

Dideoxy Sequencing of DNA

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The enzymic sequencing approach uses a DNA polymerase to synthesize transcripts that have been terminated at specific bases. A primer anneals to a complementary region on the template strand and acts as a starting point for DNA synthesis, which occurs in the presence of a mixture of deoxyribonucleotides (building blocks) and specific dideoxyribonucleotides (chain-terminators). Originally four separate reactions were set up, each with a base-specific chain-terminator. Separation of these reaction products on a DNA sequencing gel will generate a ladder of bands indicating the position of each terminated base relative to the end of the sequencing primer. A homogeneous sequencing template should generate a single band in one of the four lanes where a band in the 'A stop' lane indicates an 'A', a band in the 'C stop' lane represents a 'C' and so on. In this manner it is possible to deduce the DNA sequence of the target template by identifying consecutive bands in one of the four lanes of the ladder. Since DNA synthesis occurs in the 5' to 3' direction, the sequence is read 5' to 3' from the bottom to the top (origin) of the gel. Although it was originally suited to the sequencing of single-stranded DNA templates, there have been many advances in the chemistry of enzymatic sequencing, including the application of thermostable DNA polymerases and fluorescent dye-terminators as used in automated DNA sequencers.

Introduction

In 1977 Sanger and colleagues published an enzymatic method for DNA sequencing using dideoxynucleotides as base-specific chain-terminators (Sanger *et al.*, 1977). Within a few years, this approach became the preferred method of choice, mainly owing to ease of handling and generation of data and limited exposure to hazardous chemicals, especially compared to the chemical cleavage method of DNA sequencing (Maxam and Gilbert, 1977). Today the chemical cleavage approach is generally restricted to those cases where it may be necessary to sequence short chemically synthesized oligonucleotides, which cannot be analysed using chain terminator chemistry. Many oligonucleotides are too small for a sequencing primer to be used and the sequence at the priming site cannot be deduced.

There have been enormous advances in DNA sequencing technology, starting with a range of different DNA polymerases: Klenow (Sanger *et al.*, 1977), T4 DNA polymerase (Cameron-Mills, 1988) and AMV reverse transcriptase, finally culminating in *Taq* DNA polymerase (Innis *et al.*, 1988), one of its modified derivatives or other thermostable DNA polymerases. These thermostable polymerases facilitated the development of cycle sequencing (Lee, 1991), essentially a one-sided PCR that requires less starting template, can overcome potential secondary structure problems, and is highly suited to the direct sequencing of double-stranded PCR products or plasmids.

In all these cases of enzymic sequencing, the basic theme is to have a reference point (the sequencing primer) that is extended by the DNA polymerase in the presence of deoxyribonucleoside triphosphates (dNTP = building block) and a dideoxyribonucleoside triphosphate (ddNTP = chain-terminator). **Figure 1** shows that the crucial difference between a deoxyribonucleoside triphosphate and a dideoxyribonucleoside triphosphate is the missing hydroxyl group at the 3' position of the dideoxy analogue (indicated by an arrow).

The significance of this hydroxyl group is evident because DNA extension results from the stepwise addition of the incoming base to that 3' hydroxyl group. The incorporation of a dideoxynucleotide into a DNA strand prevents further extension of that transcript since there is no attachment site for the incoming base, so that further DNA extension of that strand is blocked (**Figure 2**).

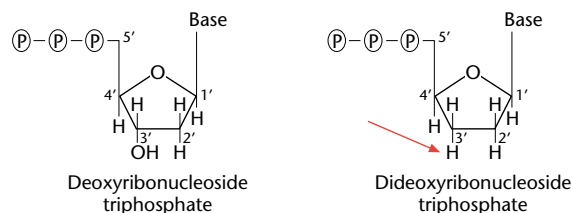


Figure 1 Deoxynucleoside triphosphate and its analogue the dideoxynucleoside triphosphate.

Secondary article

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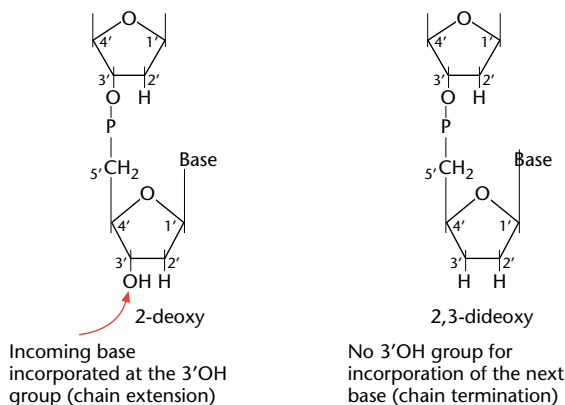


Figure 2 Chain termination upon the incorporation of a dideoxynucleoside triphosphate.

A set of sequencing reactions consists of four separate reactions, each with its own chain terminator:

- A stops owing to the incorporation of ddATP.
- C stops owing to the incorporation of ddCTP.
- G stops owing to the incorporation of ddGTP.
- T stops owing to the incorporation of ddTTP.

