# ADVANCES IN GENE TECHNOLOGY

# ADVANCES IN GENE TECHNOLOGY

*A Research Annual*

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*PHLS Centre for Applied Microbiology and Research*
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# PREFACE

A year has now elapsed since the first volume in this series was published. It is a year in which information on all aspects of gene technology has continued to be generated at an ever-increasing rate and, as noted in the preface to Volume 1, it is now almost impossible to keep abreast of the literature associated with anything but a very small sector of this specialized field. The need for state-of-the-art reviews contained within volumes such as *Advances in Gene Technology* therefore continues to grow. Even so, it is still difficult to arrange for the comprehensive review of all the major advances that may have occurred in any one 12-month period. It is a task made even more difficult because of the inevitable delay before the impact of many of these advances is fully appreciated.

It is therefore not too surprising that I have found it exceedingly difficult to select articles for publication that review some of the more obvious advances in the field and, at the same time, demonstrate the broad range of scientific approaches that are currently applied to problems in gene technology. Nevertheless, I feel that the 11 review articles eventually chosen and now contained in the second volume of this series go some way to fulfilling these objectives. Some of these articles discuss techniques that are commonly used within defined research programmes and thus provide an introduction to ways in which genes can be amplified, gene libraries can be constructed, RNA can be sequenced and gene expression can be optimized both in eukaryotic and prokaryotic organisms. The remaining articles describe the application of these and other techniques to real problems that range from the diagnosis of exotic diseases to biomining with bacteria. Hence

together these 11 articles form a broad ranging set of reviews that should be of direct or peripheral interest to many working within the expanding field of biotechnology.

Peter Greenaway
*Series Editor*

# CONTENTS

# THE POLYMERASE CHAIN REACTION

P. A. Kitchin and N. Almond

## OUTLINE



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## 1. INTRODUCTION

The polymerase chain reaction (PCR), as invented by Kary Mullis and colleagues at Cetus [1–3], is a landmark technique that has revolutionized almost all aspects of nucleic acid analysis. It is a technique that is potentially capable of detecting as little as a *single* copy of a specific target sequence. This incredible level of sensitivity increases the detection limit for specific nucleic acid sequences by about a million-fold over previous techniques. As a result the PCR is now routinely applied in a formidable range of research projects, as well as to improve the diagnosis of pathogens and disease states of medical and veterinary importance (see Table 1). This chapter will review the theoretical, practical, clinical and research applications of the PCR and where space precludes an in-depth discussion, the reader will be referred to more specific reviews or references.

## 2. THEORETICAL ASPECTS OF THE PCR

The PCR technique generates millions of copies of specific DNA sequences. This is achieved by repeated cycles of amplification (Figure 1). Each cycle causes an approximate doubling of the number of molecules of the desired

*Table 1.* Applications for the PCR.

| | |
|---|---|
| **1. Research applications** | |
| Rapid molecular cloning | |
| Rapid gene isolation | |
| Rapid sequence analysis | |
| Rapid plasmoid insert isolation | |
| Gene expression analysis | |
| Modifications or mutagenesis of a DNA sequence | |
| Identification of related, but unknown, sequences | |
| Quantification of nucleic acids | |
| Construction of chromosome linkage maps | |
| **2. Medical and Veterinary Applications** | |
| Detection of pathogens: | Viral |
| | Bacterial |
| | Protozoal |
| Disease diagnosis: | Blood related disorders |
| | Neurological disorders |
| | Metabolic disorders |
| | Neoplastic disorders |
| | Detection of oncogenes |
| | Prenatal diagnosis |
| | Genetic risk prediction |
| Miscellaneous: | HLA typing |
| | Minimal cancer residue determination |
| | Simplification of sampling techniques |
| | Population genetics and epidemiology |
| | Animal pedigree determination |
| **3. Miscellaneous Applications** | |
| Forensic analysis of biological samples | |
| Analysis of DNA from ancient and/or extinct sources | |
| Genetic fingerprinting of individuals | |

sequence. A PCR cycle is composed of three separate 'steps': denaturation, primer annealing and primer extension, as shown in Figure 1.

In its simplest form, the PCR is designed to amplify target sequences from double-stranded DNA (see Figure 2). This could be, for example, from a region of chromosomal DNA known to harbour a mutation for an inherited disease, such as sickle-cell anaemia. In this case, two oligonucleotide primers of defined sequences, flanking the $\beta$-globin gene would be synthesized. These oligonucleotides are short pieces of single-stranded DNA, normally about 20–30 nucleotides long, possessing a sequence complementary to regions flanking the target region of interest (open and closed triangles 'a' and 'b' in Figure 2).

