CHAPTER 4

Sequencing of double-stranded DNA

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1. INTRODUCTION

Since the introduction of the chain-termination method of DNA sequencing (1) the vast majority of applications of this method have probably involved single-stranded circular DNA from M13 bacteriophages (2,3,4). However, it has long been appreciated that there are considerable advantages in direct sequencing of double-stranded DNA. Long inserts, particularly of repetitive sequences, are often unstable in M13 phages but are usually stable in plasmid vectors. The sequencing of inserts in plasmids avoids the need for tedious subcloning into M13 phage and can be used to test the fidelity of constructions directly. Short inserts can often be sequenced completely by priming from both ends, avoiding the need for recloning into vectors with the polylinker in the opposite orientation or synthesis of the complementary strand of single-stranded inserts followed by repriming with primer to the complementary strand and chain-termination sequencing (5). Double-stranded DNA is also amenable to a number of directed deletion sequencing methods and the template products of such strategies can be easily and accurately screened for size, allowing a nested set of deletions to be selected.

For these reasons many attempts have been made to apply enzymatic chain-termination methods to double-stranded DNA (6−8). The number of bases of a plasmid insert which can now be sequenced by these methods is becoming comparable to that for single-stranded phages, although the overall sequencing quality, and hence accuracy, is still lower. However, because of the advantages outlined above, sequencing of plasmid DNA has become a powerful technique likely to be used increasingly as methods are improved.

DNA sequencing can also be applied to double-stranded DNA which has not been 'cloned' in the traditional sense, but obtained directly from the organisms under study, often using amplification by the 'polymerase chain reaction' (PCR). This is described in Section 3.

2. SEQUENCING OF DOUBLE-STRANDED CLONED DNA

The quality of the sequence obtained from double-stranded DNA, both in the number of bases and accuracy, depends to a very large extent on the purity of the DNA and rigorous application of the methods described below. There is often very little flexibility either in the quantity of reagents which can be added or in the timing of reactions. Where several different methods can be applied to a particular step the variations are given, but the convention used is that the first method is known to be the most efficient or productive. The other variations are included because of the natural conservatism of molecular biologists in such methods as plasmid isolation.

Table 1. Preparation of plasmid from minipreps by boiled lysis.

1	Toothpick a colony into 10 ml of LB[a] in a flat-bottomed, screw-top bottle and grow for 15–18 h at 37°C, shaking at 200–250 r.p.m. and using antibiotic selection if required.
2.	Centrifuge the cells at 1500 *g* for 10 min. Decant the supernatant and leave the inverted bottle to drain for 5 min on absorbent paper.
3.	Resuspend the cells by vortexing vigorously in 100 μl of 50 mM Tris–HCl pH 8, 25% sucrose, then transfer the suspension to a 1.5-ml microcentrifuge tube.
4.	Add 600 μl of MSTET[b] solution, then spot 14 μl of 40 mg ml^{-1} lysozyme in 50 mM Tris–HCl pH 8, 50% (v/v) glycerol[c] onto the inside of the tube.
5.	Cap the tubes, mix the contents thoroughly by shaking and transfer immediately to a boiling water bath for 1 min.
6.	Place the tubes on ice for 1 min, then centrifuge at 10 000 *g* for 30 min at 4°C.
7.	Remove the gelatinous pellet with a toothpick. Alternatively, to reduce contamination with chromosomal DNA, use a micropipette to transfer the supernatant to a fresh tube.
8	Add 60 μl of 3 M sodium acetate, pH 5, and 600 μl of isopropanol and leave on ice for 5 min before centrifuging at 10 000 *g* for 10 min. Aspirate off the supernatant, recentrifuge for 5 sec and remove all traces of liquid. Resuspend in 200 μl of TE[d].
9.	Extract the sample with 100 μl each of phenol and chloroform:isoamyl alcohol (25:1;v/v), vortexing vigorously for 1 min followed by centrifugation at 10 000 *g* for 4 min. Remove the supernatant, avoiding any contamination with the white interface, and repeat the phenol/chloroform extraction.
10.	Add 20 μl of 3 M sodium acetate, pH 5, and 550 μl of ethanol. Leave the tubes for 1 h at −70°C or at −20°C overnight. Centrifuge for 10 min at 10 000 *g*, then remove the supernatant and rinse the pellet with 500 μl of 80% ethanol stored at −20°C. Centrifuge for 2 min, aspirate off all traces of liquid and dry the pellet for 5 min under vacuum before dissolving the pellet in 50 μl of TE

[a]LB is 1% Bactotryptone, 0.5% Bacto yeast extract and 1% (w/v) NaCl.
[b]MSTET is 50 mM Tris–HCl pH 8, 50 mM Na_2EDTA, 5% (v/v) Triton X-100 and 5% (w/v) sucrose.
[c]The lysozyme solution is stable at −20°C for at least 2 weeks.
[d]TE is 10 mM Tris–HCl pH 8, 1 mM Na_2EDTA.

In the 'shotgun' approach to single-stranded DNA sequencing relatively short lengths of DNA are sequenced randomly, so that in a large sequencing project the same stretch of DNA may be sequenced up to six times. More recently methods have become available to increase the length of the oligonucleotide chains formed in a sequencing reaction to 1000 or more bases. The use of these methods provides two approaches to sequencing double-stranded DNA:

(i) to aim to generate readable sequence of 300–350 bases at a time, and when sequencing large inserts to generate a size-nested set of deletions by directed deletion techniques,

(ii) to attempt to obtain as much sequence information as possible from any insert, by synthesizing long oligomer chains and separating them on several gels of different acrylamide concentrations and with extended run times.

With either approach some additional effort has to be invested, either in generating and screening deletions or in handling long gels. Deciding which approach to use is largely subjective. However, using deletion techniques and reading 300–350 bases per template does provide security, in that as in 'shotgun' techniques the sequence may be confirmed by sequencing the same region several times. The methods initially described here favour the first approach, but alternative protocols are given for those tending towards the second.

Table 2. Preparation of plasm

A. Modified Birnboim and D

1.	Grow and centrifuge c
2.	Suspend the cells in 2 4 mg ml^{-1} lysozyme.
3.	Add 400 μl of 0.2 M leaving on ice for 5 m
4.	Add 300 μl of 3 M pot 5 min then centrifuge
5.	Remove 800 μl of the tube. Add 200 μl each for 4 min at 10 000 *g*
6.	Transfer the supernata leave on ice for 5 min centrifuge for 2 sec and 10 min.
7.	Resuspend the pellet in at 37°C.
8	Add 50 μl each of phe supernatant to a fresh tu as in *Table 1*.

