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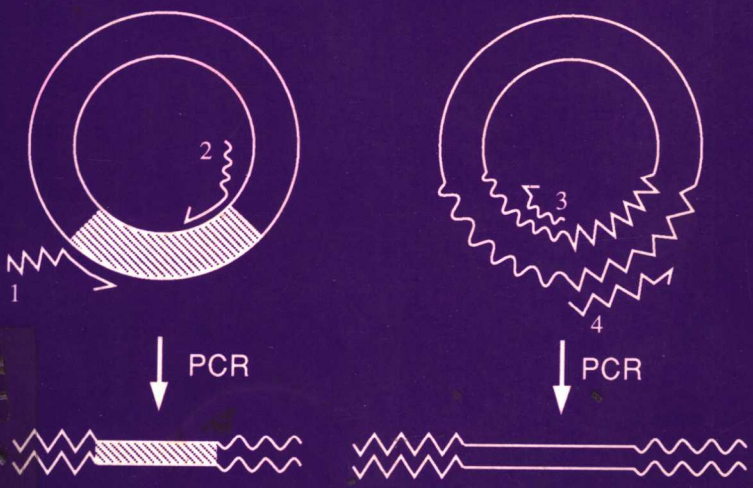
Volume 15

PCR PROTOCOLS

Current Methods and Applications

Edited by

Bruce A. White



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Preface

PCR has been successfully utilized in every facet of basic, clinical, and applied studies of the life sciences, and the impact that PCR has had on life science research is already staggering. Concomitant with the essentially universal use of PCR has been the creative and explosive development of a wide range of PCR-based techniques and applications. These increasingly numerous protocols have each had the general effect of facilitating and accelerating research. Because PCR technology is relatively easy and inexpensive, PCR applications are well within the reach of every research lab. In this sense, PCR has become the "equalizer" between "small" and "big" labs, since its use makes certain projects, especially those related to molecular cloning, now far more feasible for the small lab with a modest budget.

This new volume on *PCR Protocols* does not attempt the impossible task of representing all PCR-based protocols. Rather, it presents a range of protocols, both analytical and preparative, that provide a solid base of knowledge on the use of PCR in many common research problems. The first six chapters provide some basic information on how to get started. Chapters 7–19 represent primarily analytical uses of PCR, both for simple DNA and RNA detection, as well as for more complex analyses of nucleic acid (e.g., DNA footprinting, RNA splice site localization). The remaining chapters represent "synthetic," or preparative, uses of PCR. The use of PCR for aspects of cloning, including obtaining full-length cDNA sequence, site-directed mutagenesis, and production of synthetic genes has been emphasized in these chapters. Some duplication of important topics (e.g., sequencing, cDNA cloning, the use of degenerate oligonucleotides, and site-directed mutagenesis) has been introduced purposely to offer the reader several approaches to the same problem. As has been done in previous volumes of the *Methods in Molecular Biology* series, an emphasis has been placed on generally

applicable protocols. The description of specific experimental systems has been deemphasized, and used only when the provision of a specific example is helpful. As part of the *Methods in Molecular Biology* series, the chapters each include a "Notes" section, whose purpose is to provide a discussion of problems, tips, and alternatives. This type of discussion, not usually available in the original publications, should enhance the ability of the reader to get a procedure up and running, as well as increase experimenters' understanding of its strengths, limitations, and pitfalls. It is hoped that this collection of PCR protocols will be especially useful to young investigators, or to those new to PCR, by providing a knowledge base and encouraging the design of novel approaches and applications of PCR.

I am indebted to Jennifer Swanson for her superb secretarial skills. I also wish to thank John Walker and Humana Press for their assistance and support in putting this volume together. Finally, I want to express my appreciation to the contributing authors, who displayed a remarkable degree of enthusiasm for this volume and provided such excellent material for it.

Bruce A. White

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Contents

CHAPTER 1

Polymerase Chain Reaction

Basic Protocols

Beverly C. Delidow, John P. Lynch, John J. Peluso, and Bruce A. White

1. Introduction

The melding of a technique for repeated rounds of DNA synthesis with the discovery of a thermostable DNA polymerase has given scientists the very powerful technique known as polymerase chain reaction (PCR). PCR is based on three simple steps required for any DNA synthesis reaction: (1) *denaturation* of the template into single strands; (2) *annealing* of primers to each original strand for new strand synthesis; and (3) *extension* of the new DNA strands from the primers. These reactions may be carried out with any DNA polymerase and result in the synthesis of defined portions of the original DNA sequence. However, in order to achieve more than one round of synthesis, the templates must again be denatured, which requires temperatures well above those that inactivate most enzymes. Therefore, initial attempts at cyclic DNA synthesis were carried out by adding fresh polymerase after each denaturation step *(1,2)*. The cost of such a protocol becomes rapidly prohibitive.

