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Leland J. Cseke, Kirakosyan Ara, Peter B. Kaufman, Margaret V. Westfall

Polymerase Chain Reaction Methodologies

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Ara Kirakosyan, Peter B. Kaufman, Leland J. Cseke, E. Mitchell Seymour

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*Ara Kirakosyan, Peter B. Kaufman,
Leland J. Cseke, and E. Mitchell Seymour*

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3.1   INTRODUCTION: WHAT IS POLYMERASE CHAIN REACTION?

In nature, most organisms copy their DNA in the same way. Polymerase chain reaction (PCR) mimics this process. Only it does it in a test tube. When any cell divides, enzymes called polymerases make a copy of the entire DNA in each chromosome. The first step in this process is to "unzip" the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template.

In order to copy DNA, polymerase requires two important components: a supply of the four nucleotide bases and a primer. DNA polymerases cannot copy a chain of DNA without a short sequence of nucleotides to get it started.[1,2] So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain.

A PCR vial must contain all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, the primer sequence, and DNA polymerase. The polymerase is mostly the *Taq* polymerase isolated from *Thermus aquaticus*.

At different temperatures, the polymerase chain reaction is carried out, where the first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90°C–95°C for 30 s. Since the primers cannot bind to the DNA strands at such a high temperature, the vial is cooled to 55°C. At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 s. The final step of the reaction is to make a complete copy of the templates. Since the *Taq* polymerase works best at around 75°C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised. The *Taq* polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. This completes one PCR cycle.[3,4]

The three steps in the polymerase chain reaction—the separation of the strands, annealing the primer to the template, and the synthesis of new strands—take less than 2 min. At the end of a cycle, each piece of DNA in the vial has been duplicated. The cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template, so that after 30 cycles, 1 billion copies of a single piece of DNA can be produced! Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about 3 h.

PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications:[1–10]

- DNA cloning for sequencing
- DNA-based phylogeny or functional analysis of genes
- The diagnosis of hereditary diseases
- The identification of genetic fingerprints (used in forensics and paternity testing)
- The detection and diagnosis of infectious diseases

3.2 BASIC PCR TECHNIQUES: PROTOCOL FOR PCR WITH *TAQ* DNA POLYMERASE

3.2.1 PREPARATION OF REACTION MIXTURE

To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers, and *Taq* DNA polymerase in a single tube, which can then be aliquoted into individual tubes. $MgCl_2$ and template DNA solutions are then added. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

3.2.2 REACTION MIXTURE SETUP

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Add, in a thin-walled PCR tube, on ice:

Reagent	Final Concentration	Quantity, for 50 µL of Reaction Mixture
Sterile deionized water	—	Variable
10X *Taq* buffer	1X	5 µL
2 mM dNTP mix	0.2 mM of each	5 µL
Primer I	0.1–1 µM	Variable
Primer II	0.1–1 µM	Variable
Taq DNA polymerase	1.25 units/50 µL	Variable
25 mM $MgCl_2$	1–4 mM	Variable[a]
Template DNA	10 pg^{-1} µg	Variable

[a] Table for selection of 25 mM $MgCl_2$ solution volume:

Final concentration of $MgCl_2$ in 50 µL reaction mix, mM	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, µL	2	2.5	3	3.5	4	5	6	8

3. Gently vortex the sample and briefly centrifuge to collect all drops from the walls of the tube.
4. Overlay the sample with half volume of mineral oil or add an appropriate amount of wax. This step may be omitted if the thermal cycler is equipped with a heated lid.
5. Place samples in a thermal cycler and start PCR.

3.2.3 COMPONENTS OF THE REACTION MIXTURE

3.2.3.1 Template DNA

Usually, the amount of template DNA is in the range of 0.01–1 ng for plasmid or phage DNA and 0.1–1 µg for genomic DNA, for a total reaction mixture of 50 µL. Higher amounts of template DNA usually increase the yield of nonspecific PCR products. But if the fidelity of synthesis is crucial, maximal allowable template DNA quantities together with a limited number of PCR cycles should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification. Although even trace amounts of agents used in DNA purification procedures (phenol, EDTA, Proteinase K) strongly inhibit *Taq* DNA polymerase, ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA sample.

3.2.3.2   Primers

Guidelines for primer selection:

- PCR primers are usually 15–30 nucleotides in length. Longer primers provide higher specificity.
- The GC content should be 40%–60%. The G and C nucleotides should be distributed uniformly throughout the primer. More than three G or C nucleotides at the 3′ end of the primer should be avoided, as nonspecific priming may occur.
- The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation.
- The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly.
- All possible sites of complementarity between primers and the template DNA should be noted.
- If primers are degenerate, at least three conservative nucleotides must be located at the primer's 3′ end.
- An estimation of the melting and annealing temperatures of the primer should be made.
- If the primer is shorter than 25 nucleotides, the approximate melting temperature (T_m) is calculated by use of the following formula:

$$T_m = 4(G + C) + 2(A + T)$$

  where **G, C, A,** and **T** are the number of respective nucleotides in the primer.
- The annealing temperature should be approximately 5°C lower than the melting temperature. If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialized computer programs where the interactions of adjacent bases, the influence of salt concentration, etc. are evaluated.

3.2.3.3   MgCl$_2$ Concentration

Since Mg^{2+} ions form complexes with dNTPs, primers, and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Too few Mg^{2+} ions result in a low yield of PCR product, and too many increase the yield of nonspecific products and promote misincorporation. Lower Mg^{2+} concentrations are desirable when the fidelity of DNA synthesis is critical.

