Methods in Molecular Biology™

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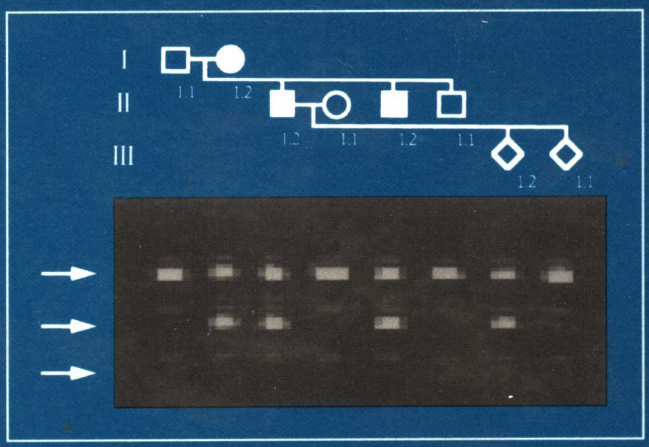
PCR IN BIOANALYSIS

Edited by

Stephen J. Meltzer, MD



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METHODS IN MOLECULAR BIOLOGY™

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Preface

The invention of the polymerase chain reaction (PCR) eventually earned Kary B. Mullis half of the 1993 Nobel Prize for Chemistry *(1–4)*. However, for several years, issues of quality control and reproducibility interfered with attempts at commercial or clinical application of PCR.

More recently, persistent work and numerous methodological innovations and refinements have resulted in the establishment of PCR as a routine, sensitive, and specific detection method in hospital and agricultural laboratories. This transformation of PCR from an experimental research technique to an established bioassay tool formed the impetus behind *PCR in Bioanalysis*.

PCR has proven particularly valuable in clinical microbiology and the diagnosis of infectious diseases in humans and animals. This large and diverse group of applications is reviewed in Chapter 1 by Gorm Lisby. Specific organisms now detectable by PCR include hepatitis C virus (protocols presented in Chapter 14), *Mycobacterium tuberculosis* (Chapter 18), *Chlamydia* and *Trichomonas* species (Chapter 20), *Toxoplasma gondii* (Chapter 17), *Legionella* species (Chapter 15), enterotoxigenic *Escherichia coli* (Chapters 9 and 10), HIV-1 subspecies (Chapter 8), bovine immunodeficiency-like virus (Chapter 6), rodent parvoviruses (Chapter 2), Ross River virus (Chapter 12), and porcine reproductive and respiratory syndrome virus (Chapter 7).

In addition, some methodologic breakthroughs are now routinely applied to clinical and industrial problems. In the analysis of human tissues, selective ultraviolet radiation fractionation (SURF) has become established as a means of selecting (or excluding) cells for subsequent PCR amplification (protocol presented in Chapter 3). In the evaluation of human tumors, new techniques for detection of amplified or deleted DNA sequences (Chapter 24) and circulating tumor cells (Chapter 19) have emerged. It is now routine to see PCR applied to the quantitative measurement of mRNA expression (Chapter 16), the examination of transgenic plants (Chapter 13) or mice (Chapter 22), the identification and cloning of differentially expressed genes (Chapter 21), and the measurement of cytokines or inducible nitric oxide synthase (Chapter 5). In vitro transcription/translation of PCR products is a successful screening technique to detect chain-terminating mutations (Chapter 11); this and other approaches are now commonly utilized to detect hereditary disease syndromes, such as familial adenomatous polyposis (Chapter 23) and Marfan's syndrome (Chapter 4).

In summary, these innovations and adaptations of existing technologies have launched a virtual bioanalytic revolution. Many more applications will probably follow in short order. We are now firmly entrenched in the PCR era, but we are still standing near that era's beginning.

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Contents

1

Application of Nucleic Acid Amplification in Clinical Microbiology

Gorm Lisby

1. Introduction

Since the discovery of the doublehelix structure of DNA *(1)*, no single event has had the same impact on the field of molecular biology as the rediscovery by Kary Mullis in the early 1980s of the polymerase chain reaction (PCR) *(2–4)*, which was first published in principle by Keld Kleppe in 1971 *(5)*. This elegant technology with its apparent simple theory has revolutionized almost every aspect of classical molecular biology, and is at the present moment beginning to make a major impact upon many medical—especially diagnostic-specialities. The field of clinical microbiology has been among the first to embrace the polymerase chain reaction technology, and the expectations of the future impact of this technology are high. First and foremost, the diagnostic possibilities of this technology are stunning, but in this era of emerging implementation, it is crucial to focus not only on the possibilities, but also on the pitfalls of the technology. Failure to do so will increase the cost of implementation manifold, and will risk to disrepute the technology in the eyes of the clinicians.