B. Rapid isolation method.

1.	Grow the cells and centr pH 8, 25 mM Na_2EDT
2.	Add 250 μl of 0.3 M Na cool to room temperatu
3.	Add 80 μl of unbuffered
4.	Carefully transfer the up and 700 μl of isopropan at 10 000 *g* for 10 min, by aspiration.
5.	Dissolve the pellet in 10 Centrifuge for 4 min an add 110 μl of isopropan of cold ethanol, drying

[a]See *Table 1* stage 9 for prepar
[b]The RNase is dissolved in 25 m slowly to room temperature. St
[c]Dissolve 5 g of phenol in 5 ml

From this point the sequ use of plasmid DNA, but it to the replicative form of M sets and ? cloning strategi

2.1 Plasmid isolation

The two most popular meth and Quigley (9) and the all these methods for the prep

Table 2. Preparation of plasmid from minipreps by alkaline lysis.

A. Modified Birnboim and Doly (10) procedure.

1. Grow and centrifuge cells as described in *Table 1*.
2. Suspend the cells in 200 μl of 25 mM Tris -HCl pH 8, 10 mM Na_2EDTA, 50 mM glucose and 4 mg ml^{-1} lysozyme. Incubate for 5 min at room temperature then mix gently.
3. Add 400 μl of 0.2 M NaOH, 1% (w/v) SDS and mix by inverting the tube several times before leaving on ice for 5 min.
4. Add 300 μl of 3 M potassium acetate, 2 M acetic acid and vortex gently to mix. Leave on ice for 5 min then centrifuge at 10 000 *g* for 10 min.
5. Remove 800 μl of the supernatant, being careful not to disturb the pellet, and transfer to a fresh tube. Add 200 μl each of phenol and chloroform: isoamyl alcohol[a], vortex for 1 min and centrifuge for 4 min at 10 000 *g*.
6. Transfer the supernatant to a fresh tube containing 800 μl of isopropanol. Mix by vortexing and leave on ice for 5 min before centrifugation at 10 000 *g* for 10 min. Aspirate off the supernatant, centrifuge for 2 sec and remove all liquid with a drawn-out pasteur pipette. Dry under vacuum for 10 min.
7. Resuspend the pellet in 100 μl of TE, add 3 μl of 10 mg ml^{-1} RNase A[b] and incubate for 20 min at 37°C.
8. Add 50 μl each of phenol and chloroform[a], then vortex and centrifuge as above. Transfer the supernatant to a fresh tube containing 10 μl of sodium acetate pH 5 and add 300 μl ethanol. Continue as in *Table 1*.

B. Rapid isolation method.

1. Grow the cells and centrifuge them as in (A). Resuspend each pellet in 250 μl of 25 mM Tris–HCl pH 8, 25 mM Na_2EDTA, 0.3 M sucrose and 2 mg ml^{-1} lysozyme and incubate on ice for 30 min.
2. Add 250 μl of 0.3 M NaOH, 2% SDS with immediate vortexing. Incubate for 15 min at 70°C then cool to room temperature in a water bath.
3. Add 80 μl of unbuffered phenol/chloroform[c], vortex for 30 sec and centrifuge at 10 000 *g* for 4 min.
4. Carefully transfer the upper phase to a fresh tube containing 70 μl of unbuffered 3 M sodium acetate and 700 μl of isopropanol. Vortex to mix and leave on ice for 5 min followed by centrifugation at 10 000 *g* for 10 min. Aspirate off the supernatant, recentrifuge briefly and remove all the liquid by aspiration.
5. Dissolve the pellet in 100 μl of TE and vortex for 1 min with 50 μl each of phenol and chloroform[a]. Centrifuge for 4 min and transfer the upper phase to 10 μl of unbuffered 3 M sodium acetate and add 110 μl of isopropanol. Leave on ice and centrifuge as above before rinsing the pellet in 500 μl of cold ethanol, drying and dissolving in 50 μl of TE.

[a]See *Table 1* stage 9 for preparation.
[b]The RNase is dissolved in 25 mM Tris–HCl pH 8 and 10 mM NaCl. Boil for 10 min and allow to cool slowly to room temperature. Store at −20°C.
[c]Dissolve 5 g of phenol in 5 ml of chloroform, 1 ml of H_2O and 5 mg of 8-hydroxyquinoline.

From this point the sequencing of double-stranded DNA will be exemplified by the use of plasmid DNA, but it should be recognized that the same techniques can be applied to the replicative form of M13 phage or lambda phage. Protocols for generating deletion sets and ? cloning strategies are given in Chapter 1.

2.1 Plasmid isolation

The two most popular methods of plasmid isolation are the boiled-lysis method of Holmes and Quigley (9) and the alkaline-lysis method of Birnboim and Doly (10). The use of these methods for the preparation of plasmid from minipreps is described in *Tables*

Table 3. Large-scale plasmid isolation.

1.	Grow cells overnight in 400 ml of LB[a] with antibiotic selection if required. Centrifuge at 1500 *g* for 10 min, decant off the supernatant and invert container to drain for 2 min on an absorbent pad.
2.	Resuspend the cells in 1 ml of 25 mM Tris−HCl pH 8, 10 mM Na_2EDTA and 50 mM glucose. Transfer to a 50-ml centrifuge tube and add 7 ml of the same solution containing 2 mg ml^{-1} lysozyme. Leave on ice for 30 min with occasional gentle mixing.
3.	Add 16 ml of 0.2 M NaOH, 1% (w/v) SDS, seal the tube and mix by inverting several times before leaving for 10 min on ice.
4.	Add 12 ml of 3 M potassium acetate, 2 M acetic acid, seal the tube and shake vigorously to mix. Leave on ice for 30 min then centrifuge at 10 000 *g* for 10 min.
5.	Decant supernatant into a 100-ml centrifuge tube through Miracloth[b] to remove any floating particles. Add ethanol to fill the tube, seal and mix by inverting. Leave at −20°C for 30 min.
6.	Collect the flocculent precipitate by centrifugation at 10 000 *g* for 5 min. Decant off the supernatant and drain the tube by leaving inverted for 5 min on an absorbent pad. Dissolve the pellet in 10 ml of TE, transfer to a 50-ml centrifuge tube on ice and add 5 ml of 7.5 M ammonium acetate. Leave for 30 min and then centrifuge at 10 000 *g* for 10 min.
7.	Pour off the supernatant into a fresh 50-ml centrifuge tube and add 150 μl of 1 M $MgCl_2$ and 30 ml of ethanol, mix and leave at −20°C for 30 min. Centrifuge for 10 min at 10 000 *g*, remove the supernatant and rinse the pellet with 5 ml of cold 80% ethanol. Dry under vacuum for 5 min.
8.	Add 2 ml of 10 × TE and dissolve the pellet before adding 3.22 g of CsCl and 0.6 ml of 10 mg ml^{-1} ethidium bromide. Make up the volume to 4.3 ml before centrifuging at 12 000 *g* for 20 min. Transfer the supernatant to an appropriate centrifuge tube, fill the tube with rebanding solution[c] and centrifuge at 200 000 g for 15 h.
9.	Visualize the plasmid band in long wave UV and remove by inserting a 21G needle on a 1-ml syringe into the tube below the plasmid band. Transfer ~ 1 ml of solution to a fresh centrifuge tube, make up the volume with rebanding solution and centrifuge for 6 h at 20 000 *g*. Remove the plasmid band as above, and transfer to a 15-ml siliconized centrifuge tube.
10.	Remove the ethidium bromide by extracting 3−4 times with 3 ml of isoamyl alcohol saturated with 50 mM Tris−HCl pH 8, 1 mM Na_2EDTA, vortexing to mix the phases. Add 3 vol of H_2O to the sample, followed by 40 μl of 1 M $MgCl_2$ and 8 ml of ethanol. Leave at −20°C for 1 h and then centrifuge at 10 000 *g* for 10 min.
11.	Dissolve the pellet in 0.4 ml of TE and add 40 μl of 3 M sodium acetate, 5 μl of 1 M $MgCl_2$ and 1.1 ml of ethanol. Leave at −20°C for 1 h, centrifuge at 10 000 *g* for 10 min. Rinse the pellet with 2 ml of cold ethanol, dry under vacuum for 5 min and dissolve in TE.