In this manner, gel fractionation of the 'A stop' products would determine the position of all the 'A' residues relative to the end of the sequencing primer, which provides the reference point from which the position of other As can be established. The position of the other nucleotides is established with the cofractionation of all stop reactions in adjacent lanes of a DNA sequencing gel, which is capable of resolving single base differences in DNA transcript length. The resultant ladder of bands allows the DNA sequence to be determined.

The products of the sequencing reactions can be visualized by autoradiography after the incorporation of some radioactive tag either on to the primer or into the sugar phosphate backbone of the DNA transcripts. ^{32}P was one of the original and commonly used radioisotopes, but there has been a trend towards other isotopes such as ^{33}P or ^{35}S . These isotopes are safer to use, have longer half-lives and can produce autoradiographs with greater resolution of the bands.

One labelling option is to incorporate the tag on to the primer (via a 5' labelling reaction using γ -labelled rATP and T4 polynucleotide kinase). Radiolabelling of chemically synthesized primers in this manner is very efficient as they are made with only a hydroxyl group at the 5' end and the kinasing efficiency is typically in excess of 90%: that is, 90% of the radiolabel becomes incorporated onto the kinased primers. The advantage of this approach is that the sequence can be deduced immediately from the primer, as shown in **Figure 3**. All fragments have one radiolabelled tag, so that the band labelling should be uniform, regardless of the size of the transcript.

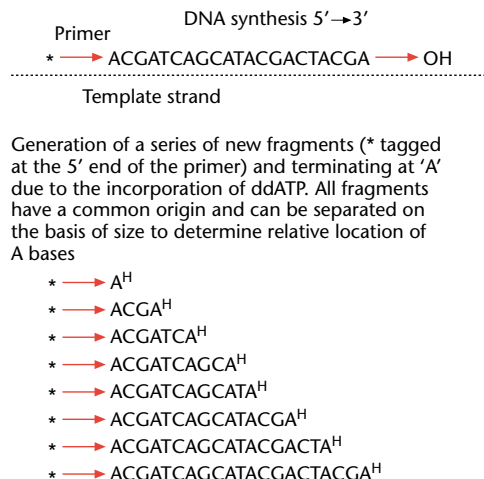


Figure 3 5'-terminal labelling of DNA sequencing fragments via the primer.

An alternate approach to labelling is to incorporate the tag into the DNA strand during the extension/termination reactions. This system can be easier than using labelled primers since it bypasses the primer labelling steps (which can become tedious if several different sequencing primers have to be used). The direct incorporation method can also offer greater labelling efficiency, especially of longer transcripts where multiple radioactive isotopes can be incorporated. One limitation of this approach is that the DNA sequence immediately adjacent to the priming site will be difficult to determine. Shorter transcripts will not be labelled until radioactive nucleotides have been incorporated. Until then, these DNA transcripts cannot be visualized on the autoradiograph, as shown in **Figure 4** where the first few bases will not be detected until the incorporation of the first labelled C.

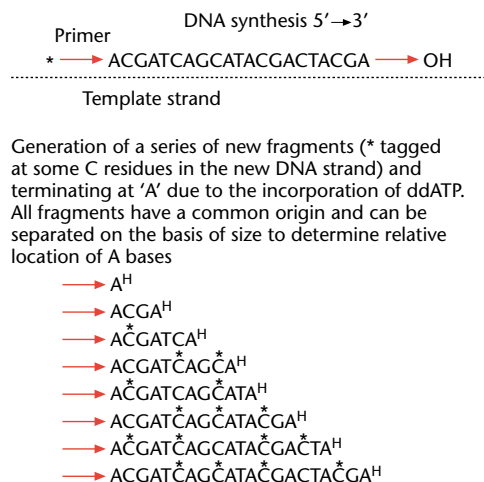


Figure 4 Incorporation of α -radiolabel into the sugar phosphate backbone.

These chain termination reactions have to be carried out separately with each set of reaction products being fractionated on four adjacent lanes of a sequencing gel. The sequencing of eight templates requires setting up eight template mastermixes, 32 separate stop reactions (eight for 'A', eight for 'C', etc.) and then loading each reaction on to a sequencing gel (i.e. 32 lanes). An example of this is shown in **Figure 5**.

The aim of automated DNA sequencing is to minimize the effort required to generate, collect and process maximum sequence data. There have been significant advances in the development of chemistry and equipment for automated sequencing. Arguably the most significant development involves different fluorescent tags that allow fractionation of the entire sequencing ladder in a single lane on a gel. It is possible to set up four separate stop reactions with four different fluorescently tagged primers (one for each base); upon completion of the sequencing reactions, the mixtures can be combined and then fractionated on a single lane of the sequencing gel. A further refinement has taken place with the development of dye-labelled chain-terminators (dye-terminators or dye-deoxynucleotides) whereby the fluorescent tag is added to the DNA strand upon incorporation of each dideoxy nucleotide. Each dideoxy nucleotide is coupled to a characteristic dye so that the incorporation of ddATP will result in a 'green' dye tagged on to the end of A-terminated transcripts.