The recommended range of $MgCl_2$ concentration is 1–4 mM under the standard reaction conditions specified. At a final dNTP concentration of 0.2 mM, $MgCl_2$ concentration ranges of 1.5 ± 0.25 mM (in *Taq* buffer with KCl) and of 2.0 ± 0.5 mM (in *Taq* buffer with $(NH_4)_2SO_4$) are suitable in most cases. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally.

3.2.3.4   dNTPs

The concentration of each dNTP in the reaction mixture is usually 200 μM. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 10–50 μM, since the fidelity of DNA synthesis is maximal in this concentration range. In addition, the concentration of $MgCl_2$ should be selected empirically, starting from 1 mM and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained.

Fermentas PureExtreme® dNTP Mixes, 2 mM (#R0241/2) and 10 mM (#R0191/2), are conveniently formulated for direct use in PCR.

3.2.3.5 *Taq* DNA Polymerase

Usually 1–1.5 units of *Taq* DNA polymerase are used in 50 μL of reaction mix. Higher *Taq* DNA polymerase concentrations may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g., if the template DNA used is not highly purified), higher amounts of *Taq* DNA polymerase (2–3 units) may be necessary to obtain a better yield of amplification products.

3.2.3.6 Reaction Overlay

If necessary, the reaction mixture can be overlaid with mineral oil or paraffin (melting temperature 50°C–60°C) of special PCR grade. One-half of the total reaction volume is usually sufficient.

3.2.4 Cycling Conditions

Amplification parameters depend greatly on the template, primers, and amplification apparatus used.

3.2.4.1 Initial Denaturation Step

- The complete denaturation of the DNA template at the start of the PCR reaction is of key importance. Incomplete denaturation of DNA results in the inefficient utilization of the template in the first amplification cycle and in a poor yield of the PCR product. The initial denaturation should be performed over an interval of 1–3 min at 95°C if the GC content is 50% or less. This interval should be extended up to 10 min for GC-rich templates.
- If the initial denaturation is no longer than 3 min at 95°C, *Taq* DNA polymerase can be added into the initial reaction mixture. If longer initial denaturation or a higher temperature is necessary, *Taq* DNA polymerase should be added only after the initial denaturation, as the stability of the enzyme dramatically decreases at temperatures over 95°C.

3.2.4.2 Denaturation Step

Usually denaturation for 0.5–2 min at 94°C–95°C is sufficient, since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and is completely denatured under these conditions. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3–4 min. Alternatively, additives facilitating DNA denaturation—glycerol (up to 10–15 vol%), DMSO (up to 10%), or formamide (up to 5%)—should be used. In the presence of such additives, the annealing temperature should be adjusted experimentally, since the melting temperature of the primer–template DNA duplex decreases significantly when these additives are used. The amount of enzyme in the reaction mix should be increased since DMSO and formamide, at the suggested concentrations, inhibit *Taq* DNA polymerase by approximately 50%. Alternatively, a common way to decrease the melting temperature of the PCR product is to substitute dGTP with 7-deaza-dGTP in the reaction mix.

3.2.4.3 Primer Annealing Step

Usually, the optimal annealing temperature is 5°C lower than the melting temperature of primer–template DNA duplex. Incubation for 0.5–2 min is usually sufficient. However, if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature should be optimized by increasing it stepwise by 1°C–2°C.

3.2.4.4 Extending Step

Usually, the extending step is performed at 70°C–75°C. The rate of DNA synthesis by *Taq* DNA polymerase is the highest at this temperature. Recommended extending time is 1 min for the synthesis of PCR fragments up to 2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 min for each 1000 bp.

3.2.4.5 Number of Cycles

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25–35 cycles are usually sufficient.

3.2.4.6 Final Extending Step

After the last cycle, the samples are usually incubated at 72°C for 5–15 min to fill in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3′ ends of PCR products. Therefore, if PCR fragments are to be cloned into T/A vectors, this step can be prolonged up to 30 min.

3.2.5 Avoiding Contamination

PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. The sensitivity of this technique means that the sample should not be contaminated with any other DNA or previously amplified products (amplicons) that may reside in the laboratory environment.

Here are some precautions that one should take to avoid contamination:

- DNA sample preparation, reaction mixture assemblage, and the PCR process, in addition to the subsequent reaction product analysis, should be performed in separate areas.
- A laminar flow hood equipped with a UV lamp is recommended for preparing the reaction mixture.
- Fresh gloves should be worn for DNA purification and each reaction setup.
- The use of dedicated vessels and positive displacement pipettes or tips with aerosol filters for both DNA sample and reaction mixture preparation, is strongly recommended.
- The reagents for PCR should be prepared separately and used solely for this purpose. Autoclaving of all solutions, except dNTPs, primers, and *Taq* DNA polymerase is recommended. Solutions should be aliquoted in small portions and stored in designated PCR areas. Aliquots should be stored separately from other DNA samples.
- A control reaction, omitting template DNA, should always be performed in order to confirm the absence of contamination.

These are only rough guidelines. Detailed instructions about PCR laboratory setup and maintenance may be found in **PCR Methods and Applications**, 3, 2, S1-S14, 1993. Cold Spring Harbor Laboratory Press, 1 Bungtown Road, Cold Spring Harbor, New York 11724.

3.3 REAL-TIME PCR*

3.3.1 Real-Time vs. Traditional PCR

Real-time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection.[5,6] Traditional methods use agarose gels for the detection of PCR amplification at the final phase or end point of the PCR reaction.

3.3.2 Limitations of End-Point PCR

Agarose gel results are obtained from the end point of the reaction. End-point detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination,

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* Modified from Applied Biosystems, Real-time PCR vs. traditional PCR.
  http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf

which may not be very precise. As seen later in the section, the end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes. Agarose gel resolution is very poor, about 10-fold. Real-time PCR can detect as little as a twofold change!

