting temperatures ($T_m$, defined as the dissociation temperature of the primer/template duplex) that are within 2 to 5°C of each other. This will assure that the proper annealing temperature for both primers is achieved. For primers shorter than 20 bases, an estimate of $T_m$ can be calculated as $T_m = 4 \times (G + C) + 2 \times (A + T)$ (12). However, for longer primers, correct estimation of $T_m$ requires a “nearest-neighbor” calculation, which takes into account thermodynamic parameters of a chosen primer and is used by most of the available computer programs for primer design (13,14). Avoid complementary sequences within a primer or between the two primers. This will reduce formation of primer-dimers that can compete with the amplification of the desired PCR product, as well as the formation of secondary structures within a primer. Primers with $T_m$ higher than...
50°C will generally provide specific and efficient amplifications. For long PCR, a $T_m$ of 62 to 70°C is recommended. In addition, a GC content of 40 to 60% is desirable for primers because this assures a higher $T_m$ and therefore increases specificity. If AT content is high, use primers of 28 to 35 bases in length for long PCR. Also, avoid continuous stretches of purines or pyrimidines, as well as multiple repeats of thymidine residues at the 3′ end of the primer. It is extremely critical when designing primers for multiplex PCR to make sure that all primers have similar melting temperatures and do not contain sequences complementary to each other. Concentrations of primers used in PCR will also influence amplification specificity and efficiency. In general, primer concentrations between 0.05 to 1.0 $\mu M$ (or 0.5–100 pmol) are routinely used in 100 $\mu L$ of PCR depending on the specific application. Higher primer concentrations can result in nonspecific priming and formation of primer-dimers, whereas lower primer concentrations may adversely affect PCR efficiency. Use the primers at a 1:1 concentration ratio to assure specificity and efficiency of amplifications. Lower concentrations of primers are more desirable for multiplex PCR because of the number of primer sets present in the reaction.

3. Magnesium concentration is critical to the success of PCR amplification because it may affect DNA polymerase activity and fidelity, DNA strand denaturation temperatures of both template and PCR product, primer annealing, PCR specificity, and primer-dimer formation. Excess magnesium results in accumulation of nonspecific amplification products seen as multiple bands on an agarose gel, whereas insufficient magnesium results in reduced yield of the desired PCR product. Common magnesium concentrations used in PCR are between 0.5 to 5 mM. It is important to optimize the magnesium concentration used for each individual PCR because DNA polymerases require free magnesium for their activity in addition to that bound by template DNA, primers, and dNTPs. Also, trace amounts of EDTA or other chelators may be present in primer stock solutions or template DNA. Therefore, each PCR should contain 0.5 to 2.5 mM magnesium over the total dNTP concentrations (15). A simple way to optimize magnesium concentration is to first perform a series of reactions in which the magnesium concentration is varied between 0.5 and 5 mM in 0.5-mM increments. After the concentration range is narrowed, perform a second round of reactions and vary the magnesium concentration in 0.2- to 0.3-mM increments.

4. Concentration of dNTPs can affect the yield, specificity, and fidelity of a PCR amplification. Concentrations of 20 to 200 $\mu M$ of each dNTP has been used to obtain successful PCR amplifications. Stock solutions of each dNTP are adjusted to pH 7.0 and diluted to a 10 mM final concentration. Commercially available premixed dNTP solutions with concentrations of 2.5 mM or individual dNTP stock solutions of 10 mM may be used. Lower concentrations of dNTPs minimize mispriming and reduce the likelihood of extending misincorporated nucleotides, which in turn increase specificity and fidelity of PCR amplifications (16). Because dNTPs are typically added in excess to a PCR, one should determine the lowest dNTPs concentration appropriate for the length and composition of the target sequence. Although 250 $\mu M$ of each of the dNTPs appears to be sufficient for long PCR (17), amplifications of sequences longer than 20 kb may require dNTP concentrations as high as 400 to 500 $\mu M$ each in a given 50-$\mu L$ reaction. It is critical not to use a large excess of dNTP because higher dNTP concentrations increase the error rate of DNA polymerases. In fact, millimolar concentrations of dNTPs actually inhibit Taq DNA polymerase (18).