2. PCR–Theory and Problems

2.1. "Classic" PCR

2.1.1. The Principle of Exponential Amplification

The hallmark of the polymerase chain reaction is an exponential amplification of a target DNA sequence. Each round of amplification is achieved by annealing specific oligonucleotides to each of the two complementary DNA

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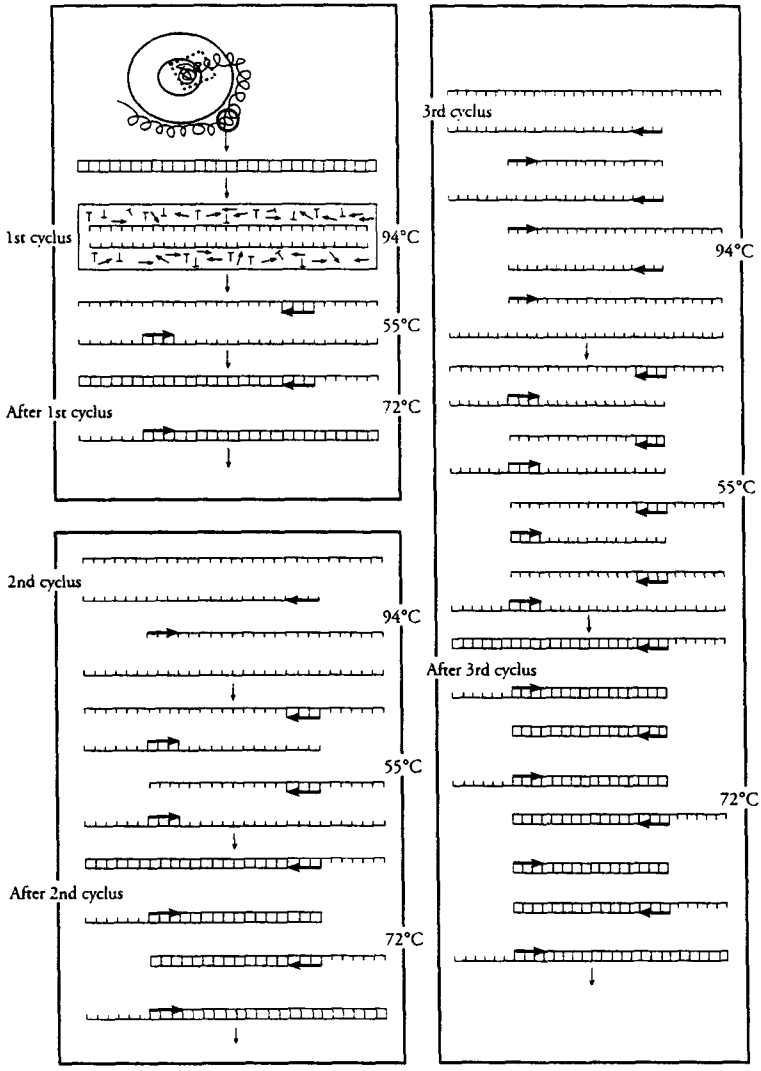

Fig. 1. The first three cycles of a standard PCR. The tentative annealing temperature of 55°C needs to be optimized for each PCR set-up.

strands after denaturation. Following annealing of the two oligonucleotides (primers), a thermostable DNA polymerase *(6)* will produce doublestranded DNA, thus in theory doubling the amount of specific DNA in each round **(Fig. 1)**. After the third round of amplification, a specific product consisting of the target DNA fragment between the two primer annealing sites (and including

the two sites) will start to accumulate. When the usual 30–40 rounds of amplification are completed, up to several hundred million fold amplification of the specific target sequence can be achieved. The amplified products can be detected by numerous methods that vary in sensitivity, accuracy, and feasibility for routine application: From the classical agarose gel electrophoresis, Southern blot and Sanger dideoxy sequencing to probe capture and visualization in microtiterplates and direct realtime detection of the product in the PCR tube by fluorescens *(7)*.