Some of the problems with end-point detection include

- Poor precision
- Low sensitivity
- Short dynamic range <2 logs
- Low resolution
- Nonautomated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post-PCR processing

3.3.3 PCR Phases

To understand why end-point PCR is limiting, it is important to understand what happens during a PCR reaction.

A basic PCR run can be broken up into three phases:

- **Exponential**: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.
- **Linear (high variability)**: The reaction components are being consumed, the reaction is slowing, and products are starting to degrade.
- **Plateau (end point: gel detection for traditional methods)**: The reaction has stopped, no more products are being made, and if left long enough, the PCR products will begin to degrade.

3.3.4 Quantitation

Theoretically, there is a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. Real-time PCR detects the accumulation of amplicon during the reaction. The data are then measured at the exponential phase of the PCR reaction. Traditional PCR methods use agarose gels or other post PCR detection methods, which are not as precise. As mentioned earlier, the exponential phase is the optimal point for analyzing data. Real-time PCR makes quantitation of DNA and RNA easier and more precise than past methods.

3.4 REAL-TIME PCR CHEMISTRIES

Currently, four different chemistries, TaqMan® (Applied Biosystems, Foster City, CA), Molecular Beacons, Scorpions®, and SYBR® Green (Molecular Probes), are available for real-time PCR[5,6,10]. All of these chemistries allow for detection of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons, and Scorpions depend on Förster resonance energy transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

3.4.1 TaqMan Probes

TaqMan probes depend on the 5′ nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5′ end and a quencher moiety coupled to the 3′ end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quencher molecules prevents the detection of the fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5′ nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. Well-designed TaqMan probes require very little optimization. In addition, they can be used for multiplex assays by designing each probe with a spectrally unique fluor/quench pair. However, TaqMan probes can be expensive to synthesize, with a separate probe needed for each mRNA target being analyzed.

3.4.2 Molecular Beacons

Like TaqMan probes, Molecular Beacons also use FRET to detect and quantitate the synthesized PCR product via a fluor coupled to the 5′ end and a quencher attached to the 3′ end of an oligonucleotide substrate. Unlike TaqMan probes, Molecular Beacons are designed to remain intact during the amplification reaction and must rebind to the target in every cycle for signal measurement. Molecular Beacons form a stem-loop structure when free in solution. Thus, the close proximity of the fluor and quench molecules prevents the probe from fluorescing. When a Molecular Beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation.

Molecular Beacons, like TaqMan probes, can be used for multiplex assays by using spectrally separated fluor/quench moieties on each probe. As with TaqMan probes, Molecular Beacons can be expensive to synthesize, with a separate probe required for each target.

3.4.3 Scorpions

With Scorpion probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5′ end and is quenched by a moiety coupled to the 3′ end. The 3′ portion of the stem also contains a sequence that is complementary to the extension product of the primer. This sequence is linked to the 5′ end of a specific primer via a nonamplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon, thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed.

3.4.4 SYBR Green

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation, emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer dimers and other nonspecific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with a spurious nonspecific background only showing up in very late cycles.

SYBR Green is the most economical choice for real-time PCR product detection. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by SYBR Green requires extensive optimization. Since the dye cannot distinguish between specific and nonspecific product accumulated during PCR, follow up assays are needed to validate results.

3.5 APPLICATIONS OF PCR

3.5.1 Isolation of Plasmid Templates for PCR

1. Harvest the bacterial cells in the block by centrifugation for 5 min at $1500 \times g$ in a centrifuge with a rotor for 96-well microplates. The block should be covered with adhesive tape during centrifugation. Remove medium by inverting the block. To remove the medium, peel off the tape and quickly invert the block over a waste container. Blot the inverted block firmly on a paper towel to remove any remaining droplets of medium.

Note: *Do this step quickly so as to avoid losing any part of the pellet, as the pellet will be loose.*

2. Resuspend each bacterial pellet in 0.3 mL Buffer R1. Use an 8-channel pipet with a large fill volume (1 mL per channel) for buffer delivery. Tape the block and mix by vortexing.

Protocol

3. Add 0.3 mL Buffer R2 to each well, seal the block with new tape, mix gently but thoroughly by inverting 10 times, and incubate at room temperature for 5 min.

Note: *Buffer R2 should be checked before use for SDS precipitation caused by low storage temperatures. If necessary, redissolve SDS by warming. Do not vortex the lysates at this step, as this may cause shearing of the bacterial genomic DNA. Do not incubate for more than 5 min. Additional incubation may result in increased levels of open circular plasmid. At the end of the incubation, the lysate should appear viscous and free of bacterial cell clumps.*

4. Add 0.3 mL Buffer R3 to each well, seal the block with new tape, and mix immediately by inverting 10 times. Gently inverting the taped block 10 times ensures uniform precipitation.

Note: *Optionally, place the block in a boiling water bath for 5 min to denature and precipitate proteins and carbohydrates that are not removed by alkaline lysis.*

5. Transfer the lysates to the wells of the QIAfilter 96-well plate (Qiagen Inc. Chatsworth, CA) using an 8-channel pipet adjusted to 1 mL fill volume. Apply vacuum (−200 to −300 mbar) until the lysates are completely transferred to the square-well block in the QIAvac base.

Note: *Occasionally, the precipitate will clog the end of a pipet tip. Lightly tapping the tip on the bottom of the well of the culture block will break the precipitate and allow the remaining material to be transferred. Any unused wells may be sealed with tape for later use.*

6. Take the square-well block containing the cleared lysates from the vacuum manifold. Add 0.7 volumes of room-temperature isopropanol to each well (0.63 mL for 0.9 mL of lysate), tape the block, and mix immediately by inverting three times.