5. Standard PCR amplifications using Taq DNA polymerase are performed in 10 mM Tris-HCl (pH 8.3–8.4 at 20–25°C) and 50 mM KCl. For Tth and Tfl DNA polymerases, and DNA polymerases with proofreading activity (for example, Pwo, Pfu, Tli, and Vent DNA polymerases (New England Biolabs), a buffer system of 50 mM Tris-HCl (pH 9.0 at 25°C) and 20 mM (NH₄)₂SO₄ is normally used. These standard buffer systems are available
commercially and have been shown to produce satisfactory PCR amplifications in most cases. However, long PCR requires a different buffer system. For example, 20 to 25 mM Tricine (pH 8.7 at 25°C) and 80 to 85 mM potassium acetate (pH 8.3–8.7 at 25°C) (19), as well as 25 mM Tris-HCl (pH 8.9 at 25°C) and 100 mM KCl (2), have been used successfully in long PCR amplifications when used in conjunction with rTth DNA polymerase (Perkin–Elmer). The use of less temperature-sensitive buffers, such as Tricine, may enhance the ability to obtain long PCR amplifications.

6. The most common enzyme used for PCR amplification is Taq DNA polymerase because of its thermostability and processivity (i.e., the number of nucleotides replicated before the enzyme dissociates from the DNA template). It was originally purified from the gram-negative thermophilic bacterium Thermus aquaticus (20). Highly purified Taq DNA polymerase exhibits a temperature optimum of 75 to 80°C (21). The half-life of Taq DNA polymerase is 40 min at 95°C, which is sufficient to remain active over 30 or more cycles, during which the enzyme is transiently exposed to extremely high denaturation temperatures. Taq DNA polymerase has an extension rate of 35 to 100 nucleotides per second at 72°C (16), which is the most common extension temperature for PCR amplifications. A recommended concentration range for Taq DNA polymerase is between 1 and 2.5 units per 100 µL of PCR. However, different concentrations of Taq DNA polymerase may be required with respect to individual target template sequences or primers. Increasing the amount of Taq DNA polymerase beyond the 2.5 units/reaction can in some cases increase PCR efficiency. However, adding more Taq DNA polymerase can sometimes increase the yield of nonspecific PCR products at the expense of the desired product. When optimizing the Taq DNA polymerase concentration for a particular PCR, testing a range of 0.5 to 5 units per 100 µL of reaction in 0.5-unit increments is recommended, followed by analysis of the PCR products by gel electrophoresis to determine the amplification specificity and efficiency.

The other important property of Taq DNA polymerase is its fidelity, which is measured as error rate. The error rate for Taq DNA polymerase, which lacks proofreading 3′ → 5′ exonuclease activity, is estimated at approx 1 to 2 × 10⁻⁵ errors (or mutation frequency) per nucleotide per duplication (22–24). For many applications, this does not present any problems. However, for some sequencing, cloning, and long PCR applications, it is essential to have few, or no incorporation errors. In situations where “high fidelity” is required, DNA polymerases with 3′ → 5′ proofreading activity (for example, Pfu or Pwo DNA polymerases) are recommended. The estimated error rates for these proofreading enzymes is approx 1 to 2 × 10⁻⁶ errors per nucleotide per duplication (23,24), representing a 10-fold improvement over standard Taq DNA polymerase. It is important to note that these proofreading enzymes with lower error rates also have lower extension rates, resulting in lower PCR efficiency. Therefore, more amplification cycles are required to achieve adequate amount of amplified DNA.

DNA polymerase characteristics, such as extension rate, processivity, fidelity, thermostability, and thermal activity profile, are important in long PCR. PerkinElmer’s rTth DNA polymerase (the recombinant form of the DNA polymerase from T. thermophilus) has been shown to perform consistent long PCR at 0.5 to 2.5 units per 50 µL of reaction (17). Use of a mixture of Taq DNA polymerase with a proofreading enzyme, such as Pfu DNA polymerase, at a 20:1 ratio (2.5 units per 50 µL of reaction) will also enhance the reliability of long PCR amplifications.