The first 'step' of an 'ideal' cycle (see Figure 1) is to denature the double-

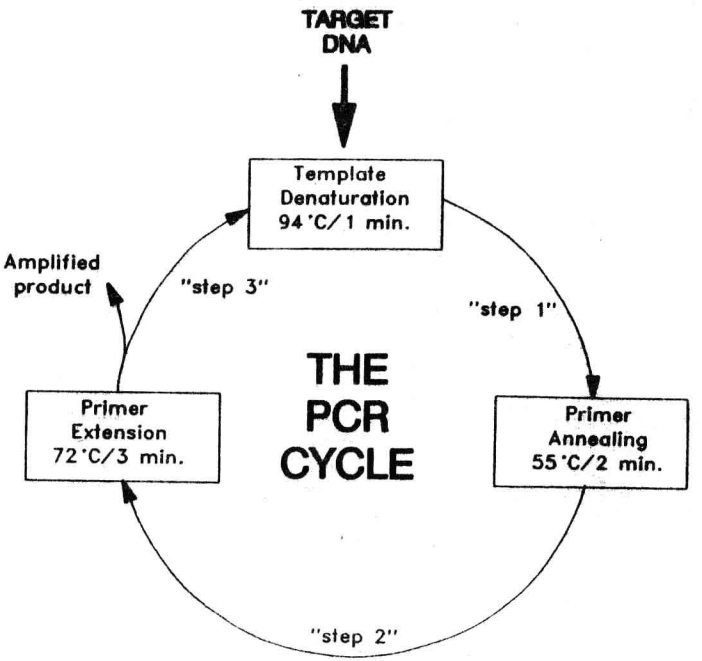


*Figure 1.* Schematic representation of the PCR thermo-cycle. See text for more details.

stranded DNA typically by heating the sample to 94°C for 1 min, causing the two strands of chromosomal DNA to unwind and separate. This step is important, because the DNA strands are now 'free' to anneal to the oligonucleotide primers. During the second 'step' of each cycle, the primers 'a' and 'b' locate and anneal to their complementary sequences flanking the β-globin gene. This step is achieved by lowering the temperature of the reaction to about 55°C for 2 min. In the third and final 'step' of each cycle, the temperature is raised to 72°C and the primers extended by an enzyme called *Taq* DNA polymerase. This heat stable enzyme now produces a complementary copy of each DNA strand of, in this example, the β-globin gene (Figure 2, cross-hatching, strands $a_1$ and $b_1$).

The product obtained by copying the original chromosomal template (strands A and B in Figure 2) will always be heterogeneous in length (Figure 2, dotted arrow heads). This length will be dictated primarily by the processivity of the *Taq* polymerase. However, the product obtained when the newly synthesized strands (Figure 2, strands $a_1$ and $b_1$) are *themselves* used as templates during subsequent PCR cycles will be of a *unique* length

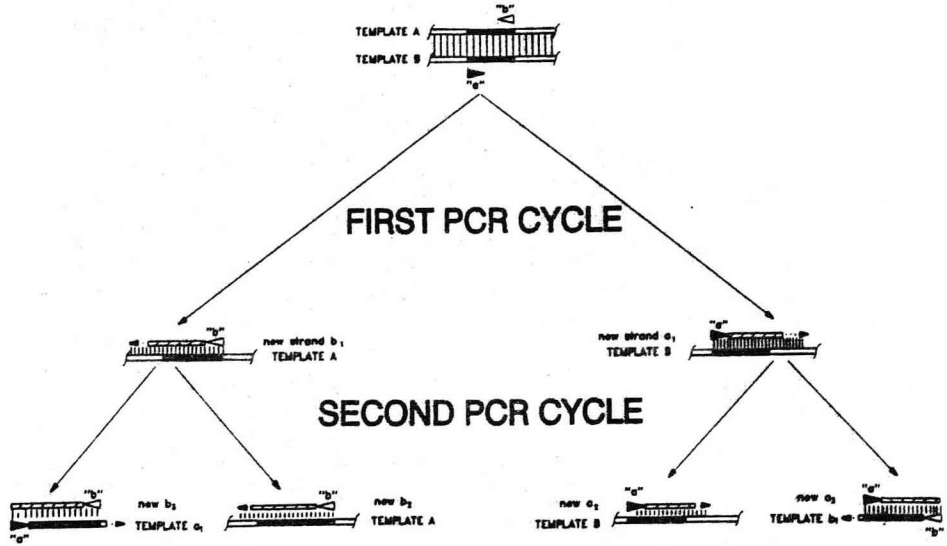


*Figure 2.* Principle of nucleic acid amplification by the PCR. The figure shows the progeny molecules for the first two cycles of PCR. The target DNA (strands A and B) is flanked by primers 'a' and 'b'. During the first cycle of PCR amplification, the strands A and B are separated by denaturation, the primers 'a' and 'b' anneal to their respective complementary regions and are extended to produce two new, heterogeneous length, progeny strands $a_1$ and $b_1$. During the second cycle of amplification the original strands A and B give rise to two more heterogeneous length progeny strands ($a_2$ and $b_2$). However, the progeny strands from the previous cycle ($a_1$ and $b_1$) when used as templates *themselves*, give rise to *unique* length progeny strands, $a_3$ and $b_3$. See text for more details. Double-stranded DNA is indicated by ||||| ; progeny strands synthesized during any given cycle are indicated by cross-hatching. The solid region indicates the target DNA. The dotted arrow heads indicate that the length of the product is variable. The lower strand of the pair in each case is the template strand and the upper strand is the progeny synthesized during that round of amplification.

