

VOLUME 2

Molecular Cloning

A LABORATORY MANUAL

THIRD EDITION

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Molecular Cloning

A LABORATORY MANUAL

THIRD EDITION

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Front cover (paperback): The gene encoding green fluorescent protein was cloned from *Aequorea victoria*, a jellyfish found in abundance in Puget Sound, Washington State. This picture of a 50-mm medusa was taken on color film by flash photography and shows light reflected from various morphological features of the animal. The small bright roundish blobs in the photograph are symbiotic amphipods living on or in the medusa. The bright ragged area in the center is the jellyfish's mouth.

Bioluminescence from *Aequorea* is emitted only from the margins of the medusae and cannot be seen in this image. Bioluminescence of *Aequorea*, as in most species of jellyfish, does not look like a soft overall glow, but occurs only at the rim of the bell and, given the right viewing conditions, would appear as a string of nearly microscopic fusiform green lights. The primary luminescence produced by *Aequorea* is actually bluish in color and is emitted by the protein aequorin. In a living jellyfish, light is emitted via the coupled green fluorescent protein, which causes the luminescence to appear green to the observer.

The figure and legend were kindly provided by Claudia Mills of the University of Washington, Friday Harbor. For further information, please see Mills, C.E. 1999–2000. Bioluminescence of *Aequorea*, a hydromedusa. Electronic Internet document available at <http://faculty.washington.edu/cemills/Aequorea.html>. Published by the author, web page established June 1999, last updated 23 August 2000.

Back cover (paperback): A portion of a human cDNA array hybridized with a red fluor-tagged experimental sample and a green fluor-tagged reference sample. Please see Appendix 10 for details. (Image provided by Vivek Mittal and Michael Wigler, Cold Spring Harbor Laboratory.)

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Protocol 1

The Basic Polymerase Chain Reaction

THIS PROTOCOL OUTLINES A PROTOTYPE AMPLIFICATION (please see Figure 8-2). Special reagents and equipment for typical amplification reactions are described below with general considerations for their preparation and use. For optimization of particular reaction conditions, please see Parameters That Affect Polymerase Chain Reactions in the introduction to this chapter. Below is a list of reagents and equipment for the basic PCR.

- **Primers.** Each primer should be 20–30 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. For further details, please see Design of Oligonucleotide Primers for Basic PCR in the introduction to this chapter. As discussed below, restriction sites are always designed into adaptor-primers; however, they can also be incorporated into the gene-specific oligonucleotides. Amplification using primers of this type generates amplified cDNAs carrying restriction sites at both ends, which can facilitate cloning.

If necessary, the yield of desired product can be improved by setting up a second round of amplification using another pair of primers that bind to sequences within the amplified segment of target DNA. After this second round of nested amplification, almost all of the product detected by ethidium bromide staining contains the desired segment of DNA.

Oligonucleotide primers synthesized on an automated DNA synthesizer can generally be used in standard PCR without further purification. However, amplification of single-copy sequences from mammalian genomic templates is often more efficient if the oligonucleotide primers are purified by chromatography on commercially available resins (e.g., NENSORB, NEN Life Science Products) or by denaturing polyacrylamide gel electrophoresis, as described in Chapter 10, Protocol 1.

- **Template DNA.** The template can be a fragment of DNA, a preparation of genomic DNA, a recombinant plasmid or bacteriophage λ , or any other DNA-containing sample. Template DNA is dissolved in 10 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM).
- **Thermostable DNA polymerase.** *Taq* DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of PCR. However, where elongation from 3'-mismatched primers is suspected, a thermostable DNA polymerase with 3'→5' proofreading activity may be preferred (Chiang et al. 1993). Please see Thermostable DNA Polymerases (p. 8.6) and the information panel on **TAQ DNA POLYMERASE**.