Note: *If you use an optional boiling method, the lysate needs to be cooled prior to addition of isopropanol. Otherwise, volatilization of isopropanol will cause the tape to detach from the wells. When preparing multiple sets of 96 samples, add isopropanol to one block, tape, and*

mix by inversion before proceeding to the next block. This will minimize separation of the tape from the block before the samples are mixed.

7. Centrifuge the block at 2500 × *g* for 15 min at room temperature to pellet the plasmid DNA. Remove the supernatants by quickly inverting the block over a waste container, then tapping the block, upside down, onto a paper towel.

Note: *Mark the orientation of the block before centrifugation so that it can be spun in the same orientation in the ethanol wash step. DNA pellets from isopropanol precipitations have a glassy appearance and may be difficult to see. Handle the block carefully to avoid dislodging the pellets.*

8. Wash each DNA pellet with 0.5 mL of 70% ethanol. Centrifuge the block (in the same orientation as before) at 2500 × *g* for 2 min. Remove the wash solutions by inverting the block, then tapping it firmly, upside down, onto a paper towel. Air dry the pellets for 15 min or dry under vacuum for 10 min. Make sure that no alcohol droplets are visible after air drying.

Note: *It is important not to overdry the DNA pellets, as this will make them difficult to dissolve. If this occurs, the DNA pellets may be heated at 50°C to completely redissolve the pellets.*

9. Redissolve the DNA pellets in 50–250 μL 10 mM Tris·Cl, pH 8.5 buffer.

Note: *The optimal volume of buffer to use will depend on the copy number of the plasmid and the desired DNA concentration. Avoid repeated pipetting, which can shear the DNA.*

3.5.2 Materials, Reagents, and Solutions

96-well R.E.A.L. miniprep kit (Qiagen Inc. Chatsworth, CA)
LB broth (Biofluids, Rockville, MD)
Superbroth (Biofluids, Rockville, MD)
96 pin inoculating block (#VP 4088, V&P Scientific, Inc, San Diego, CA)
Airpore tape sheets (# 19571, Qiagen Inc. Chatsworth, CA)
Sterile 96-well plate seals (e.g., # SEAL-THN-STR (Elkay Products, Inc., Shrewsbury, MA)
96-well U-bottom microtiter plates, #3799 and 96-well V-bottom microtiter plates, #3894 (Corning Inc., Corning, NY)
Centrifuge with a horizontal ("swinging bucket") rotor with a depth capacity of 6.2 cm for spinning microtiter plates and filtration plates (e.g., Sorvall Super T 21, Sorvall Inc., Newtown, CT)
37°C shaker incubator with holders for deep-well plates
37°C water bath
65°C incubator
Vortex mixer
−80°C freezer
−20°C freezer
Carbenicillin (Invitrogen, Carlsbad, CA)
Ethanol (200 Proof USP Ethyl Alcohol)
Isopropanol
1 M Tris-HCl (pH 8)
0.5 M NaEDTA (pH 8)

3.5.3 PCR Amplification of cDNA Inserts

cDNA inserts from plasmid templates of EST clones need to be amplified, purified, and quantified before being used for printing microarrays. The following protocols describe the general methods to obtain PCR products for printing microarrays. Because of the large number of

clones to be amplified, it is best to use 96-well formatted PCR plates for amplification, which will also facilitate printing microarrays using robotics.

1. For each 96-well plate to be amplified, prepare a PCR reaction mixture containing the following ingredients:

1000 μL	10X PCR Buffer
20 μL	dATP (100 mM)
20 μL	dGTP (100 mM)
20 μL	dCTP (100 mM)
20 μL	dTTP (100 mM)
5 μL	M13F primer[a] (1 mM)
5 μL	M13R primer[a] (1 mM)
100 μL	Ampli-*Taq* polymerase (5 u · μL^{-1})
8800 μL	H$_2$O

[a] Primers used for PCR amplification depend on the vector in which the cDNA inserts are located. M13 primers are generally useful for many commonly available cloning vectors. Keep all reagents on ice and return the enzyme tube promptly to the freezer.

2. Label 96-well PCR plates and aliquot 100 μL of PCR reaction mix to each well. Gently tap plates to insure that no air bubbles are trapped at the bottom of the wells.
3. Add 1 μL of purified EST plasmid template to each well. Mix well with a pipette.

Note: *Mark the donor and recipient plates at the corner near the A1 well to facilitate correct orientation during transfer of the template. It is important to watch that the pipette tips are all submerged in the PCR reaction mix when delivering the template. Missing the liquid is easier when multichannel pipettes are used. Always use sterile filtered tips to avoid contamination.*

4. Place PCR plate covers on plates and centrifuge the plates at 2700 rpm for 1 min.
5. Place the PCR plates in a thermal cycler (Eppendorf Master Cyler) and run the following cycling program.