This protocol on dideoxy sequencing will refer only to one style of manual sequencing in which a thermostable DNA polymerase is used in cycle sequencing reactions. There are other versions and kits available for other

methods of DNA sequencing. For example, T7 DNA polymerase is commonly used in sequencing reactions as the evenness of peak heights generated in the ladder facilitates the detection of base changes in heterozygous or mixed templates. T7 DNA polymerase and sequencing kits are available from US Biochemicals.

One of the most common sequencing approaches uses *Taq* polymerase in cycle sequencing reactions. The combination of thermostable polymerases and dideoxy chain terminators is highly suited to sequencing double-stranded DNA and PCR products. The high temperatures used in the DNA polymerization steps can overcome potential secondary structure problems in the template. The following protocol is based upon the 'fmol' kit (produced and supplied by Promega). This system recommends using kinased primers when sequencing PCR products or lambda clones, while plasmid DNA can be sequenced using an α -labelled deoxyribonucleotide that becomes incorporated into the DNA strands.

It is assumed that the plasmid DNA template has been purified to a stage suitable for DNA sequencing. This may be easily carried out using one of the many commercially available kits.

Step 1: Equipment and Solutions

Equipment

- fmol DNA Sequencing System; Promega or equivalent (components marked • are supplied in the kit): T4

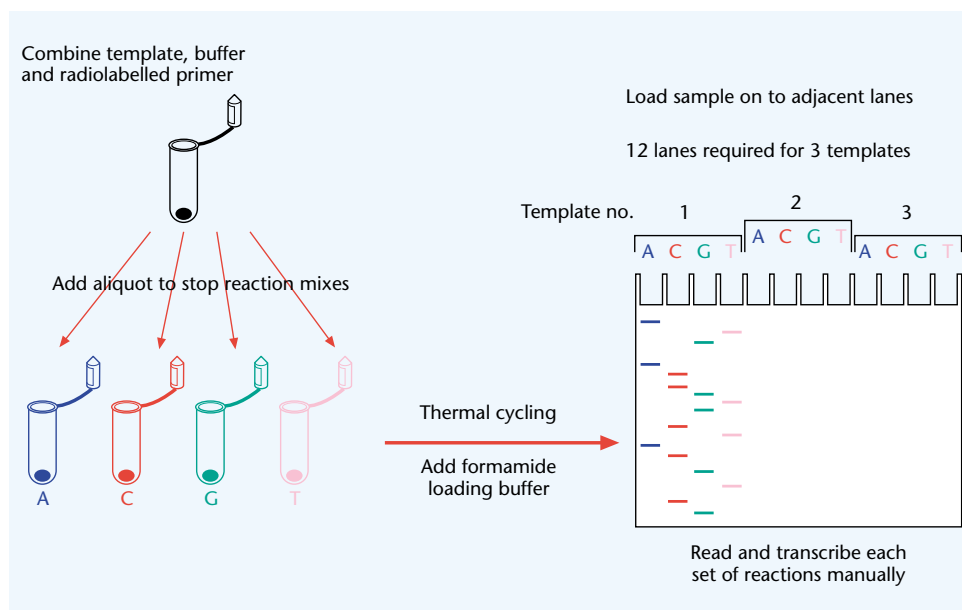


Figure 5 Overview of manual sequencing steps.

Dideoxy Sequencing of DNA

Polynucleotide kinase (PNK) 10 × buffer* (**Recipe 1**), 5 × Sequencing buffer* (**Recipe 2**), Sequencing Stop/loading buffer* (**Recipe 3**) and Extension/termination reactions* (**Table 1**)

- β-Radiation monitor
- 3MM paper sheet for gel transfer
- Darkroom facilities
- Developer
- Gel drier and vacuum pump
- Hoefer Slab Gel drier SE 1160 (or equivalent: other sequencing driers are available from Biorad, Pharmacia)
- Ice
- Microcentrifuge

Recipe 1 T4 Polynucleotide kinase (PNK) 10 × buffer

Ingredient	Final concentration	Volume/amount
Tris-HCl (1.0 mol L ⁻¹ , pH 7.5)	500 mmol L ⁻¹	500 μL
MgCl ₂ (1.0 mol L ⁻¹)	100 mmol L ⁻¹	100 μL
Dithiothreitol (1.0 mol L ⁻¹)	50 mmol L ⁻¹	50 μL
Spermidine (1.0 mol L ⁻¹)	1 mmol L ⁻¹	1 μL
Sterile distilled water to final volume		1 mL

Mix gently and store at -20°C.