2.1.2. Primer Selection and Primer Annealing

Several aspects must be considered when a primerset for PCR is designed *(8)*. Computer software programs have been constructed to deal with this problem *(9–11)*, and these programs are based on the same considerations, as one has to take during a "manual" primer design:

The primers are typically between 15 and 30 bases long and do not have to be exactly the same size. However, it is crucial that the melting temperatures of the two primer/template duplexes are identical within 1–2°C. Since a billion-fold surplus of primers may exist in the beginning of a PCR when compared to the target sequence to be amplified, the optimal primer annealing temperature of a primer may be higher than the calculated melting temperature of the primer/template duplex (where 50% of the DNA molecules are doublestranded and 50% are singlestranded). Several formulas to calculate the annealing temperature exist *(12–14)*, but eventually one has to establish the actual optimal annealing temperature by testing.

The location of the target sequence and thereby the size of the amplified product is not crucial for the sensitivity or the specificity of the analysis, and typically a fragment of 150–800 bases is amplified. Amplification of products sizing up to 42,000 basepairs has been reported *(15)*. However, when fragments above 1000–2000 basepairs are amplified, problems with template reannealing can be encountered *(15–17)*. The annealing step in a "long-range" PCR is thus a balance between keeping the templates denatured and facilitating primer annealing.

The composition of the two primer sequences must ensure specific annealing to the target sequence alone. The probability of this specificity can be made through a search in the computer databases (GeneBank or EMBL), but eventually this also has to be established empirically (absence of signals from DNA from other microorganisms than the target organism). It is of utmost importance, that the sequences at the 3' end of the two primers are not homologous, otherwise the two primers will self anneal with primer–dimer products and a possible false negative analysis as result. At the 5' end of a specific primer, a "tail" consisting of a recognition sequence for a restriction enzyme, a captur-

ing sequence or a radioactive or nonradioactive label can be added, normally without influencing the specificity of the primer annealing *(18)*.

2.1.3. Choice of Enzyme

In recent years, almost every vendor of enzymes and molecular biology products offers a thermostable DNA polymerase. No independent analysis presents a complete overview of all available enzymes, so one has to consider the specific needs in a given analysis: affinity purified vs genetic engineered enzyme, proofreading activity versus no such activity and price-per-unit, which can be difficult to determine, since the actual activity per unit may vary between different enzymes. The final choice can be determined by a price/performance study, but one should also consider the fact that only enzymes with a license to perform PCR can be used legally in a laboratory performing PCR analysis.

2.1.4. Optimization of the Variables

The components of a PCR reaction need to be optimized each time a new PCR analysis is designed *(19)*. Once the optimal annealing temperature is established, different concentrations of primers, enzyme, and $MgCl_2$ are combined, and the combination ensuring optimal sensitivity and specificity is chosen for future analysis. Whenever a variable in the analysis is changed, e.g., the DNA to be analyzed is extracted by another method, a new optimization may be needed.

2.2. Hot Start

When DNA is extracted from a sample, unavoidably some DNA will be in single-stranded form. If the components of the PCR analysis are mixed at room temperature, the primers may anneal unspecifically to the single-stranded DNA. Since the *Taq* polymerase possesses some activity at room temperature, unspecific DNA can be synthesized even before the sample is positioned in the thermal cycler. One way to avoid this is to withhold an essential component from the reaction (e.g., *Taq* polymerase or $MgCl_2$) until the temperature is at or above the optimal primer annealing temperature—the so called hot-start PCR *(20)*. This can also be achieved by inhibition of the enzymatic activity by a monoclonal antibody that denatures at temperatures above the unspecific primer annealing level *(21)* or by using an inactive enzyme, one that is activated by incubation above 90°C for several minutes *(22)*. Another method is to mix the PCR components at 0°C. At this temperature, DNA will not renature and the *Taq* polymerase has no activity. When the sample is placed directly in a preheated (94–96°C) thermal cycler, unspecific amplification is avoided—the so-called cold start PCR. If the carry-over prevention system (**Subheading 2.4.2.**) is used, a chemical hot-start is achieved, since any unspecific products

synthesized before the UNG is activated (just prior to initiation of the PCR profile) will be degraded by the UNG *(23–25)*.