[a]See *Table 1*.
[b]Calbiochem.
[c]Dissolve 76.62 g of CsCl and 9.53 ml of 10 mg ml^{-1} ethidium bromide in 10 × TE to a volume of 100 ml.

1 and *2*, which include a rapid alternative method (11), while a large-scale alkaline-lysis method employing caesium chloride gradient purification of plasmid DNA is outlined in *Table 3*. All of these methods produce sequencing-quality DNA, but the boiled-lysis method appears to produce templates which generate fewer artefacts upon sequencing. Caesium chloride gradient purified DNA provides a useful control against which other techniques can be judged, as the DNA is far less contaminated (e.g. with chromosomal DNA, RNA) than miniprep DNA. However, some background is often observed when sequencing gradient purified DNA, perhaps due to some nicking of DNA in ethidium bromide solutions when exposed to UV light.

The amount of DNA obtained from minipreps is variable and depends on plasmid copy number, but as a rough guide one would expect 25−30 μg from a 10-ml boiled-lysis preparation and about 20−25 μg using alkaline lysis.

The preparation of RF-DNA from M13 phage-transfected cells is described in *Table*

Table 4. Preparation

1.	Grow a cultu
2.	Transfer 1 ml from M13 tr
3.	The cells are inoculate 400 the cells reac a further 4 h. be 200−400

[a]Minimal medium ingr 10 ml of 20% (w/v) g 100 ml of M9 salts (7 of 100 ml).
[b]YT is 8 g of Bactotry

Table 5. Chain-termin

1	Mix the follov If using 7-dea

dCTP
dGTP
dTTP
ddATP
ddCTP
ddGTP
ddTTP
T0.1E[a]

[a]T0.1E is 10 mM Tris

4. The yields of RF number per cell; ty

2.2 Sequencing

2.2.1 *Equipment*

Sequence reactions a onto the walls of 1.5 mixed by brief cent trations are adjusted PB600 repeating dis

2.2.2 *Standard cha*

The mixes of dNTPs in *Table 5*. With the the end of the primer from the standard pr or 700−800 bases f

Table 4. Preparation of M13 RF-DNA.

1. Grow a culture of a suitable host in 10 ml of minimal medium[a] at 37°C with shaking overnight
2. Transfer 1 ml of the culture to 10 ml of YT[b] and shake at 37°C for 1 h before adding a single plaque from M13 transfected cells. Grow for 6 h and then centrifuge the cells at 1500 *g* for 10 min.
3. The cells are then treated as for a plasmid miniprep (*Table 1*). If a large-scale RF prep is required, inoculate 400 ml of YT with 4 ml of the overnight cell culture in minimal medium and grow until the cells reach an OD_{640} of 0.5–0.6. Add the supernatant from step 2 to the culture and grow for a further 4 h. Centrifuge the cells at 1500 *g* for 10 min and continue as in *Table 3*. The yield should be 200–400 μg per litre of culture.

[a]Minimal medium ingredients are autoclaved separately then mixed aseptically. Mix together 887 ml of water, 10 ml of 20% (w/v) glucose, 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 M $CaCl_2$, 1 ml of 1 M thiamine–HCl and 100 ml of M9 salts (7 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl and 0.5 g of NaCl in a total volume of 100 ml).
[b]YT is 8 g of Bactotryptone, 5 g of yeast extract and 5 g of NaCl in a volume of 1 litre.

Table 5. Chain-termination mixes for sequencing.

1. Mix the following amounts of 0.5 mM dNTPs and 5 mM ddNTPs (dissolved in T0.1E buffer[a]): If using 7-deaza-dGTP, substitute for dGTP in same molar quantities.

	A mix	*C mix*	*G mix*	*T mix*
dCTP	250 μl	12.5 μl	250 μl	250 μl
dGTP	250 μl	250 μl	12.5 μl	250 μl
dTTP	250 μl	250 μl	250 μl	12.5 μl
ddATP	1.5 μl (3 μl for [^{32}P]dATP)		–	–
ddCTP	–	8 μl	–	–
ddGTP		–	16 μl	–
ddTTP	–		–	50 μl
T0.1E[a]	250 μl	480 μl	470 μl	440 μl

[a]T0.1E is 10 mM Tris–HCl pH 8, 0.1 mM Na_2EDTA.

4. The yields of RF are lower than from plasmid minipreps because of the low copy number per cell; typically 4 μg per 10 ml of culture.

2.2 Sequencing

2.2.1 *Equipment*

Sequence reactions are performed with up to 10 templates in a set. Reagents are dispensed onto the walls of 1.5-ml lidless centrifuge tubes held in Eppendorf 10 place racks and mixed by brief centrifugation in an Eppendorf 5413 centrifuge. The reagent concentrations are adjusted so that multiples of 2 μl can be dispensed rapidly using a Hamilton PB600 repeating dispenser.

2.2.2 *Standard chain-termination mixes*

The mixes of dNTPs and ddNTPs used in the standard sequencing protocol are given in *Table 5*. With these mixes good sequence can be obtained from a few bases from the end of the primer up to 350 bases from it. Mixtures which can be used in deviations from the standard protocol for sequencing either very close to the end of the primer or 700–800 bases from it are described in Sections 2.2.6 and 2.2.7.

Table 6. Preparing templates for sequencing.