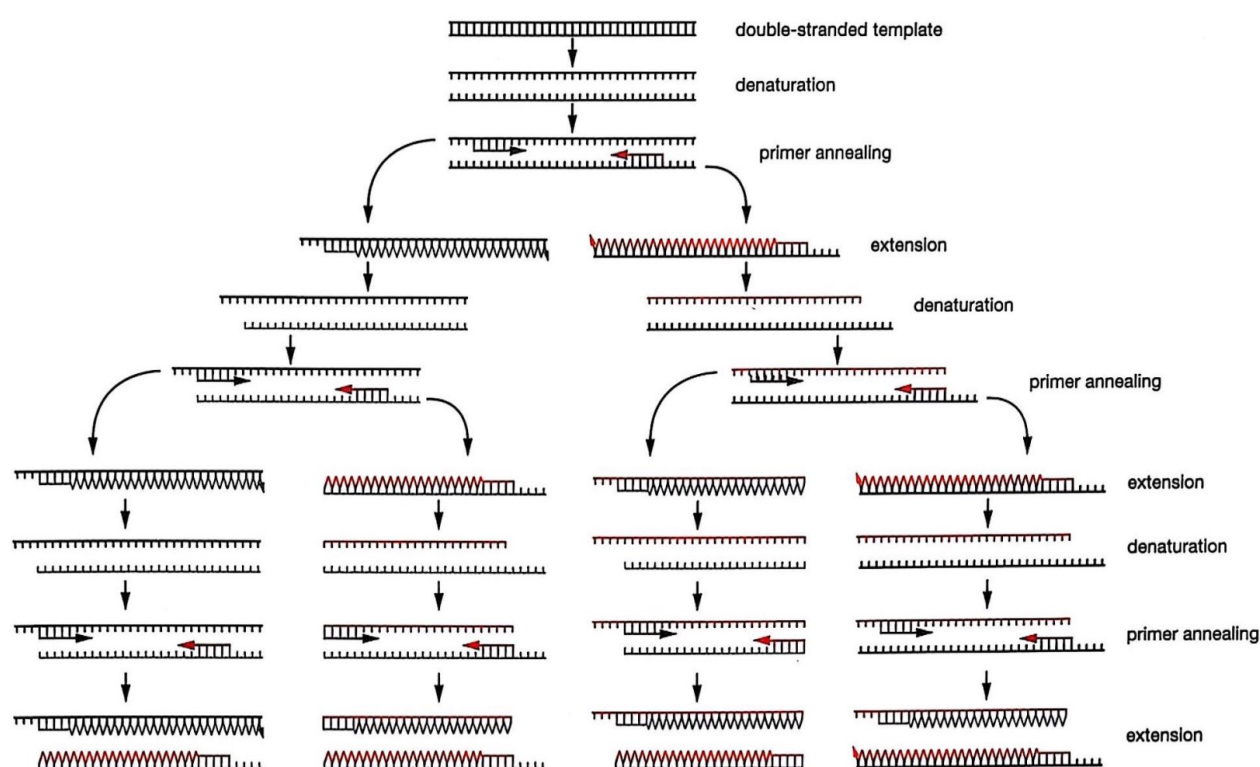


FIGURE 8-2 Sequence of Amplification in the PCR

The diagram shows the steps involved in the first few rounds of a PCR. The original template (*top line*) is double-stranded DNA and the leftward and rightward oligonucleotide primers are shown as $\leftarrow$ and $\rightarrow$, respectively. The products of the first few rounds of the amplification reaction are heterogeneous in size; however, the tract of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the amplification reaction.

Taq DNA polymerase is supplied in a storage buffer containing 50% glycerol. Because this solution is very viscous and difficult to pipette accurately, the best method is to centrifuge the tube containing the enzyme at maximum speed for 10 seconds at 4°C in a microfuge and then to withdraw the required amount of enzyme using a positive-displacement pipette.