7. Optimization of PCR thermal cycling conditions includes determination of cycle number, the temperature and incubation time period for template denaturation, primer annealing, and primer extension. The optimum number of cycles depends mainly on the starting concentration of template DNA. Because many PCRs start with very limiting amount
of template DNA, a sufficient number of cycles are required to achieve satisfactory amplifications. However, PCR amplification is not an unlimited process. A common mistake is to execute too many cycles. The exponential amplification of PCR will continue up until the point when the product reaches about $10^{-8} \text{M}$ (about $10^{12}$ molecule in a 100-µL reaction). The reaction enters a linear phase where exponential accumulation of the product is attenuated. This is termed the plateau effect (25). In most PCRs, amplifications plateau after about 20 to 40 cycles. The other major limitation of standard PCR is the amount of DNA polymerase included in the reaction. The combination of thermal inactivation of the DNA polymerase after each denaturation step, reduction in denaturation efficiency, and the reduced efficiency of primer annealing (caused by increasing competition from the template), will cause the reaction to terminate. Although too few cycles of PCR result in low product yield, most PCR amplifications are performed for no more than 20 to 40 cycles.

It is often helpful to precede the first cycling denaturation step with an initial dissociation step at 92 to 95°C for 2 to 5 min to ensure the complete separation of the DNA strands. Template denaturation temperatures range 90 to 98°C. The duration of denaturation ranges from 10 s to 1 min. Although it only takes a few seconds to denature DNA at its strand-separation temperature, it is appropriate to use a higher denaturation temperature and a longer incubation time for some templates, such as those templates with high GC content, to achieve complete denaturation. Although a higher temperature and a longer incubation period result in a more complete denaturation of the DNA template, it can also cause depurination of the DNA template, which in turn reduces amplification efficiency. It is also important to note that higher denaturation temperatures will reduce the amount of active DNA polymerase available for amplification. The half-life of Taq DNA polymerase activity is more than 2 h at 92.5°C, 40 min at 95°C, and 5 min at 97.5°C.

The optimal primer annealing temperature for a particular PCR amplification depends on the base composition, nucleotide sequence, length, and concentration of the primers (26). A typical primer annealing temperature is 5°C below the calculated Tm of the primers. Annealing temperatures from 55 to 70°C generally yield the best results. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3′ end of primers. Therefore, a higher annealing temperature increases amplification specificity. For these reasons, when using primers with higher Tm's such as for long PCR, a higher annealing temperature (i.e., 60–70°C) should be used. It is also important to note that at typical primer concentrations of 200 µM each, annealing requires only a few seconds. However, incubation times from 30 s to 1 min are generally recommended to assure successful primer annealing.

Primer extension time depends on the length and concentration of the target sequence, as well as the extension temperature. Taq DNA polymerase extends at a rate of 0.25 nucleotides per second at 22°C, 1.5 nucleotides per second at 37°C, 24 nucleotides per second at 55°C, greater than 60 nucleotides per second at 70°C, and 150 nucleotides per second at 75 to 80°C (18). Therefore, at the commonly chosen extension temperature of 72°C, Taq DNA polymerase is expected to extend at the rate of greater than 3500 nucleotides per minute. Thus, as a general rule, an extension time of 1 min per kilobase is more than sufficient to generate the expected PCR product. For PCR products up to 2 kb in length, an extension time of 1 min at 72°C is sufficient. A final extension step of 5 to 10 min at 72°C may be added in order to ensure that all amplicons are fully extended.

In addition to the conventional three-step cycling programs, two-step cycling programs that combine primer annealing and extension in one step are also widely used. Annealing and extension can be combined because most thermostable DNA polymerases can actively extend off the primers over the entire range of commonly chosen annealing and extension
temperatures. Two-step cycling programs are generally applied when a high annealing temperature is used, such as 65 to 70°C. Because a higher annealing temperature improves amplification specificity, it is argued by some investigators that better PCR results may be obtained using a two-step cycling program (27). Here is an example of a typical two-step cycling program: initial denaturation at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C and 1 min at 68°C, then held at 4°C.

It is reasonable to follow the above rationale in defining optimal cycling conditions for long PCR. However, there are a few rules that must be considered when performing long PCR. Use moderate denaturation temperatures and short incubation time periods to maintain the integrity of the long DNA templates. Choose primers with a high Tm so that a higher annealing temperature can be used to increase specificity. Two-step cycling programs are more frequently used than three-step cycling programs. For example, denaturation at 92 to 95°C for 10 to 30 s, followed by annealing and extension at 65 to 68°C for 1 min per kilobase will increase the probability of obtaining the desired product. Programming an increase in extension time automatically in later cycles may also improve the yields of the amplification. Because DNA polymerases extend primers discontinuously through a succession of reactions, increasing the extension time in each of the later PCR cycles could increase the likelihood of synthesizing long PCR products. For example, perform the extension at 1 min per kilobase for the first 10 cycles, then lengthen the extension time 10 to 20 s for each of the next 20 cycles. Here is an example of a typical cycling program used to amplify a 10-kb PCR product: initial denaturation at 94°C for 2 min, followed by 16 cycles of 30 s at 94°C and 10 min at 68°C, then 12 cycles of 30 s at 94°C and 10 mins at 68°C with a 15-s extension per cycle, then held at 4°C.