A. Spin-dialysis method

1. Mix 18 μl (12–15 μg of DNA) of a plasmid miniprep with 2 μl of RNase A (*Table 2*) and incubate for 20 min at 37°C. Add 5μl of 1 M NaOH, 1 mM Na_2EDTA and incubate for 15 min at 37°C. If using caesium chloride gradient purified DNA use 15 μg in 20 μl of TE without RNase treatment.
2. Add the sample to the top of the gel of a prepared spin-dialysis tube (*Table 7*), being careful not to disturb the gel layer. Centrifuge at 200 *g* for 4 min and use the dialysate immediately

B. Precipitation method

1. Follow the steps in (A) above, but instead of applying the sample to a spin-dialysis tube, neutralize it by the addition of 2.5 μl of 2 M ammonium acetate pH 4.5.
2. Add 100 μl of ethanol stored at −20°C and leave the sample at −70°C for 10 min. Centrifuge at 10 000 *g* for 10 min, remove the supernatant and rinse the pellet with cold ethanol.
3. Dry the pellet under vacuum for 5 min and dissolve in 25 μl of T0.1E[a] and use immediately.

C. Linearization method

1. Mix 18.5 μl of plasmid miniprep DNA with 2 μl of RNase A (*Table 2*), or use 15 μg of caesium chloride gradient purified plasmid DNA in 20.5 μl of TE, together with 2.5 μl of 10 × restriction buffer and 2 μl (20 U) of a restriction enzyme cutting the polylinker region on the opposite side of the insert to the primer site. Incubate at 37°C for 30 min.
2. Boil the sample for 5 min, cool rapidly on ice and use immediately.

[a]10 mM Tris–HCl pH 8, 0.1 mM Na_2EDTA.

Table 7. Preparation and testing of spin-dialysis tubes.

A. Preparation

1. Equilibrate Sepharose-CL6B[a] in T0.1E[b] and adjust the buffer volume to produce a packed gel:buffer supernatant ratio of 2:1.
2. Pierce the base of a 0.5-ml centrifuge tube with a 21G needle so that about 2/3 of the needle bevel emerges. Place the tube inside a 1.5-ml centrifuge tube completely pierced through the bottom with the same needle.
3. Add 25 μl of a slurry of 200 micron glass beads[c] in water to the 0.5-ml tube, followed by 300 μl of the Sepharose slurry.
4. Place the assembly into a 9-mm internal diameter tube and centrifuge at 200 *g* for 4 min. Transfer the 0.5-ml tube to an intact 1.5-ml test-tube and use within an hour to prevent drying of the gel matrix.

B. Testing

1. Prepare tubes as in A.
2. Add 25 μl of 10 mg ml^{-1} Blue Dextran 2000[a] and 10 mg ml^{-1} Orange G dye in TE to the top of the Sepharose and centrifuge as above.
3. Transfer the spin-column to a fresh tube, add 25 μl of TE and recentrifuge. More than 90% of the Blue Dextran should be present in the first dialysate, while no Orange G should pass through in the second.

[a]Pharmacia.
[b]10 mM Tris–HCl pH 8, 0.1 mM Na_2EDTA.
[c]Jencons Ballotini beads, No. 11.

2.2.3 *Denaturation of template DNA*

In order for the primer to be able to bind to the priming site the two DNA strands must first be separated. The two main methods are denaturation by alkali (8) and linearization of the plasmid by cutting with a restriction enzyme followed by denaturation

Table 8. Sequencing re

1. If sequencing wi
   1 μl of 10 μg ml
   amount of prime
2. Incubate at 37°C
3. Dispense 2.4 μl
4. For 10 templates
   Klenow fragmen
   quickly dispense
   in a water bath
5. Spot 2 μl of a ch
   tubes and centrifi
6. Terminate the rea
   formamide, 200
   blue.
7. Immediately bef
   than 2 min, then

[a]10 × TM is 100 mM T
[b]Boehringer Mannheim 5
[c][α-^{35}S]dATP at 500 Ci

by boiling (6,7), both
method involves ne
recovery of the DN
enzyme digestion or
treated DNA and re
spin-dialysis column
dialysis confers the a
of low molecular we
tions. This method i

(i) it is rapid,
(ii) precipitation c
contaminated
(iii) DNA linearize
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The amount of DN
a short insert from bo
Denatured templates
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enzyme protocols, but
to use spin-dialysis fo
80% of the volumes

2.2.4 *Chain-termina*

Following denaturati
37°C (*Table 8*). The
cooling to room tem
the complementary st

Table 8. Sequencing reactions.

1.	If sequencing with [α-^{35}S]dATP, 8.5 μl of the prepared template is added to 1 μl of 10 × TM[a] and 1 μl of 10 μg ml^{-1} primer. For sequencing with [α-^{32}P]dATP use 5 μl of template with the same amount of primer and 10 × TM and make the final volume up to 10.5 μl with water.
2.	Incubate at 37°C for 15 min and centrifuge the tubes briefly to spin down any condensation.
3.	Dispense 2.4 μl to each of four tubes, followed by 2 μl of chain-termination mix.
4.	For 10 templates mix 9 μl of 10 × TM with 1 μl of 100 mM DTT, 66 μl of H_2O, 4 μl (20 U) of Klenow fragment of DNA polymerase 1[b] and 10 μl of [^{35}S]- or [^{32}P]dATP[c]. Mix thoroughly and quickly dispense 2 μl of the mixture onto the wall of each tube. Centrifuge to mix, place immediately in a water bath at 42°C and incubate for 10 min.
5.	Spot 2 μl of a chase mix (0.5 mM in each of dATP, dCTP, dGTP and dTTP) onto the wall of the tubes and centrifuge to mix. Return the samples to the bath and continue the incubation for 5 min.
6.	Terminate the reaction by the addition of 4 μl of formamide dye mix, consisting of 10 ml of deionized formamide, 200 μl of 0.5 M Na_2EDTA pH 8 and 10 mg each of xylene cyanol and bromophenol blue.
7.	Immediately before loading onto an acrylamide gel denature the samples by boiling for no more than 2 min, then load 1.5–2 μl of each sample onto the gel.

[a]10 × TM is 100 mM Tris–HCl pH 8, 50 mM $MgCl_2$.
[b]Boehringer Mannheim 5 U μl^{-1}.
[c][α-^{35}S]dATP at 500 Ci mmol^{-1}, [α-^{32}P]dATP at 800 Ci mmol^{-1}, both at 10 mCi ml^{-1}.

by boiling (6,7), both described in *Table 6*. Conventionally, use of the alkali denaturation method involves neutralization of the sample followed by ethanol precipitation and recovery of the DNA by centrifugation. The time-consuming processes of restriction enzyme digestion or ethanol precipitation may be avoided by neutralizing the alkali-treated DNA and recovering the sample in its original volume by passage through a spin-dialysis column, the preparation of which is described in *Table 7*. The use of spin-dialysis confers the additional advantage of cleaning up the template by removing traces of low molecular weight compounds which may interfere with the sequencing reactions. This method is preferable to the two other methods in that:

(i) it is rapid,
(ii) precipitation of the DNA can cause sequencing artefacts if the DNA becomes contaminated with salt, and
(iii) DNA linearized by digestion often produces strong banding across all four lanes, the position of the artefact changing as the restriction enzyme is altered.

