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Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

Sambrook • Fritsch • Maniatis

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DIGESTING DNA WITH RESTRICTION ENZYMES

Different manufacturers of restriction enzymes recommend significantly different digestion conditions, even for the same restriction enzyme. Because most manufacturers have optimized the reaction conditions for their particular preparations, we recommend following the instructions on the information sheets supplied with the enzymes. Some manufacturers also supply concentrated buffers that have been tested for efficacy with each batch of purified enzyme. These buffers should be used whenever possible.

Buffers for different restriction enzymes differ chiefly in the concentration of NaCl that they contain. When DNA is to be cleaved with two or more restriction enzymes, the digestions can be carried out simultaneously if both enzymes work well in the same buffer. If the enzymes have different requirements, two alternatives are possible: (1) The DNA should be digested first with the enzyme that works best in the buffer of lower ionic strength. The appropriate amount of NaCl and the second enzyme can then be added and the incubation continued. (2) A single buffer (potassium glutamate buffer [KGB]) can be used in which virtually all restriction enzymes work (Hanish and McClelland 1988; McClelland et al. 1988). The dilutions of this buffer that are used to obtain maximal enzyme activity of various restriction enzymes are given in Table 5.7.

TABLE 5.7 Dilutions of KGB and Levels of Enzyme Activity Relative to Those in Manufacturer's Buffer

Enzyme	0.5×	1×	1.5×	2×
*Aat*II	+++	+++	+++	++
*Acc*I	+++	+++	+++	++
*Aha*II	–	++	+	+
*Alu*I	++	+++	++	+
*Apa*LI	+++	+	–	–
*Ava*I	++	++	++	+
*Ava*II	+	+++	++	–
*Avr*II	+++	+++	+++	++
*Bal*I	++	++	+++	+++
*Bam*HI	+++	+++	++	++
*Ban*I	+++	+++	+++	++
*Ban*II	++	+++	+++	++
*Bbv*I	+++	+++	+++	++
*Bcl*I	++	+++	+	–
*Bgl*I	++++	++++	++++	+++
*Bgl*II	++	+++	++	+
*Bsp*1286I	+	+	+	+
*Bsp*MII	+	++	+++	+++
*Bss*HII	+++	+++	++	+
*Bst*EII	+++	+++	+++	+++
*Bst*NI	++	++	++	+
*Bst*XI	+++	+++	++	+
*Cla*I	++	+++	++	++
*Dde*I	++	+++	+++	+++
*Dpn*I	++	++	++	++
*Dra*I	+++	+++	+	+
*Eae*I	+++	+++	++	+
*Eag*I	–	+	++	+++
*Eco*O109I	++	+++	+	+
*Eco*RI	+++	+++	+++	+++
*Eco*RV	++	+++	+++	+++
*Fnu*DII	++	++	+	+
*Fnu*4HI	++	++	++	+
*Fok*I	+++	+++	+	–
*Fsp*I	+++	+++	+++	+
*Hae*II	+++	+++	++	+
*Hae*III	+++	+++	++	++
*Hga*I	+++	+++	++	+
*Hgi*AI	–	+	++	+++
*Hha*I	++	+++	++	+
*Hinc*II	++++	++++	++++	+++
*Hin*dIII	+++	+++	+++	+++
*Hin*fI	++	+++	++	+
*Hpa*I	++	+++	++	+
*Hpa*II	+++	+	+	+
*Hph*I	++	+++	+	–
*Kpn*I	+++	++	–	–
*Mbo*I	++	+++	+++	++++
*Mbo*II	++	+++	+++	++
*Mlu*I	+++	+++	++++	++++
*Msp*I	++	+	+	–
*Mst*II	+++	+++	++	+
*Nar*I	++	+	–	–
*Nci*I	+++	+++	++	+

(continued)

TABLE 5.7 *(continued)*

Enzyme	0.5×	1×	1.5×	2×
*Nco*I	++	+++	+++	+++
*Nde*I	++	+++	++	+
*Nhe*I	+++	+++	+++	++
*Nla*IV	++++	++++	+++	++
*Not*I	++	++	++	++
*Nru*I	++	+++	+++	+++
*Nsi*I	+	+	++	+++
*Pfi*MI	++	+++	++++	+++
*Pst*I	+++	+++	+++	+++
*Pvu*I	++	+++	+++	+++
*Pvu*II	+++	+++	++	+
*Rsa*I	+++	+++	++	+
*Rsr*II	+++	+	–	–
*Sac*I	+++	+++	+	–
*Sac*II	+++	++	++	+
*Sal*I	–	+	+++	+++
*Sau*3AI	++	+++	+++	++++
*Sau*96I	+++	+++	++	+
*Sca*I	+	+++	+++	++
*Scr*FI	+	++	++	++
*Sfa*NI	–	+	++	+++
*Sfi*I	+++	+++	++	+
*Sma*I	+++	+++	+++	++
*Sna*BI	+++	++	+	–
*Ssp*I	++	++	+++	++
*Stu*I	+	++	+++	+++
*Sty*I	++	++	+	+
*Taq*I	++	+++	+++	+
*Xba*I	+++	+++	+++	+
*Xho*I	++	+++	+++	+
*Xmn*I	+++	+++	–	–
Bacteriophage T4 DNA polymerase	++	++	+++	+++
E. coli DNA polymerase I	++	++	+++	+++
Klenow fragment of *E. coli* DNA polymerase I	++	+++	+++	+++
Bacteriophage T4 DNA ligase (1 mM ATP)	+++	++	+	+
Reverse transcriptase	++	++	++	++

(++++) Activity greater than the activity observed in buffer recommended by the manufacturer; (+++) activity approximately equal to the activity observed in buffer recommended by the manufacturer; (++) activity approximately 50–80% of that observed in buffer recommended by the manufacturer; (+) activity approximately 20–50% of that observed in buffer recommended by the manufacturer; (–) activity less than 10% of that observed in buffer recommended by the manufacturer. Bacteriophage T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I both work well in 1.5× and 2× KGB. Bacteriophage T4 DNA ligase works well in 0.5× KGB. (Modified from McClelland et al. 1988.)

Setting Up Digestions with Restriction Enzymes

The following procedure is for a typical reaction containing 0.2–1 μg of DNA. For digestion of larger amounts of DNA, the reaction should be scaled appropriately.

1. Place the DNA solution in a sterile microfuge tube and mix with sufficient water to give a volume of 18 μl.

2. Add 2 μl of the appropriate 10 × restriction enzyme digestion buffer. (2 × KGB may also be used if the solution volumes are adjusted appropriately.) Mix by tapping the tube.

2 × KGB

200 mM potassium glutamate
50 mM Tris-acetate (pH 7.5)
20 mM magnesium acetate
100 μg/ml bovine serum albumin (Fraction V; Sigma)
1 mM β-mercaptoethanol

3. Add 1–2 units of restriction enzyme, and mix by tapping the tube.

One unit of enzyme is usually defined as the amount required to digest 1 μg of DNA to completion in 1 hour in the recommended buffer and at the recommended temperature in a 20-μl reaction. In general, digestion for longer periods of time or with excess enzyme does not cause problems unless there is contamination with DNAase or exonuclease. Such contamination is rare in commercial preparations of restriction enzymes.

4. Incubate the mixture at the appropriate temperature for the required period of time.

5. Stop the reaction by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.

6. *If the DNA is to be analyzed directly on a gel,* add 6 μl of gel-loading buffer (see Appendix B), mix by vortexing briefly, and load the digest into the gel slot.

If the volume of the restriction enzyme reaction is too large to fit into the slot of a gel, the DNA may be concentrated by the following simple procedure: After the reaction has been stopped by the addition of EDTA, add 0.6 volume of 5 M ammonium acetate and 2 volumes of ethanol. Store the tube on ice for 5 minutes, and then centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge. Discard the supernatant, which contains most of the protein. Allow the pellet to dry at room temperature. Dissolve the DNA in the appropriate volume of TE (pH 7.6).

If the digested DNA is to be purified, extract once with phenol:chloroform and once with chloroform, and precipitate the DNA with ethanol (see Appendix E).

Notes

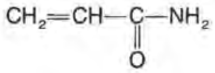
Restriction enzymes are expensive! Costs can be kept to a minimum by following the advice given below.

- Many restriction enzymes are supplied by the manufacturer in concentrated form. Often, 1 μl of many enzyme preparations is sufficient to digest 10 μg of DNA in 1 hour. To remove small quantities of enzyme from the container, briefly touch the surface of the fluid with the end of a disposable pipette tip. In this way, it is possible to remove as little as 0.1 μl of the enzyme preparation. Alternatively, a small piece of narrow-bore plastic tubing (1 cm in length) can be attached to a 1-μl Hamilton syringe and used to transfer a 0.1-μl volume. The plastic tubing is discarded after each sample is pipetted.

  Concentrated solutions of restriction enzymes may be diluted immediately before use in 1 × restriction enzyme buffer. Never dilute an enzyme in water, since it may denature.
- Restriction enzymes are stable when they are stored at −20°C in a buffer containing 50% glycerol. When carrying out restriction enzyme digestions, prepare the reactions to the point where all reagents except the enzyme have been mixed. Take the enzyme from the freezer, and *immediately* place it on ice. *Use a fresh, sterile pipette every time you dispense enzyme.* Contamination of an enzyme with DNA or another enzyme can be costly and can create time-consuming problems. Work as quickly as possible, so that the enzyme is out of the freezer for as short a period of time as possible. Return the enzyme to the freezer *immediately* after use.
- Keep reaction volumes to a minimum by reducing the amount of water in the reaction as much as possible. However, make sure that the restriction enzyme contributes less than 0.1 volume of the final reaction mixture; otherwise, the enzyme activity may be inhibited by glycerol.
- Often, the amount of enzyme can be reduced if the digestion time is increased. This can result in considerable savings when large quantities of DNA are cleaved. Small aliquots can be removed during the course of the reaction and analyzed on a minigel to monitor the progress of the digestion.
- When digesting many DNA samples with the same enzyme, calculate the total amount of enzyme (plus a small excess to allow for the losses involved in transferring several aliquots) that is needed. Remove the calculated amount of enzyme solution from the stock, and mix it with the appropriate volume of 1 × restriction enzyme buffer. Dispense aliquots of the enzyme/buffer mixture into the reaction mixtures.

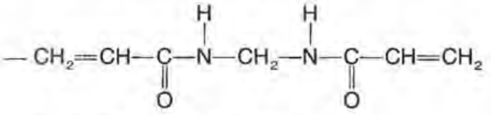
Polyacrylamide Gel Electrophoresis

Acrylamide is a monomer whose structure is



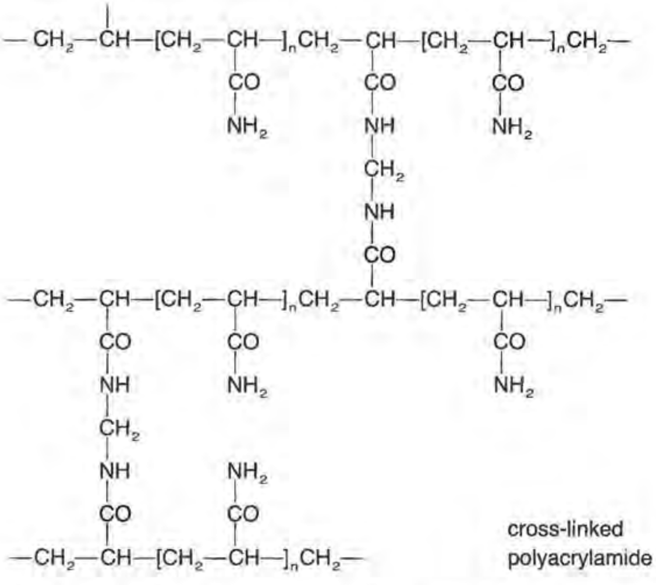
$$CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}NH_2$$

In the presence of free radicals, which are usually supplied by ammonium persulfate and stabilized by TEMED (*N,N,N′,N′*-tetramethylethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent *N,N′*-methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel, whose porosity is determined by the length of the chains and the degree of cross-linking.



$$-CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}\overset{\overset{H}{|}}{N}{-}CH_2{-}\overset{\overset{H}{|}}{N}{-}\underset{\underset{O}{\|}}{C}{-}CH{=}CH_2$$

N,N′-methylenebisacrylamide



cross-linked polyacrylamide

The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (between 3.5% and 20%): 1 molecule of cross-linker is included for every 29 monomers of acrylamide. The effective range of separation in nondenaturing gels containing different concentrations of polyacrylamide is shown in Table 6.4.

Polyacrylamide gels are more of a nuisance to prepare and run than agarose gels. They are almost always poured between two glass plates that are held apart by spacers and sealed by electrical tape. In this arrangement, most of the acrylamide solution is shielded from exposure to the air, so that inhibition of polymerization by oxygen is confined to a narrow layer at the top of the gel. Polyacrylamide gels can range in length from 10 cm to 100 cm, depending on the separation required; they are invariably run in a vertical position. However, they have three major advantages over agarose gels: (1) Their resolving power is so great that they can separate molecules of DNA whose lengths differ by as little as 0.2% (i.e., 1 bp in 500 bp). (2) They can accommodate much larger quantities of DNA than agarose gels: Up to 10 μg of DNA can be applied to a single slot (1 cm × 1 mm) of a typical polyacrylamide gel without significant loss of resolution. (3) DNA recovered from polyacrylamide gels is extremely pure and can be used for the most demanding purposes (e.g., microinjection of mouse embryos).

Two types of polyacrylamide gels are in common use:

- *Nondenaturing polyacrylamide gels for the separation and purification of fragments of double-stranded DNA.* These gels are poured and run in 1 × TBE at low voltage (1–8 V/cm) to prevent denaturation of small fragments of DNA by heat generated by the passage of electric current. Most species of double-stranded DNA migrate through nondenaturing polyacrylamide gels at a rate that is approximately inversely proportional to the $\log_{10}$ of their size. However, their electrophoretic mobility is also affected by their base composition and sequence, so that DNAs of exactly the same size can differ in mobility by up to 10%. This effect is believed to be caused by kinks that form at specific sequences in double-stranded DNA. Because it is impossible to know whether or not the migration of an unknown DNA is anomalous, electrophoresis through nondenaturing polyacrylamide gels cannot be used to determine the size of double-stranded DNAs.
- *Denaturing polyacrylamide gels for the separation and purification of single-stranded fragments of DNA.* These gels are polymerized in the presence of an agent (urea or, less frequently, formamide) that suppresses base pairing in nucleic acids. (Alkali cannot be used as a denaturing agent

TABLE 6.4 *Effective Range of Separation of DNAs in Polyacrylamide Gels*

Acrylamide (% [w/v])[a]	Effective range of separation (bp)	Xylene cyanol FF[b]	Bromophenol blue[b]
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

[a] N,N'-methylenebisacrylamide is included at 1/30th the concentration of acrylamide.

[b] The numbers given are the approximate sizes (in nucleotide pairs) of fragments of double-stranded DNA with which the dye comigrates.

because it deaminates acrylamide, and methylmercuric hydroxide cannot be used because it inhibits polymerization.) Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence. Among the uses of denaturing polyacrylamide gels are the isolation of radiolabeled DNA probes, the analysis of the products of nuclease-S1 digestions, and the analysis of the products of DNA sequencing reactions. Descriptions of denaturing polyacrylamide gels are given in Chapters 11 and 13.

PREPARATION OF NONDENATURING POLYACRYLAMIDE GELS

Most vertical electrophoresis tanks obtained from commercial sources are constructed to hold glass plates 20 cm × 40 cm. However, it is possible to run larger or smaller gels if suitable tanks are available. Spacers vary in thickness from 0.5 mm to 2.0 mm. The thicker the gel, the hotter it will become during electrophoresis; overheating results in "smiling" bands of DNA and other problems. Thinner gels are therefore preferred, since they produce the sharpest and flattest bands of DNA. However, it is necessary to use thicker gels when preparing large quantities of DNA (>1 μg/band). Below we describe the preparation and use of polyacrylamide gels.

1. Prepare the following solutions:

30% Acrylamide

acrylamide	29 g
N,N'-methylenebisacrylamide	1 g
H_2O to 100 ml	

Heat the solution to 37°C to dissolve the chemicals.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions. Stock solutions of acrylamide can easily be purified by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No. 1 paper.

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. This deamination reaction is catalyzed by light and alkali. Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature. Fresh solutions should be prepared every few months.

1 × TBE

89 mM Tris-borate
2 mM EDTA (pH 8.0)

TBE is usually made and stored as a 5 × stock solution (see Table 6.2, page 6.7). The pH of the buffer should be approximately 8.3.

TBE is used at a working strength of 1 × for polyacrylamide gel electrophoresis. This is twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small and the amount of electric current passed through them is often considerable. 1 × TBE is required to provide adequate buffering power.

10% Ammonium persulfate

ammonium persulfate	1 g
H_2O to 10 ml	

The solution may be stored at 4°C for several weeks.

2. Prepare the glass plates and spacers for pouring the gel. If necessary, clean them with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol.

Caution: Handle the KOH and the KOH/methanol solutions with great care. Use gloves and a face protector.

Then wash the glass plates and spacers in warm detergent solution and rinse them well, first in tap water and then in deionized water. Hold the plates by the edges so that oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.

Treat one surface of each plate with a silicone solution. This prevents

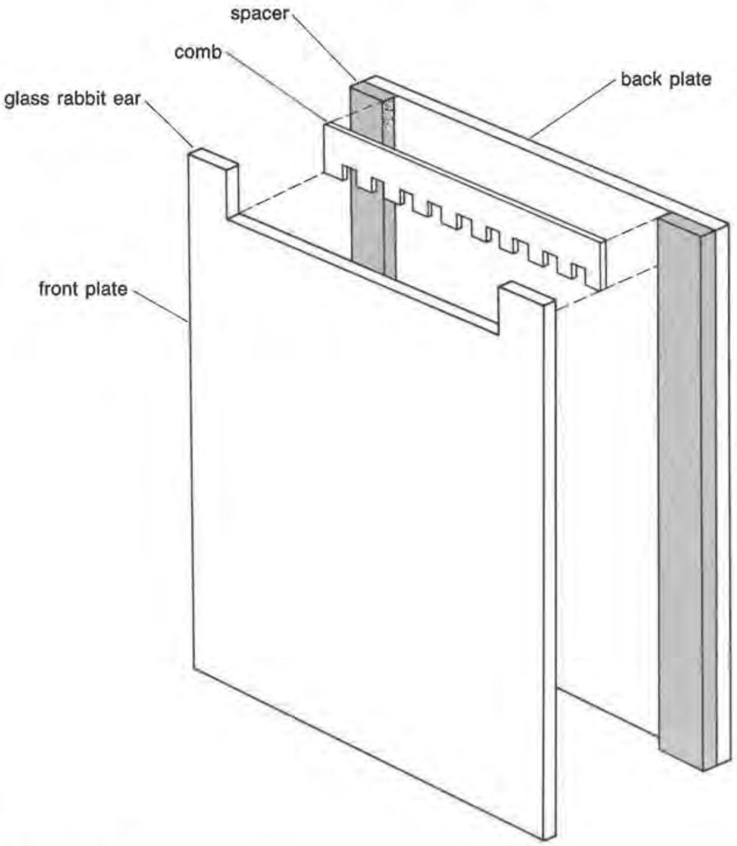


FIGURE 6.7

the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold after electrophoresis is completed.

To siliconize a plate, lay the plate on a pad of paper in a fume hood and pour a small quantity of siliconizing fluid (e.g., Sigmacote) onto it. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer. Wear gloves during siliconization.

3. There are many types of electrophoresis apparatus available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. In all cases, the aim is to form a watertight seal between the plates and the spacers so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Lay the larger (or unnotched) plate flat on the bench and arrange the spacers at each side parallel to the two edges (Figure 6.7). A couple of *minute* dabs of petroleum jelly help to keep the spacer bars in position during the next steps. Lay the inner (notched) plate in position, resting on the spacer bars. Bind the entire length of the two sides and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL catalog no. 1032ST) to make a watertight seal. Take particular care with the bottom corners of the plates, since these are the places where leaks often occur.

Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including:

- Sealing the edges with agarose
- Inserting a plastic spacer into the open space at the bottom of the mold during polymerization
- Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988)

We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available.

4. Knowing the size of the glass plates and the thickness of the spacers, calculate the volume of acrylamide solution required. (See Table 6.5 for preparation of 100 ml of solution for different gel concentrations.)

5. (*Optional*) Place the required quantity of acrylamide solution in a clean sidearm flask. Deaerate the solution by applying vacuum, gently at first. Swirl the flask during deaeration until no more air bubbles are released.

Deaeration of the acrylamide solution is not essential, but it does reduce the chance that air bubbles will form when thick gels (>1 mm) are poured.

TABLE 6.5 Volumes of Reagents Used to Cast Polyacrylamide Gels

	Milliliters of reagents to cast gels of various concentrations (%)				
Reagents	**3.5%**	**5.0%**	**8.0%**	**12.0%**	**20.0%**
30% Acrylamide (see page 6.39)	11.6	16.6	26.6	40.0	66.6
Water	67.7	62.7	52.7	39.3	12.7
5× TBE (see Table 6.2, page 6.7)	20.0	20.0	20.0	20.0	20.0
10% Ammonium persulfate (see page 6.40)	0.7	0.7	0.7	0.7	0.7

6. Wearing gloves, perform the following manipulations over a tray so that any spilled acrylamide solution will not spread over the bench.

   a. Add 35 μl of TEMED (*N,N,N′,N′*-tetramethylethylenediamine) to each 100 ml of acrylamide solution. Mix the solution by swirling.

   b. Draw the solution into the barrel of a 50-ml syringe. Invert the syringe and expel any air that has entered the barrel. Introduce the nozzle of the syringe into the space between the two glass plates. Expel the acrylamide solution from the syringe, filling the space almost to the top. Keep the remaining acrylamide solution at 4°C to reduce the rate of polymerization. If the plates were clean, there should be no trapped air bubbles, and if they were sealed well, no leaks. If air bubbles form, empty the gel mold and repour the gel, after thoroughly recleaning the glass plates.

   c. Lay the glass plates against a test-tube rack at an angle of approximately 10°. This decreases the chance of leakage and minimizes distortion of the gel.

7. Immediately insert the appropriate comb, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth should be slightly higher than the top of the glass. Clamp the comb in place with a bulldog paper clip. If necessary, use the remaining acrylamide solution to fill the gel mold completely. Check that no acrylamide solution is leaking from the gel mold.

8. Allow the acrylamide to polymerize for 60 minutes at room temperature, adding additional acrylamide solution if the gel retracts significantly. When polymerization is complete, a schlieren pattern will be visible just beneath the teeth of the comb.

Gels may be stored for 1–2 days in this state before they are used. After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1 × TBE. Then seal the entire gel in Saran Wrap and store at 4°C.

9. Carefully remove the comb. Immediately rinse out the wells with water. Using a razor blade or a scalpel, remove the electrical tape from the bottom of the gel.

It is essential to wash out the wells thoroughly as soon as the comb is removed. Otherwise, small amounts of acrylamide solution trapped by the comb will polymerize in the wells, producing irregularly shaped surfaces that give rise to distorted bands of DNA.

10. Attach the gel to the electrophoresis tank, using large bulldog paper clips on the sides and three-prong clamps on the shoulders. The notched plate should face inward toward the buffer reservoir.

11. Fill the reservoirs of the electrophoresis tank with 1 × TBE. Use a bent pasteur pipette or syringe needle to remove any air bubbles trapped beneath the bottom of the gel.

It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

12. Use a pasteur pipette to flush out the wells with 1 × TBE.

If remnants of unpolymerized acrylamide remain in the wells, diffuse, wavy bands of DNA will be observed.

13. Mix the DNA samples with the appropriate amount of 6 × gel-loading buffer (type I or type III; see Table 6.3, page 6.12). Load the mixture into the wells using a drawn-out glass micropipette or Hamilton syringe. Long, disposable, and expensive micropipette tips are also sold for this purpose by several manufacturers. Draw up all of the sample into the loading device, and then insert the tip of the device into the well. This should be done quickly, in a single movement, since the DNA sample tends to dribble out of the loading device after the tip is immersed in the electrophoresis buffer.

Usually, about 3–5 μl of DNA sample are loaded per well (0.5 cm × 0.3 cm × 1 mm). Raise the loading device as the sample is loaded into the well. The tip of the device should always be above the level of the sample in the well. Do not attempt to expel all of the sample from the loading device, since this almost always produces air bubbles that blow the sample out of the well.

The same device can be used to load many samples, provided it is thoroughly washed between each loading. However, it is important not to take too long to complete loading the gel; otherwise, the samples will diffuse from the wells.

14. Connect the electrodes to a power pack (positive electrode connected to the bottom reservoir). Nondenaturing polyacrylamide gels are usually run at voltage gradients between 1 V/cm and 8 V/cm.

If electrophoresis is carried out at a higher voltage, differential heating in the center of the gel may cause bowing of the DNA bands or even melting of the strands of small DNA fragments.

15. Run the gel until the marker dyes have migrated the desired distance (see Table 6.4, page 6.37). Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs. Detach the glass plates, and use a scalpel or razor blade to remove the electrical tape. Lay the glass plates on the bench (siliconized plate uppermost). Using a thin spatula, lift a corner of the upper glass plate. Check that the gel remains attached to the lower plate. Pull the upper plate smoothly away. Remove the spacers.

Occasionally, the gel remains attached to the siliconized plate. In this case, turn over the glass plates and remove the nonsiliconized plate.

16. Use one of the methods described on the following pages to detect the positions of bands of DNA in the polyacrylamide gel.

DETECTION OF DNA IN POLYACRYLAMIDE GELS

Staining with Ethidium Bromide

Because polyacrylamide quenches the fluorescence of ethidium bromide, it is not possible to detect bands that contain less than about 10 ng of DNA by this method.

1. Gently submerge the gel and its attached glass plate in staining solution (0.5 μg/ml ethidium bromide in 1 × TBE [see page 6.7]). Use just enough staining solution to cover the gel completely. After staining for 30–45 minutes at room temperature, remove the gel, using the glass plate as a support. Carefully blot excess liquid from the surface of the gel with a pad of Kimwipes. Do not use absorbent paper (to which the gel will stick). Cover the gel with a piece of Saran Wrap. Try to avoid creating air bubbles or folds in the Saran Wrap.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described on pages 6.16–6.17.

   Try to minimize the movement of the staining solution across the surface of the gel during staining. The aim is to keep the gel attached to its supporting glass plate. If the gel becomes completely detached, it can usually be rescued from the staining solution on a large glass plate and transferred to a shallow water bath. In most cases, the gel can then be carefully unfolded and restored to its original shape. To avoid problems, some workers use a piece of plastic mesh (mesh size 1 cm, available from garden and hardware stores) to hold the gel in place during staining.

2. To photograph the gel, place a piece of Saran Wrap on the surface of an ultraviolet transilluminator. Invert the gel, and place it on the transilluminator. Remove the glass plate, leaving the gel attached to the Saran Wrap. Photograph the gel as described on page 6.19.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

Autoradiography

UNFIXED, WET GELS

If bands of radioactive DNA are to be recovered from the gel, the gel should not be fixed or dried.

1. Wrap the gel, together with its supporting glass plate, in Saran Wrap. Try to avoid creating air bubbles or folds in the Saran Wrap. To align the gel and the film, attach adhesive dot labels marked with radioactive ink (see page 6.21) to the surface of the Saran Wrap. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with radioactive ink.

2. Invert the gel and expose it to X-ray film (Kodak XAR-2 or equivalent) as follows: In a darkroom, tape the sealed gel to a piece of X-ray film cut to the same size as the glass plate. The plate serves as a weight to ensure good contact between the Saran Wrap and the X-ray film. *Do not use a metal film cassette* (which may break the glass plate and crush the gel). Instead, wrap the gel and film in light-tight aluminum foil. Expose the film for the appropriate period of time at room temperature or at −70°C with an intensifying screen (see Appendix E).

FIXED, DRIED GELS

Analytical polyacrylamide gels containing radioactive DNA may be fixed and dried before autoradiography.

1. Immerse the gel, together with its attached glass plate, in 7% acetic acid for 5 minutes. Remove the gel from the fixative by carefully lifting the glass plate from the fluid.