8. The majority of PCR amplifications can be successfully performed after optimizing the above parameters. However, there are some PCR amplifications, for example, those using templates with high guanine/cytosine content or stable secondary structures, that may still amplify inefficiently, resulting in little or no desired product and/or nonspecific products. Incorporation of the nucleotide analog 7-deaza-2′-deoxyguanosine triphosphate (c7dGTP) in addition to deoxyguanosine triphosphate (dGTP) helps destabilize secondary structures of DNA and reduces the formation of nonspecific products (28). However, the most effective and frequently used strategy is addition of various organic additives or cosolvents. The most commonly used cosolvents and their concentration ranges are: dimethyl sulfoxide (DMSO; 1–10%), glycerol (5–20%), formamide (1.25–10%), bovine serum albumin (10–100 µg/mL), ammonium sulfate (NH₄)₂SO₄; 15–30 mM), polyethylene glycol (5–15%), gelatin (0.01%), non-ionic detergents (such as Tween 20 and Triton X-100; 0.05–0.1%), β-mercaptoethanol, tetramethylammonium chloride (TMAC), and N,N,N-trimethylglycine (betaine) (1–3 M) (15,17,29–34).

The mechanisms underlying enhancement of PCR by many of these cosolvents are not well defined. It is suggested that some cosolvents, such as DMSO, formamide, glycerol, and polyethylene glycol, may affect the Tm of the primers, the thermal activity profile of Taq DNA polymerase, as well as the degree of product strand separation (18). Gelatin, bovine serum albumin, and nonionic detergents, such as Tween-20 and Triton X-100, are thought to stabilize DNA polymerases (18). TMAC is used to eliminate nonspecific priming (34). (NH₄)₂SO₄ may increase the ionic strength of the reaction mixture, altering the denaturation and annealing temperatures of DNA, and may affect polymerase activity. Betaine has been shown to increase the thermostability of DNA polymerases, as well as to alter DNA stability such that GC-rich regions melt at temperatures more similar to AT-rich regions (29).

It is important to carefully choose the appropriate cosolvents and correct concentrations to effectively improve PCR amplifications. Cosolvent concentrations should be no greater
than absolutely necessary for optimal amplification, as they may reduce DNA polymerase activity. For example, DMSO at a final concentration of 10% can reduce Taq DNA polymerase activity by up to 50% \(15\). In addition to improving standard PCR, multiplex PCR performance has also been shown to improve when using DMSO \(34\), Tween-20 and Triton X-100 \(32\), ß-mercaptoethanol \(33\), TMAC \(34\), and betaine \(35\). Long PCR has been enhanced using glycerol, gelatin \(17\), DMSO \(19\), and betaine \(36\).

9. The “hot start” technique enhances PCR specificity by eliminating the production of nonspecific products and primer-dimers during the initial steps of PCR \(36\). This is because even a brief incubation of a PCR mix at temperatures significantly below the Tm can result in primer-dimer formation and nonspecific priming. The purpose of a hot start is to withhold one of the critical components from the reaction until the temperature in the first cycle rises above the annealing temperature. There are various methods of performing a hot start. Manual hot start is performed by withholding one of the reaction components, such as the DNA polymerase or magnesium, and adding it only after the reaction temperature rises above 80°C during the first denaturation step. Wax-mediated hot start involves addition of a wax layer separating the component being withheld from the remainder of the reaction mix. During the temperature increase in the first denaturation step, the wax melts and the withheld component is mixed with the rest of the reaction components, starting the amplification reaction. The beads for wax layer can be made in the laboratory \(37,38\) or purchased commercially (Ampliwax™ PCR Gems, PerkinElmer). Hot start Taq DNA polymerase is constructed through the addition of an anti-Taq DNA polymerase antibody (TaqStart™ Antibody, Clontech). The antibody will prevent the DNA polymerase activity until the temperature rises during the initial denaturation step. The increased temperature dissociates and degrades the bound antibody, initiating PCR amplification. Hot start is commonly used for multiplex and long PCR amplifications.