Recipe 2 5 × Sequencing buffer

Ingredient	Final concentration	Volume/amount
Tris-HCl (1.0 mol L ⁻¹ , pH 9.0)	250 mmol L ⁻¹	250 μL
MgCl ₂ (1.0 mol L ⁻¹)	10 mmol L ⁻¹	10 μL
Sterile distilled water to final volume		1 mL

Mix gently and store at room temperature.

Recipe 3 Sequencing Stop/loading buffer

Ingredient	Final concentration	Volume/amount
Formamide	98%	9.79 mL
NaOH (10 mol L ⁻¹)	10 mmol L ⁻¹	10 μL
Bromophenol blue (1%)	0.01%	100 μL
Xylene cyanol (1%)	0.01%	100 μL
Total		10 mL

Mix well, dispense into 1 mL aliquots and store at -20°C.

A good stability guide is to check that the buffer is frozen at -20°C. Discard if the buffer does not freeze. Use another de-ionized preparation of formamide.

- Micropipettes (1–20, 10–200 μL)
- Mineral oil
- Mini-monitor
- Plastic wrap
- Powerpack
- Radioisotope handling facilities (Perspex shielding)
- Radioisotope (final choice to be made by the researcher based on whether to label the primer or the DNA transcript, availability, cost, experience):

For primer radiolabelling:

γ-³²P-ATP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹)
 γ-³³P-ATP (1000–3000 Ci mmol⁻¹, 37–111 TBq mmol⁻¹)
 γ-³⁵S-ATP (~1000 Ci mmol⁻¹, ~37 TBq mmol⁻¹)

For DNA transcript labelling:

α-³²P-dATP (800 Ci mmol⁻¹, 30 TBq mmol⁻¹)
 α-³³P-dATP (1500 Ci mmol⁻¹, 56 TBq mmol⁻¹)
 α-³⁵S-dATP (~1250 Ci mmol⁻¹, 46 TBq mmol⁻¹)

- Sequencing gel apparatus
- Thermal cycler
- X-ray cassettes
- X-ray film Cronex Medical X-ray film #4 (Du Pont or equivalent)

Reagents

- 7-deaza dGTP
- Acetic acid
- Acrylamide
- Ammonium persulfate
- Bis-acrylamide
- Boric acid
- Bromophenol blue
- dATP
- dCTP
- ddATP
- ddCTP
- ddGTP
- ddTTP
- Dithiothreitol (DTT)
- dTTP
- EDTA
- Formamide
- MgCl₂
- Methanol
- Mixed Bed Resin (BioRad Mixed Bed Resin AG 501-X8(D) or equivalent)
- NaOH
- Spermidine
- Template preparation kits (QIAGEN's QIAprep Spin Plasmid Kit, Promega's Wizard Minipreps DNA Purification Systems or equivalent)
- TEMED

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Table 1 Extension/termination reactions (values are $\mu\text{mol L}^{-1}$)

Component	A stop	C Stop	G Stop	T Stop
ddATP	350			
ddCTP		200		
ddGTP			30	
ddTTP				600
dATP	20	20	20	20
dCTP	20	20	20	20
7-deaza dGTP	20	20	20	20
dTTP	20	20	20	20

- Tris-HCl
- Tris base
- Urea
- Xylene cyanol

Solutions

- $10 \times$ TBE (**Recipe 4**)
- 6% polyacrylamide sequencing gel mix (**Recipe 5**)
- 25% ammonium persulfate (**Recipe 6**)
- Gel washing/fixing solutions (**Recipe 7**)
- 1:20 dilution of dimethyldichlorosilane in chloroform (Take care when handling dimethyldichlorosilane. Always wear gloves and work in a fume hood.)

Step 2: Chain Termination Reactions – Primer Labelling

The following protocol will radiolabel 10 pmol of primer with 10 pmol of γ -ATP using T4 polynucleotide kinase (PNK). There is sufficient material for six sequencing reactions (see Hints and Tips and **Table 4**).

1. In a sterile PCR tube combine in the following order:

- 10 pmol primer (about 70 ng of a 20mer)
- 10 pmol γ - ^{32}P -ATP ($3 \mu\text{L}$ $3000 \text{ Ci mmol}^{-1}$ ($111 \text{ TBq mmol}^{-1}$) at $10 \text{ mCi } \mu\text{L}^{-1}$ ($370 \text{ MBq } \mu\text{L}^{-1}$))

Recipe 4 $10 \times$ TBE

Ingredient	Final concentration	Volume/amount
Tris base ^a	890 mmol L^{-1}	108 g
Boric acid	890 mmol L^{-1}	55 g
Disodium EDTA	25 mmol L^{-1}	9.3 g
Double-distilled water to final volume		1000 mL

^aDissolve at room temperature. Store at room temperature. Stable for several months.

Recipe 5 6% polyacrylamide sequencing gel mix

Ingredient	Final concentration	Volume/amount
Acrylamide	5.7% (w/v)	11.4 g
Bis-acrylamide	0.3% (w/v)	0.6 g
Urea	7 mol L^{-1}	84 g
Double distilled water ^a		170 mL
Mixed bed resin ^b		10 g
Double-distilled water to final volume		180 mL
$10 \times$ TBE	$1 \times$	20 mL
Final volume		200 mL

^aGentle heat (not greater than 30°C) may be applied to facilitate dissolving.