   Try to minimize the movement of fluid across the surface of the gel during fixation. The aim is to keep the gel attached to its supporting glass plate. If the gel becomes completely detached, it can usually be rescued from the acetic acid on a larger glass plate and transferred to a shallow water bath. In most cases, the gel can then be carefully unfolded and restored to its original shape. To avoid problems, some workers use a piece of plastic mesh (mesh size 1 cm, available from garden and hardware stores) to hold the gel in place during fixation.

2. Rinse the gel briefly in deionized water. Remove excess fluid from the surface of the gel with a pad of Kimwipes. Do not use absorbent paper (to which the gel will stick).

3. Wrap the gel and glass plate in Saran Wrap, and establish an autoradiograph as described above. Alternatively, dry the gel onto a piece of Whatman 3MM paper using a commercial gel dryer. Drying the gel is necessary only when the gel contains DNA labeled with weak β-emitting isotopes such as ^{35}S or such small amounts of ^{32}P-labeled DNA that long exposures (longer than 24 hours) are necessary to obtain an adequate autoradiographic image.

ISOLATION OF DNA FRAGMENTS FROM POLYACRYLAMIDE GELS

The best method to isolate DNA from polyacrylamide gels is the "crush and soak" technique originally described by Maxam and Gilbert (1977). The DNA obtained is of very high purity and is free of contaminants that inhibit enzymes or are toxic to transfected or microinjected cells. Although the method is lengthy and inefficient (<30% yield for DNA fragments >3 kb in length), it can be used to isolate both double- and single-stranded DNAs from neutral and denaturing polyacrylamide gels, respectively. A more rapid technique that is used to isolate fragments of double-stranded DNA is to embed the piece of polyacrylamide containing the DNA of interest into a slit cut in an agarose gel, and then to elute the DNA onto a sliver of DEAE-cellulose membrane (Schleicher and Schuell NA-45) as described on pages 6.24–6.27.

"Crush and Soak" Method

The following procedure is a modification of the technique described by Maxam and Gilbert (1977).

1. Run the polyacrylamide gel as described on pages 6.39–6.43. Locate the DNA of interest by autoradiography or by examination of ethidium-bromide-stained gels in long-wavelength ultraviolet light.

2. Using a sharp scalpel or razor blade, cut out the segment of the gel containing the band of interest. Do not attempt to remove the gel from the Saran Wrap before cutting; instead, cut through both the gel and the Saran Wrap, and then peel the small piece of gel containing the DNA from the Saran Wrap.

   To recover a fragment of DNA identified by autoradiography, cut out from the X-ray film a small rectangle containing the autoradiographic image of the fragment. Align the film over the gel and cut out the segment of polyacrylamide underneath the rectangular hole in the film.

   It is a good idea to photograph or to reautoradiograph the gel after the bands of DNA have been excised. This provides a permanent record of the experiment.

3. Transfer the gel slice to a microfuge tube. Use a disposable pipette tip to crush the slice against the wall of the tube.

4. Calculate the approximate volume of the slice and add 1–2 volumes of elution buffer to the microfuge tube.

Elution buffer

0.5 M ammonium acetate
10 mM magnesium acetate
1 mM EDTA (pH 8.0)
0.1% SDS

It is convenient if the volume of elution buffer is no greater than 0.5 ml, since the eluted fragment of DNA can then be precipitated with ethanol in a single microfuge tube.

5. Close the tube and incubate at 37°C on a rotating wheel or rotary platform. Small fragments of DNA (<500 bp) are eluted in 3–4 hours; larger fragments take 12–16 hours.

6. Centrifuge the sample at 12,000*g* for 1 minute at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube, being careful to avoid transferring fragments of polyacrylamide (a drawn-out pasteur pipette works well).

7. Add an additional 0.5 volume of elution buffer to the pellet of polyacrylamide, vortex briefly, and recentrifuge. Combine the two supernatants.

8. Remove any remaining fragments of polyacrylamide by passing the supernatant through a disposable plastic column or a syringe barrel containing a Whatman GF/C filter or packed siliconized glass wool.

9. Add 2 volumes of ethanol at 4°C and store the solution on ice for 30 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

   Even small quantities of DNA are efficiently precipitated by ethanol, perhaps because of the presence of small amounts of polyacrylamide in the eluate. However, 10 μg of carrier RNA can be added before precipitation, which may improve even further the recovery of small amounts of DNA. Before adding the RNA, make sure that the presence of RNA will not compromise subsequent reactions with the DNA. (For preparation of carrier RNA, see page 6.26.)

10. Redissolve the DNA in 200 μl of TE (pH 7.6), add 25 μl of 3 M sodium acetate (pH 5.2), and reprecipitate the DNA with 2 volumes of ethanol as described in step 9.

11. Carefully rinse the pellet once with 70% ethanol, and redissolve the DNA in TE (pH 7.6) to a final volume of 10 μl.

12. Check the amount and quality of the fragment by polyacrylamide gel electrophoresis.

    a. Mix a small aliquot (estimated to contain 50 ng) of the final preparation of the fragment with 10 μl of TE (pH 7.6), and add 2 μl of the desired gel-loading buffer (see Table 6.3, page 6.12).

    b. Load and run a polyacrylamide gel of the appropriate concentration, using as markers appropriate restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest.

c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA. It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

SANGER METHOD OF DIDEOXY-MEDIATED CHAIN TERMINATION

The current chain-termination method evolved from the +/− sequencing technique (Sanger and Coulson 1975), which first described (1) the use of a specific primer for extension by DNA polymerase, (2) base-specific chain termination, and (3) the use of polyacrylamide gels to discriminate between single-stranded DNA chains differing in length by a single nucleotide. Despite these advances, the +/− method was too inaccurate and clumsy to gain general acceptance, and it was not until the introduction of chain-terminating dideoxynucleoside triphosphates (ddNTPs) (Sanger et al. 1977) that enzymatic methods of DNA sequencing were used extensively.

2′,3′ ddNTPs differ from conventional dNTPs in that they lack a hydroxyl residue at the 3′ position of deoxyribose. They can be incorporated by DNA polymerases into a growing DNA chain through their 5′ triphosphate groups. However, the absence of a 3′-hydroxyl residue prevents formation of a phosphodiester bond with the succeeding dNTP. Further extension of the growing DNA chain is therefore impossible. Thus, when a small amount of one ddNTP is included with the four conventional dNTPs in a reaction mixture for DNA synthesis, there is competition between extension of the chain and infrequent, but specific, termination. The products of the reaction are a series of oligonucleotide chains whose lengths are determined by the distance between the terminus of the primer used to initiate DNA synthesis and the sites of premature termination. By using the four different ddNTPs in four separate enzymatic reactions, populations of oligonucleotides are generated that terminate at positions occupied by every A, C, G, or T in the template strand (see Figure 13.1, pages 13.4–13.5).

Reagents Used in the Sanger Method of DNA Sequencing

PRIMERS

In enzymatic sequencing reactions, priming of DNA synthesis is achieved by the use of a synthetic oligonucleotide complementary to a specific sequence on the template strand. In many cases, this template is obtained as a single-stranded DNA molecule by cloning the target DNA fragment into a bacteriophage M13 or phagemid vector. However, it is also possible to use the Sanger method to sequence denatured double-stranded DNA templates (e.g., denatured plasmid DNA) (see pages 13.70–13.72). In either case, the problem of obtaining primers that are complementary to an unknown sequence of DNA is then solved by using a "universal" primer that anneals to vector sequences that flank the target DNA. Universal primers used for the sequencing of bacteriophage M13 recombinant clones are typically 15–29 nucleotides in length and anneal to the sequences immediately adjacent to (1) the *Hin*dIII site in the polycloning region of bacteriophage M13mp18 and (2) the *Eco*RI site in the polycloning region of bacteriophage M13mp19. These primers, which can also be used for "double-stranded" sequencing of DNAs cloned into pUC plasmids, are available from a large number of commercial suppliers. In addition, several companies sell primers that have been designed to allow sequencing of target DNAs cloned into a variety of restriction sites in different plasmids.

TEMPLATES

As mentioned above, two types of DNA can be used as templates in the Sanger method of sequencing: pure single-stranded DNA and double-stranded DNA that has been denatured by heat or alkali. The best results are obtained from single-stranded DNA templates, which are usually isolated from recombinant bacteriophage M13 particles. When care is taken to optimize the ratio of single-stranded template to primer, it is possible to obtain several hundred nucleotides of sequence from each set of chain-termination reactions. Results of this quality are more difficult to obtain when denatured double-stranded DNA is used as a template. Despite the apparent simplicity and convenience of the method (Chen and Seeburg 1985), it has only recently been improved to the point where it can reliably yield unambiguous results from double-stranded DNA templates. Two factors are critical: the quality of the template DNA and the type of DNA polymerase that is used (see below). Minipreparations of plasmid DNA are always contaminated by small oligodeoxyribonucleotides and ribonucleotides, which serve as random primers, and by inhibitors of DNA polymerases. As a consequence, sequencing gels are frequently obscured by a variety of "ghost" bands, strong stops, and other artifacts. We therefore recommend that minipreparations of plasmid DNAs should not be used to determine the sequence of cloned segments of unknown DNA. However, such DNAs are often adequate templates for confirming a sequence that has already been determined by another method. Plasmid DNA that has been purified by equilibrium centrifugation in CsCl–ethidium bromide gradients yields far better results, although the labor and expense of preparing plasmid DNA in this way are considerable.

DNA POLYMERASES

Several different enzymes are commonly used for dideoxy-mediated sequencing, including the Klenow fragment of *E. coli* DNA polymerase I (Sanger et al. 1977), reverse transcriptase (see, e.g., Mierendorf and Pfeffer 1987), bacteriophage T7 DNA polymerases that have been modified to eliminate 3′→5′ exonuclease activity (Sequenase and Sequenase version 2.0) (Tabor and Richardson 1987), and the thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq* DNA polymerase). The properties of these DNA polymerases (see Table 13.1) differ greatly in ways that can considerably affect the quantity and quality of the DNA sequence obtained from chain-termination reactions.

Klenow fragment of E. coli *DNA polymerase I*

This enzyme was originally used to develop the Sanger method and is still used extensively for DNA sequencing. Two problems frequently arise:

- The low processivity of the enzyme causes the Klenow fragment to generate a high background of fragments that terminate, not by incorporation of a ddNTP, but by the random dissociation of the polymerase from the template. The inability of the enzyme to travel long distances along the template limits the length of sequence that can be obtained from standard sequencing reactions using this enzyme. Typically, such reactions generate approximately 250 to 350 nucleotides of sequence. The amount of sequence can be doubled by carrying out the reaction in two steps—an initial labeling step containing low concentrations of dNTPs, followed by a chain-extension/chain-termination reaction containing ddNTPs and a high concentration of dNTPs (Johnston-Dow et al. 1987; Stambaugh and Blakesley 1988). However, even with these improvements, the Klenow enzyme does not routinely yield as much sequence as the more processive Sequenase enzymes (see below).

- The enzyme will not efficiently copy homopolymeric tracts or other regions of high secondary structure in the template. This problem can be alleviated, but not completely solved, by increasing the temperature of the polymerization reaction to 55°C (Gomer and Firtel 1985). dNTP analogs (e.g., dITP or 7-deaza-dGTP [see page 13.10]), which are sometimes used to obtain sequence information at regions of the template that form stable secondary structures, are less effective with the Klenow enzyme than with Sequenases, perhaps because they decrease still further the already low processivity of the enzyme.

In summary, the Klenow fragment of *E. coli* DNA polymerase I is the enzyme of choice for determining the sequence of tracts of DNA that lie within 250 bases of the 5′ terminus of the primer. It is not recommended for sequencing longer segments of DNA or DNAs with dyad symmetry and/or homopolymeric tracts.

Reverse transcriptase

Although not widely used for routine sequencing, this enzyme is occasionally employed to resolve problems caused by the presence of homopolymeric regions of A/T or G/C in the template DNA. Reverse transcriptases from both avian and murine sources appear to be slightly better in this respect than the Klenow enzyme (Karanthanasis 1982; Graham et al. 1986), although perhaps not as good as the Sequenases (Cameron-Mills 1988; Revak et al. 1988).

TABLE 13.1 Properties of DNA Polymerases Used in DNA Sequencing Reactions

Enzyme	Processivity[a]	Rate of polymerization[b]
Klenow fragment of *E. coli* DNA polymerase I	10–50	45
Reverse transcriptase (AMV)	n.d.	5
Sequenase and	~2000	
Sequenase version 2.0	~3000	300
Taq DNA polymerase and AmpliTaq[c]	>7600	35–100

[a] Processivity is expressed as the average number of nucleotides synthesized before the enzyme dissociates from the template; n.d. indicates not determined.
[b] Rate of polymerization in nucleotides/second.
[c] *Taq* DNA polymerase, a highly processive DNA polymerase, is useful for determining the sequence of DNA templates that form stable secondary structures.

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Sequenases

Sequenase^{TM} is a form of bacteriophage T7 DNA polymerase that has been chemically modified to eliminate much of the enzyme's powerful 3′→5′ exonuclease activity. Sequenase version 2.0 is a genetically engineered form of Sequenase that entirely lacks 3′→5′ exonuclease activity, is extremely stable, and has a threefold higher specific activity than the chemically modified enzyme. Sequenases are the enzymes of choice for determining the sequences of long tracts of DNA because of their very high processivity, their high rate of polymerization, and their wide tolerance for nucleotide analogs such as dITP and 7-deaza-dGTP that are used to resolve regions of compression in sequencing gels. Sequenases travel such long distances along the template that several hundred nucleotides of DNA sequence can often be determined from a single set of reactions. In fact, the amount of sequence is limited more by the resolving power of polyacrylamide gels than by the properties of the enzyme.

To take full advantage of the high processivity of Sequenases, a two-step sequencing reaction is set up. In the first stage, low concentrations of dNTPs and low temperature are used to limit the extent of synthesis and to ensure efficient incorporation of a radiolabeled dNTP. The products of this reaction are primers that have been extended by only 20–30 bases. The first reaction is then divided into the standard set of four reactions, each of which contains high concentrations of dNTPs and a single ddNTP. Polymerization then continues until a chain-terminating nucleotide is incorporated into the growing chain.

Taq *DNA polymerase*

Taq DNA polymerase is useful for determining the sequence of single-stranded DNA templates that form extensive stable secondary structures at 37°C. This is because *Taq* DNA polymerase works efficiently at 70–75°C, a temperature that precludes formation of secondary structure even in templates that are rich in G + C. When used as described by Innis et al. (1988), sequencing ladders produced by *Taq* DNA polymerase demonstrate a high uniformity of band intensity for several hundred nucleotides, suggesting that the enzyme has a high degree of processivity.

RADIOLABELED dNTPs

Until a few years ago, virtually all DNA sequencing was carried out with [α-^{32}P]dNTPs. However, the strong β particles emitted by ^{32}P created two problems. First, because of scattering, the bands on the autoradiograph were far larger and more diffuse than the bands of DNA in the gel. This affected the ability to read a sequence correctly (particularly from the upper part of the autoradiograph) and limited the number of nucleotides that could be read from a single gel. Second, decay of ^{32}P caused radiolysis of the DNA in the sample. Sequencing reactions radiolabeled with ^{32}P could therefore be stored for only 1 or 2 days before the DNA was so badly damaged that it generated indecipherable sequencing gels.

The introduction of [^{35}S]dATP (Biggin et al. 1983) greatly alleviated both of these problems. Because of the decreased scatter of the weaker β particles produced by decay of ^{35}S, there is little loss of resolution between the gel and the autoradiograph. This allows unambiguous determination of several hundred nucleotides of DNA sequence from a single reaction set. Furthermore, the lower energy of ^{35}S produces less radiolysis, allowing sequencing reactions to be stored for up to 1 week at −20°C without noticeable loss of resolution. Thus, if technical problems arise with a polyacrylamide gel, the sequencing reactions can simply be reanalyzed.

S OF dNTPs

Regions of DNA with dyad symmetry (especially those with a high G + C content) can form intrastrand secondary structures that are not fully denatured during electrophoresis. This can cause an anomalous pattern of migration in which adjacent bands of DNA become compressed to the point where they are difficult to read. Compression is entirely dependent on the presence of secondary structures in DNA and cannot be alleviated by changing the type of DNA polymerase used in the sequencing reaction. However, compressed regions of gels can usually be resolved by using a nucleotide analog such as dITP (2′-deoxyinosine-5′-triphosphate) or 7-deaza-dGTP (7-deaza-2′-deoxyguanosine-5′-triphosphate). These analogs pair weakly with conventional bases and are good substrates for DNA polymerases such as the Sequenases and *Taq* DNA polymerase (Gough and Murray 1983; Mizusawa et al. 1986; Innis et al. 1988). Some compressions are not resolved by 7-deaza-dGTP; others (particularly those occurring in GC-rich regions) are not resolved by dITP. If it is necessary to use analogs, try dITP first (see pages 13.74–13.75). This analog, in contrast to 7-deaza-dGTP, does not affect the sharpness of the DNA bands in the sequencing gel. Any compression that is not resolved by either dITP or 7-deaza-dGTP can almost always be cleared up by determining the sequence of both strands of the DNA.

As discussed above, both forms of Sequenase and *Taq* DNA polymerase tolerate nucleotide analogs better than does the Klenow fragment of *E. coli* DNA polymerase I. In addition, the manufacturer claims that Sequenase version 2.0 is superior to the original enzyme when sequencing templates with strong secondary structure. Version 2.0 is more processive than Sequenase, having less tendency to pause, thereby eliminating "ghost" bands. Furthermore, version 2.0 appears to tolerate nucleotide analogs such as dITP better than does the original version.

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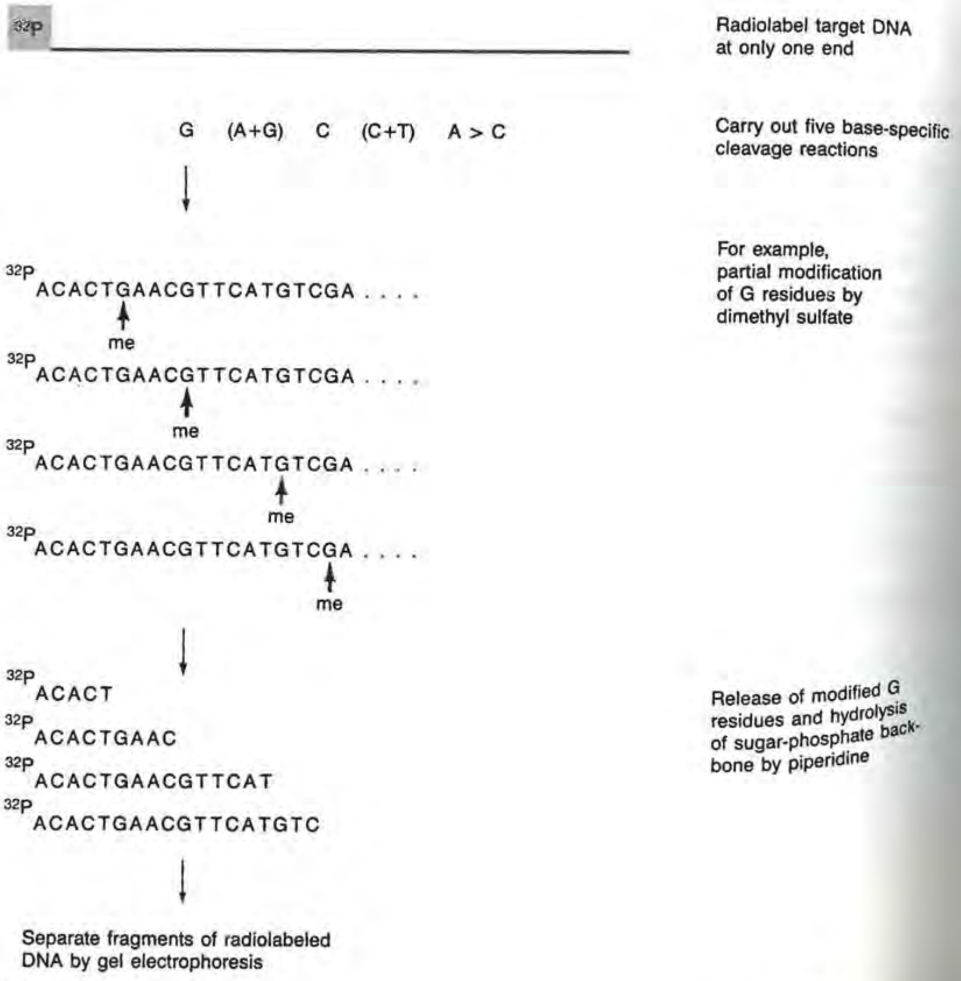
MAXAM-GILBERT CHEMICAL DEGRADATION OF DNA METHOD

Unlike the chain-termination technique, which involves synthesis, the Maxam-Gilbert method involves chemical degradation of the original DNA. This method grew out of studies of the interaction between the *lac* repressor and the *lac* operator in vitro. To this day, the ability to probe DNA conformations and protein–DNA interactions remains a unique feature of the Maxam-Gilbert method.

In this procedure (Maxam and Gilbert 1980), a fragment of DNA radiolabeled at one end is partially cleaved in five separate chemical reactions, each of which is specific for a particular base or type of base. This generates five populations of radiolabeled molecules that extend from a common point (the radiolabeled terminus) to the site of chemical cleavage. Each population consists of a mixture of molecules whose lengths are determined by the locations of a particular base along the length of the original DNA. These populations are then resolved by electrophoresis through polyacrylamide gels, and the end-labeled molecules are detected by autoradiography (see Figure 13.2).

The Maxam-Gilbert method has remained relatively unchanged since its initial development. Although additional chemical cleavage reactions have been devised (for review, see Ambrose and Pless 1987), these are generally used to supplement the reactions originally described by Maxam and Gilbert (1977, 1980). The success of the method depends entirely on the specificity of these cleavage reactions, which are carried out in two stages. In the first stage, specific bases (or types of bases) undergo chemical modification; in the second stage, the modified base is removed from its sugar and the phosphodiester bonds 5′ and 3′ to the modified base are cleaved (see Table 13.2). In every case, these reactions are carried out under carefully controlled conditions to ensure that on average only one of the target bases in each DNA molecule is modified. Subsequent cleavage by piperidine at the 5′ and 3′ sides of the modified bases yields a set of end-labeled molecules whose lengths range from one to several hundred nucleotides. The DNA sequence can then be read from an autoradiograph of a sequencing gel by comparing the G, $A+G$, $C+T$, C, and $A>C$ tracks. For a number of reasons (e.g., the use of ^{32}P as a radiolabel, the specific activity of end-labeled DNA, the statistical distribution of cleavage sites, and the limitations of gel technology), the range of the Maxam-Gilbert method is less than that of the Sanger method; the Maxam-Gilbert method works optimally for DNA sequences that lie less than 250 nucleotides from the radiolabeled end.

When the Maxam-Gilbert and Sanger methods were first developed in the 1970s, sequencing by chemical degradation was both more reproducible and more accessible to the average worker. The Sanger method required single-stranded templates, specific oligonucleotide primers, and access to high-quality preparations of the Klenow fragment of *E. coli* DNA polymerase I. The Maxam-Gilbert method used simple chemical reagents that were available to everyone. However, with the development of bacteriophage M13 and phagemid vectors, the ready availability of synthetic primers, and improvements to the sequencing reactions, the dideoxy-mediated chain-termination method is now used much more extensively than the Maxam-Gilbert method. Nevertheless, the chemical degradation approach has one clear advantage over the chain-termination method: Sequence is obtained from the original DNA molecule and not from an enzymatic copy. Therefore, with the Maxam-Gilbert method, one can sequence synthetic oligonucleotides, analyze DNA modifications such as methylation, and study both DNA secondary structure and the interaction of proteins with DNA by either chemical protection or modification interference experiments. However, because of its ease and rapidity, the Sanger technique is now the best choice for simple determination of DNA sequence. In fact, most of the current sequencing strategies have been designed for use with this method.


32P
Radiolabel target DNA at only one end
G (A+G) C (C+T) A > C
Carry out five base-specific cleavage reactions
32P ACACTGAACGTTCATGTCGA . . . .
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32P ACACTGAACGTTCATGTCGA . . . .
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32P ACACTGAACGTTCATGTCGA . . . .
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32P ACACTGAACGTTCATGTCGA . . . .
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For example, partial modification of G residues by dimethyl sulfate
32P ACACT
32P ACACTGAAC
32P ACACTGAACGTTCAT
32P ACACTGAACGTTCATGTC
Release of modified G residues and hydrolysis of sugar-phosphate backbone by piperidine
Separate fragments of radiolabeled DNA by gel electrophoresis

FIGURE 13.2
Sequencing by the Maxam-Gilbert chemical degradation of DNA method.

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Bas

G

A + (

C + T

C

A > C

[a] Hot (
sites (

TABLE 13.2 Chemical Modifications Used in the Maxam-Gilbert Method

Base	Specific modification[a]
G	Methylation of N_7 with dimethyl sulfate at pH 8.0 makes the C_8—C_9 bond specifically susceptible to cleavage by base
A + G	Piperidine formate at pH 2.0 weakens the glycosidic bond of adenine and guanine by protonating nitrogen atoms in the purine rings resulting in depurination
C + T	Hydrazine opens pyrimidine rings, which recyclize in a five-membered form that is susceptible to removal
C	In the presence of 1.5 M NaCl, only cytosine reacts appreciably with hydrazine
A > C	1.2 N NaOH at 90°C results in strong cleavage at A and weaker cleavage at C

[a] Hot (90°C) piperidine (1 M in H_2O) is used to cleave the sugar-phosphate chain of DNA at the sites of chemical modifications.

SEQUENCING STRATEGIES

Before beginning to sequence, it is important to develop an overall strategy that takes into account the size of the region to be sequenced, the accuracy of the sequence required, and the facilities that are available. Only a minor proportion of projects involve the de novo accumulation of large tracts of virgin sequence. More often, sequencing is used to map and identify mutations (e.g., point mutations and deletions) and to verify the orientation and structure of recombinant DNA constructs. The approaches used for these two purposes are very different.

Confirmatory Sequencing

Confirmatory sequencing (e.g., sequencing of mutants generated by oligonucleotide-mediated mutagenesis) often requires no more than one set of reactions that generates the nucleotide sequence of a local region of one of the two strands of DNA. This can usually be achieved by sequencing an appropriate restriction fragment that has been subcloned into a bacteriophage M13 or phagemid vector. In many cases, the region of interest will lie within the sequencing range of a universal primer; if not, the best strategy is to synthesize a priming oligonucleotide 17–19 nucleotides long that is complementary to sequences located approximately 50–100 nucleotides from the region of interest. Whenever possible, the sequence of the homologous region of the wild-type gene should be determined at the same time as that of the mutant. Direct comparison of the sequences on the same autoradiograph greatly facilitates confirmation of the sequence of the altered region and clearly reveals any unexpected, additional differences between mutant and wild-type genes.