10. In addition to all of the PCR optimization strategies discussed above, there are also commercially available buffer systems for fast and easy PCR optimization. Companies, such as Boehringer Mannheim, Stratagene, Invitrogen, and Epicentre Technologies, offer various buffer systems for PCR optimization. These buffer systems can be divided into two categories. One category (e.g., PCR optimization kit from Boehringer Mannheim) contains a set of 16 buffers that combine different pH (8.3, 8.6, 8.9, and 9.2) and various concentrations of magnesium (1.0, 1.5, 2.0, and 3.5 mM). There are also four different cosolvents, DMSO, glycerol, gelatin, and \((\text{NH}_4)_2\text{SO}_4\), provided separately for additional optimization. Because the cosolvents are not premixed in the buffers, inclusion of these cosolvents will require a second set of optimization reactions. The other category of buffer system (e.g., PCR optimization kits from Epicentre Technologies) contains variable concentrations of magnesium (1.5, 2.5, and 3.5 mM) and a betaine-containing enhancer. Because all necessary reaction components, including the cosolvent (betaine), are premixed in this buffer system, only one set of optimization reactions are performed.

11. The following conditions may lead to less than optimal PCR amplifications. The possible solutions for each condition are discussed.

   If little or not desired PCR product is detected:
   a. Too little DNA template is present in the reaction. Increase the amount of template DNA.
   b. The template DNA is damaged or degraded. Assure the purity and integrity of the DNA template by minimizing damage from nicking and shearing.
   c. Insufficient DNA polymerase is present in the reaction. Increase the DNA polymerase concentration in increments of 0.5 units per 100 µL of reaction.
   d. Insufficient number of cycles was performed. Increase cycle number by 5 to 10 cycles.
   e. Check for inhibitor(s) during template DNA preparation. Repurification of the DNA template may remove some inhibitors of PCR.
Magnesium concentration is too low. Increase magnesium concentration in increments of 0.1 mM.

The denaturation time is too long or too short. Adjust denaturation time in increments of 5 s.

Add cosolvents that enhance PCR amplification.

The denaturation temperature is too high or too low. Change denaturation temperature in increments of 1°C.

The primer annealing temperature is too high. Lower annealing temperature in increments of 2°C.

The primer extension period is too short. Increase extension time in increments of 1 minute.

Re-amplify dilutions (1:10 to 1:1000) of the first round of PCR amplification using nested primers.

Perform Touchdown (TD)/Stepdown (SD) PCR cycling program. TD or SD PCR uses a temperature cycling protocol that is performed at decreasing annealing temperatures. The cycling program begins at an annealing temperature a few degrees above the calculated Tm of the primers. This ensures that the first primer-template hybridization events involve only those sequences with the greatest specificity. The annealing temperature is decreased 1 to 4°C every other cycle to approx 10°C below the calculated Tm to permit exponential amplification (39). Here is an example of a typical TD/SD cycling program: initial denaturation at 94°C for 1 mi followed by 20 cycles of 10 s at 92°C and 20 s at 70°C with an 0.5°C decrease of temperature per cycle, then another 20 cycles of 10 s at 92°C and 30 s at 60°C with a 1-s extension per cycle and hold at 4°C. Hot start must be used with these cycling programs.

Review primer design and composition. Design new primers and try PCR again.

If multiple product bands or smear is detected:

Too much DNA template is present in the reactions. Decrease the amount of DNA template in the reaction mix.

Annealing temperature is too low. Increase annealing temperature in increments of 2°C.

DNA polymerase concentration is too high. Decrease enzyme concentration in increments of 0.5 units per 100-µL reaction.

Magnesium concentration is too high. Decrease the magnesium concentration in increments of 0.1 mM.

Denaturation time is too short. Increase the denaturation time in increments of 5 s.

Denaturation temperature is too low. Increase the denaturation time in increments of 1°C.

Cycle number is too high. Reduce the cycle number by 5 to 10 cycle.

Perform hot start.

Alter concentrations of cosolvents.

Perform TD/SD PCR.

Extension time is too long. Reduce the extension time in increments of 1 min.

Check for carry-over contamination. Set up PCR in a different area.

Review primer design and composition. Design new primers and try PCR again.

References