^bAdd resin once solids have dissolved. Gently mix for 30 min. Filter the solution through Whatman #54 (or similar) to remove mixed bed resin. NOTE: When using high-quality or premixed acrylamide preparations, the deionization step is not necessary. De-gas to remove dissolved oxygen, which can inhibit polymerization, just before use. Store at 4°C . Stable for at least one month.

Recipe 6 25% ammonium persulfate

Ingredient	Final concentration	Volume/amount
Ammonium persulfate	25% (w/v)	2.5 g
Double-distilled water to final volume		10 mL

Store at 4°C .

Recipe 7 Gel washing/fixing solutions

Ingredient	Final concentration	Volume/amount
Acetic acid	10% (w/v)	100 mL
Methanol	10% (w/v)	100 mL
Double-distilled water to final volume		1000 mL

- $1 \mu\text{L}$ $10 \times$ PNK buffer
- Water to final volume of $9 \mu\text{L}$
- $1 \mu\text{L}$ PNK ($10 \text{ U } \mu\text{L}^{-1}$)

- Incubate at 37°C for 15 min.
- Inactivate the PNK by heating to 90°C for 2 min. Centrifuge briefly to collect any condensation.
- The labelled primer may be used directly in sequencing reactions – there is no need for purification (see Hints and Tips).
- The primer may be stored frozen at this stage. The labelled primer may be kept at -20°C for up to one month for use in sequencing reactions, although

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radiolytic decay will reduce the labelling intensity of the sequencing products.

Step 3: Chain Termination Reactions – Labelled Primer: Extension/Termination Reactions (λ Clones, PCR Products)

1. Prepare a set of four stop reactions for each template.
2. Label four PCR tubes 'A#' 'C#' 'G#' and 'T#', where # designates the template preparation (see Hints and Tips).
3. Add 2 μL of the appropriate d/ddNTP stop mixture to each tube (i.e. add 2 μL d/ddATP to each A# tube, and so on). Cap the tubes and leave on ice until needed.
4. Prepare the following mastermix reaction for each template in a sterile PCR tube:
 - 4–40 fmol template DNA (10–20 ng PCR product, 1 μg λ clone)
 - 5 μL 5 $\times$ Sequencing buffer
 - 1.5 μL radiolabelled primer
 - Sterile water to a final volume of 16 μL
5. Add 1 μL *Taq* DNA polymerase (5 U μL^{-1}) to the mastermix. Mix the solution by pipetting up and down (see Hints and Tips).
6. Add 3.5 μL of the mastermix to each 2 μL stop reaction dispensed earlier. Gently mix with the pipette. Overlay with a drop of paraffin oil.
7. Once all the reactions have been set up, place into a thermal cycler that has been preheated to 94°C (see Hints and Tips) and carry out the thermal cycling conditions described in Step 5.

Step 4: Chain Termination Reactions – Direct Incorporation: Extension/Termination Reactions (Plasmid DNA)

1. Set up four stop reactions for each template.
2. Label four PCR tubes 'A#' 'C#' 'G#' and 'T#' (where # designates the template preparation) (see Hints and Tips).
3. Add 2 μL of the appropriate d/ddNTP stop mixture to each tube (i.e. add 2 μL d/ddATP to the A# tube, and so on). Seal the tubes and leave on ice until needed.
4. Prepare the following mastermix reaction for each template in a sterile PCR tube:
 - 500 fmol template DNA (about 1 μg plasmid)

- 5 μL 5 $\times$ Sequencing buffer
- 1 μL primer (30 ng μL^{-1} for a 25mer)
- 0.5 μL α - ^{32}P -dATP (10 μCi μL^{-1} ; 37 MBq μL^{-1})
- Sterile water to a final volume of 16 μL

5. Add 1 μL *Taq* DNA polymerase (5 U μL^{-1}) to the mastermix. Mix the solution by pipetting up and down (see Hints and Tips).
6. Add 3.5 μL of the mastermix to each 2 μL stop reaction dispensed earlier. Gently mix with the pipette. Overlay with a drop of paraffin oil.
7. Once all the reactions have been set up, place into a thermal cycler that has been preheated to 94°C (see Hints and Tips) and carry out the thermal cycling conditions described in Step 5.

Step 5: Thermal Cycling Conditions

Standard thermal cycling conditions are shown in **Table 2**. These conditions seem to work for most primers, although it is possible that some alternative annealing/extension temperatures could assist with some sequencing primers. Where possible, use thermal cyclers that provide tube temperature control via a thermocouple. The times indicate the duration for which the reactions are held at that temperature once the contents of the tube have reached that specified temperature.

Samples may be stored frozen at this stage, although it is advisable to fractionate the reactions as soon as convenient. End-labelled sequencing reactions may be stored for up to one month at -20°C and still generate reliable data.