Initial denaturation	96°C × 2 min
Denaturation	94°C × 30 s
Primer annealing	55°C × 30 s × 30 cycles
Primer extension	72°C × 2 min × 30 cycles
Final extension	72°C × 5 min

Note: *After PCR, plates can be held at 4°C while quality controls are performed on PCR products.*

3.5.4 MATERIALS FOR PCR AMPLIFICATION

AmpliTaq® DNA Polymerase with GeneAmp® PCR Reaction Kit (Applied Biosystems, Foster City, CA; Cat # N808-0156)
10X PCR buffer II
MgCl$_2$ solution
Platinum *Taq* polymerase
M13 forward and reverse primers (Invitrogen, Carlsbad, CA)
Forward: 5′ GTT TTC CCA GTC ACG ACG TTG 3′
Reverse: 5′ TGA GCG GAT AAC AAT TTC ACA CAG 3′
dNTP kit (100 mM of each dNTP) (Invitrogen, Carlsbad, CA; Cat # 10297-018)

MilliQ water

MicroAmp® optical 96-well reaction plate (Applied Biosystems, Foster City, CA; Cat #N801-0560)

MicroAmp 96-well full plate cover (Applied Biosystems, Foster City, CA; Cat # N801-0550)

Multiscreen® PCR filter plate (Millipore Corp. Bedford, MA; Cat # MANU3050)

Cap mat (VWR, West Chester, PA; Cat # 40002-002)

Falcon microtest U-bottom 96-well plate (BD Biosciences, Palo Alto, CA; Cat # 353077

3.5.5 CHECK PCR PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

If this is the first time the template for these cDNAs is being amplified, then it is necessary to analyze 2 μL of each PCR product on a 2% agarose gel. If amplified products from this template have been previously tested, then quality test the PCR products by analyzing one row of wells from each amplified plate. Gel imaging allows for both a rough quantitation of the product (by comparing with known DNA standards) and an excellent characterization of the product. Band size, as well as the number of bands observed in the PCR products, is necessary for the interpretation of the final results of the hybridization. The use of gel well formats suitable for loading from 96-well plates and programmable pipetters makes this form of analysis feasible on a large scale.

1. Cast a 2% agarose gel in 1X TAE (40 mM Tris base, 40 mM Acetate, 1 mM EDTA, pH 8.2) with four combs (50 teeth) and submerge in an electrophoresis apparatus with sufficient 1X TAE buffer to cover the surface of the gel.
2. Prepare a reservoir of loading buffer, using 12 wells of a microtiter plate.
3. Program pipetter (Matrix 12 channel pipetter) to sequentially carry out the following steps:
   a. Fill with 2 μL
   b. Fill with 1 μL
   c. Fill with 2 μL
   d. Mix a volume of 5 μL five times
   e. Expel 5 μL
4. Load 2 μL of PCR product from wells A1-A12 of the PCR plate using sterile filtered tips.
5. Load 2 μL of loading buffer from the reservoir. Place tips in clean wells of a disposable mixing tray and allow pipette to mix the sample and loading dye.
6. Position the pipette tip in a well row so that the tip containing the PCR product from well A1 is in the second well of the row, and the other tips are in every other succeeding well.
7. Repeat the process (changing tips for each load), loading PCR plate row B starting in the third well, interleaved with the A row, the C row starting at well 26, and the D row at well 27, interleaved with the C row.
8. Place 5 μL of 100 bp Size Standards, in wells 1 and 50. Repeat this process, loading samples from rows E, F, G, and H in the second, 50 well row of gel wells, loading samples from two 96-well PCR plates per gel, or single row samples from 16 PCR plates.

Note: *Since it will take time to load all the wells, to reduce diffusion and mixing of samples, apply voltage to the gel for a minute between loading each well strip. This will cause the DNA to enter the gel, and reduce band spreading and sample loss.*

9. Apply a voltage of 200 V to the gel and run until the bromophenol blue (faster band) has nearly migrated to the next set of wells.

Note: *For a gel that is 14 cm in the running dimension, and 3 cm between each row of wells, 200 V for 15 min is sufficient.*

10. Use a photodocumentation system or take a digital photo of the gel on a UV table and store image for future reference.

Note: *The gels should show bands of fairly uniform brightness distributed in size between 600 and 2000 base pairs depending on the sizes of cDNAs amplified. Further computer analysis of such images can be carried out with image analysis packages to provide a list of the number and size of bands. Ideally, this information can be made available during analysis of the data from hybridizations involving these PCR products.*

3.5.6 MATERIALS FOR PCR PRODUCT ANALYSIS BY AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis apparatus with capacity for four 50-well combs, (e.g., #D3, Owl Scientific, Woburn, MA)
50X Tris-acetate electrophoresis buffer (Amersham Pharmacia Biotech, Piscataway, NJ)
Agarose (Amersham Pharmacia Biotech, Piscataway, NJ)
Dye solution (Xylene Cyanol/Bromophenol Blue) (e.g., #351-081-030, Quality Biological Inc., Gaithersburg, MD)
Glycerol (enzyme grade)
Ethidium bromide solution ($10\,mg \cdot mL^{-1}$)
100 base-pair ladder size standard
Programmable, 12-channel pipetter (e.g., #2019, Matrix Technologies, Lowell, MA)
Disposable microtiter mixing trays (e.g., Falcon #353911, Becton Dickinson, Franklin Lake, NJ)
Electrophoresis power supply (Fisher Biotech, Pittsburgh, PA)
1X TAE buffer
50X TAE buffer 40 mL
Ethidium bromide ($10\,mg \cdot mL^{-1}$) 0.1 mL
Water 960 mL

Loading Buffer
Glycerol (enzyme grade) 4.0 mL
DEPC water 0.9 mL
Dye solution* 0.1 mL
5.0 mL
(*This solution is 0.25% (w/v) Xylene Cyanol and 0.25% (w/v) Bromophenol Blue)
100 bp Size standards

DNA Ladder ($1\,mg \cdot mL^{-1}$) 50 μL
1 M Tris-HCl (pH 8.0) 5 μL
0.5 M EDTA (pH 8.0) 5 μL
Loading buffer 440 μL

3.5.7 PURIFICATION AND QUANTIFICATION OF PCR PRODUCTS

1. Spin down PCR reaction plates and then transfer the PCR products (100 μL) to a Multiscreen filter plate and place the filter on a vacuum manifold filtration system (e.g., Millipore Corp. Bedford, MA; Cat # MAVM0960R).
2. Apply a vacuum pressure of approximately 250–380 mmHg for 10 min or until plate is dry.