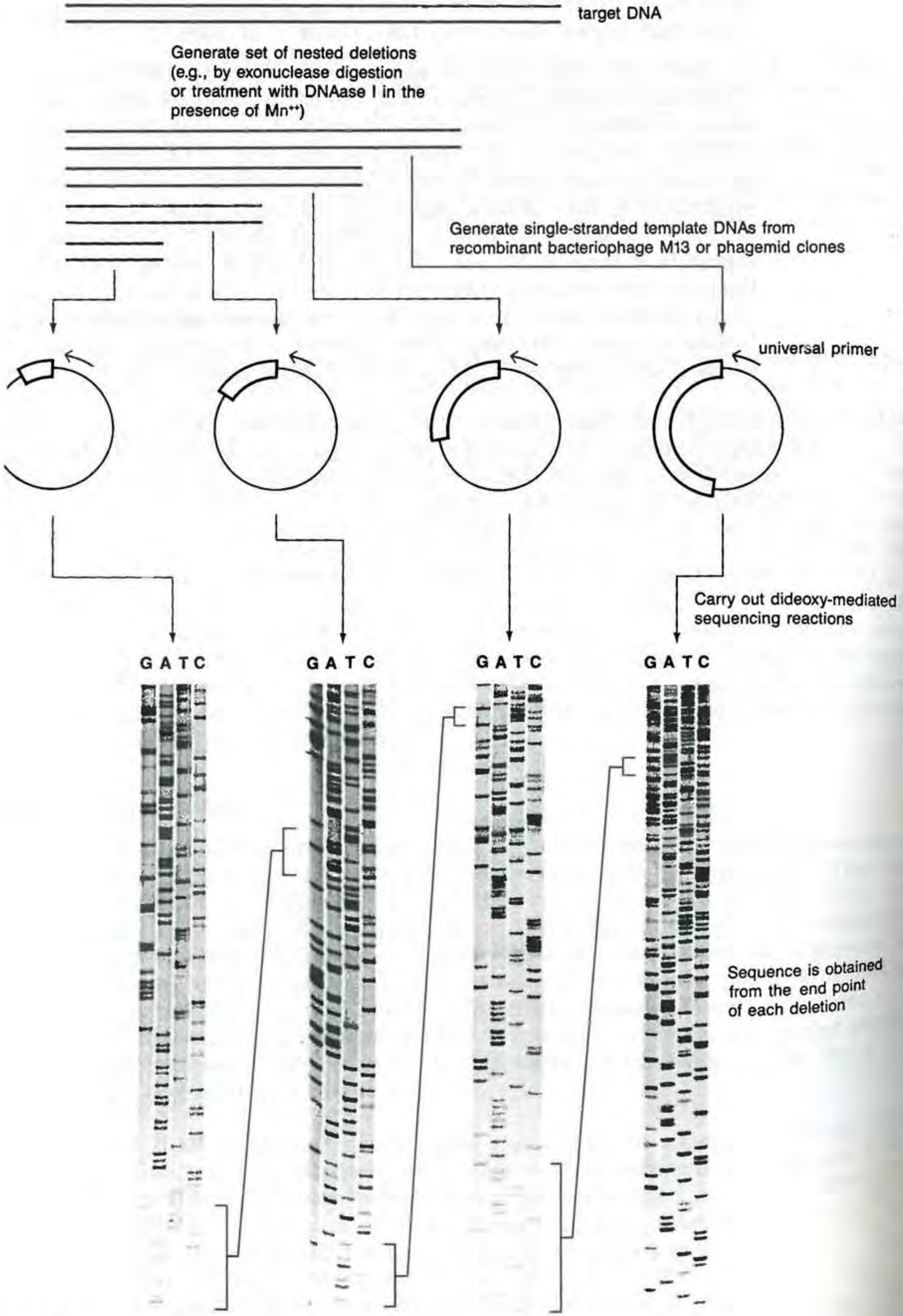
De Novo Sequencing

The aim of de novo sequencing is to provide the accurate nucleotide sequence of a virgin segment of DNA that may be many kilobases in length. This task requires careful planning because the maximum length of target DNA that can be sequenced accurately in a single set of sequencing reactions is approximately 400 bases. Target DNAs of this size can be sequenced by cloning in opposite orientations in each of two bacteriophage M13 vectors (e.g., M13mp18 and M13mp19). The entire sequence of each strand can then be determined in a single reaction set using a universal sequencing primer. To sequence longer target DNAs (e.g., several kilobases in length), one of two general strategies can be used:

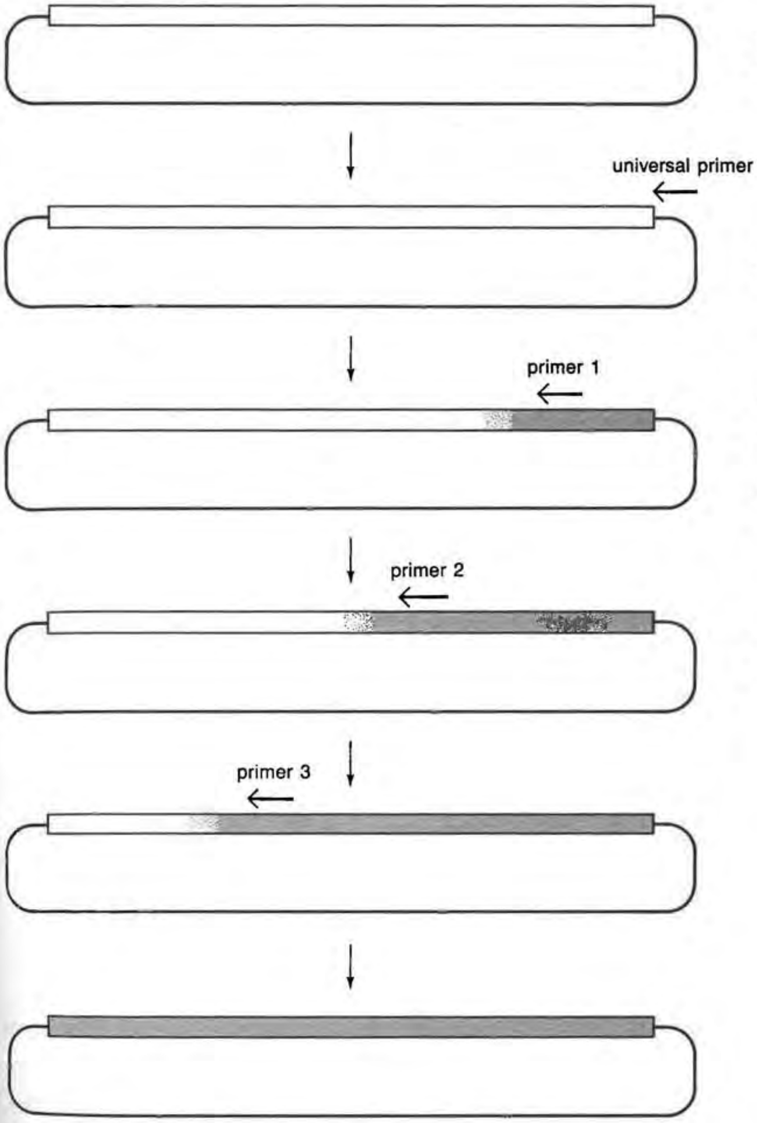
- *A random approach (or shotgun sequencing),* in which sequence data are collected from subclones containing random segments of the target DNA. No attempt is made to determine where these subclones map in the target DNA or which strand of DNA is being sequenced. Instead, the accumulated data are stored and finally arranged in order by a computer (Staden 1986). This approach, which was pioneered by the M.R.C. Laboratory in Cambridge, has been used successfully to determine the sequences of human mitochondrial DNA (Anderson et al. 1981), human adenovirus DNA (Gingeras et al. 1982; Roberts et al. 1986), bacteriophage λ DNA (Sanger et al. 1982), and Epstein-Barr virus DNA (Baer et al. 1984).

- *Directed approaches,* in which sequences of the target DNA are obtained in a systematic fashion. For example, the entire sequence of the target DNA might be obtained by sequencing a nested set of deletion mutants that begin at a common point (usually at one end of the target DNA) and penetrate various distances into the target region. They therefore bring progressively more remote regions of the target DNA into range for sequencing by universal primer (see Figure 13.3A). Alternatively, virgin segments of target DNA can be sequenced in a step-wise fashion by using the nucleotide sequences obtained from one set of reactions to design a new oligonucleotide that is then used to prime the subsequent set of reactions. In this approach, therefore, DNA sequence is accumulated by moving the priming site along the DNA in a progressive fashion (see Figure 13.3B).

Although the choice between the random and directed strategies is usually dictated by the resources and expertise that are available in the laboratory, a number of additional factors that may also influence the final selection are discussed on pages 13.18–13.20.


target DNA
Generate set of nested deletions (e.g., by exonuclease digestion or treatment with DNAase I in the presence of Mn++)
Generate single-stranded template DNAs from recombinant bacteriophage M13 or phagemid clones
universal primer
Carry out dideoxy-mediated sequencing reactions
G A T C
G A T C
G A T C
G A T C
Sequence is obtained from the end point of each deletion

IGURE 13.3A
irected sequencing with nested sets of deletion mutants.



Clone target DNA into bacteriophage M13 or phagemid vector

Sequence terminus of target DNA using universal primer

Synthesize oligonucleotide primer complementary to the most distal tract of reliable sequence.
Carry out dideoxy sequencing reaction.

Continue cycles of oligonucleotide synthesis and sequencing until entire target DNA has been sequenced

FIGURE 13.3B
Directed sequencing with progressive oligonucleotides.

FACTORS AFFECTING THE CHOICE BETWEEN RANDOM AND DIRECTED STRATEGIES

Computing facilities

Every large-scale sequencing project relies heavily on computer programs to sort and order the primary sequence data (Staden 1986). Access to adequate computing facilities becomes an overriding consideration when weighing the pros and cons of the random approach. If these facilities are not available, abandon any idea of using this strategy and turn instead to one of the two directed approaches discussed above.

Nature of the target DNA

If the target DNA is likely to contain dispersed repetitive sequences, then nested deletions should be constructed and used for sequencing. A computer may have difficulty sorting out the repetitive sequences, and oligonucleotide primers may anneal to multiple sites.

Time required to complete the project

The amount of work required to complete a sequencing project can be estimated from the following guidelines:

1. An average of 300–400 nucleotides of sequence can routinely be obtained from a single reaction set.
2. One person can comfortably handle 24–32 reaction sets in a day.
3. A typical week of sequencing, which might generate up to 15 kb of nucleotide sequence, therefore involves
   - 1 day to prepare single-stranded DNA templates
   - 1 day of DNA sequencing
   - 1 day of reading primary DNA sequences and aligning them
   - 2 days of repeating sequencing reactions and rerunning gels to resolve ambiguities and to obtain overlaps between clones

In the random approach, it is usually necessary to sequence about five to seven times more nucleotides than the actual length of target DNA contains. In most cases, a single contiguous sequence does not emerge until approximately 90% of the sequence of both strands has been determined. Because subclones for sequencing are selected at random, certain regions of the target DNA will be sequenced repeatedly before the entire region is covered, and there is no way to predict how long it will take to find and sequence the last subclones required to complete a sequence. Often, these subclones turn out to be underrepresented in the library, and it is then necessary to isolate them by screening with oligonucleotide probes corresponding to flanking sequences. These logistic problems can be alleviated by using restriction enzymes to subdivide large target DNAs into pieces of manageable size (4–5 kb). Each of these pieces is then sequenced independently by the random method.

The directed deletion approach sometimes requires a considerable investment of time to generate and characterize a complete set of nested deletions. However, once this step has been accomplished, informative DNA sequence is accumulated simultaneously from ordered regions of the target DNA. Typically, two sets of deletion mutants extending from opposite ends of the target fragment are required to determine the entire sequence of both strands of the DNA. Alternatively, a single set of deletion mutants can be used to obtain the sequence of a single strand of the target DNA. This information can then be used to synthesize a set of oligonucleotide primers that can be used to confirm the sequence of the opposite strand of DNA (see below).

Availability of an oligonucleotide synthesizer

Given unlimited access to an oligonucleotide synthesizer, custom-designed primers can be generated and synthesized rapidly and cheaply. Assuming that it takes 1–2 days to synthesize an oligonucleotide, virgin nucleotide sequence can be accumulated at a maximum rate of 600–800 nucleotides per week from a designated entry point to the target DNA. This rate of progress can be accelerated by the simultaneous use of several entry points, for example, by cloning the target fragment into bacteriophage M13mp18 and M13mp19 vectors and using a universal primer to begin sequencing from both termini simultaneously or by sequencing internal restriction fragments that have been subcloned into appropriate vectors.

When designing primers for DNA sequencing, observe the following rules:

1. Make sure that the oligonucleotide is complementary to the correct strand of the target DNA and to sequences that have been determined unambiguously to be present in the target DNA. This is particularly important when sequencing virgin DNA by the progressive oligonucleotide method (see Figure 13.3B).

   There is a natural tendency to design an oligonucleotide that is complementary to the furthest limits of known sequence. However, in most circumstances, this sequence has been obtained from closely spaced bands at the top of a sequencing gel, where reading errors frequently occur. It is therefore better to be conservative and to design the primer to be complementary to sequences that lie some distance behind the advancing front, within a region of the gel that can be read with confidence.

2. The primer should have a balanced base composition (40–55% G + C) and should be a least 18 nucleotides in length. If the %(G + C) lies outside these limits, design an oligonucleotide whose length is $18 + n/2$ nucleotides, where $n = 50 - \%(\mathrm{G} + \mathrm{C})$ for A/T-rich regions and $n = \%(\mathrm{G} + \mathrm{C}) - 50$ for G/C-rich regions.

3. Check the sequence of the proposed primer to ensure that

   a. It does not contain regions of dyad symmetry. Oligonucleotides that can self-hybridize to form hairpin or stem-loop structures are inefficient primers.

   b. It is not complementary either to the vector DNA or to regions of the target DNA that have already been sequenced. This greatly reduces the possibility that the oligonucleotide might prime DNA synthesis from more than one location on the template DNA. Most commercially available computer programs for the analysis of DNA have the ability to search sequences for regions that are complementary to synthetic oligonucleotides.

Accuracy of the sequence

When DNA sequencing is carried out carefully, the error rate is less than 0.1%. However, to achieve this high rate of accuracy, it is necessary to sequence both strands of the target DNA completely and to resolve all ambiguities and discrepancies. In this respect, random sequencing has an advantage, since the gradual accumulation of redundant primary sequences greatly improves the accuracy of the final assembled sequence. However, there may be regions of the target DNA that cannot be sequenced accurately by either the random method or directed methods. Resolving these difficult sequences often takes a surprisingly long time and sometimes requires the use of base analogs (to eliminate compressions) or Maxam-Gilbert sequencing.

Future direction of the project

Different sequencing strategies yield different types of material that can be used in later experiments. For example, nested sets of deletions generated for DNA sequencing can be used to study the domains within a promoter region or sets of oligonucleotides complementary to different regions of the target fragment can be used to sequence mutant forms of the target sequence. Random subclones created for shotgun sequencing provide a store of material that can subsequently be used for site-directed mutagenesis or for the generation of radiolabeled probes.

Random Sequencing

Three methods can be used to break large target DNAs into segments that are optimal in size for random sequencing: digestion with restriction enzymes, sonication, and digestion with pancreatic DNAase I in the presence of Mn^{++}. Sonication and digestion with DNAase I are superior to digestion with restriction enzymes in a number respects. First, they cleave DNA in a random fashion that is relatively independent of sequence, and therefore potential difficulties caused by uneven distribution of restriction sites are avoided. Second, both methods yield a collection of overlapping fragments—something that is impossible when DNA is digested to completion with a single restriction enzyme. In theory, sets of overlapping fragments can be obtained by partial digestion of the target DNA with a cocktail containing several frequently cutting restriction enzymes. In practice, this turns out to be difficult to achieve because different restriction enzymes cleave at vastly different rates. In contrast, random fragmentation follows a predictable time course once the system has been calibrated. The only major disadvantage of fragmentation either by sonication or by digestion with DNAase I in the presence of Mn^{++} is that the resulting small pieces of DNA carry frayed ends that must be repaired before cloning into bacteriophage M13 or phagemid vectors. This step is inefficient and, in the past, it was sometimes difficult to obtain enough clones to saturate the target DNA. This problem was largely solved when bacteriophage T4 DNA polymerase of high quality became available from commercial sources. Typically, fragmented DNA that has been repaired with this enzyme yields between 500 and 1000 subclones per microgram of starting DNA. This is far in excess of the number of subclones required to assemble a complete sequence of a target DNA less than 10 kb in length. The steps involved in fragmentation, repair, and cloning are outlined below and illustrated in Figure 13.4.

1. *Purification of the target DNA.* The target DNA is separated from vector sequences by digestion with restriction enzymes and polyacrylamide or agarose gel electrophoresis. If the target DNA and the vector are similar in size and cannot be separated by gel electrophoresis, try to find a restriction enzyme that will cleave the vector into several pieces without digesting the target DNA. This alleviates the problem of sequencing clones that contain only fragments of the vector. Alternatively, the collection of recombinant clones can be screened by hybridization to radiolabeled DNA of the parental vector. Only those clones that fail to hybridize (i.e., which carry inserts consisting entirely of target DNA) are selected for sequencing. The sequences of the terminal regions of the target DNA that formed junctions with the original vector can be obtained by subcloning appropriate restriction fragments into bacteriophage M13 or phagemid vectors.
2. *Ligation.* The purified target DNA is ligated to itself, generating a series of concatenated and/or circular molecules that are then subjected to

fragmentation. This ligation step is important because it ensures that all segments of the target DNA have an equal chance of being fragmented by sonication. Without ligation, monomeric linear target DNA will tend to be sheared preferentially near its center during sonication.

The ligation step is less important if the DNA is fragmented by digestion with DNAase I in the presence of Mn^{++}. However, it is still worthwhile because it helps to ensure that all segments of the target DNA will be equally represented in the final library. Without ligation, the ends of the target DNA will be cloned preferentially and will be overrepresented in the resulting library.

3. *Fragmentation.* When fragmenting DNA for the first time, either by sonication or by digestion with DNAase I in the presence of Mn^{++}, it is

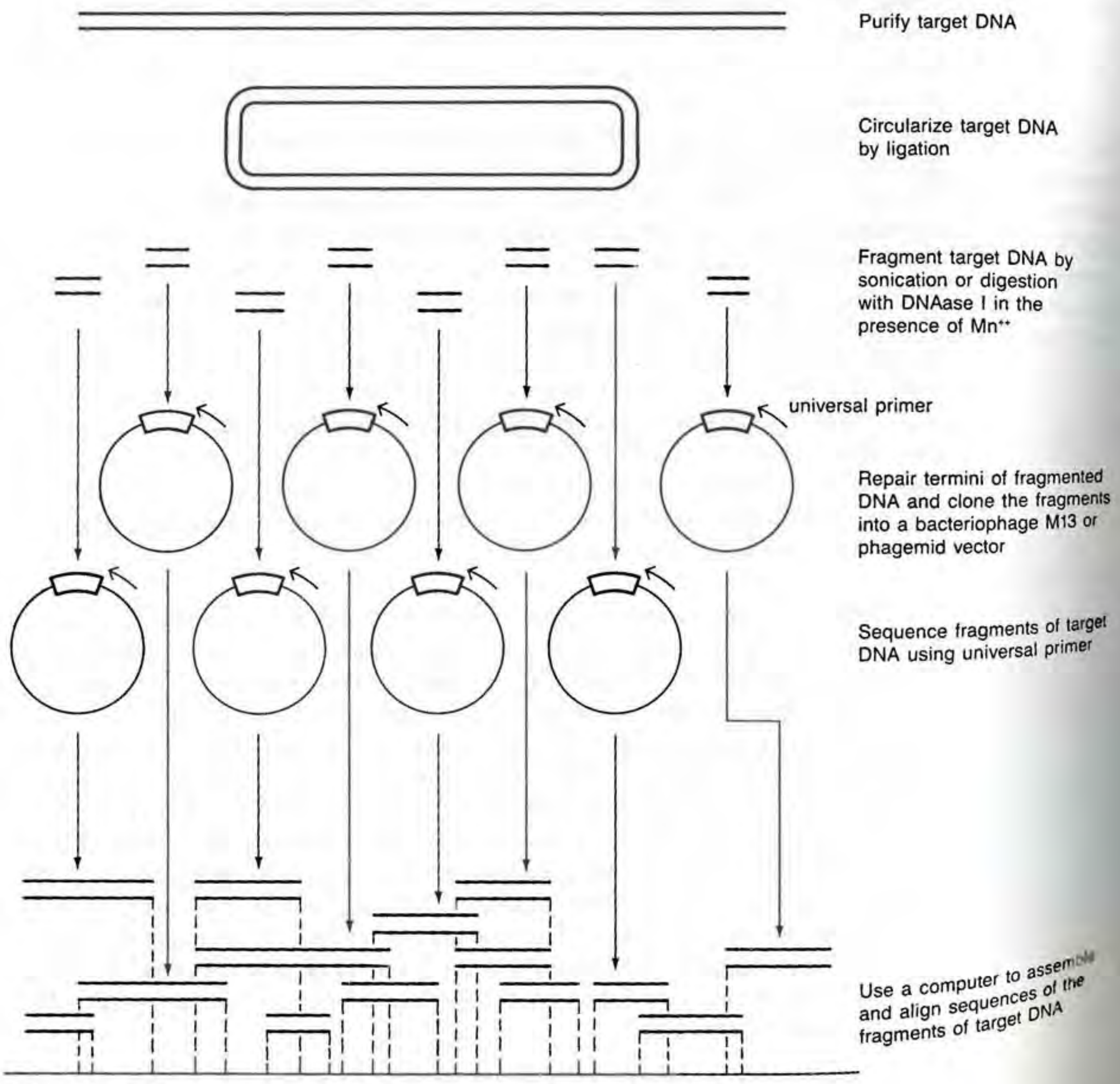

Purify target DNA
Circularize target DNA by ligation
Fragment target DNA by sonication or digestion with DNAase I in the presence of Mn⁺⁺
universal primer
Repair termini of fragmented DNA and clone the fragments into a bacteriophage M13 or phagemid vector
Sequence fragments of target DNA using universal primer
Use a computer to assemble and align sequences of the fragments of target DNA

Figure 13.4
Random sequencing.

essential to carry out a series of calibration experiments to establish conditions that yield acceptable quantities of fragments of the desired size. This is best done by performing a series of preliminary experiments with a control DNA of defined size, for example, bacteriophage λ DNA.

4. *Size selection, repair, and cloning.* DNA fragments of the desired size (500–800 bp) are isolated by gel electrophoresis and their termini are repaired with bacteriophage T4 DNA polymerase. The DNAs are then ligated to an appropriately prepared vector (e.g., bacteriophage M13mp18 or M13mp19 that has been linearized with a restriction enzyme that generates blunt ends). Finally, the ligated DNA is transfected into a suitable strain of *E. coli* (see Chapter 4), and single-stranded DNAs prepared from plaque-purified bacteriophages are used as templates in the Sanger dideoxy-mediated chain-termination method of sequencing.

GENERATION OF A LIBRARY OF RANDOMLY OVERLAPPING CLONES

Purification and Ligation of the Target DNA

1. Using the appropriate restriction enzymes, digest a sufficient amount of recombinant vector to yield 10–15 μg of purified target DNA. Separate the target DNA from the vector by preparative gel electrophoresis, and recover the target DNA from the gel by one of the methods described in Chapter 6. Redissolve the purified DNA in 15 μl of TE (pH 7.6). Check the integrity and recovery of the purified DNA by analyzing an aliquot (0.5 μl) by agarose gel electrophoresis.

   Because the next step of the procedure requires the target DNA to be ligated to itself, it is best to use restriction enzymes that generate compatible termini. If this is not possible, an acceptable alternative is to use restriction enzymes that generate incompatible protruding termini and then to alter the ligation conditions as described in the note to step 2. If it is not possible to avoid restriction enzymes that generate blunt ends, the ligation (step 2) should be carried out in the presence of substances that increase macromolecular crowding (e.g., polyethylene glycol [Pheiffer and Zimmerman 1983]; see Chapter 1, pages 1.70–1.71).

2. Transfer 5–10 μg of the purified DNA to a fresh microfuge tube. Add:

10 × bacteriophage T4 DNA ligase buffer	2.5 μl
5 mM rATP	2.5 μl
30% w/v polyethylene glycol (PEG 8000) (optional—see note to step 1)	5.0 μl
H_2O to 25 μl	
bacteriophage T4 DNA ligase	
for cohesive termini	1.0 Weiss unit
for blunt termini	5.0 Weiss units

   Incubate the mixture at 16°C for 4 hours, and then inactivate the ligase by heating the mixture to 68°C for 15 minutes.

10 × Bacteriophage T4 DNA ligase buffer

200 mM Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

If the termini of the purified target DNA are compatible, the products of ligation will be a series of oligomeric linear and circular DNA molecules in which adjacent segments of target DNA are arranged as either direct or inverted repeats. Up to 50% of the sonicated fragments carrying junction sequences will contain inverted repetitions and will be difficult to clone in bacteriophage M13 vectors. This problem becomes much worse if the termini of the purified target DNA are incompatible. In this case, the major products of ligation will be circular DNA molecules containing only inverted repetitions of the target DNA, and virtually all of the sonicated fragments containing junction sequences will carry inverted repetitions. To solve this problem, ligate only two thirds of the original target DNA and mix the products of ligation with the remaining one third of the unligated DNA before proceeding to the sonication step.

Several different assays have been used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

3. Add 175 μl of TE (pH 7.6), and purify the ligated DNA by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube. Add 50 μl of 10 M ammonium acetate and 2 volumes of ethanol at room temperature. Store the microfuge tube on ice for 10 minutes, and then collect the precipitate of DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Remove the supernatant, wash the pellet with 0.5 ml of 70% ethanol at room temperature, and recentrifuge. Remove as much as possible of the supernatant, and allow the last traces of ethanol to evaporate at room temperature. Redissolve the DNA in 25–50 μl of TE (pH 7.6) to a final concentration of 200 μg/ml.

4. Fragment the DNA either by sonication or by digestion with DNAase I in the presence of Mn^{++} (see pages 13.26–13.29).

ymentation of the Target DNA

As discussed above (see page 13.21), two methods—sonication and digestion with DNAase I in the presence of Mn^{++}—are commonly used to fragment the target DNA randomly into pieces whose sizes are convenient for sequencing in bacteriophage M13 or phagemid vectors. If a suitable sonicator is available, sonication is the method of choice. Once the sonicator is calibrated, fragmentation proceeds at a predictable rate that varies little from preparation to preparation of target DNA. On the other hand, the rate of fragmentation with DNAase I in the presence of Mn^{++} is more variable, often proceeds asynchronously, and is sensitive to trace contaminants in the DNA (e.g., sulfated polysaccharides from agarose gels).

NICATION

The following method is a modification of a procedure described by Bankier et al. (1987). In almost all cases, the segment of DNA that is to be sequenced will already have been cloned and propagated in a plasmid, cosmid, or bacteriophage λ vector. To avoid generating subclones that carry vector sequences, it is necessary to recover the target DNA from the parental clone.

1. Calibrate the sonication apparatus. The times of sonication given below are for a cup-horn sonicator with a nominal peak output energy of 475 watts. However, because the actual output of different sonicators varies widely, it is necessary to calibrate each instrument as follows:

   a. Transfer 25 μl of a solution containing 200 μg/ml of bacteriophage λ DNA (or some other large DNA of defined molecular weight) to a microfuge tube.

   b. Fill the cup horn of the sonicator with ice water, and clamp the microfuge tube containing the DNA just above the probe.

   c. Sonicate at maximum power for a total of four 30–40 second bursts. After each burst, transfer 3 μl of the DNA to a fresh microfuge tube. Store this tube on ice. Between bursts, centrifuge the original microfuge tube for 2 seconds to deposit the DNA solution at the bottom of the tube, and add fresh ice to the sonicator horn to maintain the temperature of the DNA solution at 0°C.

   e. After sonication is completed, analyze the size of the DNA fragments in each sample stored in step c by electrophoresis through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

   f. Stain and photograph the gel, and determine the amount of sonication required to produce a reasonable yield of fragments of the desired size (300 bp–1 kb).

By far the best type of apparatus is a cup-horn sonicator (e.g., W-385 Sonicator fr Heat Systems Ultrasonics) in which the DNA samples, dissolved in small volumes of buffer, are enclosed in microfuge tubes and do not come into contact with the probe.

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a cup-horn sonicator is not available, a conventional sonicator equipped with a microtip probe can be used. However, in this case, it is essential to remove contaminating DNA by cleaning the probe fastidiously before and after it is immersed in each sample. This is best done by boiling the sonicator probe in 1% SDS, 0.001 M EDTA for 10 minutes and then rinsing it thoroughly in sterile water. In addition, the volume of the DNA sample must be increased to at least 250 μl to accommodate the probe. After sonication is completed, the DNA is reconcentrated by precipitation with 2 volumes of ethanol and redissolved in 25 μl of TE (pH 7.6).

2. Sonicate the purified, ligated target DNA (from step 3, page 13.25) for exactly the same length of time calculated to produce a reasonable yield of fragments of the desired size (300 bp–1 kb).