The discovery and isolation of a heat-stable DNA polymerase from a thermophilic bacterium, *Thermus aquaticus (Taq)*, enabled Saiki et al. *(3)* to synthesize new DNA strands repeatedly, exponentially amplifying a defined region of the starting material, and allowing the birth of a new technology that has virtually exploded into prominence. Not

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since the discovery of restriction enzymes has a new technique so revolutionized molecular biology. There are scores of journal articles published *per month* in which PCR is used, as well as an entire journal (at least one) devoted to it. To those who use and/or read about PCR every day, it is remarkable that this method is not yet 10 years old.

One of the great advantages of PCR is that, although some laboratory precaution is called for, the equipment required is relatively inexpensive and very little space is needed. The only specialized piece of equipment needed for PCR is a thermal cycler. Although it is possible to perform PCR without a thermal cycler—using three water baths at controlled temperatures—the manual labor involved is tedious and very time-consuming. A number of quality instruments are now commercially available. A dedicated set of pipets is useful, but not absolutely necessary. If one purchases oligonucleotide primers, all of the other equipment required for PCR is readily found in any laboratory involved in molecular biology. Thus, a very powerful method is economically feasible for most research scientists.

The versatility of PCR will become clear in later chapters, which demonstrate its use in a wide variety of applications. Additionally, the reader is referred to several recent reviews *(4,5)*. In this chapter, we outline the preparations required to carry out PCR, the isolation of DNA and RNA as templates, the basic PCR protocol, and several common methods for analyzing PCR products.

2. Materials

2.1. Preparation for PCR

2.1.1. Obtaining Primers

1. Prepared oligonucleotide on a cartridge. Cap ends with parafilm and store horizontally (the columns contain fluid, which can leak) at –20°C until the oligo is to be purified.
2. Ammonium hydroxide, reagent grade. Ammonium hydroxide should be handled in a fume hood, using gloves and protective clothing.
3. 1-mL tuberculin syringes (needles are not required).
4. 1.25-mL screw-cap vials, with O-rings (e.g., Sarstedt #D-5223, Sarstedt, Inc., Pennsauken, NJ).
5. Parafilm.
6. Sterile water, filter deionized distilled water through a 0.2-µm filter, store at room temperature.

7. 1*M* $MgSO_4$. Filter through a 0.2-µm filter and store at room temperature.
8. 100% Ethanol.
9. 95% Ethanol; for precipitations store at –20°C.

2.1.2. Isolation of DNA

1. Source of tissue or cells from which DNA will be extracted.
2. Dounce homogenizer.
3. Digestion buffer: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 25 m*M* EDTA, 0.5% SDS.
4. Proteinase K, 20 mg/mL.
5. a. Buffered phenol *(6,7)*: Phenol is highly corrosive, wear gloves and protective clothing when handling it. Use only glass pipets and glass or polypropylene tubes. Phenol will dissolve polystyrene plastics.
   b. Buffering solutions: 1*M* Tris base; 10X TE, pH 8.0 = 100 m*M* Tris-HCl, pH 8, 10 m*M* EDTA; 1X TE, pH 8 = 10 m*M* Tris, pH 8, 1 m*M* EDTA. To a bottle of molecular biology grade recrystallized phenol add an equal volume of 1*M* Tris base. Place the bottle in a 65°C water bath and allow the phenol to liquify (approx 1 h). Transfer the bottle to a fume hood and allow it to cool. Cap the bottle tightly and shake to mix the phases, **point the bottle away** and vent. Transfer the mix to 50-mL screw-top tubes by carefully pouring or using a glass pipet. Centrifuge at 2000 rpm for 5–10 min at room temperature to separate the phases. Remove the upper aqueous phase by aspiration. To the lower phase (phenol) add an equal volume of 10X TE, pH 8. Cap tubes tightly, shake well to mix, and centrifuge again. Aspirate the aqueous phase. Reextract the phenol two or three more times with equal volumes of 1X TE, pH 8.0, until the pH of the upper phase is between 7 and 8 (measured using pH paper). Aliquot the buffered phenol, cover with a layer of 1X TE, pH 8, and store at –20°C.
6. $CHCl_3$.
7. 100% Ethanol.
8. 70% Ethanol.
9. TE buffer, pH 8.0: 10 m*M* Tris-HCl, pH 8.0, 1m*M* EDTA.
10. Phosphate-buffered saline (PBS): 20X stock = 2.74*M* NaCl, 53.6 m*M* KCl, 166 m*M* Na_2HPO_4, 29.4 m*M* KH_2PO_4, pH 7.4. Make up in deionized distilled water, filter through a 0.2-µm filter, and store at room temperature. For use, dilute 25 mL of 20X stock up to 500 mL with deionized distilled water and add 250 µL of 1*M* $MgCl_2$. Sterile-filter and store at 4°C.
11. 7.5*M* Ammonium acetate.
12. RNase A. Prepare at 10 mg/mL in 10 m*M* Tris-HCl, pH 7.5, 15 m*M*

NaCl. Incubate at 100°C for 15 min and allow to cool to room temperature. Store at –20°C.