Step 6: Gel Fractionation of the Sequencing Products – Introduction

Upon completion of the thermal cycling, the reactions are ready for fractionation on a sequencing gel. One of the more critical steps of successful sequencing is the preparation, electrophoresis and treatment of the polyacrylamide gel. A poorly run or poorly treated gel will compromise the results and may seriously limit the amount of data generated.

Table 2 Standard thermal cycling conditions

94°C	2 min	15 s	15 s	
55°C		15 s		
72°C		2 min	2 min	
4°C				HOLD
		25 cycles	10 cycles	

Step 7: Gel Fractionation of the Sequencing Products – Gel Type

Sequencing gels must be able to differentiate between single-stranded DNA transcripts that differ in length by a single nucleotide. The DNA fragments from the sequencing reaction are separated on the basis of length under denaturing conditions in order to minimize any anomalous migration due to secondary structures occurring within the sequencing products.

The gel described in this protocol is designed for the fractionation of products up to about 400 bases long; this is what may be expected from the enzymatic sequencing of a cloned template. If more sequence information is required, either double loadings can be carried out (see Hints and Tips) or the acrylamide concentration of the gel can be reduced accordingly (a 4% acrylamide gel may be used to separate fragments in excess of 400 bases).

Step 8: Gel Fractionation of the Sequencing Products – Preparing the Gel

There is a wide choice of gel apparatus that can be used for DNA sequencing. Most gels are 0.4 mm thick with an average length of 40–50 cm and width ranging from 20 to 40 cm. The 20 cm wide gels are easier to handle, but the larger gels are capable of processing more samples. The final choice must be made by the researcher on the basis of throughput of samples and what is currently available in the laboratory. The number of samples that can be fractionated on a gel may be increased by utilizing a sharktooth comb. Another advantage of these combs over

the standard well formers is that the lanes on a sharktooth gel are much closer to each other, which can facilitate reading the sequencing ladder (Figure 6).

1. Mark and identify the outside surface of the plates using waterproof tape. In this way, you can concentrate on thoroughly cleaning one surface of each plate.
2. Remove all traces of old gel, grease, etc. with a plastic scourer pad and Pyroneg detergent or equivalent.
3. Rinse thoroughly in hot water. Rinse with ethanol (use a squirt or spray bottle) and allow to air dry.
4. To facilitate subsequent gel handling steps, the notched backing plate should be treated with a silane solution so that the gel does not adhere to that plate. Wearing gloves and working in a fume hood, prepare 1–2 ml of a 1:20 dilution of dimethyldichlorosilane in chloroform and use a tissue to spread/wipe over the glass plate. Allow to dry for at least 30 min in the fume hood before use. One plate treatment is normally sufficient for 10 gel runs.
5. Once the plates are clean and dry, assemble the gel cassette with the spacers (0.4 mm thick) on each side and clamp.
6. If the plates have been properly cleaned, it is not necessary to tape or seal the bottom. When the gel cassette is poured along one edge up a slight incline, the gel solution will flow into the cassette and will not leak out because of surface tension.
7. Determine the amount of gel solution required to fill the cassette (and then allow an extra 20% for any spillage).
8. Pour the gel solution into a beaker and de-gas to remove dissolved oxygen. Add 1.5 μL TEMED per mL of gel solution (i.e. 70 mL of gel solution will need 105 μL TEMED).

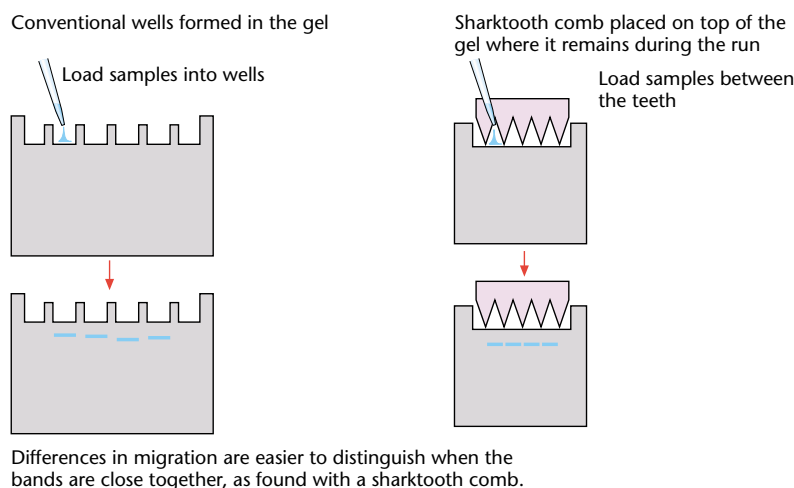


Figure 6 Comparison of well-forming and sharktooth combs for sequencing gels.