Note: *Filter until no more fluid is visible in the well. The filter may appear wet and shiny even when dry, so do not use the appearance of the filter as a guide. It is also important to check all the wells in the plate before removing from vacuum; some wells filter more slowly than others.*

3. Remove plate from manifold filtration system and add 100 μL of MilliQ water to each well. Place filter plate on a shaker and shake vigorously for 20 min to resuspend the DNA.

4. Pipet the purified PCR product to a new Falcon U-bottom 96-well plate. Seal PCR storage plates with a plastic cap mat or adhesive foil lid and store at −20°C until needed for making microarray printing plates.

Note: *For long-term storage after filtration, aliquot equal volumes of purified PCR product into multiple plates. Store one plate with a cap mat at −20°C (for short term use) and dry down the remaining plates in a speedvac and store at 4°C in a desiccator.*

5. Resuspend dried PCR product in MilliQ water just before microarraying printing plates.

3.5.8 Fluorometric Determination of PCR DNA Concentration

It is impractical for most labs to determine the exact quantification of PCR products, especially if thousands of cDNAs must be prepared. However, it is possible to use a strategy where excess DNA is spotted, so that the exact quantities used do not produce much variation in the observed results. When using this strategy, it is necessary to track the productivity of the PCR reactions. Fluorometry methods using 96-well plates provide a simple way to obtain an approximate concentration of the double-stranded PCR product from many thousands of samples.

1. Label 96-well plates for fluorescence assay.
2. Add 200 μL of Fluor Buffer to each well. Add 1 μL of PCR product from each well in a row of a PCR plate to a row of the fluorometry plate. Samples can be added to rows A through G of the fluorometry plate.
3. In the final row of the fluorometry plate, add 1 μL of each of the series of dsDNA standards 0 μg·mL^{-1} (TE only), 50, 100, 250, and 500 μg·mL^{-1} dsDNA. Repeat this series twice in the final row.
4. Set the fluorometer for excitation at 346 nm and emission at 460 nm. Adjust, as necessary, to read the plate. If the fluorometer does not support automated analysis, export the data table to Excel.
5. Establish that the response for the standards is linear and reproducible from the range of 0–500 μg·mL^{-1} of dsDNA.
6. Calculate the concentration of dsDNA in the PCR reactions using the following equation after subtracting the average value obtained from the sample containing 0 μg·mL^{-1} from all other sample and control values:

$$[dsDNA(\mu g \times mL^{-1})] = ((PCR\ sample\ value)/(average\ 100\ \mu g \times mL^{-1}\ value) \times 100$$

Note: *Constantly tracking the yields of the PCRs makes it possible to rapidly detect many ways in which PCR can fail or perform poorly.*

3.5.9 Materials and Reagents for PCR Product Quantification

Reference double-stranded DNA (0.5 μg·mL^{-1}) (e.g., #15612-013 Invitrogen, Carlsbad, CA))
96-well plates for fluorescent detection (e.g., #7105, Dynex, Chantilly, VA)
Fluorometer (e.g., #LS50B, Perkin Elmer, Norwalk, CT)
FluoReporter Blue dsDNA quantitation kit (#F-2962, Molecular Probes, Eugene, OR)
TE
12 channel multipipettes
Computer equipped with Microsoft Excel™ software

dsDNA standards: $0\,\mu g$ mL^{-1}, $10\,\mu g$ mL^{-1}, $20\,\mu g \cdot$mL^{-1}, $50\,\mu g \cdot$mL^{-1}, $100\,\mu g \cdot$mL^{-1}, $250\,\mu g \cdot$mL^{-1}, $500\,\mu g \cdot$mL^{-1}
Fluor buffer
Hoechst 33258 solution (from kit) $25\,\mu L$ TNE buffer (from kit) $10\,mL$

Notes: *Hoechst 33258 solution contains the dye at an unspecified concentration in a 1:4 mixture of DMSO:H$_2$O. TNE buffer is 10 mM Tris-HCl (pH 7.4), 2 M NaCl, and 1 mM EDTA.*

3.6 GENOMIC CLONING USING PCR

Under some circumstances, one may be able to take advantage of genomic PCR cloning techniques to isolate new genomic clones. This is usually the case when a good amount of sequence informa-tion is known about the cDNA of interest, such as nucleotide sequence, relatedness of the cDNA sequence to other genes, and possible intron positions as determined by comparison with related gene sequences. There is a wide variety of PCR techniques that address this issue. In this section, we suggest two possibilities that have worked well in our laboratories.

3.6.1 Selection of Oligonucleotides

The same rules apply to the oligonucleotide primer design as detailed in Chapters 7 and 12. However, when dealing with the isolation of genomic clones, one must make some special considerations.

1. If possible, any new cDNA sequence should be compared with other related genes whose intron positions have already been determined. This will help the researcher avoid possible intron junctions that could interrupt the primer sequence and prevent the primer from bind-ing in that region. These regions should be avoided during primer design.
2. Based on the sequence of the cDNA, selection of primer sets at the very beginning of the gene of interest and at the very end may allow cloning in only one PCR amplifica-tion. However, since genomic clones in animals, and especially in plants, often have large introns, it is wise to create additional primer sets that can be used to amplify the 5′ and 3′ ends of the genomic sequence separately. If an overlapping region is included, then these two regions can later be assembled using unique restriction sites or the use of PCR to extend the ends of the clones in reactions containing a mixture of the 5′ and 3′ clones. We recommend setting up reactions for the 5′ end, 3′ end, and the entire clone at the same time using several sets of primers at each location. This gives better odds of successful amplification.
3. Due to the low abundance of each specific genomic sequence, especially for single copy genes, the initial cycle in the PCR reactions is critical. To provide the best results, primers should be carefully designed to have almost identical annealing temperatures. A melting temperature of between 60°C and 70°C usually works well. A discussion of the calculation and design of primer melting temperatures can be found in Chapter 12.