- **Automatic pipetting devices.** Automatic micropipetting devices equipped with barrier tips should be used to assemble the components of PCRs. Disposable barrier tips are fitted with a hydrophobic barrier to prevent the accidental passage of liquids into a micropipetting device. This arrangement reduces the potential for cross-contamination of PCRs and DNA samples. Barrier micropipette tips are sold by several commercial companies (e.g., ART Barrier tips from Molecular Bioproducts, Inc.).
- **Positive-displacement pipette.** Positive-displacement pipetting, in which the piston is in direct contact with the liquid, is used for accurate transfer of high-viscosity liquids.
- **Microfuge tubes or microtiter plates.** Thin-walled plastic tubes that fit snugly in the block of the thermal cycler are used for amplification. Their use facilitates heat transfer and greatly reduces much of the time lag in reaching programmed temperatures.
- **Thermal cycler programmed with desired amplification protocol.** A large number of programmable thermal cyclers marketed by different commercial companies are licensed by Perkin-Elmer for use in PCR. The choice among these instruments depends on the investiga-

tor's inclination, the available budget, and the range of uses to which the machine will be put. Before purchasing a thermal cycler, we recommend soliciting as many opinions as possible to find out the pros and cons of different machines. If the machine is not licensed for use in PCR, investigators must negotiate their own financial arrangements with Perkin-Elmer. *The Scientist* profiles thermal cyclers every year (www.the-scientist.com).

MATERIALS

▲ **IMPORTANT** To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 15°C and autoclave all plasticware. For more information, please see the panel on **CONTAMINATION IN PCR** in the introduction to this chapter.

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

10x Amplification buffer

Chloroform <!.>

dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Enzymes and Buffers

Thermostable DNA polymerase

Gels

Polyacrylamide <!.> or agarose gel

Please see Step 4.

Nucleic Acids and Oligonucleotides

Bystander DNA

Please see the panel to Step 1.

Forward primer (20 μM) in H₂O and Reverse primer (20 μM) in H₂O

Please see the discussion on Primers in the protocol introduction.

Use the following formula to calculate the molecular weight of the oligonucleotides:

$$M_r = (C \times 289) + (A \times 313) + (T \times 304) + (G \times 329)$$

where C is the number of C residues in the oligonucleotide, A is the number of A residues, T is the number of T residues, and G is the number of G residues. The molecular mass of a 20-mer will be ~6000 daltons; 100 pmoles of the oligonucleotide will be equivalent to ~0.6 μg.

Template DNA

Dissolve template DNA in 10 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM) at the following concentrations: mammalian genomic DNA, 100 μg/ml; yeast genomic DNA, 1 μg/ml; bacterial genomic DNA, 0.1 μg/ml; and plasmid DNA, 1–5 ng/ml.

Special Equipment

Barrier tips for automatic micropipetting device

Microfuge tubes (0.5 ml, thin-walled for amplification reactions) or Microtiter plates

Positive-displacement pipette

Thermal cycler programmed with desired amplification protocol

If the thermal cycler is not equipped with a heated lid, use either mineral oil or paraffin wax to prevent evaporation of liquid from the reaction mixture during PCR. Paraffin wax not only prevents evaporation, but also maintains separation between components (e.g., primer and template) until the reaction mixture is heated. This separation prevents nonspecific binding of primers during the initial phase of the reaction (please see the information panel on **HOT START PCR**).

Additional Reagents

Step 4 of this protocol may require the reagents listed in Chapter 6, Protocol 10, and/or Chapter 12, Protocol 6.

METHOD

1. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, mix in the following order:

10x amplification buffer	5 μ l
20 mM solution of four dNTPs (pH 8.0)	1 μ l
20 μ M forward primer	2.5 μ l
20 μ M reverse primer	2.5 μ l
1–5 units/ μ l thermostable DNA polymerase	1–2 units
H ₂ O	28–33 μ l
template DNA	5–10 μ l
Total volume	50 μ l

The table below provides standard reaction conditions for PCR.

Mg ²⁺	KCl	dNTPs	Primers	DNA polymerase	Template DNA
1.5 mM	50 mM	200 μ M	1 μ M	1–5 units	1 pg to 1 μ g

The pH of the reaction buffer should be 8.3 when measured at 25°C. Because of the high temperature dependence of the pK_a of Tris, the pH of the reaction will drop to ~7.2 at 72°C (Good et al. 1966; Ferguson et al. 1980). The amount of template DNA required varies according to the complexity of its sequence. In the case of mammalian DNA, up to 1.0 μ g is used per reaction. Typical amounts of yeast, bacterial, and plasmid DNAs used per reaction are 10 ng, 1 ng, and 10 pg, respectively.