The amount of DNA used in the denaturation provides enough material to sequence a short insert from both ends, leaving sufficient DNA for a further reaction if required. Denatured templates can be stored frozen at −20°C for at least several weeks. The amount of starting material can be adjusted easily within the precipitation or restriction enzyme protocols, but because of possible problems of low recovery of DNA it is unwise to use spin-dialysis for samples smaller than a final volume of 20 μl i.e. using less than 80% of the volumes given in *Table 6*.

2.2.4 *Chain-termination reaction*

Following denaturation the template is annealed to the primer by brief incubation at 37°C (*Table 8*). There is no advantage in annealing at higher temperatures and slow cooling to room temperature, since this may cause problems through reannealing of the complementary strands and premature termination. The amount of primer indicated

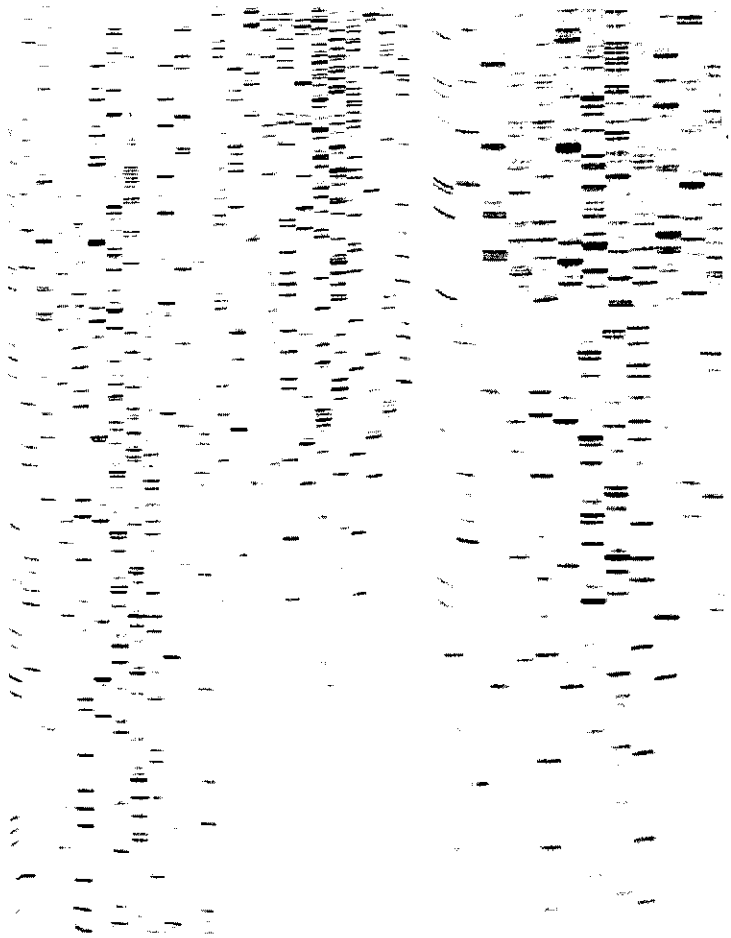

Figure 1. Supercoiled DNA sequencing gel. Three templates were loaded in the order A-C-G-T onto a 6% acrylamide gel and electrophoresed for 2 h before a second loading and electrophoresing for a further 90 min. The **left panel** is the entire gel with the first set of samples in the first 12 tracks. The **bottom right panel** shows the region centred on 50 bases from the gel bottom, the **middle right panel** the region centred on 150 bases and the **top right panel** the region centred on 250 bases.

is equimolar for 5 μl of a plasmid of 4 kb. Do not use larger amounts of primer, because at higher concentrations priming to sequences of lower specificity may occur, generating ghost bands on the gel (see Chapter 3, Section 4.2).

The reactions are performed at 42°C to reduce secondary structure formation in the template, particularly in regions of high G−C content. If such artefacts are observed the reaction temperature may be increased to 50°C, but if higher temperatures are used additional Klenow fragment of DNA polymerase I should be included in the chase-mix.

Table 9. Hig

1.	Prepa
	1 mM dCTP
	1 mM dGTP
	1 mM dTTP
	T0 1E buffer
	Followed by:
(i)	4 × S
	A mix
	C mix
	G mix
	T mix
(ii)	8 × St
	A mix
	C mix:
	G mix:
	T mix:
2.	Perforn
	chain-t
3.	Separat
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	blue in
4.	Run the
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2.2.5 *Gel el*

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Table 9. High ddNTP/dNTP ratio chain-termination mixes.

1. Prepare stock solutions by mixing:

	A0	*C0*	*G0*	*T0*
1 mM dCTP	100 µl	5 µl	100 µl	100 µl
1 mM dGTP	100 µl	100 µl	5 µl	100 µl
1 mM dTTP	100 µl	100 µl	100 µl	5 µl
T0.1E buffer	–	100 µl	100 µl	100 µl

Followed by:

(i) 4 × Stock
A mix: 37.5 µl A0 + 2 µl 1 mM ddATP + 60 µl T0.1E
C mix: 37.5 µl C0 + 3.2 µl 5 mM ddCTP + 60 µl T0.1E
G mix: 37.5 µl G0 + 6.4 µl 5 mM ddGTP + 56 µl T0.1E
T mix: 37.5 µl T0 + 20 µl 5 mM ddTTP + 40 µl T0.1E

(ii) 8 × Stock
A mix: 37.5 µl A0 + 4 µl 1 mM ddATP + 60 µl T0.1E
C mix: 37.5 µl C0 + 6.4 µl 5 mM ddCTP + 55 µl T0.1E
G mix: 37.5 µl G0 + 12.8 µl 5 mM ddGTP + 50 µl T0.1E
T mix: 37.5 µl T0 + 40 µl 5 mM ddTTP + 20 µl T0.1E

2. Perform the sequencing reactions as in *Table 8*, but substituting one of the above mixes for the standard chain-termination mix, and omitting bromophenol blue from the loading buffer.
3. Separate the samples on a 20 × 50 cm gel using an 8% w/v acrylamide mix and wedge-shaped gel spacers varying from 0.4 mm at the top to 1.2 mm at the bottom. Use loading buffer with bromophenol blue in adjacent lanes to act as markers.
4. Run the gel until the bromophenol blue is 5 cm from the bottom. Increase the fixing and drying times to 60 min each.