*Table 2.* Theoretical accumulation of PCR products by type.

| Cycle number | Number of double-stranded progeny molecules | | | |
|---|---|---|---|---|
| | heterogeneous length (H) | unique length | H as % of total | total progeny molecules |
| 1 | 1 | 0 | 100 | 1 |
| 2 | 2 | 1 | 67 | 3 |
| 3 | 3 | 4 | 42 | 7 |
| 4 | 4 | 11 | 27 | 15 |
| 5 | 5 | 26 | 16 | 31 |
| 6 | 6 | 57 | 9.5 | 63 |
| 7 | 7 | 120 | 5.5 | 127 |
| 8 | 8 | 247 | 3.1 | 255 |
| 9 | 9 | 502 | 1.8 | 511 |
| 10 | 10 | 1013 | 1.0 | 1023 |
| 20 | 20 | $\sim 1 \times 10^6$ | negligible | $\sim 1 \times 10^6$ |
| 25 | 25 | $\sim 33 \times 10^6$ | negligible | $33 \times 10^6$ |
| 30 | 30 | $\sim 1 \times 10^9$ | negligible | $\sim 1 \times 10^9$ |
| 40 | 40 | $\sim 4 \times 10^{14}$ | negligible | $\sim 4 \times 10^{14}$ |

See Figure 2 for examples of heterogeneous length molecules ($a_1$, $b_1$, $a_2$ and $b_2$) and unique length molecules ($a_3$ and $b_3$). The accumulation of double-stranded unique length molecules is given by $(2)^n - (n + 1)$, where $n$ is the number of cycles.

(Figure 2, strands $a_3$ and $b_3$). This is because, unlike the chromosomal templates, these template strands now have unique ends, derived from the original primer during the previous cycle. The expected length of this PCR product is thus defined by the separation of the two oligonucleotide primers.

The heterogeneous length PCR product theoretically accumulates in a linear fashion with each cycle of the PCR (Table 2). This is because its template can only be the original chromosomal DNA (strands A and B, Figure 2). However, the unique length product theoretically accumulates in an exponential manner with each cycle of the PCR, since every newly synthesized strand acts as a template during the next cycle of PCR amplification. The theoretical accumulation of each molecular type is shown in Table 2. Initially, the proportion of the heterogeneous length molecules in comparison to the total number of amplified molecules is high. However, after 10 cycles of PCR, these molecules represent less than 1% of the total and thereafter become negligible. If the efficiency of the reaction is 100%, then a million-fold amplification of the target sequence can be achieved in just 20 cycles of the PCR (Table 2). However, in practice, the efficiency is less than 100% and most amplifications are therefore performed with 25–40 cycles, to compensate for the loss of potential product at each cycle.

## 3. PRACTICAL ASPECTS OF THE PCR

### 3.1 Optimization of the Assay

For many applications, the initial reaction conditions shown in Table 3 will provide a satisfactory starting point. The PCR technique is almost as straight forward in practice as it appears to be in theory. However, it is sometimes desirable to 'fine tune' the reaction to remove any unwanted, non-specific PCR products and/or to improve the yield of the desired product.

There is often a temptation with PCR to continue to increase sensitivity almost for its own sake. This is not necessary and is in fact frequently undesirable. A laboratory using PCR to analyse inserts in plasmids (inserts for analysis) does not need a PCR with a million-fold or greater amplification level. Increased sensitivity will certainly bring with it increased problems of contamination which were previously undetectable. Consequently, it is sensible to perform PCR amplifications only to the levels necessary. With this in

*Table 3.* Suggested initial conditions for the PCR assay.

*I Assay Components*

| Reaction component | Concentration (in 50 $\mu$l) | |
|---|---|---|
| | Initial | Range |
| Tris–HCl buffer pH 8.3 | 10 mM | NT |
| $MgCl_2$ | 1.5 mM | 0–15 mM |
| dNTPs | 200 $\mu$M | 10–400 $\mu$M |
| Primers | 0.1 $\mu$M | 0.05–1 $\mu$M |
| *Taq* polymerase | 1.25 units | 0.6–5 units |
| Gelatin | 0.01% w/v | NT |

*II Thermal cycling*

| Step | Initial | | Range | |
|---|---|---|---|---|
| | Temperature | Time | Temperature | Time |
| 1. Denaturation | 94°C | 60 s | NT | NT |
| 2. Annealing | 55°C | 120 s | 30–70°C | 30–120 s |
| 3. Extension | 72°C | 180 s | NT | 30–180 s |

NT: Not tested

The 'initial' concentrations have been found to work well for most applications with the *Taq* polymerase obtained from Cetus. The 'range' of concentrations represent the minimal concentrations found to support PCR and guidance to the highest values tested in our own lab. Note that the dNTP and $MgCl_2$ concentrations will be inter-dependent to some extent. These reaction conditions are not suited to all sources of *Taq* polymerase and users should refer to manufacturers protocols as well.

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