9. Mix gently with a glass rod (do not introduce bubbles, as dissolved oxygen will inhibit the polymerization process).
10. Add 1.5 μL 25% ammonium persulfate per mL of the gel solution and mix gently (i.e. 70 mL of gel solution will require 105 μL 25% ammonium persulfate).
11. Draw the solution into a 60 mL syringe and gently introduce the gel mix into the cassette. Take care not to introduce bubbles into the gel (see Hints and Tips).
12. Place the cassette at a slight incline and try to run the solution down one side first. The solution front should move smoothly and evenly across the glass (**Figure 7a**).
13. Grease, dust or impurities on the glass will retard the movement of the gel solution and may act as a focal point for a bubble. Gentle tapping (a plastic microfuge rack is ideal) can assist the gel solution getting past these imperfections (**Figure 7b**).
14. In some cases, bubbles may form despite vigorous tapping or can even be introduced via the syringe. It is possible to remove these bubbles with the aid of a hook made out of plastic sheeting that is slightly thinner than the spacers (**Figure 7c**).
15. Once the gel solution is in the cassette, insert the well-forming comb into the top of the gel. If a sharktooth comb is to be used, it is necessary to place a single slot-forming insert into the top of the cassette so that the top of the gel will have a smooth and even surface. It is often convenient to invert the sharktooth comb and use the top to form a smooth surface on the gel.
16. Clamp the top of the gel and leave the gel to set (about 10–20 min, depending on the temperature). Never clamp the bottom of the gel as this can draw the plates together, making the gel paper thin and adversely affecting electrophoresis. The gel can be used after an hour or so. In areas of low humidity, make sure the top and bottom of the gels are kept moist to stop them drying out.

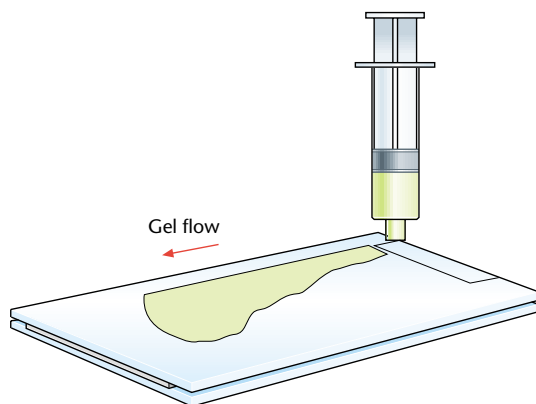


Figure 7(a) Inserting the gel into the gel apparatus.

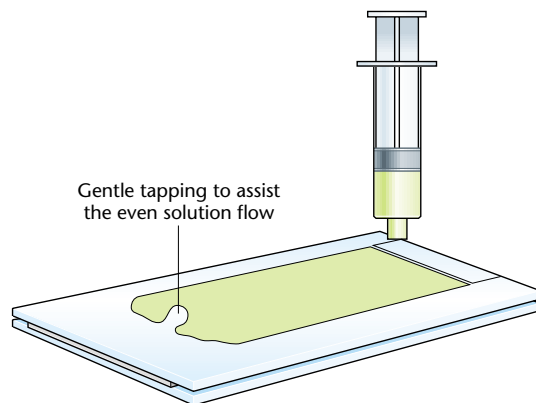


Figure 7(b) Pouring acrylamide mix into the gel cassette.

A plastic hook can be used to 'fish out' and remove bubbles in the gel

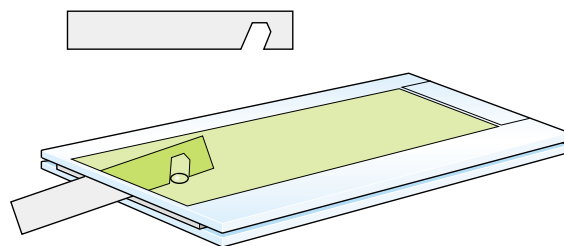


Figure 7(c) Bubble formation during gel pouring.

Sequencing gels may be stored for days if wet paper towels are placed over each end and the gel is well sealed in plastic wrap to prevent it drying out.

Step 9: Electrophoresis Setup

1. Once the gel has set, use a damp paper towel to wipe away any polyacrylamide that could interfere with the sealing of the gel on to the reservoir tank.
2. Assemble the gel in the electrophoresis unit.
3. Remove the comb and, using a syringe, flush the wells out with the running buffer ($1 \times \text{TBE}$).

Step 10: Fractionation of the Sequencing Reactions

1. Pre-electrophorese the gel for 10 min. The running conditions will depend upon the size and type of the gel apparatus.
2. Mark the layout A C G T for each set of sequencing reactions on the front glass plate (see Hints and Tips).
3. Record the gel layout in a laboratory book: e.g. ACGT// Template #1/Template #2/Template #3, and so on.

4. Gels 35 cm × 40 cm × 0.4 mm thick can be run at 1300 V. The gel should heat up to around 45°C to maintain denaturing conditions. If the gel becomes too hot, there can be a loss in resolution and the dye front may 'smile' as a result of the middle of the gel becoming hotter than the sides. In extreme cases, the gel plates may even crack because of excessive heating.