2.2.5 *Gel electrophoresis*

With the methods described in Section 2 and electrophoresis conditions as in Chapter 2, good, readable and unambiguous sequence extending to about 325 bases from the primer should be observed on a 50-cm, 6% acrylamide buffer gradient gel (12). However, strong bands are observed in regions above which the bands are not well separated (*Figure 1*). The number of readable bases can be increased by:

(i) separating part of the sample on a 50-cm 6% acrylamide non-gradient gel for about 100 min,

(ii) separating another part of the sample on a similar gel or a 5% acrylamide gel for about 4 h.

Using these methods the amount of readable sequence can be extended to around 450–500 bases. When extended separations are performed a region of blurred bands is frequently observed at around 350–400 bp. This is caused by a reaction between borate ions in the buffer and glycerol from the Klenow storage buffer and can be eliminated by passing the diluted enzyme mixture through a spin-dialysis column as described in *Table 7*, using Sepharose equilibrated in the reaction buffer, before the addition of [^{35}S]dATP.

Table 10. Sequencing in the kilobase range with the Klenow enzyme.

1. Prepare a primed template and dispense 2.4 μl into each of four tubes as described in Sections 2.2.3 and 2.2.4.
2. Prepare an extension and labelling mix 7.5 μM in dCTP, dGTP and dTTP (EL mix). Mix for the following approximate range of bases synthesized:

	10–500 bases	80–1000 bases	300–1200 bases
1 × TM buffer[a]	68 μl	65 μl	65 μl
0.1 M DTT	11 μl	11 μl	11 μl
EL mix	2.2 μl	4.5 μl	9 μl
Klenow enzyme (5 U μl^{-1})	4 μl	4 μl	4 μl
[^{35}S]dATP[b]	5 μl	5 μl	5 μl

3. Dispense 2 μl of the appropriate mix into each reaction tube. Mix by centrifugation and incubate at 42°C in a water bath for 5 min.
4. Add 2 μl of the appropriate termination mix per tube, centrifuge and return to the water bath for 5 min. Termination mixes:

	A mix	*C mix*	*G mix*	*T mix*
5 mM dATP	7 μl	70 μl	70 μl	70 μl
5 mM dCTP	70 μl	7 μl	70 μl	70 μl
5 mM dGTP	70 μl	70 μl	7 μl	70 μl
5 mM dTTP	70 μl	70 μl	70 μl	7 μl
5 mM ddATP	84 μl	–	–	–
5 mM ddCTP	–	28 μl	–	–
5 mM ddGTP	–	–	42 μl	–
5 mM ddTTP	–	–	–	140 μl
T0.1E	700 μl	750 μl	740 μl	640 μl

5. Add 4 μl of formamide dye (*Table 8*) and mix by centrifugation. Denature by boiling for 2 min and load 2 μl onto a suitable gel.

[a]*Table 8*.
[b]500 Ci mmol^{-1}, 10 mCi ml^{-1}.

Table 11. Sequencing in the kilobase range with Sequenase.

1. Proceed as in *Table 10*, with the exception that:
(i) the volume of enzyme added is reduced to 3 μl (36 U Sequenase),
(ii) the initial labelling reaction should be at room temperature;
(iii) the size ranges obtained with the three labelling mix dilutions are around 10–250, 20–450 and 100–1000 bases respectively.
2. The termination mixes are:

	A mix	*C mix*	*G mix*	*T mix*
5 mM dATP	25 μl	25 μl	25 μl	25 μl
5 mM dCTP	25 μl	25 μl	25 μl	25 μl
5 mM dGTP	25 μl	25 μl	25 μl	25 μl
5 mM dTTP	25 μl	25 μl	25 μl	25 μl
5 mM ddATP	2.5 μl	–	–	–
5 mM ddCTP	–	2.5 μl	–	–
5 mM ddGTP	–	–	2.5 μl	–
5 mM ddTTP	–	–	–	2.5 μl
T0.1E	900 μl	900 μl	900 μl	900 μl

Table 12. Resolving sequencing artefacts caused by secondary structure formation.

A. Use of dITP and Sequenase

1. Substitute 15 μM dITP in the extension and labelling mix of *Table 10*, in place of the dGTP.
2. Replace termination mix with that below.

	A mix	*C mix*	*G mix*	*T mix*
5 mM dATP	25 μl	25 μl	25 μl	25 μl
5 mM dCTP	25 μl	25 μl	25 μl	25 μl
5 mM dITP	50 μl	50 μl	50 μl	50 μl
5 mM dTTP	25 μl	25 μl	25 μl	25 μl
5 mM ddATP	2.5 μl	–	–	–
5 mM ddCTP	–	2.5 μl	–	–
0.5 mM ddGTP	–	–	5 μl	–
5 mM ddTTP	–	–	–	2.5 μl
T0 1E	875 μl	875 μl	875 μl	875 μl

3. Start the labelling reaction and immediately begin to dispense aliquots of the termination mixes onto the wall of the tubes. The labelling reaction should be carried out for as short a time as possible – ideally no more than 2 min.
4. Spin to mix and transfer immediately to a 37°C bath, then incubate for 3 min before the addition of formamide dye.

B. Stabilizing template with single-stranded DNA-binding protein

1. After denaturation of the template, spin-dialysis and priming, add between 1 and 5 μg (depending on the severity of the problem) of T4 Gene 32 protein[a] to the sample.
2. Following the sequencing reactions and the addition of formamide dye, add 0.5 μg of proteinase K[b] and incubate the mixture at 65°C for 20 min before samples are loaded onto the gel.

[a]Pharmacia.
[b]Boehringer.

2.2.6 *Sequencing close to the primer site*

When sequence adjacent to the primer is required, for example when it is necessary to sequence through the junction of vector and insert DNA to check that the sequence is in the correct protein coding frame, some modifications to the standard protocol are necessary (*Table 9*). By increasing the ddNTP/dNTP ratio chain termination occurs more easily, generating strong bands on the autoradiograph next to the primer site. To sharpen the bands the acrylamide concentration is increased to 8% and a wedge-shaped gel, 0.4 mm at the top and 1.2 mm at the bottom, is employed. Bromophenol blue should not be included in the formamide dye, as it has a deleterious effect on the separation and resolution of short oligomers. Instead a small amount of dye containing bromophenol blue should be loaded in spare tracks on the outer edges of the gel.