DNA that has been sonicated for excessive periods of time is extremely difficult to clone. Most sonicators will not shear DNA to a size smaller than 300—500 bp, and it is tempting to continue sonication until the entire population of DNA fragments has been reduced to this size. However, the yield of subclones is usually greater if sonication is stopped when the fragments of target DNA first reach a size of 300 bp–1 kb.

3. Proceed with repair and size selection of DNA as described on page 13.30.

Pancreatic DNAase I normally introduces single-strand nicks into double-stranded DNA. In the presence of Mn^{++}, however, the enzyme cleaves both strands of DNA (Melgar and Goldthwait 1968) to generate molecules that are blunt-ended or have protruding termini only 1 or 2 nucleotides in length. Before the following protocol is used to fragment valuable target DNA, it should be tested with bacteriophage λ DNA or another large DNA of defined molecular weight.

1. Make up stock solution of DNAase I (1 mg/ml in 0.01 N HCl). Divide the enzyme solution into small aliquots (10 μl), and store them in small microfuge tubes at −70°C.

2. When you are ready to start the digestion, remove an aliquot of DNAase I from storage at −70°C and dilute it 1:1000 into ice-cold 1 × DNAase/Mn^{++} digestion buffer. The concentration of the diluted enzyme should be 1 ng/μl.

10 × DNAase/Mn^{++} digestion buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MnCl_2$
1 mg/ml bovine serum albumin (Fraction V; Sigma) (optional)

3. Immediately prepare the following reaction mixture:

target DNA (200 μg/ml)	25 μl
H_2O	65 μl
10 × DNAase/Mn^{++} digestion buffer	10 μl

Transfer 10 μl of the reaction mixture to a fresh microfuge tube containing 5 μl of 50 mM EDTA (pH 8.0). Store the tube on ice.

To the remainder of the reaction mixture, add 1.5 μl of diluted DNAase I (1 ng/μl) prepared in step 2. Incubate this reaction at 15°C.

4. After 1, 2, 5, 10, and 30 minutes of incubation, transfer 15 μl of the reaction to a fresh microfuge tube containing 5 μl of 50 mM EDTA (pH 8.0). Store the tubes on ice until all of the samples have been collected.

5. Analyze the size of the DNA fragments in each sample by electrophoresis of an aliquot (2 μl) through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

6. Stain and photograph the gel, and pool the samples that contain a reasonable amount of fragments of the desired size (300 bp–1 kb).

7. Purify the DNA in the pooled sample by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 5

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volumes of H_2O (to reduce the concentration of Mn^{++} and EDTA). Add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2 volumes of ethanol at room temperature. Store the microfuge tube on ice for 10 minutes, and then collect the precipitate of DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the supernatant, wash the pellet with 0.5 ml of 70% ethanol at room temperature, and recentrifuge. Remove as much as possible of the supernatant, and allow the last traces of ethanol to evaporate at room temperature. Redissolve the DNA in 25 μl of TE (pH 7.6).

8. Verify the size of the target DNA by analyzing an aliquot (2 μl) by electrophoresis through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

9. Proceed with repair and size selection of DNA as described on page 13.30.

pair and Size Selection of DNA

1. To DNA (23 μl) that has been fragmented by sonication or by digestion with DNAase I in the presence of Mn^{++}, add:

a solution containing all four dNTPs, each at a concentration of 0.5 mM	3.0 μl
50 mM $MgCl_2$	3.0 μl
bacteriophage T4 DNA polymerase (10 units)	1.5 μl

   Incubate the reaction for 15 minutes at room temperature, and then add 1 μl (~ 5 units) of the Klenow fragment of *E. coli* DNA polymerase I. Continue incubation for a further 15 minutes at room temperature.

   The termini produced by sonication are extremely heterogeneous, consisting of both blunt-ended and frayed molecules with and without phosphate residues. Only a fraction of these molecules can be repaired by DNA polymerases, and the efficiency with which sonicated DNA can be cloned in bacteriophage M13 or phagemid vectors is therefore very low. Usually, however, 5–10 μg of sonicated, repaired, and size-selected target DNA yields several thousand recombinant clones. If insufficient clones are obtained, the problem is almost always due to the inability of DNA polymerases to repair the termini of the sonicated DNA. In this case, we recommend that a fresh batch of sonicated DNA be prepared and treated with the following enzymes in turn (1) calf intestinal alkaline phosphatase, (2) mung-bean nuclease, (3) bacteriophage T4 DNA polymerase in the presence of all four dNTPs, and finally (4) bacteriophage T4 polynucleotide kinase. The DNA should be purified by extraction with phenol and precipitation with ethanol after the addition of enzymes 1, 2, and 3.

2. Purify fragments of DNA of the desired size (300–600 bp) by electrophoresis through a 1.2% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

   To minimize the possibility of contamination, several lanes should be left empty between the fragmented target DNA and the marker and a fresh pipette tip should be used to load each lane of the gel.

3. Recover the target DNA from the gel by one of the elution methods described in Chapter 6. Redissolve the purified DNA in 10 μl of TE (pH 7.6). Check the integrity and recovery of the purified DNA by analyzing an aliquot (0.5 μl) by gel electrophoresis.

4. Ligate the target to the vector as described on page 13.33.

Preparation

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Preparation of Vector DNA

1. In a total volume of 100 μl, digest 10 μg of vector DNA (phagemid or bacteriophage M13 replicative form DNA) with an enzyme that cleaves once within the polycloning site and generates blunt termini (e.g., digest M13mp18 and/or M13mp19 with *Sma*I). Analyze an aliquot of the digest in parallel with a sample of undigested DNA by electrophoresis to be sure that no circular forms of the DNA remain. Continue incubating the remainder of the digestion mixture at the appropriate temperature while the gel is running.

2. When digestion with the enzyme(s) is complete, add 10 μl of 100 mM Tris · Cl (pH 8.3), 10 mM $ZnCl_2$. Add 5 units of calf intestinal alkaline phosphatase (CIP), and incubate the reaction for 15 minutes at 37°C.

3. Add 2 μl of 0.5 M EDTA (pH 8.0) and 5 μl of 10% SDS. Add proteinase K to a final concentration of 50 μg/ml. Incubate for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method is to heat the reaction (at the end of step 2) to 65°C for 1 hour (or 75°C for 10 minutes) and then to extract once with phenol:chloroform.

4. Extract the reaction mixture once with phenol and once with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 2 μl of 5 M NaCl. Add 2 volumes of ethanol at 0°C, and store the tube on ice for 10 minutes.

   NaCl is used instead of sodium acetate (pH 5.2) to prevent precipitation of EDTA.

5. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Remove the supernatant by aspiration, and add 1 ml of 70% ethanol at room temperature to the tube. Recentrifuge for a further 5 minutes. Carefully discard the supernatant, and store the open tube on the bench until the last traces of ethanol have evaporated. Redissolve the DNA in 200 μl of TE (pH 7.6). Check the recovery and integrity of the vector DNA by analyzing an aliquot (2 μl) by electrophoresis through a 0.8% agarose gel.

6. Carry out a series of test ligations to determine the effectiveness of the phosphatase treatment. Set up five reactions as follows:

	Type of DNA[a]					
Reaction	**A**	**B**	**C**	**H_2O (μl)**	**10× Ligation buffer (μl)**	**Bacteriophage T4 DNA ligase (μl)**
1	+			6.5	1.0	0.5
2	+			7.0	1.0	
3		+		6.5	1.0	0.5
4		+		7.0	1.0	
5			+	5.0	1.0	0.5

[a] A = 100 ng of linearized, nonphosphatase-treated vector (in a volume of 2 μl); B = 100 ng of linearized, phosphatase-treated vector (in a volume of 2 μl); and C = 100 ng of bacteriophage λ DNA cleaved to completion with *Alu*I (in a volume of 2 μl).

Incubate the ligation reactions for 12–16 hours at 16°C.

10 × Ligation buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

The efficiency of ligation can be increased by adding polyethylene glycol (PEG 8000; 30% w/v) to a concentration of 5% in the final ligation mixture (Pheiffer and Zimmerman 1983) (see Chapter 1, pages 1.70–1.71).

7. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors; see Chapter 4 for test ligations) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The following day, count the numbers of blue and white colonies (or colorless plaques).

   Dephosphorylation should reduce the number of plaques or colonies by a factor of 50–100 (reaction 3 vs. reaction 1). Ligation of dephosphorylated vector to a standard preparation of blunt-ended fragments should result in a 10- to 50-fold increase in colorless plaques or white colonies.

8. If the results of the test ligations are satisfactory, dispense the dephosphorylated DNA into aliquots and store them at −20°C.

Ligation to Vector DNA

1. Using the results of the test ligations (see pages 13.31–13.32) as a guide, set up a second series of test ligations containing 100 ng of dephosphorylated vector DNA and increasing concentrations of fragmented, blunt-ended target DNA.

2. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors; see Chapter 4 for test ligations) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing IPTG or X-gal. The following day, count the numbers of blue and white colonies (or colorless plaques).

   The number of recombinants obtained with fragmented, blunt-ended target DNA will be approximately tenfold lower than the number obtained with a standard preparation of blunt-ended DNA.

   The aim of this test ligation is to find a concentration of fragmented target DNA that minimizes the number of recombinants containing artificially fused target fragments, which can greatly complicate random sequencing. It is therefore essential not to use saturating quantities of target DNA. Instead, determine the amount of target DNA that will produce a modest increase (approximately fivefold) in the number of recombinant clones over background.

3. When the results of the second series of test ligations are available, set up a large-scale ligation that will yield sufficient recombinant clones to complete the sequencing project.

4. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing IPTG and X-gal. The following day, count the numbers of blue and white colonies (or colorless plaques).

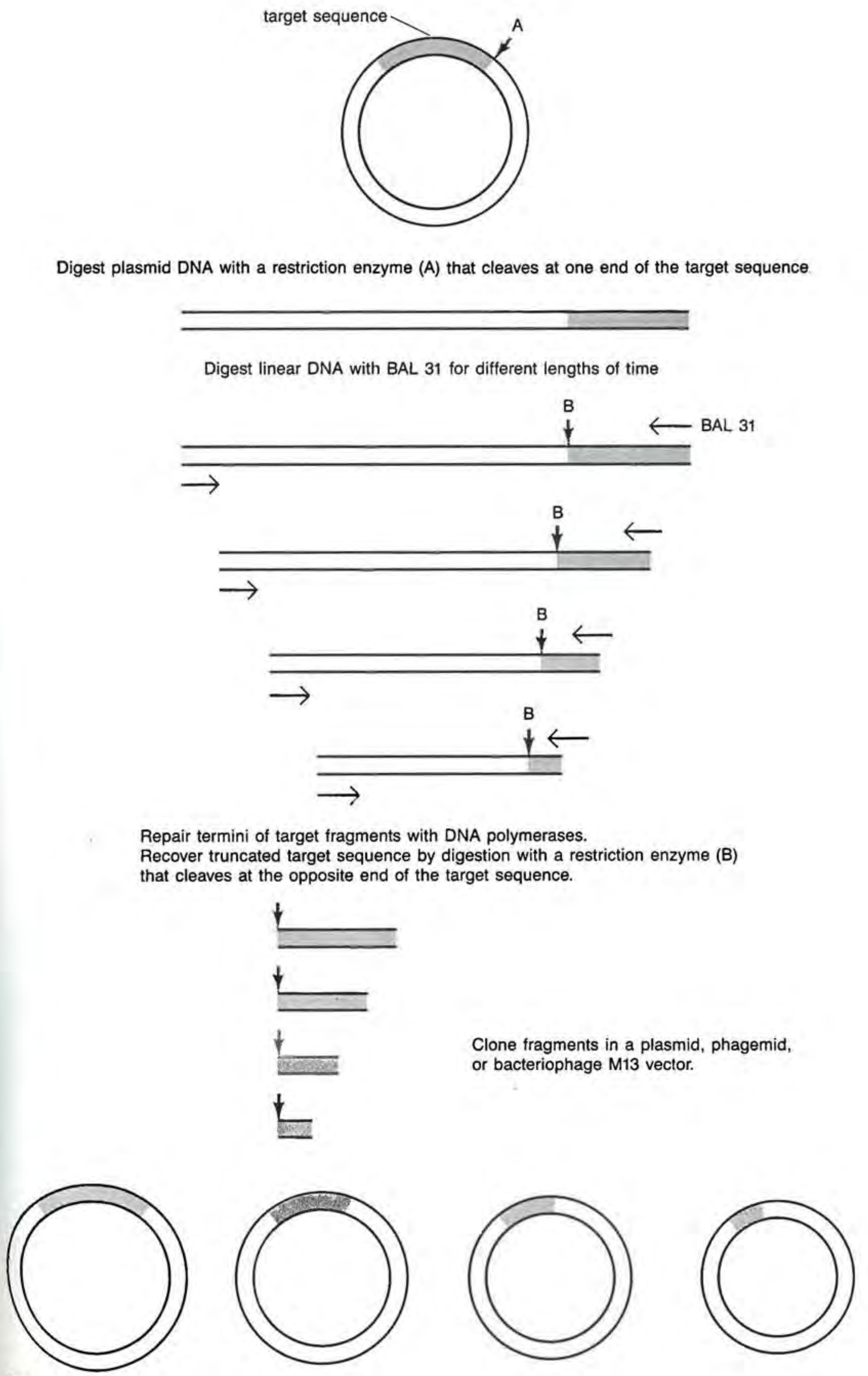
   Bacterial colonies carrying phagemids can be stored at 4°C for a few weeks on the original agar plates. However, recombinant bacteriophage M13 plaques should be picked as soon as possible, since the bacteriophage particles can diffuse considerable distances through top agar. Plaques grown for extended periods of time (>12–16 hours) at 37°C or stored for a few days at 4°C frequently become contaminated. There is an increase in both the intensity and number of background bands when single-stranded DNAs prepared from old plaques are used in sequencing reactions. To minimize the possibility of cross contamination, it is important to pick plaques that are well-separated from their neighbors.

irected Sequencing

ENERATION OF NESTED SETS OF DELETION MUTANTS

Several methods have been developed to generate nested sets of deletion mutants that lack progressively more nucleotides from one end or the other of the target DNA. These methods all rely on the following nucleases that digest DNA in a predictable fashion:

- *BAL 31*. This enzyme carries both an exonucleolytic activity that progressively degrades double-stranded linear DNA by liberating mononucleotides from the 3′ termini and a weaker single-strand-specific endonucleolytic activity. Thus, the products of digestion of double-stranded DNA with BAL 31 are truncated molecules carrying blunt ends or short, protruding 5′ termini. To generate deletion mutants, a recombinant plasmid (or bacteriophage M13 replicative form DNA) is linearized with a restriction enzyme that cleaves at one end of the target sequence. The double-stranded linear DNA is digested with BAL 31 for different lengths of time and the truncated fragments of target DNA are then cleaved from the remainder of the vector with a second restriction enzyme and recloned (see Figure 13.5).
- *Pancreatic DNAase I*. In the presence of Mg^{++}, DNAase I behaves like an endonuclease that introduces nicks independently into each strand of double-stranded DNA. However, in the presence of a transition metal ion, such as Mn^{++} or Co^{++}, the enzyme cuts both strands of DNA at approximately the same place (Melgar and Goldthwait 1968). This property can be used to create deletions in a segment of target DNA that has been cloned in a plasmid or bacteriophage M13 vector (Frischauf et al. 1980; Hong 1982, 1987; Labeit et al. 1987). Because closed circular DNA is cleaved more efficiently than linear molecules and because the sites of cleavage are distributed in a statistically random fashion, the population of closed circular DNAs is converted by DNAase I into a permuted set of linear molecules. These are then digested with a restriction enzyme whose unique site of cleavage lies at one end of the target DNA. Recircularization of the resulting population generates clones that lack sequences lying between the site of DNAase I cleavage and the restriction site (see Figure 13.6).
- *Exonuclease III*. This enzyme catalyzes the stepwise removal of 5′ mononucleotides from the recessed or blunt 3′-hydroxyl termini of double-stranded DNA (Weiss 1976). However, protruding 3′ termini are completely resistant to the activity of the enzyme (Rogers and Weiss 1980). This property of exonuclease III has been used to develop a protocol that allows construction of unidirectional deletions without subcloning the target DNA. To create unidirectional deletion mutants (Henikoff 1984, 1987), the double-stranded DNA of a recombinant plasmid or bacteriophage M13 replicative form DNA is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the primer-binding site. The enzyme that cleaves nearer the target sequences must generate a blunt



Digest plasmid DNA with a restriction enzyme (A) that cleaves at one end of the target sequence

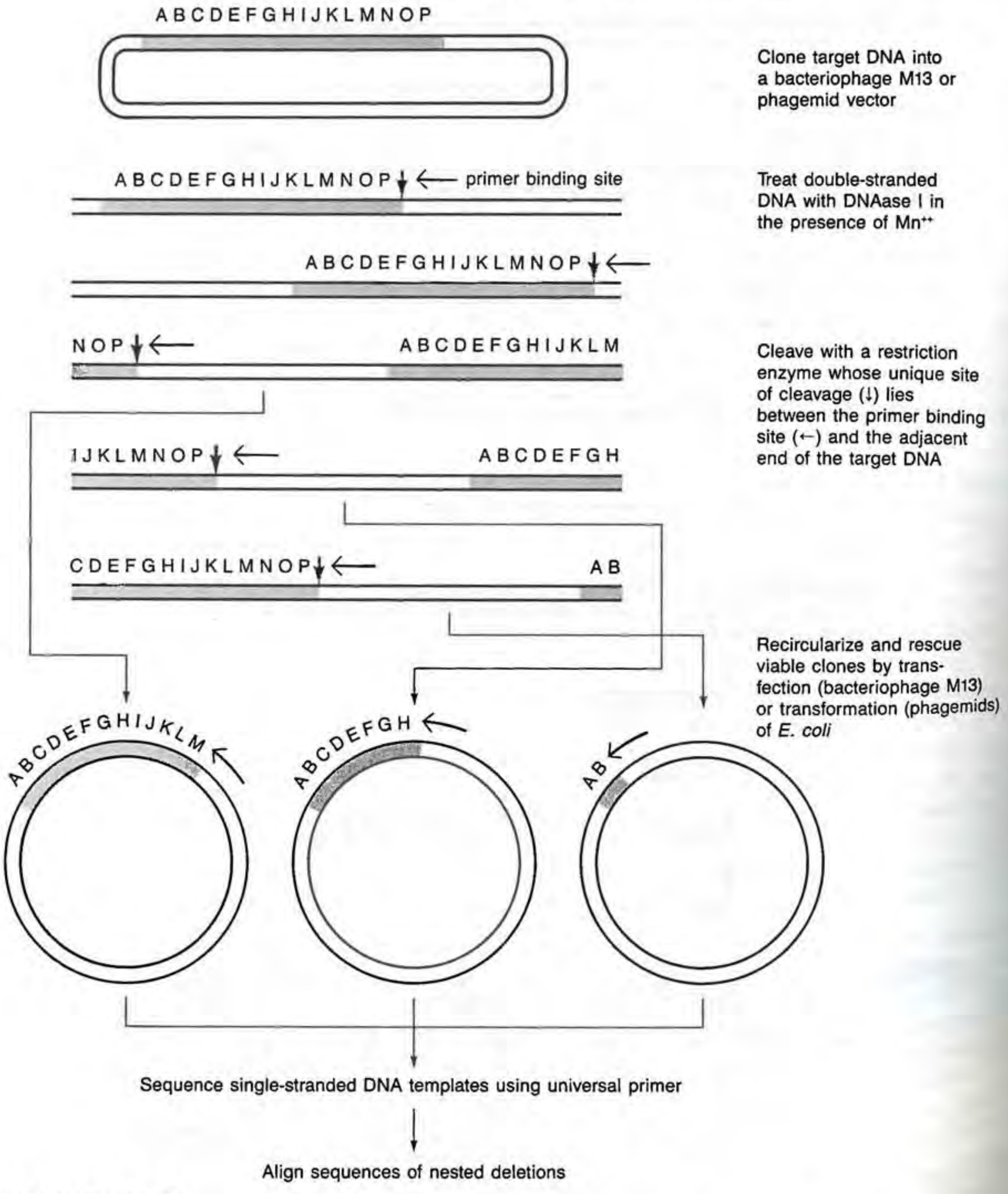
Digest linear DNA with BAL 31 for different lengths of time

Repair termini of target fragments with DNA polymerases.
Recover truncated target sequence by digestion with a restriction enzyme (B)
that cleaves at the opposite end of the target sequence.

Clone fragments in a plasmid, phagemid,
or bacteriophage M13 vector.

FIGURE 13.5
Generation of nested deletion mutants with BAL 31.

The enzyme that cleaves nearer the target sequences must generate a blunt end or a recessed 3′ terminus; the other enzyme must generate a 3- or 4-nucleotide protruding 3′ terminus. Because only one end of the resulting linear DNA is susceptible to exonuclease III, digestion proceeds unidirectionally away from the site of cleavage into the target DNA sequences (see Figure 13.7). The exposed single strands are removed by digestion with nuclease S1, and the DNA is then recircularized. If desired, a synthetic linker can be inserted at the site of recircularization.


A B C D E F G H I J K L M N O P
A B C D E F G H I J K L M N O P
primer binding site
A B C D E F G H I J K L M N O P
N O P
A B C D E F G H I J K L M
I J K L M N O P
A B C D E F G H
C D E F G H I J K L M N O P
A B
A B C D E F G H I J K L M
A B C D E F G H
A B

Clone target DNA into a bacteriophage M13 or phagemid vector

Treat double-stranded DNA with DNAase I in the presence of Mn⁺⁺

Cleave with a restriction enzyme whose unique site of cleavage (↓) lies between the primer binding site (←) and the adjacent end of the target DNA

Recircularize and rescue viable clones by transfection (bacteriophage M13) or transformation (phagemids) of *E. coli*

Sequence single-stranded DNA templates using universal primer

Align sequences of nested deletions

FIGURE 13.6
Generation of nested deletion mutants with DNAase I in the presence of Mn^{++}.

FIGURE 13.7
Generation of n

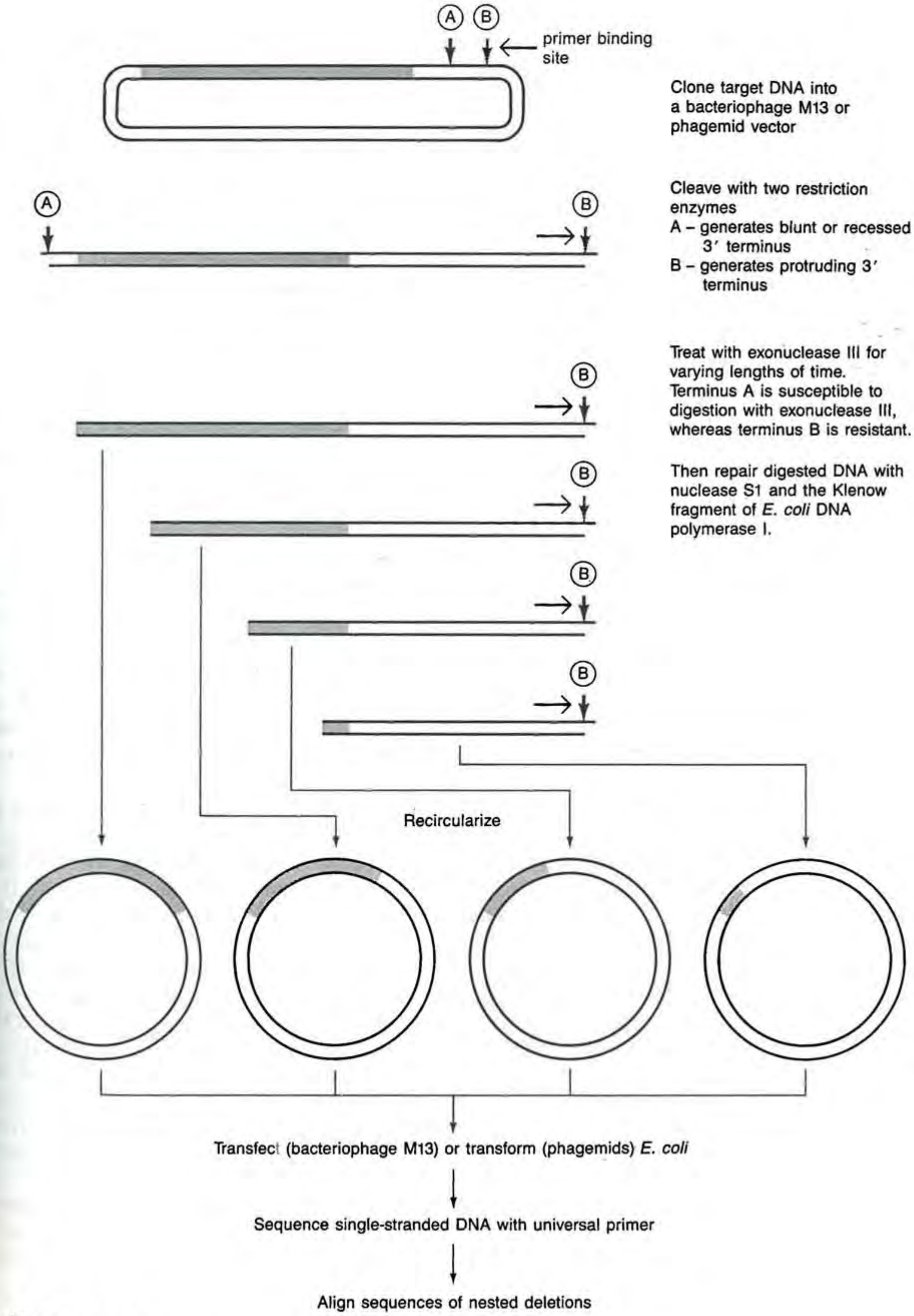

A
B
primer binding site
Clone target DNA into a bacteriophage M13 or phagemid vector
A
B
Cleave with two restriction enzymes
A – generates blunt or recessed 3′ terminus
B – generates protruding 3′ terminus
B
Treat with exonuclease III for varying lengths of time. Terminus A is susceptible to digestion with exonuclease III, whereas terminus B is resistant.
B
Then repair digested DNA with nuclease S1 and the Klenow fragment of E. coli DNA polymerase I.
B
B
Recircularize
Transfect (bacteriophage M13) or transform (phagemids) E. coli
Sequence single-stranded DNA with universal primer
Align sequences of nested deletions

FIGURE 13.7
Generation of nested deletion mutants with exonuclease III.

Of these three methods, digestion with exonuclease III is by far the best for several reasons. (1) The exonucleolytic reaction proceeds at a remarkably uniform and predictable rate. This means that it is possible to isolate not only nested deletions, but also groups of deletions whose endpoints are tightly clustered. (2) The method is not restricted to any particular vector. (3) The entire series of enzymatic reactions (exonuclease digestion, treatment with nuclease S1, and recircularization) can be carried out in a single set of tubes without purification of intermediate products (Henikoff 1987).

In contrast, making deletions with the other two enzymes is a more difficult proposition. First, BAL 31 is processive and tends to digest the population of double-stranded DNA in an asynchronous fashion. The resulting deletions are therefore far more heterogeneous in size than those created by exonuclease III. In addition, because BAL 31 degrades both ends of double-stranded DNA simultaneously, both the target fragment and the flanking vector sequences are degraded. To obtain viable deletion mutants, it is therefore almost always necessary to purify the truncated target fragments by gel electrophoresis and to reclone them into an appropriate vector. These steps are time-consuming, and the recovery of fragments from the gel is frequently poor.

Conversion of closed circular DNA to full-length linear molecules by digestion with DNAase I in the presence of Mn^{++} is an inefficient reaction. Although the enzyme works more efficiently on closed circular templates (which are under torsional strain), it also degrades linear DNAs at an appreciable rate. It is therefore not possible to convert the closed circular molecules quantitatively to full-length linear molecules: A fraction of the linear molecules undergo secondary cleavages, and a proportion of the closed circular molecules are never converted. The fraction of clones that contain usable mutants may therefore be quite low.

