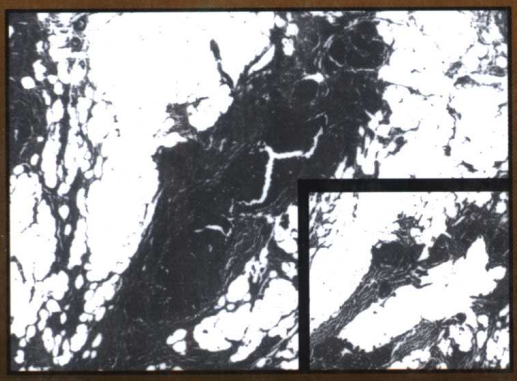
METHODS IN MOLECULAR MEDICINE™

Clinical Applications of PCR

PCR 临床应用

Edited by

Y. M. Dennis Lo



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Preface

The polymerase chain reaction (PCR) is one of the most important molecular biological methods ever devised, with numerous applications to clinical molecular medicine. Since its description in 1985, PCR has undergone tremendous improvements, and many variations on the basic PCR theme have been published. With such a large volume of PCR-related literature, a clinical scientist wishing to use the technique will have a difficult task locating the relevant information to implement it effectively. There is thus clearly a need for an up-to-date volume with detailed protocols to facilitate the setting up of those techniques most relevant to clinical applications.

Unlike some other books on this topic, *Clinical Applications of PCR* includes only methods that are of direct relevance in clinical settings. The book is organized in three parts: an introductory section, a section on general methodology, and a final section with specific clinical applications. The first section covers the basic principles of PCR and is most useful to those new to molecular diagnosis. The next chapter includes useful tips for setting up a PCR laboratory. Section 2 then outlines some of the most commonly used PCR-based techniques in molecular diagnosis. Section 3 includes carefully chosen examples that represent typical applications of PCR in diverse clinical fields, encompassing hematology, oncology, genetics, and microbiology.

For clinical applications, two attributes of PCR are especially important: its sensitivity and its ability to detect sequence variations rapidly. Consequently, a significant part of the book is devoted to PCR applications that take advantage of these characteristics. The sensitivity of PCR allows single cell analysis and the detection of minority cell/DNA populations to be carried out. Important clinical applications in those areas covered here include the detection of circulating cancer cells in oncology patients and noninvasive prenatal diagnosis using fetal cells in maternal blood. The analysis of sequence variations by PCR is covered extensively in Section 2.

Our hope is that clinical scientists across the many specialties that increasingly require the application of PCR will find *Clinical Applications of PCR* the highly useful book we have striven to create.

Y. M. Dennis Lo

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I

INTRODUCTION

1

Introduction to the Polymerase Chain Reaction

Y. M. Dennis Lo

1. Introduction

The polymerase chain reaction (PCR) is an in vitro method for the amplification of DNA that was introduced in 1985 *(1)*. The principle of the PCR is elegantly simple but the resulting method is extremely powerful. The adoption of the thermostable *Taq* polymerase in 1988 greatly simplifies the process and enables the automation of PCR *(2)*. Since then a large number of applications have been developed that are based on the basic PCR theme. The versatility and speed of PCR have revolutionized molecular diagnostics, allowing the realization of a number of applications that were impossible in the pre-PCR era. This chapter offers an introductory guide to the process.

2. Principle of the PCR

PCR may be regarded as a simplified version of the DNA replication process that occurs during cell division. Basic PCR consists of three steps: thermal denaturation of the target DNA, primer annealing of synthetic oligonucleotide primers, and extension of the annealed primers by a DNA polymerase (**Fig. 1**). This three step cycle is then repeated a number of times, each time approximately doubling the number of product molecules. The amplification factor is given by the equation $n(1 + E)^x$ where n = initial amount of target, E = efficiency of amplification, and x = number of PCR cycles. After a few cycles, the resulting product is of the size determined by the distance between the 5'-ends of the two primers. With the performance of a previous reverse transcription step, PCR can also be applied to RNA (*see* Chapter 14).

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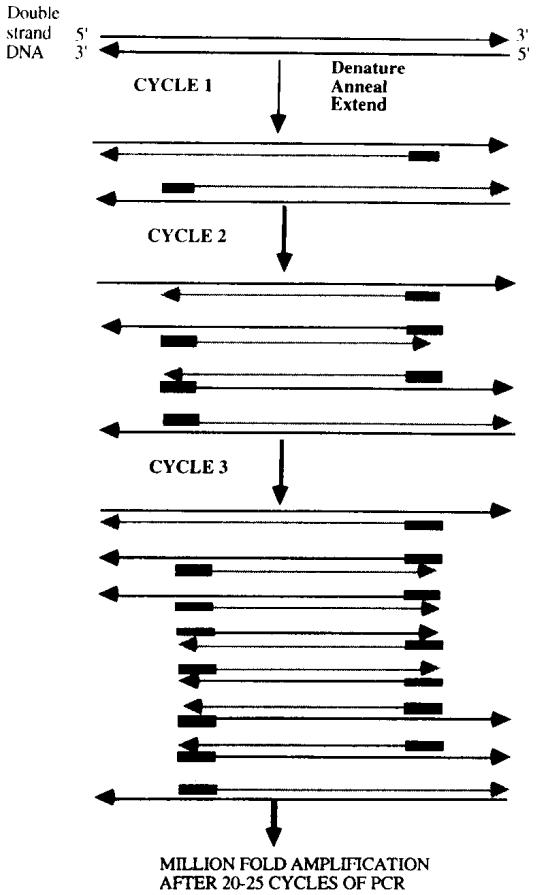