13. 20% SDS.

2.1.3. Isolation of RNA

2.1.3.1. Isolation of RNA by CsCl Centrifugation (*see* Note 1)

1. Source of tissue or cells from which RNA will be extracted.
2. PBS (*see* Section 2.1.2., item 10).
3. 2-mL Wheaton glass homogenizer.
4. Guanidine isothiocyanate/β-mercaptoethanol solution (GITC/βME): 4.2*M* guanidine isothiocyanate, 0.025*M* sodium citrate, pH 7.0, 0.5% *N*-laurylsarcosine (Sarkosyl), 0.1*M* β-mercaptoethanol. Prepare a stock solution containing everything except β-mercaptoethanol in deionized distilled water. Filter-sterilize using a Nalgene 0.2-µm filter (Nalge Co., Rochester, NY) (*see* Note 2). Store in 50-mL aliquots at –20°C. To use, thaw a stock tube, transfer the required volume to a fresh tube, and add 7 µL of β-mercaptoethanol/mL of buffer. Guanidine isothiocyanate and β-mercaptoethanol are strong irritants, handle them with care.
5. 1-mL tuberculin syringes, with 21-g needles.
6. Ultraclear ultracentrifuge tubes, 11 × 34 mm (Beckman #347356).
7. Diethylpyrocarbonate, 97% solution, store at 4°C.
8. Diethylpyrocarbonate (DEPC)-treated water *(6,7)*. Fill a baked glass autoclavable bottle to two-thirds capacity with deionized distilled water. Add diethyl pyrocarbonate to 0.1%, cap and shake. Vent the bottle, cap loosely, and incubate at 37°C for at least 12 h (overnight is convenient). Autoclave on liquid cycle for 15 min to inactivate the DEPC. Store at room temperature.
9. 200 m*M* EDTA, pH 8.0. Use molecular biology grade disodium EDTA. Make up in deionized distilled water and filter through a 0.2-µm filter. Place in an autoclavable screw-top bottle. Treat with DEPC as described in the preceding step for DEPC water. Store at room temperature.
10. CsCl: molecular biology grade. For 20 mL, place 20 g of solid CsCl in a sterile 50-mL tube. Add 10 mL of 200 m*M* EDTA, pH 8.0 (DEPC-treated). Bring volume to 20 mL with DEPC water. Mix to dissolve. Filter through a 0.2-µm filter and store at 4°C.
11. TE buffer, pH 7.4: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA. Make a solution of 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 7.4, in DEPC water (*see* Note 3). Filter through a 0.2-µm filter, autoclave 15 min on liquid cycle, and store at room temperature.

12. TE-SDS: Make fresh for each use. From a stock solution of 10% SDS in DEPC water, add SDS to a concentration of 0.2% to an aliquot of TE, pH 7.4.
13. Buffered phenol (*see* Section 2.1.2., item 5).
14. $CHCl_3$.
15. 4*M* NaCl. Make up in deionized distilled water and DEPC treat. Autoclave 15 min on liquid cycle and store at room temperature.
16. 95% Ethanol, stored at –20°C.
17. Polyallomer 1.5-mL microcentrifuge tubes, for use in an ultracentrifuge (Beckman #357448, Beckman Instrument Inc., Fullerton, CA).
18. RNasin RNase inhibitor, 40 U/µL (Promega, Madison, WI). Store at –20°C.
19. Beckman TL-100 table-top ultracentrifuge, TLS 55 rotor, and TLA-45 rotor.

2.1.3.2. Isolation of RNA by Guanidine/Phenol (RNAzol™) Extraction

1. RNAzol reagent (TEL-TEST, Inc., Friendswood, TX). This reagent contains guanidine isothiocyanate, β-mercaptoethanol, and phenol; handle with care.
2. Glass-Teflon homogenizer.
3. Disposable polypropylene pellet pestle and matching microfuge tubes (1.5 mL) (Kontes Life Science Products, Vineland, NJ).
4. $CHCl_3$ (ACS grade).
5. Isopropanol (ACS grade). Store at –20°C.
6. 80% Ethanol. Dilute 100% ethanol with DEPC-treated H_2O and store at –20°C.
7. TE buffer, pH 7.4, in DEPC-treated water (*see* Section 2.1.3.1.).

2.1.4. Synthesis of Complementary DNAs (cDNAs) from RNA

1. RNA in aqueous solution.
2. Oligo dT_{18-20} primer (Pharmacia, Piscataway, NJ). Dissolve 5 OD U in 180 µL of sterile water to give a concentration of 1.6 µg/µL.
3. Specific primer, optional. Choose sequence and obtain as for PCR primers (*see* Section 3.1.1.).
4. MMLV reverse transcriptase (200 U/µL) with manufacturer-recommended buffer and 0.1*M* DTT.
5. Deoxynucleotides dATP, dCTP, dGTP, and dTTP. Supplied as 10 mg solids. To make 10 m*M* stocks: Resuspend 10 mg of dNTP in 10% less