2.3. Quantitative Amplification

In the clinical setting, not only information regarding the presence or absence of a microorganism, but also information regarding the level of infection can be of great value. Since the PCR technology and the other nucleic acid amplification technologies (except bDNA signal amplification) comprises an exponential amplification followed by a linear phase, several built-in obstacles must be overcome in order to gain information about the initial target level. First, the final linear phase must be avoided by limiting the number of amplification cycles. Second, a known amount of an internal standard amplifiable by the same primerset as the target—but different from the target in sequence length and composition—must be included *(26–28)*. However, since the amplification efficiency varies not only from cycle to cycle, but also between different targets *(29)*, a semiquantitative rather than an absolute quantitative amplification seems to be the limit of the PCR technology (and LCR/3SR, *see* **Subheadings 5.1.** and **5.2.**) *(30)*. Calculations of the tolerance limits of a quantitative HIV assay showed that an increase in HIV DNA copies of 60% or less, or a decrease in HIV DNA copies of 38% or less, could be explained by random and not by an actual increase or decrease in the number of HIV DNA copies *(26)*. If an absolute quantitation is to be achieved, the bDNA signal amplification assay (**Subheading 5.3.**) can be implemented at the cost of a substantially lower sensitivity.

2.4. Sources of Error

2.4.1. False Negative Results

If the extraction procedure applied does not remove inhibitory factors present in the clinical material, even a high copy number of the target gene will not produce a positive signal. In theory, the PCR reaction can ensure a positive signal from just one copy of the target gene hidden in an infinite amount of unspecific DNA. In practical terms, however, 3–10 copies of the specific target gene sequence are needed to reproducibly give a positive signal, and more than 0.5–1 μg unspecific DNA will inhibit the analysis. If the primers are not specific, the primer annealing temperature is not optimized, or the concentration of the components of the reaction is not optimized, a false negative result can occur because of inefficient or unspecific amplification. Products only consisting of primer sequences can arise if the two primers have complementary sequences, but can also be seen if the primer and/or enzyme concentration is too high—even if the primers are not complementary. These primer–dimer artifacts will dramatically reduce the efficiency of the specific amplification and will likely result in a false negative result.

2.4.2. False Positive Results

If the primers are homologous to other sequences than the target gene or if products from previous similar PCR analysis are contaminating the reaction, a false positive signal will be the result. Primers crossreacting with other sequences can be a problem when conserved sequences (e.g., the bacterial ribosomal RNA gene) are amplified. The problem can be avoided by a homology search in GeneBank or EMBL combined with a screening test using DNA from a number of related as well as, unrelated microorganisms. Contamination has in the past been considered the major problem of the PCR technology *(31,32)*, but this problem can be minimized by rigorous personnel training, designing the PCR laboratory according to the specific needs of this technology (*see* **Subheading 4.1.**) and application of the carryover prevention system already included in commercial PCR kits. This system substitutes uracil for thymine in the PCR, and if the following PCR analyses are initiated with an incubation with a uracil-degrading enzyme such as uracil-*N*-glycosylase, contaminating–but not wild-type–DNA will be degraded *(23–25)*. Implementation of this technology in the PCR analysis has reduced the problem of contamination in most routine PCR laboratories.

3. Detection of Microorganisms

3.1. Relevant Microorganisms

At the present time, PCR cannot be considered as a substitution but rather a supplement for the classic routine bacteriology. The PCR is clearly inferior in terms of sensitivity to classic methods such as blood culture when fast-growing bacteria such as staphylococci are present. Moreover, although antibiotic resistance can be identified by PCR *(33–38)*, the sequence still has to be known, whereas the classical disk methods will reveal the susceptibility and resistance no matter what genetic sequence (chromosomal or plasmid) the underlying mechanism is based upon. Even though PCR has been applied to detect a great number of bacteria (**Table 1**, **refs.** *39–132*), only the detection of slowly or poorly growing bacteria (e.g., *Legionella* spp., *Mycobacterium* spp., or *Borrelia* spp.) are relevant in the clinical setting. In contrast, all pathogenic viruses and especially all pathogenic fungi would be candidates to detection by PCR or related technologies, because of the problems with speed and/or sensitivity of the current diagnostic methods.