The only limitation of the exonuclease III method is the requirement for appropriate restriction sites at one end of the target DNA. However, in practice, this limitation is not severe. The range of cloning sites in bacteriophage M13, phagemid, and plasmid vectors is now so large that it is almost always possible to find a vector that is suitable for the particular target fragment under study. If necessary, recessed 3′ termini can be protected from attack by filling with the Klenow fragment of *E. coli* DNA polymerase I and α-thiophosphate dNTPs (Guo and Wu 1983; Jasin et al. 1983). Alternatively, a large "buffer" fragment can be inserted between the site of cleavage and the flanking vector sequences (Haltiner et al. 1985). As long as the buffer fragment is long enough, the progressive removal of the target DNA is unlikely to be accompanied by loss of essential vector sequences. However, to facilitate the separation of the target fragment from the buffer fragment, it is advisable to insert a linker at the site of recircularization.

Detailed protocols for the generation of unidirectional deletion mutants with BAL 31 and for the linearization of closed circular DNA with DNAase I in the presence of Mn^{++} are given in Chapter 15. Here we present a protocol for the generation of nested deletion mutants with exonuclease III.

eneration of Nested Sets of Deletions with Exonuclease III

1. Digest 10 μg of recombinant bacteriophage M13 replicative form DNA or recombinant phagemid DNA with two restriction enzymes that cleave in the polycloning site between the primer-binding site of the vector and the target DNA. The enzyme that cleaves nearer the target sequences must generate a blunt end or a recessed 3′ terminus; the other enzyme must generate a 3- or 4-nucleotide protruding 3′ terminus. Neither enzyme should cleave elsewhere in the recombinant DNA.

Because exonuclease III will initiate digestion from single-strand nicks, it is important to begin with DNA that contains a high proportion of closed circular molecules. Before starting digestion, check an aliquot of the DNA by electrophoresis through an agarose gel. If the preparation contains more than 10% relaxed circular molecules, it should be purified by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, pages 1.42–1.45). This step has the added advantage of removing small pieces of DNA and RNA from the closed circular DNA preparation. These can interfere with digestion by exonuclease III.

To maximize the efficiency of cleavage, avoid using restriction enzymes whose recognition sites are immediately adjacent to each other in a polycloning site.

Digest first with the restriction enzyme that generates the blunt end or recessed 3′ terminus. When all of the closed circular DNA has been converted to linear DNA, as confirmed by gel electrophoresis, adjust the buffer and add the second enzyme. Any DNA that escapes cleavage by the second enzyme will be digested in both directions by exonuclease III and will therefore be unlikely to generate viable clones. To adjust the restriction buffer, it may be necessary to purify the DNA by extraction with phenol:chloroform and precipitation with ethanol or by spun-column chromatography (see Appendix E).

The efficiency of cleavage by the second restriction enzyme can be checked by end-labeling as discussed in Chapter 1, page 1.56.

2. Purify the DNA by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2 volumes of ethanol, and store the tube on ice for 10 minutes. Collect the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Carefully remove the supernatant, and add 0.5 ml of 70% ethanol to the pellet. Recentrifuge for 2 minutes at 4°C, and then carefully remove the supernatant. Store the open tube on the bench until the last traces of ethanol have evaporated, and then dissolve the DNA in 60 μl of 1 × exonuclease III buffer. Store the dissolved DNA on ice.

10 × Exonuclease III buffer

0.66 M Tris · Cl (pH 8.0)
66 mM $MgCl_2$

3. Place 7.5 μl of S1 reaction mixture (see page 13.40) in each of 25 0.5-ml microfuge tubes or in 25 wells in a 96-well microtiter plate with U-shaped wells (e.g., Baxter B1190-17). Store the microtiter plate or microfuge tubes on a bed of ice.

S1 reaction mixture contains:

H_2O	172 μl
10 × S1 buffer	27 μl
nuclease S1	60 units

10 × S1 buffer

5 M NaCl	5.0 ml
3 M potassium acetate (pH 4.5)	1.1 ml
glycerol	5.0 ml
1 M $ZnSO_4$	20.0 μl

4. Incubate the DNA solution prepared in step 2 for 5 minutes at 37°C. Transfer 2.5 μl of the solution to the first microfuge tube or well of the microtiter plate containing the S1 reaction mixture. To the remainder of the DNA solution, add 150 units of exonuclease III per picomole of recessed 3′ termini. Vortex briefly to mix the contents of the tube, and immediately return the tube to the 37°C water bath.

5. At 30-second intervals, remove 2.5-μl samples of the DNA solution and place in successive microfuge tubes or wells of the microtiter plate containing the S1 reaction mixture.

Under these conditions, the amount of exonuclease III is saturating, and approximately 200 nucleotides per minute are removed from the blunt end or recessed 3′ terminus of each of the DNA molecules in the solution. More or less DNA can be removed by varying the intervals between successive samples.

6. After all of the samples have been taken, incubate the microfuge tubes or microtiter plate for 30 minutes at 30°C. Add 1 μl of S1 stop mixture to each of the microfuge tubes or wells, and incubate for 10 minutes at 70°C. This inactivates nuclease S1 and any residual exonuclease III.

S1 stop mixture

0.3 M Tris base
50 mM EDTA (pH 8.0)

7. Transfer the microfuge tubes or microtiter plate to a bed of ice. Analyze aliquots of each of the samples by agarose gel electrophoresis. Choose a concentration of agarose that will allow maximum discrimination between the original DNA and fragments that are smaller in size by up to 2 kb.

8. Pool the samples containing DNA fragments of the desired size. Add 1 μl of Klenow mixture for each 10 μl of pooled sample. Incubate the pooled sample for 5 minutes at 37°C.

Klenow mixture (sufficient for 30 samples) contains:

H_2O	20 μl
1 M $MgCl_2$	6 μl
0.1 M Tris·Cl (pH 7.6)	3 μl
Klenow fragment of *E. coli* DNA polymerase I	3 units

During this brief incubation in the absence of dNTPs, the 3′→5′ exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase I removes any remaining protruding 3′ termini from the digested DNA.

9. For each 10 μl of pooled sample, add 1 μl of a solution containing the four dNTPs, each at a concentration of 0.5 mM. Continue incubation for a further 15 minutes at room temperature.

10. Add 40 μl of ligase mixture for each 10 μl of pooled sample. Mix, and continue incubation at room temperature for a further 2 hours.

Ligase mixture (sufficient for 24 samples) contains:

H_2O	550 μl
10× ligation buffer	100 μl
5 mM rATP	100 μl
30% w/v polyethylene glycol (PEG 8000)	250 μl
bacteriophage T4 DNA ligase	5 Weiss units

10× Ligation buffer

500 mM Tris·Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

For definition of Weiss unit, see page 13.25.

11. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors) competent *E. coli* of the appropriate strain (see Chapter 4). Using 5–10 μl of the pooled sample, prepare minipreparations of bacteriophage M13 replicative form DNA or phagemid DNA from at least 18 randomly selected plaques or colonies (see Chapter 1, pages 1.25–1.31). Linearize the DNAs by digestion with an appropriate restriction enzyme and analyze their sizes by electrophoresis on a 1% agarose gel. Use as marker the original plasmid DNA that has been linearized by restriction enzyme digestion. Choose clones of an appropriate size for sequencing.

Approximately 80–90% of the deletions retain the primer-binding site and can be sequenced using a universal primer.

It is best to select clones for sequencing that differ in size by approximately 200 bp. Such clones are likely to form an overlapping set.

Sequencing by the Sanger Dideoxy-mediated Chain-termination Method

SETTING UP DIDEOXY-MEDIATED SEQUENCING REACTIONS

Preparation of Single-stranded DNA

Small-scale preparations of single-stranded bacteriophage M13 or phagemid DNAs should be isolated from bacteriophage particles synthesized by individual plaques or from bacterial colonies as described in Chapter 4. The yields of DNA are normally sufficient for 5–10 sets of sequencing reactions. Before setting up sequencing reactions, it is advisable to check the amount of single-stranded template in each preparation of single-stranded bacteriophage M13 or phagemid DNA. This can be done by analyzing an aliquot of each preparation of DNA by electrophoresis through a 1% agarose gel, using as marker a standard preparation of bacteriophage M13 DNA. Although single-stranded bacteriophage M13 DNA runs on an agarose gel as a broad fuzzy band that stains poorly with ethidium bromide, it is nevertheless quite easy to estimate the relative quantities of DNA in different preparations.

Preparation of Primers

Many companies supply universal primers as lyophilized powders, which should be dissolved at a concentration of 2 μg/ml in 10 mM Tris · Cl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA (pH 8.0). Some companies supply universal primers already in solution. Solutions of primers should be stored at −20°C. (*Note*: 1 μg of universal primer [19-mer] is approximately equal to 160 pmoles.)

Primers of several different lengths (from 15 to 26 nucleotides) are available from a number of different commercial suppliers. There is no detectable difference in the quality of the sequence obtained from recombinant bacteriophage M13 templates with primers of different lengths. However, when denatured double-stranded DNA templates such as bacteriophage λ or plasmids are used, longer primers (from 25 to 29 nucleotides) give rise to fewer artifactual bands.

Primers synthesized in the laboratory generally need not be purified by gel electrophoresis before use. The amount of contamination by "off-size" oligonucleotides is usually too small (< 10%) to affect the quality of the DNA sequence. However, it is often necessary to experiment with laboratory-synthesized primers to determine the optimum ratio of primer to template in the sequencing reaction. Laboratory-synthesized primers should be dissolved at a concentration of 2 μg/ml as described above and stored at −20°C.

Microtiter Plates

Dideoxy-mediated sequencing reactions using the Klenow fragment of *E. coli* DNA polymerase I or reverse transcriptase are carried out in two stages: an *annealing reaction*, in which the oligonucleotide primer is hybridized to the template DNA, and four *chain-extension/chain-termination reactions*, in which the annealed oligonucleotide is extended and the newly synthesized chains of DNA are terminated by incorporation of ddATP, ddGTP, ddCTP, or ddTTP. In sequencing reactions using Sequenases, a preliminary step—an *incorporation reaction*—is included to ensure efficient incorporation of radioactive precursor in the newly synthesized DNA.

Until a few years ago, chain-extension/chain-termination reactions were carried out in conventional microfuge tubes—a procedure that works well when only one or two sets of reactions are being processed but is extremely tedious and inefficient when larger numbers of templates are sequenced. This problem can be solved by carrying out the chain-extension/chain-termination reactions in disposable microtiter plates with 96 U-shaped wells (e.g., Baxter B1190-17). The reagents are deposited as separate droplets on the side of each well, toward the rim, and the reactions are initiated simultaneously by briefly centrifuging the plates in a low-speed centrifuge equipped with microtiter plate buckets. This sediments the droplets on the bottom of the well and mixes the components of the reaction. Microtiter plates have the following advantages: (1) Up to 24 sets of chain-extension/chain-termination reactions can be processed simultaneously, (2) reagents can be added using repeating dispenser pipettes, and (3) many sets of sequencing reactions can be carried out synchronously.

Some laboratories carry out all stages of sequencing reactions—from annealing to chain termination—in microtiter plates. In our experience, this is not advisable. During annealing, when small volumes of solutions are heated to 55°C, evaporation from the wells of a microtiter plate can be a problem. We therefore recommend that annealing of primer to template be carried out in closed microfuge tubes and that the subsequent enzymatic reaction be set up in microtiter wells.

Chain-extension/Chain-termination Reaction Mixtures

The competition between chain elongation and termination is determined by the ratio of dNTP to ddNTP in each of the four sequencing reactions. Each of the DNA polymerases used for dideoxy-mediated sequencing has a different affinity for both conventional dNTPs and chain-terminating ddNTPs. Thus, reaction mixtures that give good results with one enzyme are unlikely to work well with another. It is almost always necessary to "fine-tune" the concentration of ddNTPs to obtain optimal results for the particular task at hand. When sequencing for the first time with a particular enzyme (either Sequenases or the Klenow fragment of *E. coli* DNA polymerase I), we recommend purchasing a kit from a commercial manufacturer that contains the relevant DNA polymerase, pretested chain-extension/chain-termination mixtures, single-stranded templates, and oligonucleotide primers. As components of the kit are used up, we recommend that they be replaced with homemade materials, which are less expensive and can be made in a wide range of concentrations. Each new batch of reagents should be checked by setting up a series of test reactions with a standard template and primer and a range of concentrations of dNTPs and ddNTPs. Initially, it is best to use fixed concentrations of dNTPs and variable concentrations of ddNTPs. Often, several series of test reactions are necessary to define optimal conditions.

STOCK SOLUTIONS OF dNTPs AND ddNTPs

The following stock solutions are used to prepare the working solutions of dNTPs and ddNTPs (see page 13.60) for sequencing reactions using either the Klenow fragment of *E. coli* DNA polymerase I or Sequenase.

Stock solutions of dNTPs contain a single dNTP (sodium salt) at a concentration of 0.5 mM in TE (pH 8.0):

0.5 mM dATP	302 μg/ml
0.5 mM dCTP	284 μg/ml
0.5 mM dGTP	304 μg/ml (*or* 0.5 mM dITP 288 μg/ml)
0.5 mM dTTP	291 μg/ml

Stock solutions of ddNTPs contain a single ddNTP at a concentration of 10 mM in TE (pH 8.0):

10 mM ddATP	5.7 mg/ml
10 mM ddCTP	5.4 mg/ml
10 mM ddGTP	5.7 mg/ml
10 mM ddTTP	5.5 mg/ml

DENATURING POLYACRYLAMIDE GELS

Both the amount and the accuracy of the nucleotide sequence are determined by the quality of the polyacrylamide gels used to display the radiolabeled DNA fragments generated by chemical and enzymatic sequencing reactions. Under optimal conditions, between 300 and 400 nucleotides of reliable sequence can be obtained from a denaturing polyacrylamide gel. However, by electrophoresing aliquots of the sequencing reaction for different lengths of time, it is possible to read up to 500 nucleotides of sequence from a single gel. This high degree of resolution can be obtained reproducibly only if care is taken in the preparation and handling of the gels.

The concentration of acrylamide used to prepare the gel depends on the size of the DNA fragments that are to be analyzed. To read sequence within 50 nucleotides of the 5′ terminus of the primer (or within 50 nucleotides of the radiolabeled terminus in the case of Maxam-Gilbert sequencing), gels should be cast with high (12–20%) concentrations of acrylamide. Sequences lying between 25 and 400 nucleotides from the terminus of the primer can be read from gels containing 6% acrylamide. More distant sequences can be determined from gels cast with 4% or 5% polyacrylamide.

Sequencing gel apparatuses come in many sizes and shapes to suit individual tastes. The major variables are:

- *The length of the gel.* A length of 40–50 cm is standard, but apparatuses that accommodate gels up to 100 cm in length are available. However, long gels are extremely difficult to handle and require special, large containers for fixation and exposure to X-ray film.
- *The width of the gel.* Narrow gels (20 cm) can contain up to 40 lanes and are easier to handle, but wide gels (40 cm) will accept twice as many samples.
- *The thickness of the gel.* The thickness of sequencing gels is defined by the thickness of the thin plastic strips that are used as spacers between the front and back glass plates. Standard sequencing gels are 0.3–0.4 mm thick. Thinner gels (0.2 mm) give excellent resolution, but they are fragile; thicker gels (0.6 mm) will accept larger volumes of samples, but they are more difficult to fix and dry.
- *The cross-sectional shape of the gel.* Most sequencing gels are of constant thickness. On such gels, the logarithmic relationship between the length of a fragment of DNA and its mobility produces widely spaced bands at the bottom of the gel and crowded bands at the top. This limits the amount of reliable nucleotide sequence that can be read from a single gel. One way to alleviate this problem is to use tapered or wedge-shaped gels that are thicker at the bottom (0.6–0.75 mm) than at the top (0.25 mm) (Bankier and Barrell 1983; Reed et al. 1986). Wedge-shaped gels compress the spacing between adjacent bands of DNA by lowering the electrical resistance, and thus the voltage drop, per unit length of the gel. The DNA therefore slows down as the thickness of the gel increases. Despite their increased resolution, wedge-shaped gels are not used routinely for sequencing in most laboratories. Wedge-shaped gels take much longer to dry than normal gels, and they often crack on the drying apparatus. Furthermore,

the resolution of bands at the bottom of the gel is sometimes poor. Fortunately, there is another way to reduce the spacing between the bands of DNA at the bottom of the gel, i.e., by progressively increasing the concentration of buffer in the lower part of the gel (Biggin et al. 1983; Hong 1987). Because the voltage carried through the gel decreases with increasing ionic strength, DNA moves more slowly as it approaches the bottom of the gel. This decreases the spacing between adjacent bands of DNA and increases the total number of nucleotides that can be read from a single gel by approximately 30%.

- *The loading slots.* Two types of slots can be used in sequencing gels:
  1. Conventional wells that are formed by inserting a plastic template into the top of the acrylamide solution before it polymerizes. After the template is removed from the gel, the slots are washed with buffer to remove any unpolymerized acrylamide and the samples are then loaded. The samples are therefore separated from each other by fingers of polyacrylamide.
  2. Slots that are created by inserting a shark's tooth comb into the top of a gel after it has polymerized. The points of the teeth serve as barriers to prevent the mixing of adjacent samples of DNA. This design reduces the risk of torn or deformed wells and generates a flatter, more uniform surface for loading. Furthermore, the shark's tooth comb leaves virtually no separation between adjacent lanes, facilitating accurate reading of the sequence. The disadvantages are that loading of samples is more difficult and leakage is sometimes a problem. The first of these difficulties can be overcome with practice. The second problem arises when the spacers used to form the gel are slightly thicker than the shark's tooth comb. Combs and spacers should therefore be kept as matched sets.
- *The temperature gradient across the gel.* During electrophoresis, a temperature gradient is established as the gel heats up. Unless the apparatus has a mechanism to diffuse the heat evenly, the center of the gel becomes hotter than the edges, resulting in curved bands of DNA. The simplest temperature-control systems are plastic-coated metal plates that are clamped to the glass plates holding the gel. In more elaborate apparatuses, the electrophoresis buffer is used to cool the back of the gel, which can then be run at a predetermined temperature.

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Preparation of Buffer-gradient Polyacrylamide Gels

The method given below describes how to pour and run sequencing gels that contain increasing concentrations of buffer toward the base of the gel (Biggin et al. 1983; Hong 1987). It can easily be adapted to all commonly used makes of apparatuses. Sequencing gels should be made at least 2 hours before use and can be made up to 24 hours before they are needed.

1. Prepare the following stock solutions:

40% Acrylamide solution

acrylamide (DNA-sequencing grade)	380 g
N,N'-methylenebisacrylamide	20 g
distilled H_2O to 600 ml	

Heat the solution to 37°C to dissolve the chemicals. Adjust the volume to 1 liter with distilled water. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size), and store in dark bottles at room temperature.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions. Stock solutions of acrylamide can easily be purified by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration though Whatman No. 1 paper.

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. This deamination reaction is catalyzed by light and alkali. Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature. Fresh solutions should be prepared every few months.

5 × TBE

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
deionized H_2O to 1 liter	

TBE is used at a working strength of 1 × (89 mM Tris-borate, 2 mM EDTA) for polyacrylamide gel electrophoresis. This is twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1 × TBE is required to provide adequate buffering power. The pH of the buffer should be ~8.3.

The same stock of 5 × TBE should be used to prepare both the gel and the running buffers. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the DNA bands.

10% Ammonium persulfate

Ammonium persulfate	1 g
H_2O to 10 ml	

The solution may be stored at 4°C for several weeks.

6% Acrylamide/urea top solution

40% acrylamide solution	75 ml
5 × TBE	50 ml
urea (ultrapure)	230 g

Adjust the volume to 500 ml with deionized water, and filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). The 6% acrylamide/urea top solution may be stored for several weeks at 4°C.

6% Acrylamide/urea bottom solution

40% acrylamide solution	30 ml
5 × TBE	100 ml
urea (ultrapure)	92 g
sucrose	20 g
bromophenol blue	10 mg

Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). The 6% acrylamide/urea bottom solution may be stored for several weeks at 4°C.

The sucrose is added to increase the density of the bottom solution and to facilitate pouring of the buffer gradient. The bromophenol blue serves as a visible marker to check that the buffer gradient has formed correctly.

2. Prepare the number of glass plates required to accommodate the sequencing reactions. If it is necessary to remove old silicon from the plates, swab them with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol.

   Caution: Handle the KOH and the KOH/methanol solutions with great care. Use gloves and a face protector.

   Then wash the glass plates and spacers in warm detergent solution, and rinse them thoroughly in tap water, followed by deionized water. Hold the plates by their edges so that material from the gloves or oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry. The plates must be cleaned meticulously to ensure that air bubbles do not form when the gel is poured.

Treat one surface of each plate with silicone solution. This prevents the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold when electrophoresis is completed.

To siliconize a plate, lay the plate on a pad of paper in a chemical hood, and pour a small quantity of siliconizing fluid (e.g., Sigmacote) onto it. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer. Wear gloves during siliconization.

3. There are many types of electrophoresis apparatuses available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. In all cases, the aim is to form a watertight seal between the plates and spacers, so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Spacers are made of thin (usually 0.4-mm) flexible plastic.

Lay the larger (or unnotched) plate flat on the bench and arrange the two spacers in place along the sides (see Figure 13.8). A couple of *minute* dabs of petroleum jelly help to keep the spacers in position during the next steps. Make sure there is no dust on the plates, and then lay the smaller (or notched) plate in position, resting on the spacers. Clamp together one side of the plates with several large (5-cm length) bulldog binder clips. Bind the entire length of the other side and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL) to make a watertight seal. Take particular care with the bottom corners of the plates, since these are the places where leaks most often occur. Transfer the bulldog clips to the sealed side of the plates. Bind the remaining side of the plates with gel-sealing tape.

Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including:
- Sealing the edges with agarose.
- Inserting a plastic spacer into the open space at the bottom of the mold, sealing with tape, and then clamping the plates together with bulldog clips. (*Note:* A bulldog clip should be used on the bottom of the gel mold *only* when this third spacer is in place; otherwise, the glass plates may crack.)
- Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988).

We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available.

4. Place the comb into the open end of the gel mold and test to see that it fits snugly. Remove the comb and lay the empty gel mold on the bench.

5. Cover the working area with plastic-backed protective paper. It is almost impossible to pour sequencing gels without dripping acrylamide solutions onto the bench. Prepare a sequencing gel as described on pages 13.52–13.53. The preparation of the gel must be done without interruption.

Caution: Wear gloves when preparing and handling the gel solutions.

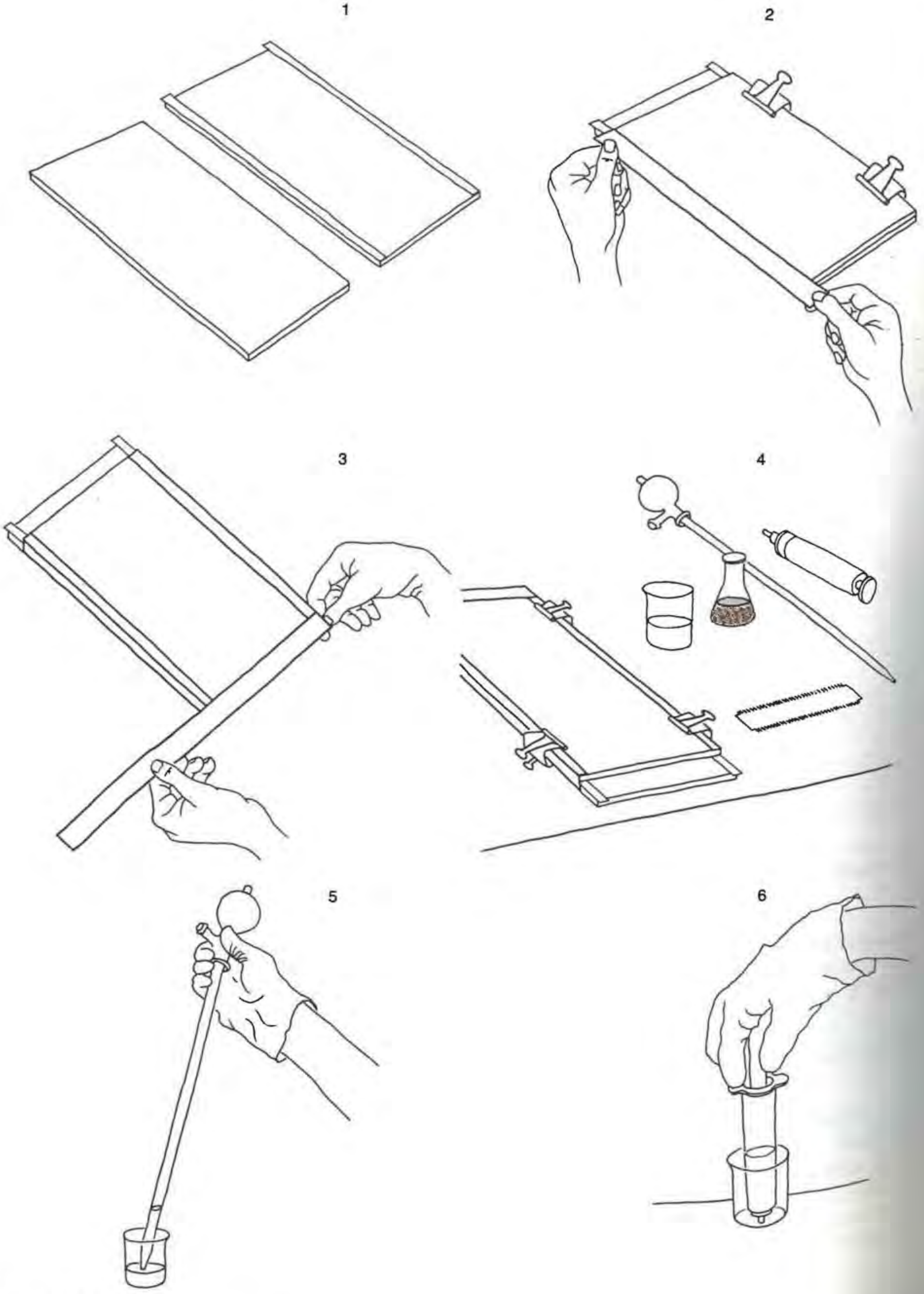

FIGURE 13.8
Preparation of a sequencing gel.