2.2.7 *Kilobase sequencing*

In conventional chain-termination sequencing the processes of extension, labelling and termination are occurring at the same time. Although the mean size of the oligomers formed can be varied by altering the ddNTP/dNTP ratio, the range spanned is around 450–500 bases. More recent sequencing techniques employ a two step-process involving separate labelling and extension stages. In the first labelling step a broad but adjustable range of strand sizes is created by varying the substrate concentration, then the nascent

labelled chains are elongated and terminated during the second stage. This can be performed either with the Klenow fragment of DNA polymerase I (13) or by modified T7 DNA polymerase (Sequenase; 14). The use of these methods using either enzyme is outlined in *Table 10* and *11*.

2.2.8 *Troubleshooting poor sequencing reactions*

More artefacts appear to be observed on an autoradiograph after double-stranded DNA sequencing than would be seen if the same insert were sequenced using ssDNA derived from an M13 vector (see Chapter 3). The majority of these artefacts are probably caused by the greater propensity of the denatured DNA to form interchain cross-links through reannealing. Many of the problems are caused through the use of poor quality templates, due to degradation of the sample during preparation or contamination with RNA or other materials, or by storing unprimed denatured DNA for long periods at temperatures where reannealing is likely to occur.

Where compressions are observed in the resulting autoradiograph these can often be eliminated by reducing the formation of hairpin loops in regions of dyad symmetry by using dITP or 7-deaza-dGTP (15) in the sequencing reactions (Chapter 2, Section 3.2). If 7-deaza-dGTP is used it can be substituted directly in the reaction mixture for the dGTP.

Strong bands occurring in all four lanes, often at several points in the sequence ladder, appear to be produced more frequently during double-stranded sequencing. These can often be eliminated by performing the chain extension and termination reactions at higher temperatures to overcome secondary structures. Perhaps the most effective way, however, is to use dITP rather than dGTP and to use Sequenase rather than the Klenow enzyme. If Sequenase is used with dITP, the initial labelling reaction should be performed briefly at room temperature, followed by a rapid extension and termination reaction. A chain-termination mixture for reactions employing Sequenase and dITP is given in *Table 12*. When 7-deaza-dGTP or dITP are used it is important to include a reaction employing the normal mixes on the same gel, as the substitutes may generate artefacts in other regions of the sequence.

If secondary structures are thought to be occurring and the methods described above do not overcome the problem an alternative is to use single-stranded DNA-binding protein in the sequencing reactions. This will prevent secondary structures being formed, but it must be removed by digestion with proteinase K before acrylamide gel separation, as it will otherwise retard the migration of the oligonucleotides. The procedure is also outlined in *Table 12*.

Some of the artefacts that are observed after autoradiography are described in *Table 13*, along with possible causes and remedies. It should be clear from this that the majority of these problems can be avoided by precise adherence to the protocols described above, together with the use of freshly prepared reagents and newly purchased enzymes.

3. GENOMIC SEQUENCING

There are numerous instances where it is desirable to have methods of rapidly determining sequences directly from genomic DNA, circumventing the time-consuming steps involved in cloning the DNA which is being analysed. There is therefore a need for

Table 13. Troubleshoo

Symptom
Faint bands on develop
High background on fi fuzzy bands
Occasional bands in all lanes

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are also discussed.

3.1 **Direct sequen**

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Table 13. Troubleshooting

Symptom	*Possible Causes*	*Remedies*
Faint bands on developed film.	Loss of DNA on spin-dialysis.	Check recoveries as described in *Table 7*.
	Insufficient primed template.	Increase amounts of DNA or primer; prime for 15 min.
High background on film, with fuzzy bands.	Templates contaminated with RNA.	Check quality of RNase; incubate template in 0.2 M NaOH at 37°C for 15 min before spin-dialysis.
	NaOH passing through spin-dialysis column.	Test efficiency of spin-dialysis as in *Table 7*.
	Template degraded by nucleases	Process minipreps as rapidly as possible. Phenol extract at the earliest opportunity.
	Template reannealing.	Add primer to samples as quickly as possible; do not prime for longer than 15 min; do not store unprimed templates.
Occasional bands in all four lanes.	Secondary structure in template or synthesized strand.	Perform reactions at 50°C; use 7-deaza-dGTP with the Klenow enzyme, or dITP with Sequenase; use single-stranded DNA binding protein in reactions (see *Table 12* and Section 2.2.8).

techniques to facilitate these analyses. The following sections are concerned with a brief description of the method of Church and Gilbert (16) for direct sequencing of genomic DNA, and the use of the polymerase chain reaction (PCR) to amplify enzymatically a selected DNA fragment (17,18). Current techniques for sequencing the amplified DNA are also discussed.

3.1 Direct sequencing of genomic DNA

The direct genomic sequencing method (16) is limited by the requirement for sequence information to identify specific primer and probe segments in the vicinity of the region to be sequenced, in addition to knowledge of adjacent restriction sites. The method involves the following procedures.

(i) Restriction of isolated genomic DNA to completion.
(ii) Random chemical cleavage of the restricted DNA using Maxam–Gilbert methodology (19).
(iii) Electrophoretic separation of the DNA fragments on a denaturing acrylamide gel.
(iv) Transfer of the DNA fragments onto a Nylon membrane followed by probing with relatively short single-stranded ^{32}P-labelled probe which is specific for one end of the genomic fragment.

In addition to providing sequence information, this method enables the methylation levels of cytosines in vertebrates and plants to be quantitated, as hydrazine (the reagent

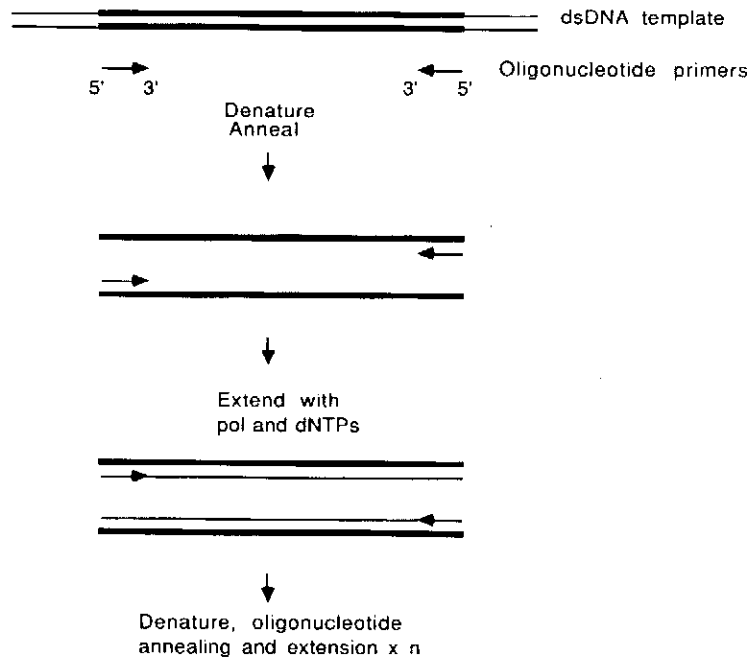

Figure 2. Schematic representation of the PCR. Thickened lines represent the region of DNA to be amplified.