Each set of PCRs must always include positive and negative controls. Positive controls are required to monitor the efficiency of the PCR, whereas negative controls are required to detect contamination with DNAs that contain the target sequence.

	Bystander DNA ^a	Template DNA ^b	Target DNA ^c	Specific Primers ^d
Positive Controls				
1	+	–	+	+
2	–	–	+	+
Negative Controls				
3	–	–	–	+
4	+	–	–	+

^aBystander DNA does not contain target sequences. It should resemble the template DNA in all other respects: complexity, size, and concentration.

^bTemplate DNA is the DNA under test.

^cTarget DNA contains the target sequence. It can be a recombinant DNA clone, a purified DNA fragment, or a sample of genomic DNA. It should be added to the positive control at concentrations equivalent to those expected in the template DNA. It is often necessary to set up a series of positive controls containing different amounts of target DNA spanning the amount predicted in the template DNA. An appropriate dilution of the target sequence should be prepared ahead of time in an area of the laboratory different from that used for the preparation of other PCR reagents. This precaution reduces the risk of contaminating equipment and plasticware in the area of the laboratory set aside for PCRs.

^dSpecific Primers are oligonucleotide primers specific for the segment of target DNA.

2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (~50 μ l) of light mineral oil to prevent evaporation of the samples during repeated cycles of heating and cooling. Alternatively, place a bead of wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler.
3. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed below.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	30 sec at 94°C	30 sec at 55°C	1 min at 72°C
Last cycle	1 min at 94°C	30 sec at 55°C	1 min at 72°C

These times are suitable for 50- μ l reactions assembled in thin-walled 0.5-ml tubes and incubated in thermal cyclers such as the Perkin-Elmer 9600 or 9700, Master Cycler (Eppendorf), and PTC 100 (MJ Research). Times and temperatures may need to be adapted to suit other types of equipment and reaction volumes.

Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.

Most thermal cyclers have an end routine in which the amplified samples are incubated at 4°C until they are removed from the machine. Samples can be left overnight at this temperature, but should be stored thereafter at -20°C.

4. Withdraw a sample (5–10 μ l) from the test reaction mixture and the four control reactions and analyze them by electrophoresis through an agarose or polyacrylamide gel. Be sure to include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.

A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), Southern hybridization (please see Chapter 6, Protocol 10), and/or restriction mapping.

If all has gone well, lanes of the gel containing samples of the two positive controls (Tubes 1 and 2) and the template DNA under test should contain a prominent band of DNA of the appropriate molecular weight. This band should be absent from the lanes containing samples of the negative controls (Tubes 3 and 4). If, on the other hand, all is not well, please see Tables 8-4 and 8-5.

5. If mineral oil was used to overlay the reaction (Step 2), remove the oil from the sample by extraction with 150 μ l of chloroform.

The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.

For many purposes, e.g., purification of the amplified DNA using a Centricon microconcentrator or cloning amplification products, it is desirable to remove the oil from the sample before proceeding.

▲ IMPORTANT Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.

OPTIMIZATION OF PCRs

When setting up PCRs for first time with new template DNA, new primers, or a new preparation of thermostable DNA polymerase enzyme, amplification will generally be less than optimal. Fine tuning of the reaction is usually required to suppress nonspecific amplification and/or to enhance the yield of the desired DNA product. Table 8-4 lists some commonly observed problems and suggests ways in which the reaction can be optimized.