Fig. 1. Schematic representation of the polymerase chain reaction. The newly synthesized DNA is indicated by dotted lines in each cycle. Oligonucleotide primers are indicated by solid rectangles. Each DNA strand is marked with an arrow indicating the 5' to 3' orientation.

3. Composition of the PCR

PCR is usually performed in a volume of 25–100 μL. Deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) at a concentration of 200 μM each, 10 to 100 pmol of each primer, the appropriate salts, buffers, and DNA polymerase are included. Many manufacturers have included reaction buffer with their DNA polymerase and this practice is convenient to newcomers to the PCR process.

4. Primers

Primers are designed to flank the sequence of interest. Oligonucleotide primers are usually between 18 and 30 bases long, with a GC content of about 50%. Complementarity at the 3'-ends of the primers should be avoided to decrease the likelihood of forming the primer–dimer artifact. Runs of three or more C's or G's at the 3'-ends of the primers should be avoided to decrease the probability of priming GC-rich sequences nonspecifically. A number of computer programs are available to assist primer design. However, for most applications PCR is sufficiently forgiving in that most primer pairs seem to work. The primers are generally positioned between 100 to 1000 bp apart. It should be noted, however, that for high sensitivity applications, shorter PCR products are preferred. For most applications, purification of the PCR primers are not necessary. To simplify subsequent operations, it is recommended that all primers are diluted to the same concentration (e.g., 50 pmol/μL) such that the same volume of each primer is required for each reaction. Some primer pairs seem to fail without any obvious reason, and when difficulty arises, one simple solution is to change one or both of the primers.

The use of primers for allelic discrimination (Chapters 7 and 8) and the application of labeled primers (Chapters 6, 20, and 23) are described later on in the book.

5. Steps of the PCR

5.1. Thermal Denaturation

A common cause of failed PCR is inadequate denaturation of the DNA target. We typically use an initial denaturation temperature of 94°C for 8 min. For subsequent cycles, 94°C for 1–2 min is usually adequate. As the targets of later PCR cycles are mainly PCR products rather than genomic DNA, it has been suggested that the denaturation temperature may be lowered after the first 10 cycles so as to avoid excessive thermal denaturation of the *Taq* polymerase *(3)*. 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.

5.2. Primer Annealing

The temperature and length of time required for primer annealing depends on the base composition and the length and concentration of the primers. Using primers of 18–30 bases long with approx 50% GC content, and an annealing step of 55°C for 1–2 min is a good start. In certain primer–template pairs, a small difference in the annealing temperature of 1–2°C will make the difference between specific and nonspecific amplification. If the annealing temperature is >60°C, it is possible to combine the annealing and extension step together into a two step PCR cycle.

5.3. Primer Extension

Primer extension is typically carried out at 72°C, which is close to the temperature optimum of the *Taq* polymerase. An extension time of 1 min is generally enough for products up to 2 kb in length. Longer extension times (e.g., 3 min) may be helpful in the first few cycles for amplifying a low copy number target or at later cycles, when product concentration exceeds enzyme concentration.

6. Cycle Number

The number of cycles needed is dependent upon the copy number of the target. As a rule of thumb, to amplify 10^5 template molecules to a signal visible on an ethidium bromide stained agarose gel, requires 25 cycles. Assuming that we use 1 min each for denaturation, annealing and extension, the whole process can be completed in approx 2–3 h (with extra time allowed for the lag phase taken by the heat block to reach a certain temperature). Similarly, 10^4, 10^3, and 10^2 target molecules will require 30, 35, and 40 cycles, respectively. Careful optimization of the cycle number is necessary for quantitative applications of PCR (*see* Chapter 4).

7. PCR Plateau

There is a limit to how many product molecules a given PCR can produce. For a 100 µL PCR, the plateau is about 3–5 pmol ***(4)***. The plateau effect is caused by the accumulation of product molecules that result in a significant degree of annealing between complementary product strands, rather than between the primers and template. Furthermore, the finite amount of enzyme molecules present will be unable to extend all the primer–template complex in the given extension time.

8. Sensitivity

The sensitivity of PCR is related to the number of target molecules, the complexity of nontarget molecules, and the number of PCR cycles. Since the introduction of the *Taq* polymerase, it has been known that PCR is capable of amplification from a single target molecule ***(2,5)***. This single-molecule capability has allowed the development of single sperm typing ***(5,6)*** and preimplantation diagnosis ***(7–9)*** (*see* Chapters 20 and 22). In these applications, the single target molecule is bathed, essentially, in PCR buffer—in other words, in a low complexity environment. In situations where the complexity of the environment is high, the reliability of single molecule PCR decreases and strategies such as nesting and Hot Start PCR ***(10,11)*** are necessary for achieving maximum sensitivity (*see* Chapters 11, 15, 18, 19, and 21). The sensitivity of PCR has also allowed it to be used in situations where the starting materials have been partially degraded (*see* Chapter 3).