3.2. Identification of Microorganisms

3.2.1. Identification by Ribosomal RNA PCR

The classical detection of microorganisms by PCR is based on the amplification of a sequence specific for the microorganism in question. If a broad

Table 1
Examples of Microorganisms Detected by PCR (refs. *39–132*)

Mycobacterium tuberculosis
Mycobacterium paratuberculosis
Mycobacterium leprae
Mycobacterium species
Legionella pneumophila
Legionella species
Borrelia burgdorferii
Listeria monocytogenes
Listeria species
Haemophilus influenzae
Bordetella pertussis
Neisseria meningitidis
Treponema pallidum
Helicobacter pylori
Vibrio vulnificus
Aeromonas hydrophilia
Yersinia pestis
Yersinia pseudotuberculosis
Clostridium difficile
Escherichia coli
Shigella flexneri
Shigella dysenteriae
Shigella boydii
Shigeila sonnei
Mycoplasma pneumoniae
Mycoplasma genitalium
Mycoplasma fermentas
Chlamydia trachomatis
Chlamydia psittaci
Chlamydia pneumoniae
Whipple's disease bacillus
(Trophyryma whipelii)
HIV 1/2
HTLV I/II
Endogenous retrovirus
Cytomegalovirus
Herpes simplex 1/2
HHV 6/7/8
Varicella-Zoster virus
Epstein-Barr virus
Hepatitis virus A/B/C/D/E/F/G/H
Human papilloma virus
Rubella virus
Morbilli virus
Parotitis virus
Influenza virus A
Rhino virus
Coxsackie virus
Polio virus 1-3
Echovirus
Enterovirus 68/70
Adeno virus type 40/41
Rota virus
Rabies virus
Parvo virus B19
Dengue virus
St. Louis encephalitis virus
Japanese encephalitis virus
Yellow fever virus
Lassa virus
Hanta virus
JC/BK virus

Rickettsia rickettsii
Rickettsia typhi
Rickettsia prowazekii
Rickettsia tsutsugamushi
Rickettsia conorii
Rickettsia canada
Toxoplasma gondii
Taenia saginata
Schistosoma mansoni
Echinococcus multilocularis
Pneumocystis carinii
Plasmodium falciparum
Plasmodium vivax
Leishmania
Trypanosoma cruzi
Trypanosoma brucei
Trypanosoma congolense
Entamoeba histolytica
Naegleria fowleri
Giardia lamblia
Babesia microti

Candida albicans
Candidae species
Cryptococcus species
Trichosporon beigelii

range of bacterial pathogens is to be detected in a clinical sample, conserved genetic sequences must be sought. The bacterial 16S ribosomal gene contains variable as well as conserved regions *(133)*, and is well suited for this strategy. By 16S RNA PCR, it is not only possible to detect all known bacteria (at kingdom level, *[134]*), identification can also be performed at genus or species level (e.g., *mycobacterium* spp., *Mycobacterium tuberculosis [135,136]*). Moreover, since some conserved sequences are present in all bacteria, it is now possible to detect unculturable bacteria. By application of this approach, the cause of Whipple's disease *(137)* as well as bacillary angiomatosis *(138)* has been identified. It is likely that more diseases of unknown etiology in the future will be correlated to the presence of unculturable bacteria by the application of 16S RNA PCR. Furthermore, since the typing and identification of bacteria at the present time are based upon phenotypical characterization (shape, staining, and biochemical behavior), typing at the genetypic level (e.g., by 16S RNA PCR) would most likely result in altered perception of the relation between at least some bacteria.

3.2.2. Identification by Random Amplification of Polymorphic DNA (RAPD)

Classical detection of microorganisms by PCR as well as amplification of bacterial 16S RNA sequences relies upon specific primer annealing. However, if one or two oligonucleotides of arbitrarily chosen sequence with no known homology to the target genes were used as primers during unspecific primer annealing conditions in a PCR assay, arrays of DNA fragments would be the result *(139–141)*. Under carefully titrated conditions of the PCR, empirical identification of primers generating an informative number of DNA fragments can be made. By analyzing the pattern of DNA fragments, bacterial isolates can be differentiated, not only on genus level, but also on species and subspecies level *(142–147)*. This method could prove to be an efficient tool for monitoring the epidemiology of infections such as hospital infections *(148)*.

3.3. Sample Preparation

Once the variables of a PCR analysis have been optimized, the actual clinical performance is determined by the efficiency of the extraction method applied to the clinical material as well as the handling of the clinical material. Different clinical materials contain different levels of factors capable of inhibiting the PCR—some acting by direct inhibition of the enzyme, some by binding to other components of the PCR (e.g., the $MgCl_2$).

The optimal extraction method for any clinical material is a method that extracts and concentrates even a single target molecule into a volume that can be analyzed in a single PCR. Because of the loss of material during the extrac-