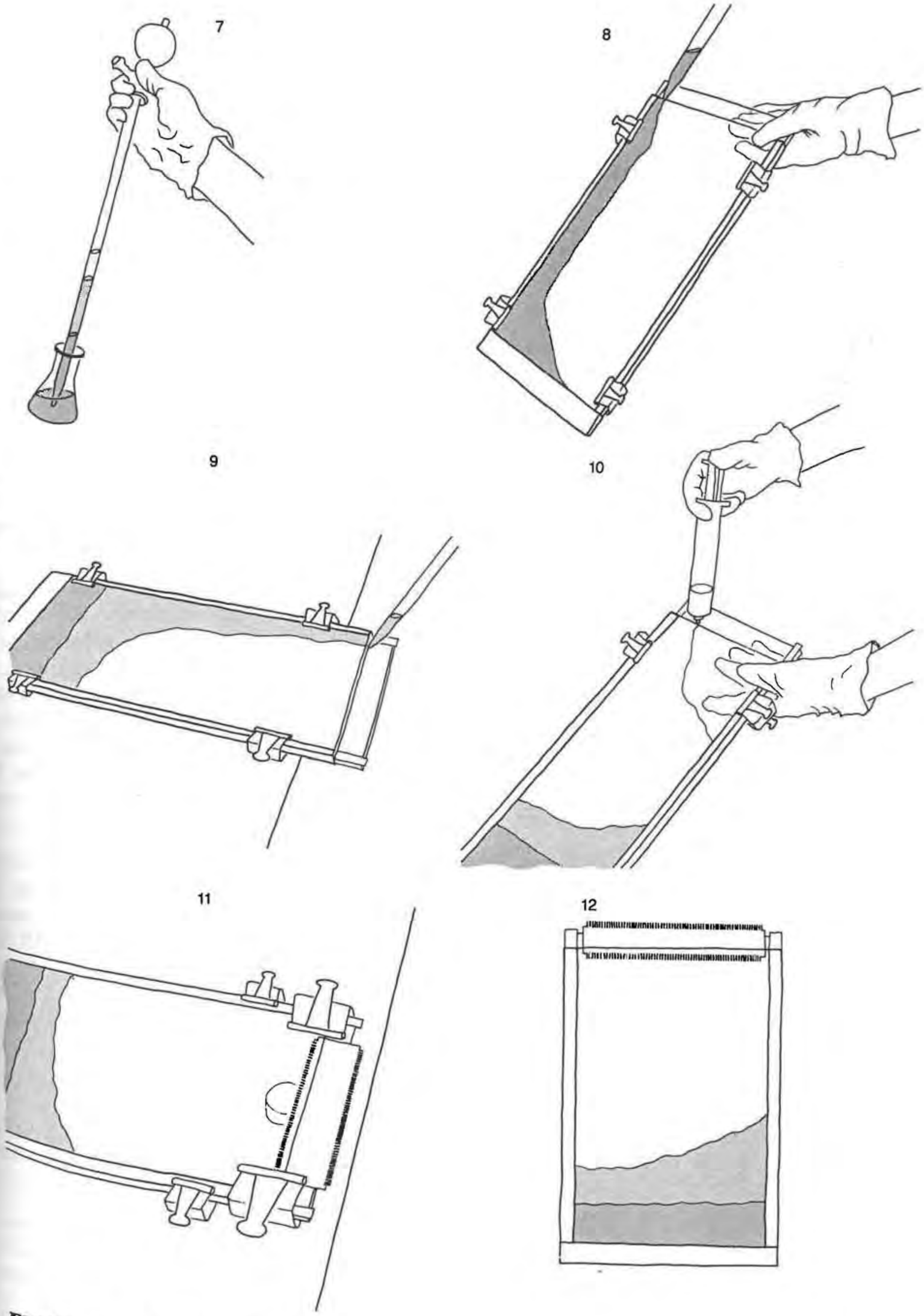

FIGURE 13.8 *(continued)*

For a 20-cm × 40-cm sequencing gel:

a. Place 10 ml of 6% acrylamide/urea bottom solution in a small Erlenmeyer flask.

b. Place 35 ml of 6% acrylamide/urea top solution in a 100-ml beaker.

c. Add 40 μl of 10% ammonium persulfate to the 6% acrylamide/urea bottom solution. Mix the solution by rapid swirling.

d. Add 120 μl of 10% ammonium persulfate to the 6% acrylamide/urea top solution. Mix the solution by rapid swirling.

e. Add 50 μl of TEMED (*N,N,N′,N′*-tetramethylethylenediamine) to the top solution and mix. Draw 7 ml of the solution into a 25-ml pipette fitted with a propipette bulb. Draw the remainder of the solution into a 50-ml hypodermic syringe.

f. Add 15 μl of TEMED to the bottom solution and mix. Draw 7 ml of the bottom solution into the same 25-ml pipette used for the top solution. Allow 2–3 air bubbles to pass upward through the two acrylamide solutions in the pipette. This establishes a crude buffer gradient.

g. Slowly pour the solution from the pipette down one side of the gel mold while holding the mold at an angle of approximately 45° to the horizontal. To avoid producing air bubbles, pour the solution in a continuous stream. When the pipette is nearly empty, lower the mold slowly to the horizontal position. Quickly resume pouring the gel using the top solution in the 50-ml syringe. Take care that no air bubbles form and that the bottom solution is not pushed up the opposite side of the gel mold by the incoming top solution. This can be avoided by tilting the gel at an angle and pouring the top solution down the lower side.

h. Carefully examine the gel for air bubbles. If any are present in the buffer gradient toward the bottom of the gel, it may be necessary to pour another gel. Bubbles in the upper portion of the gel can sometimes be moved with a thin spacer to a position where they will not interfere with the migration of the DNA samples. However, this is possible only when the full width of the gel is not to be used for loading samples. The presence of bubbles is a sure sign that the gel plates were not thoroughly cleaned before the mold was assembled.

A well-poured gel is illustrated in Figure 13.8.

Invariably, the gradient of bromophenol blue is not completely even from one side of the gel to the other. Nevertheless, upon electrophoresis, the samples will fractionate properly.

6. Lay the mold down at an angle so that the top of the mold rests on a support about 5-cm high (e.g., an empty pipette-tip rack) (see Figure 13.8). This reduces the hydrostatic pressure at the base of the mold and prevents leaks and bowing of the gel plates.

7. Immediately insert the *flat* side of a shark's tooth comb approximately 0.5 cm into the gel solution. Insert both ends of the comb into the fluid to an equal depth so that the flat surface is level when the gel is standing in a vertical position. Clamp the comb into position using bulldog binder clips (see Figure 13.8). Use the remaining 6% acrylamide/urea top solution in the hypodermic syringe to form a bead of acrylamide across the top of the gel. Allow the gel to polymerize for 1–2 hours.

8. Wash out the 25-ml pipette and the syringe so that they do not become clogged with polymerized acrylamide.

   Caution: A small amount of unpolymerized acrylamide is released during this washing step. Wear gloves and a mask.

9. After 45 minutes of polymerization, examine the gel for the presence of a schlieren line just underneath the flat surface of the comb. This is a sign that polymerization has occurred satisfactorily. When polymerization is complete, the gel can be stored for up to 24 hours at room temperature. To prevent dehydration during storage, leave the comb in the gel and surround the top of the gel with paper towels dampened with 1 × TBE. Cover the paper towels with Saran Wrap.

oading and Running Gradient Sequencing Gels

An oven or a water bath set at 80°C is required to denature the sequencing reactions just before they are loaded on the gel. Check that the temperature of the oven or water bath is correct before starting the following procedure.

1. When polymerization of the gel is complete, use damp paper towels to wipe away any dried polyacrylamide/urea from the outside of the gel mold. Carefully remove the shark's tooth comb from the top of the gel, and strip the electrical tape from the bottom of the gel mold. This is best done by cutting the tape into several segments with a scalpel blade and then removing each segment in turn. Many workers also remove the electrical tape from the sides of the gel. If this is done, transfer the bulldog binder clips from one side of the mold to the other while stripping the tape. This prevents the glass plates from moving.

2. Attach the gel mold to the electrophoresis apparatus with bulldog binder clips or plastic-coated laboratory clamps. Depending on the design of the electrophoresis apparatus, it may be necessary to clamp plastic-covered metal plates to the gel mold to ensure even diffusion of the heat produced during electrophoresis. Fill the top reservoir with 0.5 × TBE and the bottom reservoir with 1 × TBE (see page 13.47 for 5 × TBE stock solution).

3. Remove the cover from the microtiter plate containing the completed sequencing reactions. Incubate the open plate in the oven (or float it in the water bath) for 10 minutes at 80°C.

   Microtiter plates melt when heated to 85°C.

4. While the plate is incubating, fill a 10-ml hypodermic syringe fitted with a 22-gauge needle with 1 × TBE. Squirt the TBE across the submerged flat loading surface of the gel to remove any fragments of urea and polyacrylamide.

5. Reinsert the shark's tooth comb with its teeth just sticking into the loading surface of the gel. Remove the comb, and once again wash out the slots of the gel with 1 × TBE.

6. Remove the microtiter plate from the 80°C oven or water bath, and immediately transfer it onto packed ice. Keep the plate at 0°C until the samples have been loaded onto the gel. This retards renaturation of the template and radiolabeled strands.

7. Load 1–2 μl of each sequencing reaction onto adjacent slots of the gel. Keep a record of the order of the templates and load the samples in every reaction set in the same order. TCGA is the best order because the two tracks (G and C) that suffer most from abnormal patterns of migration (e.g., compression) are then adjacent to one another and can be easily compared. In addition, if the gel has been loaded in the order TCGA, the sequence of the complementary strand ($3' \rightarrow 5'$) can be read by flipping the

autoradiograph over and reading the gel from the bottom. (The tracks on the flipped autoradiograph are read from left to right and their order is assumed to be TCGA.)

If there are any spare slots at the edge of the gel, load an aliquot of an old sequencing reaction into one of them. This unambiguously distinguishes right from left on the final autoradiograph.

There are several different methods that can be used to load the samples into the wells of the gel. The choice among them is a matter of personal preference.

- *A Hamilton syringe equipped with a 30-gauge needle.* This needle is narrow enough to enter the space between the two glass plates, and the sample can therefore be layered directly on the surface of the gel. Take care not to touch the gel with the needle. The syringe and needle must be washed out with 1 × TBE (which may be taken from the bottom buffer reservoir) between samples. Excess TBE should be ejected before the next sample is drawn into the syringe and needle.
- *An automatic micropipettor (20 μl) equipped with a standard tip.* Because the tip is too large to fit between the glass plates, the sample must be expelled while the tip is resting on the sill formed by the lower (back) plate, just above the well. The tip need not be washed out between samples as long as no electrophoresis buffer becomes trapped in the tip.
- *A drawn-out glass capillary attached to a mouthpiece that is equipped with a barrier to prevent the sample from being drawn into the user's mouth.* The capillary must be washed out with 1 × TBE between samples. Excess TBE should be blown from the capillary before the next sample of DNA is loaded.

8. When all of the samples are loaded, connect the electrodes to the power pack and the electrophoresis apparatus (anode to the bottom reservoir). The gel should be run at constant power (35–40 W for a 20-cm × 40-cm gel; ~1700 V) for the time required to achieve optimal resolution of the sequence of interest. The time required can be estimated by monitoring the migration of the marker dyes in the formamide/EDTA/XC/BPB gel-loading buffer (see Table 13.3).

Depending on the distance between the sequence of interest and the oligonucleotide primer, a second loading of the sequencing samples can be applied to the gel approximately 15 minutes after the bromophenol blue from the first loading buffer has migrated from the gradient gel (~3 hours). The sequence obtained from the first loading will be more distal to the primer, whereas that obtained from the second loading will be more proximal. Samples used for the second loading should be denatured at 80°C for 2–3 minutes just before loading.

TABLE 13.3 Migration Rates of Marker Dyes through Denaturing Polyacrylamide Gels

% Polyacrylamide	Bromophenol blue[a]	Xylene cyanol FF[a]
5	35	130
6	26	106
8	19	76
10	12	55
20	8	28

[a] The numbers are the approximate sizes of DNA (in nucleotides) with which the marker dyes will comigrate.

Autoradiography of Sequencing Gels

1. At the end of the electrophoresis run, turn off the power and disconnect the sequencing apparatus from the power pack. Dispose of the electrophoresis buffer, and then remove the gel mold from the apparatus.

2. Lay the gel mold flat on plastic-backed protective bench paper with the smaller (notched) plate uppermost. Remove any remaining pieces of electrical tape. Using the end of a metal spatula, slowly pry apart the plates of the mold. With luck, the gel will remain attached to the lower plate. If it does not, place the plate back on the gel, invert the plates, and try again.

3. When the glass plates have been separated, cut off the bottom corner of the side of the gel that was loaded first. This serves to orient the gel during the subsequent manipulations. Transfer the gel (together with its supporting plate) to a shallow bath containing 10% methanol and 10% acetic acid in water. This fixes the gel and removes the urea and sucrose, which otherwise prevent the gel from drying completely and causes it to stick to the autoradiographic film. There is no need to agitate the fluid while the gel is being fixed.

   The time required for fixation varies according to the thickness of the gel:

Thickness of gel (mm)	Fixation time (minutes)
0.2	5
0.4	15
0.6	40

   The same batch of fixation fluid can be used to fix several gels.

   If the gel shows signs of detaching from the supporting glass plate, cover it with a piece of stiff plastic netting (available from many hardware stores) to prevent it from escaping and forming a crumpled mass.

4. Pick up the supporting plate by its edges, and lift the gel out of the fixation fluid. Take care that the gel does not slide off the plate. When the plate is clear of the fixation fluid, hold the gel in place with a gloved hand and tilt the plate to allow excess fixation fluid to drain away.

5. Lay the plate (gel uppermost) on a piece of protective paper. Remove any folds or distortions by gently kneading the gel with gloved fingers. Wipe excess fixation fluid from the glass plate with Kimwipes. Try not to touch the surface of the gel with the Kimwipes.

6. Place a piece of Whatman 3MM paper on top of the gel. The paper should be slightly larger (2–3 cm) than the gel in both length and width and should be centered over the gel. Apply gentle pressure so that the gel becomes firmly attached to the rough surface of the paper. Hold the paper in place with one hand and pick up the supporting glass plate with the other. Quickly flip the plate over, and lay it on a dry piece of protective paper.

7. Slide the piece of protective paper to the edge of the bench. Take hold of the leading edge of the 3MM paper and pull it downward while moving the glass plate slowly toward the edge of the bench. The gel will stick to the 3MM paper as it is peeled from the glass plate,

8. Lay the 3MM paper (gel uppermost) on two other pieces of 3MM paper of the same size. Cut a piece of Saran Wrap slightly longer and wider than the gel and lay it on top of the gel. Try to avoid creases and bubbles. This is best done with the help of another person. Hold the corners of the Saran Wrap and pull outward so that it is tightly stretched. Lower the stretched Saran Wrap onto the surface of the gel. Once the Saran Wrap has touched the gel, do not attempt to remove it, since this will cause the gel to tear.

9. Using a paper cutter, trim all three pieces of 3MM paper and the Saran Wrap so that they are nearly the same size as the gel.

10. Dry the gel for 30–40 minutes under vacuum on a commercial gel dryer set at 80°C.

11. Remove the gel from the dryer and peel off the Saran Wrap. The dried gel should feel smooth to the touch but not sticky. To orient the autoradiograph, attach a small adhesive label marked with radioactive ink to the 3MM paper in the space created by cutting the bottom corner of the gel (step 3).

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black ink. We find it convenient to make the ink in three grades: very hot ($>$2000 cps on a hand-held minimonitor), hot ($>$500 cps on a hand-held minimonitor), and cool ($>$50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive label. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

12. Establish an autoradiograph by exposing the gel to X-ray film (Kodak XAR-2, XAR-5, or equivalent) for 16–24 hours at room temperature. If possible, use spring-loaded metal cassettes to ensure direct contact between the entire surface of the dried gel and the film emulsion.

13. Develop the autoradiograph, and read the sequence of the DNA as described on page 13.58.

Reading the Sequence

Reading sequences from gels is not as easy as it looks and is an acquired skill. The following list of tips may help to simplify the process and minimize problems.

1. As soon as the autoradiograph is developed, label it with the date and the names of the templates. Mark each set of sequencing reactions clearly.
2. Be sure that you can distinguish the left and the right sides of the gel. The image of the radioactive ink should appear at the bottom of the sequencing reaction that was loaded on the first track of the gel.
3. When searching for correspondence between the new sequence and one that is already known, look for obvious "signatures" such as homopolymeric runs (e.g., consecutive T residues) or alternating purine and pyrimidines (e.g., GTGTGT). Once found, these signatures can be used to locate the sequence of interest quickly.
4. An unknown sequence should be read and recorded at least twice, preferably by different people. The two readings should then be compared and discrepancies should be resolved, if necessary, by further sequencing.
5. If the gel has been loaded in the order TCGA, the sequence of the complementary strand (3'→5') can be read by flipping the autoradiograph over and reading the gel from the bottom. (The tracks on the flipped autoradiograph are read from left to right and their order is assumed to be TCGA.)
6. The following guidelines are useful when reading gels:
   - Single C bands are generally weaker than single bands of the three other nucleotides.
   - The first A in a homopolymeric run of As is generally stronger than the rest.
   - The first C in a homopolymeric run of Cs is usually much weaker than the second.
   - G bands are weak when they are preceded by a T.

DIDEOXY-MEDIATED SEQUENCING REACTIONS USING THE KLENOW FRAGMENT OF *E. coli* DNA POLYMERASE I

Preparation

1. Prepare oligonucleotide primers (0.3 pmole/μl ≅ 2 ng/μl) and single-stranded DNA templates (0.1–0.5 μg/μl) as described on page 13.42.

2. Prepare working solutions of dNTPs and ddNTPs. We recommend making up three sets of working solutions:

   - A chase solution that contains all four dNTPs, each at a concentration of 0.5 mM. Dispense the chase solution into aliquots and store them at −70°C in tightly closed microfuge tubes.
   - A set that consists of working solutions of dTTP, dCTP, and dGTP. Each of these solutions contains high concentrations of two of these dNTPs and a lower concentration of one of these dNTPs. The names of these three working solutions identify the particular dNTP that is present in low concentration. For example, the working solution of dCTP contains a lower concentration of dCTP than of the other two dNTPs.
   - A set that consists of four working solutions of ddNTPs, each of which contains a single ddNTP.

   Before use, the appropriate amounts of the dNTP and ddNTP working solutions are mixed together to generate four chain-extension/chain-termination mixtures. For example, the working solution of ddCTP is added to the working solution of dCTP. By varying the relative amounts of the two working solutions in the mixture, it is possible to alter the balance between chain extension and chain termination to suit the particular sequencing task at hand.

   Working solutions of dNTPs and ddNTPs are prepared as described on page 13.60.

3. Pour the required number of polyacrylamide sequencing gels as described on pages 13.45–13.54.

PREPARATION OF WORKING SOLUTIONS OF dNTPs

1. Prepare working solutions of dNTPs by mixing the four 0.5 mM stock solutions of dNTPs (see page 13.44) in the ratios shown below. For example, the working solution of dTTP is prepared by mixing 2 ml of 0.5 mM dCTP, 2 ml of 0.5 mM dGTP, 0.1 ml of 0.5 mM dATP, and 0.1 ml of 0.5 mM dTTP.

dNTP working solution	0.5 mM Stock solutions of dNTPs (ratio)			
	dTTP	dCTP	dGTP	dATP
dTTP	1	20	20	1
dCTP	20	1	20	1
dGTP	20	20	1	1

It is not necessary to make a working solution containing high concentrations of dATP, which is supplied to the reaction as [^{35}S]dATP.

2. Dispense the working solutions of dNTPs into aliquots and store them at −70°C in tightly closed microfuge tubes.

PREPARATION OF WORKING SOLUTIONS OF ddNTPs

1. Prepare working solutions of ddNTPs by diluting the four 10 mM stock solutions of ddNTPs (see page 13.44) with TE (pH 8.0) and water as shown below.

ddNTP working solution	10 mM ddNTP (µl)	TE (pH 8.0) (µl)	H_2O (µl)
ddTTP	60	40	900
ddCTP	8	92	900
ddGTP	20	80	900
ddATP	9	91	900

2. Dispense the working solutions of ddNTPs into aliquots and store them at −70°C in tightly closed microfuge tubes.

Sequencing Reactions

1. If *N* different templates of DNA are to be sequenced, add to a microfuge tube:

primer (0.3 pmole ≅ 2 ng = 1 μl)	$N+1$ μl
Tris-Mg solution	$N+1$ μl
H_2O	$7N+7$ μl

The final number of microliters of annealing mixture in the tube should equal $9N+9$.

Tris-Mg solution

100 mM Tris · Cl (pH 8.5)
100 mM $MgCl_2$

Separate annealing mixtures should be prepared for each primer that is used.

2. Place 9 μl of annealing mixture in a 450-μl microfuge tube, and then add 2 μl of single-stranded DNA template. Close the top of the tube, and incubate it for 30 minutes at 55°C.

The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 μg/μl to 0.5 μg/μl, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for DNA sequencing, the template DNA is present in excess. Small variations in the amount of template added to different sets of reactions therefore do not generally affect the quality of the results (however, see the Troubleshooting Guide, Table 13.5, pages 13.76–13.77).

3. While the primer and template are annealing at 55°C, thaw

the dNTP working solutions
the ddNTP working solutions
[^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)

Store the thawed solutions on ice.

4. Make up four chain-extension/chain-termination mixtures as follows:

a. Label four microfuge tubes ddA, ddC, ddT, and ddG.

b. For each template to be sequenced, add to the appropriately labeled tube:

the appropriate ddNTP working solution	2.5 μl
the appropriate dNTP working solution	7.5 μl
[^{35}S]dATP (4 μCi)	0.4 μl

The total number of microliters in each tube should equal 10.4 times the number of templates.

5. In a fresh microfuge tube, make up a Klenow mixture. For each template to be sequenced, mix:

10 mM Tris · Cl (pH 8.0)	8 μl
0.1 M dithiothreitol	1 μl

The total number of microliters in the tube should equal 9 times the number of templates. Store the Klenow mixture at room temperature.

6. At the end of the incubation (step 2), remove the primer-template mixture from the 55°C incubator. Centrifuge the tube briefly in a microfuge to return any condensation to the bottom of the tube.

   The primer-template mixture can be stored at −20°C at this stage, if desired.

7. For each template, designate a group of four contiguous wells (labeled T, C, G, A) in a 96-well disposable microtiter plate (e.g., Baxter, B1190-17) (see Figure 13.9).

   Using an automatic pipettor, transfer 4 μl of the annealed primer-template mixture to the bottom of each of these four U-shaped wells. Continue in the same fashion until all of the annealed template-primers have been dispensed into wells.

8. Dispense 2 μl of the appropriate chain-extension/chain-termination mixture prepared in step 4 into each well. The well designated A should receive the ddA mixture; the well marked C should receive the ddC mixture, and so on. The fluid should be deposited as a droplet on the wall of each well, near the rim.

   The most accurate and rapid device for transferring the chain-extension/chain-termination mixtures to each well is a Hamilton repeating dispenser (model PB-600-1; Baxter B6732-11) and a 100-μl gas-tight syringe (model 1710-TLL; Baxter S9662-81) fitted with a Luer-lock adapter (Baxter B6732-21) that will accept standard disposable pipette tips. Use a fresh tip for each chain-extension/chain-termination mixture. Work quickly to avoid problems caused by evaporation.

9. Add the Klenow fragment of *E. coli* DNA polymerase I (6 units for each template sequence; i.e., 1.5 units for each of four sequencing reactions) to the Klenow mixture prepared in step 5. Mix the contents of the tube thoroughly by gently tapping the side of the tube with a finger.

10. Using a Hamilton repeating dispenser (see note to step 8), immediately dispense 2 μl of the Klenow reaction mixture into each well. The fluid should be deposited as a droplet on the wall of each well, near the rim. Work quickly to avoid problems caused by evaporation. When all of the wells have received the Klenow reaction mixture, cover the plate with a plastic lid.

    To prevent accidental transfer of fluid from one well to another, the lid may be lined with Whatman 3MM paper.

11. To mix the reagents and start the chain-extension/chain-termination reaction, centrifuge the droplets to the bottom of the U-shaped wells. Place the covered microtiter plate in a suitable holder (e.g., Sorvall PN11065) in a swing-out centrifuge. Turn on the centrifuge at maximum acceleration. When the rotor speed reaches 2000 rpm, immediately turn off the centrifuge.

12. Remove the plate from the centrifuge, and incubate the reactions for 15 minutes at room temperature.

13. While the reactions are incubating, thaw the chase solution which contains all four dNTPs, each at a concentration of 0.5 mM.

In most cases, there is no need to add additional Klenow enzyme to the reaction during the chase period. However, if there is a persistent problem with bands appearing in all four tracks, a more elaborate chase solution should be used containing all four dNTPs (each at a concentration of 0.5 mM), 10 mM Tris · Cl (pH 8.5), 10 mM $MgCl_2$, and the Klenow fragment of *E. coli* DNA polymerase I (1 unit per 8 μl of chase solution).

14. After 12 minutes of incubation, begin adding the chase solution (2 μl) to the wall of each well. After a total of 15 minutes of incubation, centrifuge the microtiter plate briefly as described in step 11 to mix the solutions. Continue incubation for a further 12 minutes at room temperature.

15. After a total of 27 minutes of incubation, begin adding formamide/EDTA/XC/BPB gel-loading buffer (4 μl) to the wall of each well. After a total of 30 minutes of incubation, centrifuge the plate as described in step 11 to mix the solutions. The plate may be covered with a lid, sealed with Parafilm, and stored overnight at −20°C.

Formamide/EDTA/XC/BPB gel-loading buffer

formamide	10 ml
xylene cyanol FF	10 mg
bromophenol blue	10 mg
0.5 M EDTA (pH 8.0)	200 μl

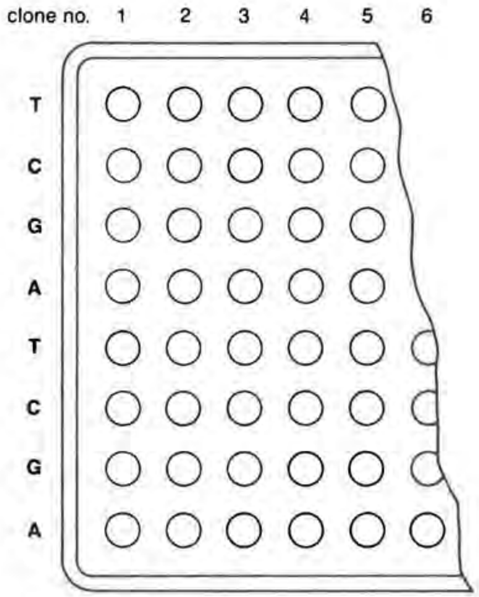

FIGURE 13.9
Microtiter plate.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

Several companies sell formamide distilled and packaged under nitrogen that need not be purified before use (e.g., BRL 5515UA).

16. Separate the products of the sequencing reactions by electrophoresis through denaturing polyacrylamide gels as described on pages 13.45–13.58.

DIDEOXY-MEDIATED SEQUENCING REACTIONS USING SEQUENASES

Sequencing with Sequenase or Sequenase version 2.0 is carried out in two steps:

- A brief polymerization reaction in which limiting concentrations of dNTPs (including one radiolabeled dNTP) are used to extend the primer by an average of 25 nucleotides

- A set of four chain-extension/chain-termination reactions in which the radiolabeled chains are rapidly extended and then terminated by incorporation of a ddNTP

Preparation

1. Prepare oligonucleotide primers (0.3 pmole/μl ≅ 2 ng/μl) and single-stranded DNA templates (0.1–0.5 μg/μl) as described on page 13.42.

2. Prepare the following reaction mixtures from stock solutions of dNTPs (0.5 mM) and ddNTPs (10 mM) (see page 13.44):

   a. *Stock labeling mixture*

dGTP (0.5 mM)	15 μl
dCTP (0.5 mM)	15 μl
dTTP (0.5 mM)	15 μl
deionized H_2O to a final volume of 1 ml	

   Dispense the stock labeling mixture into aliquots (20 μl) in small microfuge tubes and store them at −20°C.

   b. *Chain-extension/chain-termination mixtures*

   Dilute stock solutions of ddNTPs (10 mM) with water to a final concentration of 0.5 mM. Mix 160 μl of *each* dNTP working solution (see page 13.60), 10 μl of 5 M NaCl, 334 μl of H_2O, and 16 μl of the appropriate diluted ddNTP working solution (0.5 mM) for the mixture being made (e.g., for the ddG mixture, use 16 μl of the diluted ddGTP working solution [0.5 mM]).

   Dispense the chain-extension/chain-termination mixtures into aliquots (50 μl) in small microfuge tubes and store them at −20°C.

3. Prepare 5 × Sequenase buffer.

5 × Sequenase buffer

1 M Tris · Cl (pH 7.5)	200 μl
1 M $MgCl_2$	100 μl
5 M NaCl	25 μl
H_2O to 1 ml	

4. Have available a fresh solution of 0.1 M dithiothreitol, TE (pH 7.5) and water baths or incubators set at 37°C and 55°C.

5. Pour the required number of polyacrylamide sequencing gels as described on pages 13.45–13.54.

Sequencing Reactions

1. If *N* different templates of DNA are to be sequenced, add to a microfuge tube:

primer (0.5 pmole)	$2.5N + 2.5$ μl
5 × Sequenase buffer	$2.0N + 2.0$ μl
H_2O	$4.5N + 4.5$ μl

The total number of microliters of annealing mixture in the tube should equal $9N + 9$.

2. Place 9 μl of annealing mixture in a 450-μl microfuge tube, and then add 2 μl of single-stranded DNA template. Close the top of the tube and incubate it for 30 minutes at 55°C.

The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 μg/μl to 0.5 μg/μl, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for DNA sequencing, the template DNA is present in excess. Small variations in the amount of template added to different sets of reactions therefore do not generally affect the quality of the results (however, see the Troubleshooting Guide, Table 13.5, pages 13.76–13.77).