3.7 PCR AMPLIFICATION OF GENOMIC DNA AND GENOMIC LIBRARIES

Some of the most important factors in performing successful genomic PCR are the selection of primers, the quality of the DNA being used, the amount of DNA used, and the quality of the *Taq* polymerase used. Primers that are too far apart due to large introns or those that fall on intron junctions will not work. Only highly pure DNA works well in the PCR process, and too much DNA often inhibits amplification. Procedures for the preparation of high quality genomic DNA are found in Chapter 1. Finally, the enzyme used is critical. It must be capable of producing large DNA fragments up to 10 kb in length. We prefer to use either the Advantage™ 2 Polymerase Mix or the

Advantage™ Genomic Polymerase Mix from CLONTECH, Palo Alto, CA. Both enzymes are a mixture of a robust *Taq* polymerase and a proof-reading enzyme that helps to provide longer and more accurate amplifications. The Advantage™ Genomic Polymerase Mix also comes with a bound antibody that prevents activity until the first heat cycle, which gives it an intrinsic "hot start." Other companies have similar high-quality enzymes.

Due to the low abundance of most genomic sequences and the number of cycles required in this method, even very slight contamination with the cDNA clone may cause difficulties in the amplification of the genomic clone. Usually, optimization of the PCR reactions is necessary before the reactions are highly efficient. The following procedure covers these criteria and can be applied to any genomic DNA template or λDNA isolated from a prepared genomic library.

1. Using procedures detailed in Chapter 1, isolate genomic DNA or λDNA from one of the genomic DNA libraries mentioned previously.
2. Carry out PCR amplification as follows:
    a. Design oligonucleotide primers that are specific and complementary to two different regions of the gene of interest, following the criteria detailed previously. Degenerate primers designed accordingly from protein sequences can also be used, but many non-specific bands are usually amplified.
    b. In a 0.2-mL thin-walled PCR tube on ice, set up the reactions by adding the following in the order listed. It is also important to set up negative controls using no DNA template and only one primer at a time.
        i.   10X Advantage™ genomic polymerase buffer (Clonetech) (5 μL)
        ii. ddH$_2$O (10 μL)
        iii. Mixture of four dNTPs (1.25 mM each) (8.5 μL)
        iv. Primer 1 (50–100 ng) in ddH$_2$O (5 μL)
        v.   Primer 2 (50–100 ng) in ddH$_2$O (5 μL)
        vi. Genomic DNA or λDNA in TE buffer (0.2–2 μg) (2 μL)
        vii. Advantage™ genomic polymerase mix (0.5 μL)
        viii. Add ddH$_2$O to a final volume of 50 μL

Notes: *(1) Thin-walled PCR tubes are highly recommended to allow better heat transfer between the thermocycler block and the sample. (2) Depending on the number of samples (including negative controls), a cocktail containing all reagents with the exception of the DNA template should be made to establish uniform conditions between all reactions.*

    c. Overlay the mixture with 50 μL of light mineral oil (Sigma or equivalent) to prevent evaporation of the sample if a thermocycler having a heated lid is not available.
    d. Carry out "touchdown" PCR amplification in a PCR machine. The conditions of PCR, including primer annealing temperatures, extension times, and number of cycles, will have to be optimized. However, a good starting example is as follows:

Cycle	Denaturation	Annealing	Polymerization	
First: T1	3 s at 94°C	15 s at 70°C	5 min at 70°C	2 Cycles
T2	3 s at 94°C	15 s at 68°C	5 min at 70°C	2 Cycles
T3	3 s at 94°C	15 s at 66°C	5 min at 70°C	2 Cycles
T4	3 s at 94°C	15 s at 64°C	5 min at 70°C	2 Cycles
T5	3 s at 94°C	15 s at 62°C	5 min at 70°C	2 Cycles
Subsequent	3 s at 94°C	15 s at 60°C	5 min at 70°C	29 Cycles
Last	3 s at 94°C	15 s at 60°C	10 min at 72°C	

Finally, hold at 4°C until the sample is removed.

Notes: *(1) When using thin-walled PCR tubes, it is generally not necessary to denature the genomic DNA template for longer than 3 s during each cycle. In fact, exposure to high temperatures tends to ruin the integrity of the template. An initial heat denature step is also not necessary (2) The initial 10 cycles are at higher temperatures, making primer annealing more stringent. This enriches the reaction with the template of interest prior to the final 30 cycles. Final primer annealing temperatures will have to be optimized. However, a temperature 3°C below the calculated Tm of the primer having the lowest Tm is a good starting point. (3) Other conditions that must be optimized are the extension times and the number of cycles used. Shorter extension times should be used for the amplification of shorter genes. We recommend using as few cycles as possible to amplify the DNA fragment of interest. Taq polymerases (even with a proof-reading enzyme) have higher mutation rates, and each subsequent cycle increases the chance of introducing error. (4) Proof-reading enzymes also tend to remove the T residues at the end of the synthesized fragments. Consequently, the fragments must be cloned immediately after amplification.*

    e. If using mineral oil, remove the reaction mixture from the mineral oil using a pipette with a relatively long tip. Slowly insert the tip into the bottom of the tube and then carefully take up the sample leaving the oil phase behind. Withdraw the tip from the tube, wipe the outside of the tip with a clean paper towel, and transfer the sample into a fresh tube.