Table 14. Polymerase chain reaction.

1.	Mix in a 0.5-ml Eppendorf tube: 5 μl of 10 × amplification buffer (500 mM KCl, 100 mM Tris−HCl pH 8.4, 25 mM $MgCl_2$, 2 mg ml^{-1} gelatine) 5 μl of a mix of 5 mM each of dATP, dCTP, dGTP and TTP 1 μg of genomic DNA 1−2 units Taq pol Sterile distilled H_2O to 100 μl.
2.	Spin briefly in a microcentrifuge and overlay with approximately 100 μl of liquid parafin. Subject the reaction to 25−30 cycles of amplification in a programmable heating block, 1 min at 95°C (denaturation of DNA) 1 min at 50°C (annealing of primers) 2 min at 72°C (polymerase extension of primers)
3.	Analyse PCR products by agarose or acrylamide gel electrophoresis.

used in Maxam−Gilbert sequencing to modify cytosines and thymines prior to cleavage) reacts poorly with 5-Me cytosine.

The limitation in direct genomic sequencing is lack of resolution, however, due to the fact that the DNA of interest is present in extremely low concentrations compared to the level of other DNA.

3.2 Polymerase chain reaction

The virtue of the recently developed PCR (17,18) is that it allows the amplification of specific regions of (genomic) DNA by a factor of approximately 10^6. For PCR, suitable primer design relies on knowledge of the sequences associated with the region of DNA to be amplified. Thus the method is of particular use in the diagnosis of genetic disorders (18,20–22), the analysis of allelic sequence variations (23), or any project where the rapid cloning and sequence determination of homologous DNA fragments is required. The enzymatic amplification is directed by sequence-specific primers, and involves repeated cycles of heat denaturation of the DNA, annealing of complementary primers and extension of the annealed primer with a DNA polymerase (*Figure 2*). This results in the exponential increase (2^n) of the target DNA, and over a million copies of the desired DNA sequence may be generated in several hours. Moreover, the error rate over many cycles of amplification is sufficiently low to enable reliable genomic sequence information to be obtained from the amplified product (24).

The earlier reports of the PCR (17,18) involved the use of Klenow fragment of *Escherichia coli* polymerase I. As this enzyme is heat-labile, it was necessary to add fresh polymerase at each cycle. The Klenow polymerase amplification also resulted in the generation of a heterogeneous set of products in addition to the target fragment, probably due to non-specific priming and non-processivity of the enzyme under the conditions of the PCR (23). The recent development of the use of a thermostable polymerase isolated from *Thermus aquaticus* has overcome these problems (25), as synthesis of DNA at a higher temperature allows less non-specific priming. Using this enzyme ('Taq polymerase'), detection of, for example, a single copy of target DNA following amplification from 10 μg of genomic DNA is possible (26). The amplification of RNA transcripts using Taq polymerase can also be carried out using PCR (27); the method involves conversion of the mRNA to a cDNA copy using reverse transcriptase, and then amplification of the cDNA using PCR. Further improvements in the PCR reaction have made the direct amplification of target fragments from genomic DNA routinely possible (26), thus superseding the more time-consuming method involving cDNA synthesis in many instances.

3.2.1 *Equipment and protocol*

Buffers, primers and enzymes for the PCR should be stored at −20°C. It is advisable to store buffers and primers in aliquots to avoid excessive freeze-thawing. Gel purification (28) of oligonucleotide primers is recommended. Programmable Dri-Block PHC-1 may be obtained from Techne (Cambridge) Ltd, Duxford, Cambridge CB2 4PZ, UK. Taq polymerase may be obtained from a variety of commercial suppliers. We have found the Cetus, New England Biolabs and Koch-Light products give satisfactory results.

Reaction. The optimal PCR conditions will depend on the source of polymerase, the DNA to be amplified, and the length of the target sequence and primers. Thus it is not possible to give conditions which are universally appropriate for all PCR reactions, but typically a PCR would be set up as in *Table 14*. Optimization of the reaction conditions for a given amplification is recommended, for example by varying the cycle times and temperatures. The reader is referred to the references cited, and product profiles, for details of suitable conditions.

3.2.2 *Sequencing the amplified products*

The nucleotide sequence of the fragments generated by PCR can be determined in one of several ways. The amplified product can either be cloned into the M13mp sequencing vectors (23,24), or directly sequenced (29,30).

(i) *Cloning into M13 phage.* To facilitate cloning the amplified DNA into M13 (see Chapter 1 for details concerning the use of these vectors), selected restriction sites can be incorporated at the 5′ ends of the amplification primers (23,24). Thus amplification followed by restriction and purification of the target DNA generates a fragment which can be readily cloned into M13 RF DNA. The presence of unpaired bases near the 5′ ends of the primers does not appear to affect the efficiency of the amplification (23,24), and during later cycles these oligonucleotides anneal to the amplified products with 100% complementarity, rather than the original genomic sequences.

(ii) *Direct sequencing.* Direct sequencing of the amplified DNA is possible using the methodology for sequencing dsDNA in Section 2 of this chapter.

3.2.3 *Generation of ssDNA for sequencing*

A recent development in the use of PCR for genomic sequencing is the generation of ssDNA copies of the target DNA in a single (31−33), or at most, two enzymatic steps (34), thus circumventing the need for M13 cloning. Two methods can currently be used to do this. The more straightforward involves the use of unequal molar amounts of the two amplification primers so that an excess of ssDNA of the selected strand is produced. The ssDNA can be used directly in chain-termination sequencing reactions. In addition, Taq polymerase has been used in chain-termination sequencing reactions containing the ssDNA generated by 'asymmetric PCR' as template (33). The processivity and heat stability of this enzyme make it particularly suitable for sequencing GC-rich DNA, where the formation of secondary structures in the template may cause problems with heat-labile polymerases such as Klenow fragment or Sequenase.

An alternative PCR method involves two steps to generate an RNA copy of the amplified material. This method uses a PCR primer with a phage promoter attached at the 5′ end (34). Following amplification, the PCR products are transcribed into RNA and sequenced using reverse transcriptase and chain-termination sequencing (see Chapter 6).

3.3. **Prospects**

Methodology for the sequencing of genomic DNA is developing rapidly. The PCR provides a convenient and rapid way of producing relatively large quantities of sequenceable material from a selected locus in genomic DNA, and enables work which would formerly take weeks to be performed in a several hour automated reaction. The methods for generating ssDNA using PCR will also facilitate the development of automated sequencing systems.

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