9. PCR Fidelity

The fidelity of amplification by PCR is dependent upon several factors: annealing/extension time, annealing temperature, dNTP concentration, $MgCl_2$ concentration, and the type of DNA polymerase used. In general, the rate of misincorporation may be reduced by minimizing the annealing/extension time, maximizing the annealing temperature, and minimizing the dNTP and $MgCl_2$ concentration *(12)*. Eckert and Kunkel reported an error rate per nucleotide polymerized at 70°C of 10^{-5} for base substitution and 10^{-6} for frameshift errors under optimized conditions *(12)*. The use of a DNA polymerase with proofreading activity reduces the rate of misincorporation. For example, the DNA polymerase from *Thermococcus litoralis*, which has proofreading activity, misincorporates at 25% of the rate of the *Taq* polymerase, which lacks such activity *(13)*. Interestingly, the combination of enzymes with and without proofreading activity has enabled the amplification of extremely long PCR products (*see* Chapter 9).

For most applications, product molecules from individual PCR are analyzed as a whole population and rare misincorporated nucleotides in a small proportion of molecules pose little danger to the interpretation of data. However, for sequence analysis of cloned PCR products, errors due to misincorporation may sometimes complicate data interpretation. Thus, it is advisable to analyze multiple clones from a single PCR or to clone PCR products from several independent amplifications. Another application where misincorporation may result in error in interpretation is in the amplification of low copy number targets (e.g., single molecule PCR). In these situations, if a misincorporation happens in an early PCR cycle (the extreme case being in cycle 1), the error will be passed onto a significant proportion of the final PCR products. Hence, in these applications, the amplification conditions should be carefully optimized.

10. PCR Thermocyclers

One of the main attractions of PCR is its ability to be automated. A number of thermocyclers are available from different manufacturers. These thermocyclers differ in the design of the cooling systems, tube capacity, number of heating blocks, program memory, and thermal uniformity. In our opinion, units using the Peltier system are fast and have a uniform thermal profile across the block. Units with multiple heating blocks are very valuable for arriving at the optimal cycling profile for a new set of primers, as multiple conditions can be tested simultaneously. Tube capacity generally ranges from 48 to 96 wells and should be chosen with the throughput of the laboratory in mind. Some thermocyclers have heated covers and, thus, allow the omission of mineral oil from the reaction tubes. Specially designed thermal cyclers are required for *in situ* amplification (*see* Chapter 12) that accommodate glass slides.

11. Analysis and Processing of PCR Product

The amplification factor produced by PCR simplifies the analysis and detection of the amplification products. In general, analytical methods for conventional DNA sources are also applicable to PCR products. Some of these methods for studying sequence variation are covered in this volume (*see* Chapters 5, 6, and 13)

11.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis followed by ethidium bromide staining represents the most common way to analyze PCR products. A 1.5% agarose gel is adequate for the analysis of PCR products from 150 to 1000 bp. A convenient molecular weight marker for this size range is ΦX174 DNA digested by *Hae*III.

11.2. Restriction of PCR Products

Restriction mapping is a commonly used way of verifying the identity of a PCR product. It is also a simple method of detecting restriction site polymorphisms and for detecting mutations that are associated with the creation or destruction of restriction sites. There is no need to purify the PCR product prior to restriction and most restriction enzymes are functional in a restriction mix in which the PCR product constitutes up to half the total volume.

11.3. Sequence-Specific Oligonucleotide Hybridization

This is a powerful method for detecting the presence of sequence polymorphisms in a region amplified by PCR. Short oligonucleotides are synthesized and labeled (either radioactively or nonradioactively), allowed to hybridize to dot blots of the PCR products *(5)*, and washed under conditions that allow the discrimination of a single nucleotide mismatch between the probe and the target PCR product.

For the detection of a range of DNA polymorphisms at a given locus, the hybridization can be performed "in reverse," that is, with the oligonucleotides immobilized onto the filter. Labeled amplified products from target DNA are then hybridized to the filters and washed under appropriate conditions *(14)*. The reverse dot-blot format is now available for many multi-allelic systems *(15,16)*.

11.4. Cloning of PCR Product

PCR products may be cloned easily using conventional recombinant DNA technology. To facilitate cloning of PCR products into vectors, restriction sites may be incorporated into the primer sequences. Digestion of the PCR products with the appropriate restriction enzymes will then allow "sticky end" ligation into similarly restricted vector DNA.

12. Conclusion

The versatility of PCR has made it one of the most widely used methods in molecular diagnosis. The number of PCR-based applications have continued to increase rapidly and have impacted in oncology (*see* Chapters 15 and 17–19), genetics (*see* Chapters 16 and 20–23), and microbiology (*see* Chapters 24 and 25). In this book we attempt to present some of the most important clinical applications of PCR.

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