2. Check the purity of the amplified cDNA on an agarose gel and elute the fragment of interest if required.

    a. Add DNA loading buffer to 10 μL of the amplified cDNA sample, and load the sample into 1% agarose gel, which contains EtBr for staining, including positive and negative controls as well as DNA standard markers. Carry out electrophoresis at 120 V.

    b. Under UV light, inspect the resulting bands to see if an appropriate fragment was amplified.

    c. If a single, clearly resolved band is visualized in the sample of interest, then this DNA can be cloned directly from the PCR reaction using 1–7 μL of the PCR reaction and a variety of PCR cloning kits, such as the pGEM-T® easy vector (Promega) or the TOPO TA Cloning kits (Invitrogen) (Figure 12.9). We highly recommend this procedure; however, it must be performed immediately after the PCR reactions are finished.

    d. If too many bands are resolved in the sample of interest, then the band of interest will have to be eluted from the gel. Run an appropriate amount of the remaining PCR reaction on a gel, and slice the individual sharp band(s) using a clean razor blade and trim away excess agarose gel as much as possible.

    e. Procedures for the elution of the bands are detailed in Chapter 1.

3. Ligate the fragment to an appropriate TA cloning vector, and transform an appropriate *E. coli* host in preparation for minipreps.

    a. Set up the ligation reaction as follows:

        i. Ligase 10X buffer (2 μL)

        ii. Vectors (0.1 μg)

        iii. Amplified genomic DNA fragment (0.5 μg)

        iv. T4 DNA ligase (10 Weiss units)

        v. Add ddH$_2$O to a final volume of 20 μL

    b. Incubate the ligation reactions at room temperature (22°C–24°C) for 4 h and proceed to subcloning as described earlier in this chapter.

4. Identify correct clones and verify the sequence.

    a. Prepare miniprep DNA of at least 20 colonies after *E. coli* transformation. Cut these preps with flanking restriction enzymes, and run fragments on a 1% agarose gel to identify clones with the correct size fragment. A Southern blot can be prepared using this gel if an appropriate probe can be generated, and restriction maps can be prepared using the miniprep DNA from potential positive clones.

Notes: *When performing Southern blots on cloned PCR fragments, it is important to design a probe that lies between, but does not include, the primers used to create the products. This will prevent the hybridization of primer sequences that may flank nonspecific amplified DNA fragments. This is especially important if the fragment was directly cloned out of the PCR reaction without gel elution due to the higher probability of the presence of nonspecific fragments.*

   b. Carry out sequencing of the putative clones having the amplified cDNA, as described in Chapter 7.

   c. Compare the nucleotide sequence and the deduced amino acid sequence with the known genes from which the primers were designed.

   d. If it is determined that a given clone is correct, then glycerol stocks and large-scale DNA preps should be prepared and stored for future use.

3.8   ISOLATION OF FLANKING SEQUENCES BY INVERSE PCR

In some cases, only a small portion of a gene sequence is known. In these situations, the researcher needs to make efforts to obtain the sequence of the regions of the gene that flank the known sequence. There is a wide variety of approaches to this problem, many of which include PCR. In this section, we describe one technique that has worked well in our hands.

Inverse PCR makes use of primers that are designed in the opposite orientation from the direction that they are normally prepared. In other words, the primers face away from each other and are used to amplify surrounding genomic DNA that was cut with restriction enzymes and religated to form a collection (or library) of circular DNAs. The resulting fragments can be as large as 6 kb. The protocol is as follows:

1. Using procedures detailed in Chapter 1, isolate genomic DNA from the organism of interest. This DNA must be of high quality.
2. Perform single enzyme digestions each with 10 μg of genomic DNA using several different enzymes. Since unknown sequences are being dealt with, it is not known which enzyme will provide the best results. Four and six cutter enzymes will result in different size fragments.
3. Run a small portion of each digest on a 1% agarose gel to confirm that the cutting was complete.
4. If the digestion was successful, extract with 1 volume of TE-saturated phenol/chloroform. Mix well by vortexing and centrifuge at $11,000 \times g$ for 5 min at room temperature.
5. Carefully transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Mix well and centrifuge as in Step 4.
6. Transfer the top, aqueous phase to a fresh tube and add 0.1 volume of 3 M sodium acetate buffer (pH 5.2) or 0.5 volume of 7.5 M ammonium acetate. Mix and add 2–2.5 volumes of chilled 100% ethanol. Allow to precipitate at −70°C for 30 min.
7. Centrifuge at $12,000 \times g$ for 10 min at room temperature. Carefully decant the supernatant and briefly rinse the DNA pellet with 1 mL of 70% ethanol. Dry the pellets under vacuum for 10 min. Dissolve the DNA in a small amount of ddH$_2$O in preparation for ligation.
8. Carry out ligations as described for **Large-Scale Ligations** in Section 6.2.1.7 in Chapter 6. This will create a collection of circular DNA molecules upon which some will have the partial known sequence of the gene of interest.
9. Repeat Steps 4–7 to prepare the DNA for PCR. If a unique restriction site is available within the region of known sequence and between the two primers, the circular DNA can be linearized prior to PCR; however, while this may prevent the polymerase from looping around the circular DNAs, we have found that it is not strictly necessary.

10. Design oligonucleotide primers that are specific, but in opposite orientations to each other, within the region of known sequence following the criteria detailed earlier in this chapter. This should be done well in advance.

11. Perform PCR as described in PCR Amplification of Genomic DNA and Genomic Libraries using the prepared primers in each of the ligated samples of cut genomic DNA. Analysis is also done as described previously. The same primers can be used to sequence the region lying between the primers.

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