3. While the primer and template are annealing at 55°C, thaw:

the stock labeling mixture (see page 13.65)
the chain-extension/chain-termination mixtures (see page 13.65)
[^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)

Store the thawed solutions on ice.

The reaction conditions have been optimized to obtain the sequence of DNA lying up to 300–400 nucleotides from the 5′ terminus of the primer. Reading of sequences closer to or further away from the primer can be facilitated by the modifications presented in Table 13.4.

TABLE 13.4 Modification of Sequenase Reactions to Optimize Sequencing of Different Lengths of DNA

Distance of target DNA from 5' terminus of primer (nucleotides)	Modification
20–100	Dilute the labeling mixture tenfold more. Halve the time of incubation of both the labeling and chain-extension/chain-termination reactions.
100–350	Normal reaction conditions.
>350	Increase the concentrations of unlabeled dNTPs and [^{35}S]dATP in the labeling reaction fourfold. Double the time of incubation of the labeling reaction.

4. After the primer and template have been incubating for 25 minutes, prepare the Sequenase/[^{35}S]dATP mixture. For each DNA template that is to be sequenced, mix:

stock labeling mixture	0.5 μl
TE (pH 7.4)	8.0 μl
[^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)	0.5 μl
0.1 M dithiothreitol	0.5 μl
Sequenase (or Sequenase version 2.0)	0.5 μl

   The total number of microliters in the enzyme/[^{35}S]dATP mixture should be 10 times the number of DNA templates to be sequenced. Store the tube on ice.

   Because the specific activities of Sequenase and Sequenase version 2.0 are different from each other, the manufacturer (United States Biochemical) uses different definitions for the units of activity of the two enzymes. Approximately 2.5 units of Sequenase are equivalent to 1 unit of Sequenase version 2.0. Both enzymes are supplied by the manufacturer at a concentration that generally is optimal for sequencing after dilution by a factor of 8- to 20-fold. Dilution is required to reduce the concentration of glycerol, which can cause distortions in the region of the sequencing gel that contains fragments of DNA in the 400–600-nucleotide range.

5. After 30 minutes of incubation, remove the primer-template mixture from the 55°C incubator. Centrifuge the tube briefly in a microfuge to return any condensation to the bottom of the tube.

   The primer-template mixture can be stored at −20°C at this stage, if desired.

6. For each template, designate a group of four contiguous wells (labeled T, C, G, A) in a 96-well disposable microtiter plate (e.g., Baxter B1190-17) (see Figure 13.9, page 13.63).

   Using an automatic pipettor, transfer 4 μl of the annealed primer-template mixture to the bottom of each of these four U-shaped wells. Continue in the same fashion until all of the annealed template-primers have been dispensed into wells.

7. Transfer 2 μl of the enzyme/[^{35}S]dATP mixture to the side of each well. The fluid should be deposited as a droplet on the wall of each well, near the rim. Work quickly to avoid problems caused by evaporation. When all of the wells have received the enzyme/[^{35}S]dATP mixture, cover the plate with a plastic lid.

8. To mix the reagents and start the labeling reaction, centrifuge the droplets to the bottom of the U-shaped wells. Place the covered microtiter plate in a suitable holder (e.g., Sorvall PN11065) in a swing-out centrifuge. Turn on the centrifuge at maximum acceleration. When the rotor speed reaches 2000 rpm, immediately turn off the centrifuge.

9. Incubate the plate for 10 minutes at room temperature.

10. Dispense 2 μl of the appropriate chain-extension/chain-termination mixture into each well. The fluid should be deposited as a droplet on the

wall of each well, near the rim. The well designated A should receive the ddA mixture; the well marked C should receive the ddC mixture, and so on. When all of the wells have received the appropriate chain-extension/chain-termination mixture, cover the plate with a plastic lid.

The most accurate and rapid device for transferring the chain-extension/chain-termination mixtures to each well is a Hamilton repeating dispenser (model PB-600-1; Baxter B6732-11) and a 100-μl gas-tight syringe (model 1710-TLL; Baxter S9662-81) fitted with a Luer-lock adapter (Baxter B6732-21) that will accept standard disposable pipette tips. Use a fresh tip for each chain-extension/chain-termination mixture. Work quickly to avoid problems caused by evaporation.

To prevent accidental transfer of fluid from one well to another, the lid may be lined with Whatman 3MM paper.

11. To mix the reagents and start the chain-extension/chain-termination reaction, centrifuge the droplets to the bottom of the U-shaped wells as described in step 8.

12. Incubate the plate for 10 minutes at 37°C.

13. Add formamide/EDTA/XC/BPB gel-loading buffer (4 μl) to the wall of each well. Centrifuge the plate as described in step 8 to mix the solutions.

The plate may be covered with a plastic lid, sealed with Parafilm, and stored overnight at −20°C.

Formamide/EDTA/XC/BPB gel-loading buffer

formamide	10 ml
xylene cyanol FF	10 mg
bromophenol blue	10 mg
0.5 M EDTA (pH 8.0)	200 μl

For preparation of deionized formamide, see page 13.64

14. Separate the products of the sequencing reactions by electrophoresis through denaturing polyacrylamide gels as described on pages 13.45–13.58.

SEQUENCING DENATURED DOUBLE-STRANDED DNA TEMPLATES

Dideoxy-mediated sequencing can be performed on denatured plasmid (for recent references, see Chen and Seeburg 1985; Hattori and Sakaki 1986; Mierendorf and Pfeffer 1987) or bacteriophage λ templates (Mehra et al. 1986) using oligonucleotide primers that are complementary to sequences flanking restriction sites commonly used for cloning. Frequently, these primers are longer (25–29 nucleotides) than primers normally used for sequencing single-stranded templates. Longer primers give rise to fewer artifactual bands when used with denatured double-stranded DNA templates such as bacteriophage λ or plasmids.

Until recently, the quality of the sequence obtained from such templates left much to be desired. However, if care is taken with the preparation of the template, it is now possible to obtain up to 200 nucleotides of virgin sequence per reaction set. The best results are obtained using plasmid DNA that has been purified by equilibrium centrifugation in CsCl–ethidium bromide gradients. Large-scale preparations of plasmid DNA purified by precipitation with polyethylene glycol also give acceptable results, although less reproducibly. Minipreparations of plasmid DNA must be treated with RNAase or LiCl to remove contaminating small fragments of RNA that can serve as primers in the sequencing reaction. This improves the quality of the sequence to the point where it is usually possible to verify a construct or to confirm the site of a mutation. Large-scale sequencing of virgin DNA is, however, not yet advisable.

The Klenow fragment of *E. coli* DNA polymerase I is sensitive to contaminants in many plasmid preparations and is unable to extend growing chains for long distances. The resulting elevated level of nonspecific termination leads to the appearance of artifactual bands in all lanes of the sequencing gel. Reverse transcriptase is slightly better, but it is not as good as either version of Sequenase for the sequencing of denatured double-stranded DNA templates. Below we outline the use of Sequenase to obtain nucleotide sequence from large-scale preparations of plasmids purified by equilibrium centrifugation or minipreparations of plasmid DNA that have been treated with RNAase or LiCl. The method can be easily adapted to sequencing DNAs cloned in bacteriophage λ vectors (see, e.g., Mehra et al. 1986; Hardwick and Huberman 1988).

Sequencing of Plasmid DNAs Purified by Equilibrium Centrifugation in CsCl–Ethidium Bromide Gradients

This method is a modification of procedures published by Zhang et al. (1988) and by the manufacturer of Sequenase (United States Biochemical).

1. Purify the plasmid DNA from a large-scale culture of bacteria as described in Chapter 1, pages 1.42–1.45. Remove the ethidium bromide by extraction with isoamyl alcohol as described in Appendix E.

2. Add 4 μl of 1 N NaOH to 2.5 μg of superhelical plasmid DNA (in a volume of 16 μl of TE [pH 7.6]). Incubate the solution for 5 minutes at room temperature.

3. Add 2 μl of 5 M ammonium acetate (adjusted to pH 4.6 with acetic acid). Immediately mix the solution by vortexing briefly, and then quickly add 50 μl of ethanol at 0°C. Mix again, and store the solution on ice for 15 minutes.

4. Recover the denatured plasmid DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove the supernatant by gentle aspiration, and then add 1 ml of 70% ethanol (4°C) to the tube. Recentrifuge for a further 2 minutes.

5. Carefully remove and discard the ethanol. Leave the open tube on the bench until the last traces of ethanol have evaporated. Dissolve the DNA in 9 μl of H_2O and store it on ice until needed.

6. Carry out sequencing reactions as described on pages 13.67–13.69 with the following modifications:

   - Use all of the plasmid DNA (2.5 μg) in a single set of sequencing reactions.
   - Titrate the amount of primer. Although denatured double-stranded DNA templates require more primer than single-stranded templates, the addition of too much primer will lead to an unacceptably high level of background. In general, between 1 and 5 pmoles of oligonucleotide primer will be optimal for 2.5 μg of denatured plasmid template.
   - The primer should be complementary to sequences lying 50–100 nucleotides upstream of the target sequence. The average length of sequence that can be determined from denatured double-stranded templates is approximately 250 nucleotides.
   - Anneal the primer and template for 5 minutes at 60°C, and then allow the solution to cool to room temperature for 20–30 minutes before setting up sequencing reactions.
   - Use the standard labeling mixture and the standard chain-extension/chain-termination mixtures. However, the amount of [^{35}S]dATP in the enzyme/[^{35}S]dATP mixture should be increased to 1 μl (10 μCi) per set of four sequencing reactions.

Note

Target DNAs cloned in bacteriophage λ can be sequenced essentially as described above. However, improved results are obtained if the bacteriophage DNA is cleaved with restriction enzymes that release the target DNA from the vector sequences. The DNA is then purified by extraction with phenol and precipitated with ethanol. Approximately 3 μg of DNA is used for each set of sequencing reactions. If background problems arise, 5′-end-labeled primers can be used for sequencing.

moval of RNA from Minipreparations of Plasmid DNA by ecipitation with Lithium Chloride

1. Purify plasmid DNA from small-scale bacterial cultures as described on page 13.71.
2. After the last precipitation with ethanol (step 5, page 13.71), redissolve the DNAs in 100 μl of TE (pH 7.6).
3. To each minipreparation of plasmid DNA, add 300 μl of an ice-cold solution of 4 M LiCl. Store the minipreparations for 30 minutes on ice.
4. Centrifuge the plasmid DNAs at 12,000*g* for 10 minutes at 4°C in a microfuge.
5. Transfer the supernatants to fresh microfuge tubes. Add 600 μl of isopropanol. Store the tubes for 30 minutes at room temperature.
6. Recover the plasmid DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.
7. Carefully remove the supernatants by aspiration, and add 1 ml of 70% ethanol to each tube. Vortex the tube briefly, and then recentrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.
8. Carefully remove the supernatants by aspiration, and leave the open tubes on the bench until the last traces of ethanol have evaporated.
9. Resuspend the pellets in 10 μl of TE (pH 7.6). Check the recovery of plasmid DNAs by analyzing 1 μl of the solution by electrophoresis through a 0.8% agarose gel.
10. Denature the plasmid DNAs as described on page 13.71, and carry out sequencing reactions as described on pages 13.67–13.69.

PROBLEMS T

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PROBLEMS THAT ARISE WITH DIDEOXY-MEDIATED SEQUENCING

Although dideoxy-mediated sequencing usually goes smoothly, it is hardly ever perfect. For example, there is a constant need to adjust the ratio of ddNTPs to dNTPs in the chain-extension/chain-termination reactions to maximize the amount of information that can be obtained. In addition, more serious problems occasionally arise that compromise the extent or quality of the DNA sequence obtained. Most of these problems can be solved by minor adjustments to the techniques used to prepare templates or by small alterations in the conditions used for chain-extension/chain-termination reactions or polyacrylamide gel electrophoresis. An important first step is to determine whether the problem is systematic (i.e., affects all sequencing reactions) or whether it is template-specific. If this is not immediately obvious from a comparison of the results obtained from different templates that were sequenced on the same day, it can be rapidly ascertained by carrying out sequencing reactions with a reliable template (i.e., one that has given good results previously or one that is supplied as part of a commercially produced sequencing kit).

Template-specific Problems

The vast majority of template-specific problems have one of the four causes listed below. The diagnostic symptoms of each of these problems and possible suggestions for methods to solve them are listed in Table 13.5.

1. The presence of contaminating DNA (usually bacterial chromosomal DNA) in the preparation.
2. The presence of residual polyethylene glycol or high concentrations of EDTA in the preparation.
3. Low concentrations of template DNA in the chain-extension/chain-termination reactions.
4. The absence of a primer-binding site. This problem arises chiefly when sequencing deletion mutants generated by digestion with exonuclease III or BAL 31. Occasionally, these deletions extend back into the primer-binding region, thereby eliminating the ability to obtain DNA sequence. This problem can sometimes be corrected by using a primer complementary to sequences that lie further upstream of the target DNA.

Systematic Problems

Systematic problems with chain-extension/chain-termination reactions can, in theory, be due to deficiencies in any of the components in the reactions (primer, dNTPs, ddNTPs, [^{35}S]dATP, etc.). In practice, however, the most common cause of difficulty is the DNA polymerase, particularly when sequencing is carried out with the Klenow fragment of *E. coli* DNA polymerase I. This enzyme needs to be handled with great care. It should never be vortexed, and it should be kept cold at all times. The enzyme should be diluted immediately before use and should not be stored in a diluted state. The Klenow fragment expressed from a cloned gene, which lacks all $5' \rightarrow 3'$

exonuclease activity, is superior to the fragment produced by subtilisin treatment of *E. coli* DNA polymerase I.

The most common types of systematic sequencing problems are listed in Table 13.5 together with suggestions for solving them. When problems of this type arise, it is often useful to use the reagents provided in commercial sequencing kits to identify the defective component of the chain-extension/chain-termination reaction.

blems with Polyacrylamide Gels

Problems with polyacrylamide gels are of two types: (1) technical difficulties arising from the use of poor reagents (see Table 13.5) and, more rarely, (2) problems caused by secondary structure within the template or radiolabeled product. Problems due to secondary structure appear in two forms:

- *Blocks* caused by regions of secondary structure (e.g., homopolymeric tracts) in the template strand that severely impede the progress of the DNA polymerase. These blocks can sometimes be relieved by using reverse transcriptase, *Taq* DNA polymerase, or Sequenase instead of the Klenow fragment of *E. coli* DNA polymerase I or by performing the sequencing reactions at 55°C (Gomer and Firtel 1985). In a few cases, however, the blocks and the sequences that lie beyond them can be sequenced only by the Maxam-Gilbert method.
- *Compressions,* in which bands in a particular location within the gel are crowded together, resulting in unreadable sequence. In the region of the gel above the compression, the space between the bands frequently becomes abnormally large. These are gel artifacts caused by short stretches of dyad symmetry—especially those containing a high proportion of G and C residues—at the 3′ terminus of the radiolabeled strand. These regions of ambiguity can often be resolved by sequencing the opposite strand. If the problem persists, try substituting the base analog dITP for dGTP in the chain-extension/chain-termination reaction. This analog forms I-C base pairs that contain only two hydrogen bonds instead of the three normally formed by G-C base pairs. Although the Klenow fragment of *E. coli* DNA polymerase I will accept dITP, Sequenases incorporate the analog more efficiently and are therefore the enzymes of choice when carrying out sequencing reactions with dITP. Because the use of dITP accentuates pauses in the chain-extension/chain-termination reaction, sequencing reactions containing the base analog should always be run in parallel with reactions containing dGTP. The concentrations of dNTPs and ddNTPs in the stock labeling mixture and chain-extension/chain-termination mixtures when dITP is substituted for dGTP are given below.

dITP stock labeling mixture

dITP (0.5 mM)	30 μl
dCTP (0.5 mM)	15 μl
dTTP (0.5 mM)	15 μl

deionized H_2O to a final volume of 1 ml

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Dispense the dITP stock labeling mixture into aliquots in microfuge tubes and store them at −20°C.

ddITP chain-extension/chain-termination mixtures

Dilute stock solutions of ddNTPs (10 mM) (see page 13.44) with water to a final concentration of 0.5 mM. Use the diluted stock solutions of ddNTPs and the 0.5 mM stock solutions of dNTPs (see page 13.44) to make up chain-extension/chain-termination mixtures as shown below. Dispense the ddITP chain-extension/chain-termination mixtures into aliquots in microfuge tubes and store them at −20°C.

ddNTP mixture	**0.5 mM Stock solutions (µl)**				**Diluted working solution of ddNTP**[a] **(0.5 mM)(µl)**	**5 M NaCl (µl)**	**H_2O (µl)**
	dITP	**dATP**	**dTTP**	**dCTP**			
ddI	320	160	160	160	3	10	187
ddA	320	160	160	160	16	10	174
ddT	320	160	160	160	16	10	174
ddC	320	160	160	160	16	10	174

[a] Use the appropriate ddNTP for the mixture being made; for example, for the ddI mixture, use 3 µl of the diluted working solution of ddITP (0.5 mM).

The quality of sequence obtained is never as good when base analogs are used instead of conventional dNTPs. It is therefore helpful to load each set of four sequencing reactions into eight adjacent lanes of a polyacrylamide gel in the order IATCITAC. This ensures that each of the four sequencing reactions is adjacent to the other three and allows the order of closely spaced bands to be determined more easily.

Compressions not resolved by dITP can occasionally be resolved by using 7-deaza-dGTP (Mizusawa et al. 1986). However, dITP is usually the more effective base analog and is therefore the first choice for resolving compressions. If problems persist, the addition to the chain-extension/chain-termination reactions of 0.5 µg of single-stranded DNA-binding protein from *E. coli* usually eliminates the difficulty. When using single-stranded DNA-binding protein (United States Biochemical 70032), it is necessary to treat the reactions with proteinase K (0.1 µg/reaction) for 20 minutes at 65°C after adding the formamide/EDTA/XC/BPB gel-loading buffer. This allows the DNA to enter the sequencing gel and prevents smearing of bands.

The substitution of dITP for dGTP causes nucleic acids to migrate slightly faster through denaturing polyacrylamide gels.

TABLE 13.5 ***Troubleshooting Guide***

Problem	Potential causes	Possible solutions
Entire film blank or nearly so	Essential component (enzyme, dNTPs, primer, radioactive precursor) omitted from reaction Saran Wrap not removed from gel after drying	Check that the correct labeled dNTP was used Make a new batch of labeling and chain-extension/chain-termination mixtures Use a new batch of [^{35}S]dATP
One set of sequencing reactions yields four blank tracks	No template DNA Primer-binding site deleted from template High concentrations of EDTA present Incorrect primer used	Check concentration of template on agarose gel Repeat sequencing reactions after reprecipitating the template DNA with ethanol Resynthesize primer Check that the primer does not display a high degree of self-complementarity If tracks are still blank, abandon this particular template
All sequencing reactions on gel contain the same blank track	Problems with a specific chain-extension/chain-termination mixture	Make a fresh batch of chain-extension/chain-termination mixture
Variation in incorporation of radiolabel from template to template	Variation in template concentration Dirty templates	Check concentrations of template DNAs on agarose gels; make sure all sequencing reactions contain approximately equal amounts of template Purify a new batch of template DNAs
Generalized high background in all four lanes makes sequence difficult to read	Dirty templates, perhaps contaminated by chromosomal DNA	Replate recombinant bacteriophage on a fresh culture of indicator bacteria grown from a single colony; make sure that single-stranded DNA is prepared from an infected culture grown for not more than 4.5 hours
Shadow bands or double bands present in one set of sequencing reactions	More than one template present Primer is binding to more than one site Heterogeneity in primer length	Plaque-purify recombinant bacteriophage; make sure that single-stranded DNA is prepared from an infected culture grown for not more than 4.5 hours Titrate primer or use an alternative primer Repurify primer
Shadow bands in all sequencing reactions	Primer concentration is too high Klenow fragment is deteriorating or is in limiting concentration Heterogeneity in primer length	Titrate primer Increase amount of Klenow fragment to 2 units per template Add additional Klenow fragment during the chase (see note to step 13, page 13.63) Try a new batch of enzyme (see pages 13.73–13.74 for precautions in handling Klenow fragment)

Bands in all four tracks of all sequencing reactions	Klenow fragment is badly deteriorated or in limiting concentration Contaminated templates	Increase amount of Klenow fragment to 2 units per template Try a new batch of enzyme (see pages 13.74–13.75 for methods to resolve compressions) Repurify primer
Bands in all four tracks in particular regions of sequence	Homopolymeric tracts in the template, especially Gs	Sequence the complementary strand
Smeary bands	Polyacrylamide gel is defective	Make up new gel mixtures using freshly purified components Make sure that the gel mixture is at room temperature when gel is poured; otherwise, urea may precipitate
Wavy bands	Wells not thoroughly washed before loading	Pour new gel and wash wells thoroughly
Black dots on autoradiograph	Precipitation of TBE or urea in gel mixtures	Make up new gel mixtures using freshly purified components Make sure that the gel mixture is at room temperature when gel is poured; otherwise, urea may precipitate
Radioactivity remains in well	Samples not properly denatured	Transfer samples to microfuge tubes and denature by boiling for 5 minutes; chill samples to 0°C and load gel within 20 minutes
Bands are faint at top of gel	Ratio of ddNTP to dNTP is too high	Adjust ratio of ddNTP to dNTP
Bands are faint at bottom of gel	Ratio of ddNTP to dNTP is too low	Adjust ratio of ddNTP to dNTP
	Sequencing with Sequenases	
Bands are faint at bottom of gel	Concentration of template DNA is limiting, resulting in the synthesis of long DNA molecules during the elongation reaction	Increase the amount of template Use a primer more distant from the target site
Bands are faint at top of gel	Concentration of dNTP, most probably dATP, is limiting	Check that specific activity of [^{35}S]dATP is 600 Ci/mmole Add 1 μl of [^{35}S]dATP to the labeling reaction
Bands in all four tracks of sequencing reactions at bottom of gel	Sequenase is inactivated during elongation reaction	Incubate the elongation reaction for 5 minutes at *20°C* (check temperature!)
Bands in all four tracks of sequencing reactions at top of gel	Termination reaction is working inefficiently	Repurify template DNA Check that termination reaction is carried out at *37°C*

APPLICATIONS OF PCR AMPLIFICATION

Although a relatively new technique, PCR amplification has already found extensive application in the diagnosis of genetic disorders (Wong et al. 1987; Engelke et al. 1988), the detection of nucleic acid sequences of pathogenic organisms in clinical samples (Kwok et al. 1987; Ou et al. 1988), the genetic identification of forensic samples (including DNA extracted from individual hairs [Almoguera et al. 1988; Higuchi et al. 1988] or a single sperm [Li et al. 1988]), and the analysis of mutations in activated oncogenes (Bos et al. 1987; Farr et al. 1988). In addition, as discussed below, PCR amplification is being used to carry out a variety of tasks in molecular cloning and analysis of DNA, including:

- Generation of specific sequences of cloned double-stranded DNA for use as probes
- Generation of probes specific for uncloned genes by selective amplification of particular segments of cDNA
- Generation of libraries of cDNA from small amounts of mRNA
- Generation of large amounts of DNA for sequencing
- Analysis of mutations
- Chromosome crawling

During the next few years, the technique is likely to find increasing application in many aspects of molecular cloning (for review, see Erlich et al. 1988; Marx 1988; Oste 1988). However, a limitation of the current method is its relatively high rate of misincorporation. The *Taq* DNA polymerase lacks editing functions and incorporates an incorrect nucleotide at a rate of 2×10^{-4} nucleotides per cycle in polymerase chain reactions, a rate about four times higher than that obtained with the Klenow fragment of *E. coli* DNA polymerase I. This rate of misincorporation translates into an overall error frequency of 0.25% in a 30-cycle amplification (Saiki et al. 1988b). The error frequency appears to increase in the presence of higher concentrations of dNTPs and Mg^{++}. These misincorporations occur throughout the length of the amplified product and consist of both transitions and transversions (but not large deletions, mosaics, or insertions). Such occasional errors are not a problem when the products of the entire amplification reaction are used as hybridization probes or as templates for direct DNA sequencing. However, the sequence of an *individual* DNA molecule cloned from an amplified pool is unreliable. Any sequence obtained in this manner should be confirmed either by sequencing a number of independent recombinant clones, preferably generated from at least two separate amplification reactions, or by sequencing a pool of single-stranded DNAs derived from 100–200 recombinant clones. This eliminates the possibility that misincorporation(s) occurring during the early rounds of amplification might appear in every clone derived from a particular amplification reaction.

Generation of Specific Sequences of Cloned Double-stranded DNA for Use as Probes

The polymerase chain reaction can be used, for example, to amplify cDNAs carried in bacteriophage λ vectors by using oligonucleotide primers that anneal to the flanking vector sequences (Saiki et al. 1988b). In some cases, this can eliminate the need for subcloning into plasmid or bacteriophage M13 vectors or for the isolation of specific segments of DNA prior to subcloning. Because the termini of the amplified segment are defined by the sequences of the priming oligonucleotides, the polymerase chain reaction can be used to eliminate unwanted sequences flanking the target DNA. It is therefore possible to generate a series of precisely defined deletion mutants in a set of polymerase chain reactions that are primed by a progressive series of nested oligonucleotides. In addition, useful sequences (e.g., restriction sites) can be inserted at the termini of the amplified fragment(s) by incorporating additional nucleotides at the 5′ termini of the priming oligonucleotides. The polymerase chain reaction therefore provides a very versatile tool to increase the speed and precision of many of the methods used to manipulate DNA in molecular cloning.

Generation of Probes Specific for Uncloned Genes by Selective Amplification of Particular Segments of cDNA

Several methods have been devised to use the polymerase chain reaction to generate probes for genes or cDNAs that have not yet been cloned. For example, when a limited amount of amino acid sequence is available from the protein of interest, degenerate pools of oligonucleotides can be used to amplify target sequences contained in a pool of first-strand cDNA. One pool consists of oligonucleotides that encompass all possible ways of coding for a particular tract of amino acids in a protein. The other consists of sequences complementary to all possible sequences coding for a second tract of amino acids some distance away in the protein. If desired, one or both pools of oligonucleotides can contain inosine at positions of high degeneracy. mRNA extracted from cells that synthesize the protein is used as a template for synthesis of the first strand of cDNA catalyzed by reverse transcriptase. Both pools of oligonucleotides are then used to prime a polymerase chain reaction that uses this first strand of cDNA as a template. The amplified products of the reaction can then be cloned in a suitable vector, sequenced, and used as probes in Southern or northern hybridizations or for screening cDNA and genomic DNA libraries (Lee et al. 1988).

The obvious advantage of this method (MOPAC, *m*ixed *o*ligonucleotide *p*rimed *a*mplification of *c*DNA) is that it rapidly yields a probe of unique sequence. In most cases, this probe will be long enough to allow hybridization to be carried out under conventional conditions rather than under conditions established empirically for pools of redundant oligonucleotides (see Chapter 11). However, the method has another, potentially greater advantage, namely, the selection for priming by the "correct" oligonucleotides in each of the two pools. Oligonucleotides that are not exactly complementary to the cDNA encoding the protein of interest will be less effective as primers over the course of many rounds of amplification. Lee et al. (1988) used polymerase chain reactions catalyzed by the Klenow fragment of *E. coli* DNA polymerase I and analyzed the sequences of seven independent clones that contained an amplified DNA fragment of porcine urate oxidase cDNA 112 bp in length. Although the sequences of all seven clones of amplified DNA corresponded exactly to the sequence of the authentic porcine urate oxidase gene, each of them carried a different combination of upstream and downstream primers. Thus, the polymerase chain reaction catalyzed by the Klenow fragment of *E. coli* DNA polymerase I can generate amplified copies of a desired target sequence even when there is mismatch between the primer and the template.

It is likely that the heterogeneity in sequence at the termini of the amplified DNA will be greatly reduced when the amplification reaction is catalyzed by *Taq* DNA polymerase rather than by the Klenow fragment. The higher temperatures used during annealing and/or extension of oligonucleotides by *Taq* DNA polymerase should suppress hybridization between mismatched primers and the target sequence, generating an amplified product that carries the "correct" oligonucleotides at its termini. However, when using mixed pools of oligonucleotides as primers for *Taq* DNA polymerase, it is essential to give some thought to both the temperature of annealing and the "ramping time" (i.e., the time required to raise the temperature of the annealing mixture to 72°C, the temperature routinely used for polymerization). The temperature chosen for annealing should be low enough to ensure that the oligonucleotide with the lowest content of G + C in the pool has an opportunity to hybridize efficiently to its target. The ramping time should be short enough to suppress elongation of oligonucleotides that hybridize to false target sequences.