TABLE 8-4 Troubleshooting Amplifications

PROBLEM	EXPLANATION	SUGGESTED REMEDY
Bands of the desired product are sharp but very faint in both positive controls <i>and</i> test PCR. Bands of the desired product smear into the portion of the gel containing lower-molecular-weight DNAs.	Inefficient priming or inefficient extension.	<p>Set up a series of PCRs containing different concentrations of the two primers. Find the optimal concentration and then set up a series of touchdown PCRs containing different concentrations of Mg^{2+} to find the optimal concentration.</p> <p>Include GC-Melt (CLONTECH) in the reaction mixture</p> <p>Use the minimum possible temperature during the annealing step.</p> <p>Consider adding adjuvants such as BSA (0.2–0.6 mg/ml), DMSO (5%), or glycerol (5%) to the reaction mixture.</p>
Bands of the desired product are very faint in the test sample and in positive control 2. The band in positive control 1 is much stronger.	Dirty template DNA.	Repurify the template DNA by extraction (twice) with phenol:chloroform and ethanol precipitation. Dissolve the purified DNA in H_2O (no EDTA).
Bands in the negative controls.	Contamination of solutions or plasticware with template DNA.	Please see the panel on CONTAMINATION IN PCR (Introduction) and then make up new reagents.
Distinct band(s) of the wrong molecular weight.	Nonspecific priming by one or both primers.	Decrease the annealing time and/or increase the annealing temperature.
A generalized smear of amplified DNA that may obscure the desired product.	Amplification of primer dimers. Too much template DNA.	<p>Check that neither primer has homology with highly repetitive sequences in the template DNA. Optimize the concentrations of each primer independently.</p> <p>Use touchdown PCR and/or hot start PCR (please see the information panels).</p> <p>Reduce the amount of template DNA by factors of 2.5 and 10.</p>

TROUBLESHOOTING MAJOR PROBLEMS THAT OCCUR IN PCRs

Table 8-5 describes problems that may be encountered in amplification reactions and provides guidance for how to address major difficulties.

TABLE 8-5 Troubleshooting PCRs

SYMPTOM	POSSIBLE CAUSES	POSSIBLE REMEDIES
Amplification weak or nondetectable.	Defective reagent(s); defective thermal cycler; programming error.	Compare the yields obtained from fresh and old reagents in PCRs incubated in two different thermal cyclers.
	Suboptimal annealing conditions.	Recalculate T_m of primers. Use touchdown PCR, preferably in combination with hot start PCR. Verify the concentration of primers and, if necessary, optimize their concentration. If the primers appear to be the cause of the problem, design and synthesize new primers.
	Suboptimal extension of annealed primers.	Optimize the concentration of $MgCl_2$, template DNA, and dNTPs. Test a range of pH values in the PCR. Consider replacing Tris with tricine, bicine, or EPPs which have a lower temperature coefficient than Tris (Cheng et al. 1994). Use a fresh preparation of thermostable DNA polymerase. Repurify the template DNA to remove inhibitors. Increase the number of cycles at constant annealing temperature (55°C). If problems persist, add an enhancer (e.g., 10% DMSO, 5% PEG 6000, or 10% glycerol). If problems still persist, <i>either</i> reamplify a 1:100 dilution of the PCR in fresh PCR buffer and primers for 30 cycles at a constant annealing temperature <i>or</i> carry out nested PCR. Use GC-Melt (CLONTECH) in the reaction mixture.
	Ineffective denaturation.	Increase the time or temperature of denaturation.
	Distance between primers too large.	Use preparations of thermostable polymerases capable of amplifying long segments of DNA.
Multiple amplification products.		Use touchdown PCR, preferably in combination with hot start or booster PCR. Optimize the concentration of $MgCl_2$, template DNA, thermostable DNA polymerase, and dNTPs. Test a range of pH values. <i>Either</i> reamplify a 1:100 dilution of the PCR in fresh PCR buffer and primers for 30 cycles at a constant annealing temperature (55°C) <i>or</i> carry out nested PCR <i>or</i> recover the desired band of DNA from a gel and reamplify. Verify the concentration of primers and, if necessary, optimize their concentration. If the problem persists, design and synthesize new primers.
Excessive amounts of primer-dimers.		Use touchdown PCR, preferably in combination with hot start or booster PCR. If the problem persists, design and synthesize new primers, paying particular attention to the sequences of the 3' ends.