In a variation of the MOPAC technique, called the *anchored polymerase chain reaction* (Loh et al. 1989), the first strand of cDNA is synthesized in a conventional way using as a primer either oligo(dT) or a synthetic oligonucleotide complementary to the sequence of mRNA that codes for a known tract of amino acids. Although the original experiments used only a single synthetic oligonucleotide for this purpose, degenerate pools of oligonucleotides would also be expected to work well. A poly(dG) tail is then added to the 3′ termini of the resulting cDNAs with terminal transferase, and the desired product is amplified in a polymerase chain reaction primed by (1) an oligonucleotide consisting of a poly(dC) tail attached to a sequence with convenient restriction sites (termed the anchor) and (2) a single synthetic oligonucleotide (or a degenerate pool of oligonucleotides) corresponding to a known tract of amino acids in the protein of interest. If desired, these oligonucleotides can also be anchored. This simplifies cloning of the amplified product.

The central benefit of both of these methods is that a unique primer is not needed at both ends of the sequence to be amplified. Furthermore, because the resulting amplified DNAs are highly enriched for the sequences of interest, there is no need to establish or screen large cDNA libraries.

Generation of Libraries of cDNA from Small Amounts of mRNA

The polymerase chain reaction can be used to amplify first-strand cDNA that has been synthesized in vitro by DNA-dependent RNA polymerase from mRNA templates using oligo(dT) as a primer. The addition of a homopolymeric tract of G residues to the 3′ terminus of the cDNA allows the subsequent polymerase chain reaction to be primed by oligo(dT) and oligo(dC) primers (see, e.g., Belyavsky et al. 1989). If desired, these primers can contain a potential restriction site at their 5′ termini to facilitate cloning of the amplified double-stranded cDNA into an appropriate vector. The main advantage of this method is that it allows large cDNA libraries to be established from mRNA extracted from as few as 1 or 2 mammalian cells. However, it is important to bear in mind that the amplified sequences will accumulate a high proportion of errors (0.25% if the cDNA is amplified through ~35 cycles). It is therefore essential to compare the sequences of several independent isolates of any clones of interest obtained from such amplified libraries. In addition, smaller fragments of cDNA will accumulate preferentially during the amplification process, and this method is therefore not recommended for the isolation of full-length cDNAs corresponding to mRNAs more than 1–2 kb in length.

Generation of Large Amounts of DNA for Sequencing

Sequencing can be carried out either by the Maxam-Gilbert chemical degradation method or, more commonly, by the Sanger dideoxy-mediated chain-termination method (see Chapter 13). Three types of polymerase chain reaction are used to generate templates for sequencing by the Sanger method:

- *A conventional reaction in which both priming oligonucleotides are present in vast excess.* At the end of the reaction, the amplified DNA is desalted and excess dNTPs are removed by centrifuge-driven spin dialysis on a microconcentrator (Amicon). The amplified DNA is then sequenced using a ^{32}P-labeled oligonucleotide primer complementary to an appropriate region of the DNA. A description of this method is given on pages 14.22–14.27.

  Although this method can work well, the presence of oligonucleotide primers (remaining from the amplification reaction) can complicate the dideoxy-mediated sequencing reaction. These unlabeled primers may either compete directly with the radiolabeled primer for binding sites on the template DNA or they may generate extension products that efficiently exclude the radiolabeled oligonucleotide. These problems can be avoided by purifying the amplified DNA by gel electrophoresis (see Chapter 6).

- *A conventional reaction in which at least one of the primers is designed to include a promoter for a bacteriophage-encoded DNA-dependent RNA polymerase.* At the end of the reaction, one strand of the amplified DNA is transcribed in vitro, and the resulting RNA is sequenced using reverse transcriptase and an oligonucleotide complementary to the 3′ terminus of the RNA (Stoflet et al. 1988).

  The chief disadvantage of this method is the necessity of generating long oligonucleotides that contain bacteriophage promoters to prime the amplification reaction. When both strands of the amplified DNA are to be sequenced, each of the two oligonucleotides must contain a separate promoter sequence. The expense of generating such long oligonucleotides can become burdensome when this method is used routinely.

- *A reaction in which one of the two primers is present in limiting concentration.* After approximately 12–15 cycles of amplification, one primer is essentially depleted and the final stages of the amplification reaction therefore generate copies of only one strand of the original DNA (Gyllensten and Erlich 1988; Mihovilovic and Lee 1989). Sequencing is carried out using unlabeled primers complementary to an appropriate region of the amplified single-stranded DNA. A description of this method is given on pages 14.28–14.29.

  This method generally works well. However, to optimize the yield of single-stranded product, it is sometimes necessary to carry out several amplification reactions that contain varying amounts and ratios of the priming oligonucleotides. In addition, separate sets of amplification reactions must be set up if both strands of the amplified DNA are to be sequenced.

Analysis of Mutations

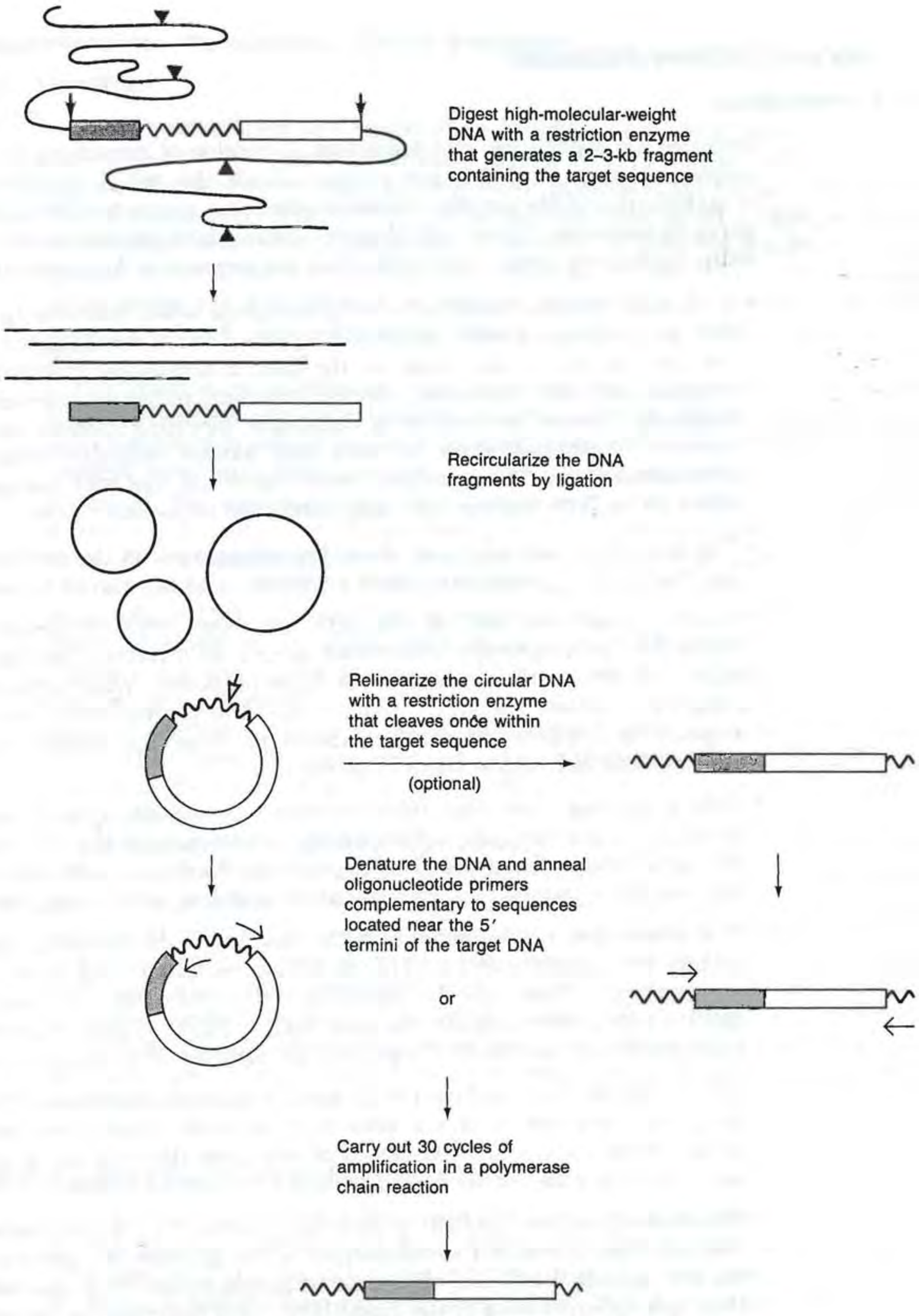
The polymerase chain reaction has been adapted in a number of ways to facilitate the identification and analysis of mutations in eukaryotic DNA. For example, deletions and insertions at defined locations can be detected by a change in the size of the amplified product. Alternatively, deletions can be recognized by their failure to amplify when one of the priming oligonucleotides maps within the deleted sequence. The polymerase chain reaction can also be useful in the identification of point mutations in the following ways:

- Hybridizing amplified DNA to radiolabeled RNA probes (Almoguera et al. 1988). Perfectly matched heteroduplexes can be distinguished from mismatched duplexes by digestion with RNAase A (see Chapter 7, pages 7.71–7.78).
- Using mixtures of synthetic oligonucleotides as primers for DNA synthesis (Gibbs et al. 1989). A DNA template is mixed with two oligonucleotides that differ in sequence by a single base and therefore compete for the same site in the target DNA. During the course of a polymerase chain reaction, the perfectly matched oligonucleotide is favored over its mismatched competitor and is therefore incorporated into the amplified DNA product. In this technique, which is called *competitive nucleotide priming*, the two oligonucleotides are included in two separate reactions. In one reaction, the first primer is radiolabeled; in the second reaction, the other primer is radiolabeled. Both reactions generate an amplified product, but only the reaction primed by the perfectly matched oligonucleotide yields a product radiolabeled to high specific activity. It is therefore a simple matter to determine which primer correctly matches the sequence in the original unamplified sample.
- Using allele-specific oligonucleotide probes to discriminate between amplification products that differ by a single base pair (Saiki et al. 1988a). This method often requires the application of a comprehensive panel of oligonucleotide probes in order to check for each possible change (see, e.g., Hirai et al. 1987; Liu et al. 1987; Rodenhuis et al. 1987; Farr et al. 1988).

Chromosome Crawling

Although the polymerase chain reaction is used frequently to amplify DNA segments lying between the two priming oligonucleotides, it can also be used to amplify sequences that lie outside the boundary of known sequences (Ochman et al. 1988; Triglia et al. 1988). As shown in Figure 14.2, genomic DNA is digested to completion with a restriction enzyme that has no sites of cleavage within the target sequence. The fragment carrying the target sequence should be no larger than 2–3 kb in length. The DNA is then diluted and ligated under conditions that favor the formation of monomeric circles. Because *Taq* DNA polymerase works slightly more efficiently with linear DNA than with circular DNA, some workers (Triglia et al. 1988) relinearize the template DNA by digesting with a restriction enzyme that cleaves once within the known sequence of the target DNA (see Figure 14.2). No matter whether linear or circular templates are used, the regions flanking the target sequence can then be amplified in a polymerase chain reaction using oligonucleotide primers that are complementary to the 5′ termini of the target fragment. The major product of the amplification reaction is a linear double-stranded DNA molecule that consists of a head-to-tail arrangement of sequences flanking the original target DNA. The junction between the upstream and downstream sequences is marked by the presence of a restriction site for the enzyme originally used to digest the genomic DNA. This procedure, which is known as *inverse PCR* (Ochman et al. 1988), permits the rapid amplification of uncharacterized segments of DNA that immediately flank a target sequence. Inverse PCR is therefore useful as a method ("chromosome crawling" [Triglia et al. 1988]) to explore the chromosomal sequences that are contiguous to a known segment of DNA.

FIGURE 14.2
A diagram of the major steps involved in an inverse polymerase chain reaction. The target DNA is shown as 〰 and the flanking sequences are shown as □ and □. Restriction sites are marked ▲ and △. Arrows show the positions of complementarity between the priming oligonucleotides and the template DNA.



Digest high-molecular-weight DNA with a restriction enzyme that generates a 2–3-kb fragment containing the target sequence

Recircularize the DNA fragments by ligation

Relinearize the circular DNA with a restriction enzyme that cleaves once within the target sequence

(optional)

Denature the DNA and anneal oligonucleotide primers complementary to sequences located near the 5′ termini of the target DNA

or

Carry out 30 cycles of amplification in a polymerase chain reaction

The major product of the amplification reaction consists of a head-to-tail arrangement of the sequences that originally flanked the target region. The junction between the two sets of flanking sequences is marked by a restriction site that can be cleaved by the enzyme used in step 1 to digest the original high-molecular-weight DNA.

FIGURE 14.2 *(See facing page for legend.)*

AMPLIFICATION METHODS

Precautions

Because the polymerase chain reaction is capable of amplifying as little as a single molecule of DNA, precautions should be taken to guard against contamination of the reaction mixture with trace amounts of DNAs that could serve as templates (Kwok and Higuchi 1989). Such precautions are essential when amplifying target sequences that are present at low concentrations.

- If possible, assemble and carry out polymerase chain reactions in a laminar flow hood equipped with ultraviolet lights. Turn on these lights whenever the hood is not in use. Keep in the hood a microfuge, disposable gloves, supplies, and sets of pipetting devices used only to handle polymerase chain reactions. Since the barrels of automatic pipetting devices are common sources of contamination, prepare and handle reagents using positive-displacement pipettes equipped with disposable tips and plungers. Autoclave all buffers, pipette tips, and centrifuge tubes before use.
- Put on a fresh pair of gloves when beginning work in the area designated for assembly of polymerase chain reactions. Change gloves frequently.
- Prepare your own sets of reagents and store them in small aliquots, preferably in a specially designated section of a freezer located near the hood. Do *not* use these reagents for other purposes. When preparing these reagents, use new glassware, plasticware, and pipettes that have not been exposed to DNAs in use in the laboratory. After use, discard aliquots of reagents; do not return them to storage.
- Before opening microfuge tubes containing reagents used in polymerase chain reactions, centrifuge them briefly (10 seconds) in the microfuge located in the laminar flow hood. This deposits the fluid in the base of the tube and reduces the possibility of contamination of gloves or pipetting devices.
- It is best to add *all* components of the reaction to the microfuge tube *before* adding the template DNA. This includes the mineral oil used to prevent evaporation. Then add the template DNA, close the tube, and mix the contents by gently tapping the side with a gloved finger. Centrifuge the tube briefly (10 seconds) to separate the aqueous and organic phases.
- When adding the template DNA to an assembled polymerase chain reaction, take care not to create aerosols that could contaminate other reactions. Keep the tops tightly closed on all tubes that are not in immediate use. Change gloves after handling tubes containing template DNA.
- Whenever possible, include a positive control (i.e., a polymerase chain reaction that contains a small amount of the appropriate target sequence). An appropriate dilution of the target sequence should be prepared ahead of time in a different area of the laboratory. This prevents the introduction of concentrated solutions of target DNA into the area of the laboratory set aside for polymerase chain reactions.
- Always include a control that contains all the components of the polymerase chain reaction except the template DNA. Assemble this control after all other polymerase chain reactions have been set up.

OLIGONUCLEOTIDES

Oligonucleotides used for priming the polymerase chain reaction should be at least 16 nucleotides and preferably 20–24 nucleotides in length. These oligonucleotides are too short to form stable hybrids at the temperature used for polymerization (usually 72°C). It is assumed that the *Taq* DNA polymerase begins to work, albeit sluggishly, as soon as the priming oligonucleotides become bound to their templates at low temperature (37–55°C). The extended products are long enough to remain attached to the template as the temperature of the polymerase chain reaction is raised rapidly to 72°C.

The products of polymerase chain reactions are blunt-ended DNA molecules that can be phosphorylated by bacteriophage T4 polynucleotide kinase and cloned into standard vectors by conventional techniques. However, many workers have found that the efficiency with which amplified molecules can be cloned in this way is much lower than normal. The reasons for this are unknown. A solution to the problem is to use oligonucleotide primers that carry the recognition sequence for a restriction enzyme at their 5′ termini. The site chosen should be for a restriction enzyme that rarely cleaves mammalian DNA. The presence of unpaired bases at the 5′ termini does not affect the ability of the oligonucleotides to prime DNA synthesis. However, during successive cycles of the amplification reaction, these unpaired sequences are converted to double-stranded DNA. The final products of the reaction therefore consist of target sequences flanked by novel restriction sites. After these sites have been cleaved with the appropriate enzyme(s), the amplified DNA can be cloned with high efficiency into vectors that carry compatible termini.

Usually, oligonucleotides are used at a concentration of 1 μM in polymerase chain reactions. This is usually sufficient for at least 30 cycles of amplification. The presence of higher concentrations of oligonucleotides can cause priming at ectopic sites, with consequent amplification of undesirable nontarget sequences. Conversely, the polymerase chain reaction is extremely inefficient when the concentration of primers is limiting. If the yield of amplified product is poor or if contamination by nontarget sequences is unacceptably high, set up a series of control experiments to determine the minimum amounts of the two primers required to generate the desired amount of amplified product.

BUFFERS USED FOR POLYMERASE CHAIN REACTIONS

The standard buffer for polymerase chain reactions contains 50 mM KCl, 10 mM Tris · Cl (pH 8.3 *at room temperature*) and 1.5 mM $MgCl_2$. When incubated at 72°C, the pH of the reaction drops by more than a full unit, producing a buffer whose pH is approximately 7.2. The presence of divalent cations is critical. Magnesium ions are superior to manganese, and calcium ions are ineffective (Chien et al. 1976). Because the optimal concentration of Mg^{++} is quite low (1.5 mM), it is important that the preparation of template DNA does not contain high concentrations of chelating agents such as EDTA or of negatively charged ionic groups such as phosphates. DNAs to be used as templates should therefore be dissolved in 10 mM Tris · Cl (pH 7.6), 0.1 mM EDTA (pH 8.0).

The standard buffer works well for a wide range of templates and oligonucleotide primers, but it may not be optimal for any particular combination (see, e.g., Krawetz et al. 1989). The conditions given should therefore be regarded as a point of departure to explore modifications and potential improvements. In particular, the concentration of Mg^{++} should be optimized whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is altered. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available Mg^{++}. We recommend setting up a set of reactions containing fixed concentrations of Tris · Cl (10 mM) and KCl (50 mM) and varying concentrations of $MgCl_2$ (0.05–5 mM in 0.5 mM increments). After completion of the reactions, compare the yields of the amplified products by electrophoresis through an agarose gel in the presence of ethidium bromide.

Taq DNA POLYMERASE

Two forms of *Taq* DNA polymerase are now available: the native enzyme purified from *Thermus aquaticus* and a genetically engineered form of the enzyme synthesized in *E. coli* (AmpliTaq^{TM}). Both forms of the polymerase carry a $5' \rightarrow 3'$ polymerization-dependent exonuclease activity, but they lack a $3' \rightarrow 5'$ exonuclease activity (S. Stoffel, pers. comm.). The properties of the two polymerases are essentially equivalent (see Chapter 5, page 5.50), and they can be used interchangeably in polymerase chain reactions. Approximately 2 units of either of the enzymes are required to catalyze a typical polymerase chain reaction. Addition of excess enzyme may lead to amplification of nontarget sequences.

DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

dNTPs are used at saturating concentrations (200 μM for each dNTP). A stock solution of dNTPs (50 mM) should be adjusted to pH 7.0 with 1 N NaOH to ensure that the pH of the final reaction does not fall below 7.1.

Prepared solutions of dNTPs sold by commercial manufacturers are generally not adjusted to pH 7.0.

TARGET SEQUENCES

DNA containing the target sequences can be added to the polymerase chain reaction mixture in a single- or double-stranded form. Although the size of the DNA is generally not a critical factor, amplification is improved if very-high-molecular-weight DNAs (e.g., genomic DNAs) are digested with a rarely cutting restriction enzyme (e.g., *Sal*I or *Not*I). Target sequences are amplified slightly less efficiently when they are carried in closed circular DNAs rather than in linear DNAs. It is therefore preferable to linearize plasmid DNAs before they are used as templates in polymerase chain reactions. The concentration of target sequences in the template DNA obviously varies according to circumstances and is often not under the control of the experimenter. However, it is worthwhile setting up a series of control reactions that contain decreasing amounts of known target sequences (1 ng, 0.1 ng, 0.001 ng, etc.) to check that the amplification reaction is working at the required sensitivity.

Amplification Reactions

1. In a sterile 0.5-ml microfuge tube, mix in the following order:

sterile H_2O	30 µl
10 × amplification buffer	10 µl
mixture of four dNTPs, each at a concentration of 1.25 mM	16 µl
primer 1 (in 5 µl of H_2O)	100 pmoles
primer 2 (in 5 µl of H_2O)	100 pmoles
template DNA (up to 2 µg, depending on the concentration of target sequences)	
H_2O to a final volume of 100 µl	

10 × Amplification buffer

500 mM KCl
100 mM Tris · Cl (pH 8.3 at room temperature)
15 mM $MgCl_2$
0.1% gelatin

The standard conditions given above work well for a wide range of templates and oligonucleotide primers, but they may not be optimal for any particular combination. Methods to optimize the reaction conditions are discussed on pages 14.14–14.17.

The pH of the mixture of dNTPs should be 7.0 (see page 14.16).

For amplification of single sequences from mammalian genomic DNA, use 0.2–2 µg of DNA. The reaction mixture will then contain approximately 0.03–0.3 pg of a target sequence 500 bp in length. For amplification of target DNA cloned in a plasmid vector (e.g., for DNA sequencing), add 20 ng of linearized plasmid DNA.

Because the optimal concentration of Mg^{++} in the reaction is quite low, DNAs to be used as templates should be dissolved in 10 mM Tris · Cl (pH 7.6), 0.1 mM EDTA (pH 8.0) (see pages 14.15–14.16).

2. Heat the reaction mixture for 5 minutes at 94°C to denature the DNA completely.

3. While the mixture is still at 94°C, add 0.5 µl of *Taq* DNA polymerase (5 units/µl; Perkin Elmer Cetus N801-0046).

Taq DNA polymerase is supplied in a storage buffer containing 50% glycerol. This solution is very viscous and is difficult to pipette with accuracy. The best method is to centrifuge the tube containing the enzyme at 12,000*g* for 10 seconds at 4°C in a microfuge and then to withdraw the required amount of enzyme using a positive-displacement pipetting device. Automatic pipetting devices are not recommended for handling reagents used in polymerase chain reactions (see page 14.14).

4. Overlay the reaction mixture with 100 µl of light mineral oil (Sigma M-3516 or equivalent). This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Carry out amplification as described below. Typical conditions for denaturation, annealing, and polymerization are as follows:

Cycle	**Denaturation**	**Annealing**	**Polymerization**
First cycle	5 minutes at 94°C	2 minutes at 50°C	3 minutes at 72°C
Subsequent cycles	1 minute at 94°C	2 minutes at 50°C	3 minutes at 72°C
Last cycle	1 minute at 94°C	2 minutes at 50°C	10 minutes at 72°C

The denaturation step is omitted after the last cycle of amplification and the samples are transferred to −20°C for storage.

Timing of individual steps should begin only after the reaction mixtures have reached the required temperatures. Between 30–60 seconds are required for reaction mixtures to reach the desired temperature after a temperature shift. The length of the lag depends on several factors, including the type of tube used, the volume of the reaction mixture, the source of the heat (water bath or heating block), and the temperature differential between successive steps. It is important to adjust the length of the incubation steps to compensate for these lags.

The temperature (50°C) chosen for annealing of the oligonucleotide primers to the target DNA is a compromise. Amplification is more efficient if annealing is carried out at lower temperatures (37°C), but the amount of mispriming is significantly increased (Saiki et al. 1988b). At higher temperatures (55°C), the specificity of the amplification reaction is increased, but its overall efficiency is reduced. Ideally, a series of control reactions should be set up to determine the annealing temperature that gives optimal results for a given amplification reaction.

Polymerase chain reactions can be automated with a thermal cycler (available from Perkin Elmer Cetus or Ericomp). These machines, although expensive, allow the individual cycles of the reactions to be standardized and relieve the experimenter of the burden of transferring tubes from one temperature to another for many hours.

The longer the distance between the oligonucleotide primers, the longer the time required for the complete synthesis of the entire sequence of the target sequence. The times given above are optimized for a target sequence approximately 500 nucleotides in length.

The number of amplification cycles depends on the concentration of target DNA in the reaction mixture. At least 25 cycles are required to amplify single-copy target sequences in mammalian genomic DNA to the point where they can be detected by direct examination of agarose or polyacrylamide gels. However, the amount of *Taq* DNA polymerase usually becomes limiting after 25–30 cycles of amplification (i.e., after an $\sim 10^6$-fold amplification has been obtained). If further amplification is required, a sample of the amplified DNA should be diluted 1000- to 10,000-fold and used as the template for further rounds of synthesis in a fresh polymerase chain reaction.

6. Withdraw a sample of the amplified DNA from the reaction mixture and analyze it by gel electrophoresis, Southern hybridization, or DNA sequencing. If necessary, the oil can be removed from the sample by extraction with 150 μl of chloroform. The aqueous phase, which contains the amplified DNA, forms a micelle near the meniscus. This micelle can be transferred to a fresh tube with an automatic micropipettor.

Amplification of DNA Generated by Reverse Transcription of mRNA

The first strand of cDNA generated by reverse transcription of mRNA can be used as a template for polymerase chain reactions as described below.

1. Set up a reaction to synthesize the first strand of cDNA:

10 × amplification buffer	2 μl
mixture of four dNTPs, each at a concentration of 10 mM	2 μl
oligo(dT)$_{12-18}$ (100 μg/ml)	1 μl
placental RNAase inhibitor	20 units
mRNA	1–2 μg
50 mM MgCl$_2$	1 μl
H$_2$O to 20 μl	
murine reverse transcriptase	100–200 units

Incubate the reaction for 30 minutes at 37°C.

10 × Amplification buffer

500 mM KCl
100 mM Tris · Cl (pH 8.3 at room temperature)
15 mM MgCl$_2$
0.1% gelatin

10–50 pmoles of a synthetic oligonucleotide complementary to a desired sequence of the mRNA may be used in place of oligo(dT) as a primer.

Bovine serum albumin (Fraction V, Sigma; final concentration 100 μg/ml) may be substituted for gelatin in the reverse transcription reaction.

The amount of MgCl$_2$ in the amplification buffer needs to be supplemented with additional MgCl$_2$ in the reverse transcriptase reaction mixture because reverse transcriptase requires higher concentrations of divalent cations than does *Taq* DNA polymerase.

A method for rapidly preparing RNA suitable for chain reactions is given on p. 14.33.

2. Inactivate the reverse transcriptase by heating the reaction to 95°C for 5 minutes.

This step improves the efficiency of amplification and the "cleanness" of the product.

3. Add:

"upstream" oligonucleotide primer (i.e., primer complementary to the original mRNA)	10–50 pmoles
"downstream" oligonucleotide primer (i.e., primer complementary to the first strand of cDNA)	10–50 pmoles
1 × amplification buffer to a final volume of 79 μl	
Taq DNA polymerase (5 units/μl) (Perkin Elmer Cetus N801-0046)	1–2 units

The total amount of "upstream" primer in the amplification reaction should be 10–50 pmoles. This includes any "upstream" oligonucleotide primer that is used instead of oligo(dT) to prime the synthesis of the first strand of cDNA (see note to step 1).

The presence of excess oligonucleotide primers in the reaction often leads to amplification of undesirable nontarget sequences. It is therefore desirable to carry out preliminary experiments to determine the minimum amounts of the two primers that are required for satisfactory amplification.

4. Overlay the reaction mixture with 100 μl of light mineral oil (Sigma M-3516 or equivalent). This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Carry out the desired number of cycles of amplification as described on pages 14.18–14.19, step 5.