Exercise care in the electrophoresis conditions and follow the manufacturer's recommendations.

5. Once the gel is set up and ready for use, add an equal volume of formamide loading buffer to each sequencing reaction. It is not necessary to remove the oil overlay; simply add the formamide loading buffer through the paraffin oil directly into the reaction.
6. Heat the samples at 94°C for 2 min.
7. Place the tubes in a rack in a predetermined order to facilitate loading. Use the same order as the layout on the gel and when that sample has been loaded on to the gel, place the tube one row down in the rack to avoid misloading.
8. Use a syringe to flush the wells with running buffer. Urea will diffuse out of the gel and form a dense cushion in the wells, which can make loading very difficult or impossible.
9. When loading the sample on to the gel, make sure that only the blue sample, and no oil, is drawn up the tip. It may help to load after wiping oil off the tip with some paper tissue. Remember that these samples are radioactive, so take care and use thick tissue for this step.
10. Load 2 µL of the heat-denatured sequencing reaction into the appropriate lane. After a sample has been loaded on to the gel, that tube should be moved back one row (or to another rack) to ensure there is no mix-up in the loading order. The gel must be electrophoresed under conditions in which the gel heats up (to about 45–50°C) and maintains the denaturing conditions.
11. Apply 1250–1400 V until the bromophenol blue has migrated a suitable distance through the gel. Total run times can be up to 3 h.

On a 6% gel, the bromophenol blue marker migrates in the position of a 26mer and the xylene cyanol runs as a 106mer.

Step 11: Gel Manipulations

Gel manipulations should be carried out as soon as possible upon completion of electrophoresis to minimize diffusion of the bands.

1. Upon completion of electrophoresis, remove the gel from the apparatus. Take care because the bottom tank

and the bottom of the gel have been in contact with unincorporated radioactive nucleotides.

2. Discard the bottom reservoir under appropriate conditions (radioactive flushing sink, storage, etc.) and rinse the bottom of the gel cassette with tap water to remove excess radioactive isotope.
3. Gently prise the glass plates apart (use a broad spatula of which one end has been ground down). **CAUTION:** Do not prise the plates apart near the 'ears' of a notched glass plate as this will damage the glass. The plates should come apart with the gel remaining on the nonsiliconized (front) plate.

Step 12: Fixing and Drying the Gel

1. Fix and then dry the gel to get high resolution of the bands.
2. Place strips of tissue paper around the edges of the gel and gently flood the gel surface with the gel washing/fixing solution. (The tissue minimizes the risk of the gel wash getting under the gel and causing wrinkles.)
3. Wash the gel with at least 500 mL gel washing/fixing solution over a period of 30 min. Allow excess gel washing/fixing wash to drain away from the gel.
4. Transfer the gel to 3MM paper by placing a sheet of 3MM paper (slightly larger than the gel) on top of the gel. (Start from one end of the gel and roll the paper across the surface of the gel.) Avoid getting bubbles or wrinkles forming between the paper and the gel. Pat down to ensure good contact between the gel and the paper.
5. Start from one corner and peel the paper back from the glass plate. The gel should remain attached to the paper.
6. Cover with plastic wrap, avoiding the formation of bubbles or wrinkles between the plastic and the gel.
7. Dry in a gel drier under vacuum at 80°C for 1–2 h (follow the manufacturer's recommendations).
8. Expose to X-ray film for 4–16 h. Exposure times can be estimated by scanning the gel with a radiation monitor to gauge the extent of incorporation.

Avoid using any cassette that has an intensification screen, as the enhancement of the radioactive signals will result in fuzzy bands, thereby decreasing the resolution of the sequencing ladder.

Step 13: Reading the Sequence

Read the ladders from the first clear bands on the bottom of the autoradiograph. Since DNA synthesis occurs in the 5' to 3' direction, the sequence is read 5' to 3' as you move up the gel to the higher molecular weight products. Remember that the sequence you are reading is complementary to the template sequence!

Each nucleotide should be represented as a band in either the A, C, G or T lanes with the subsequent band up the ladder corresponding to the next nucleotide away from the sequencing primer. As the ladder is read towards the top of the gel, the relative difference between the bands decreases and they become closer together. It is for this reason that the sequencing gels should be fixed and dried down in order to enable differentiation of fragments in the upper area of the gel (Figures 8 and 9).

The relative difference between a 49mer and a 50mer is about 2%, while a 200mer and a 201mer differ by only 0.5%.

Hazards

The hazards associated with the chemicals/apparatus used in this protocol are detailed in Table 3.

Hints and Tips

Step 2

2.1

2.2

It is often wise to check that the kinase reaction has been completed to a satisfactory level. (The kinase reaction is very quick and simple, but the enzyme T4 polynucleotide kinase (PNK) is very sensitive to ammonium ions and will be inhibited even by $7 \text{ mmol L}^{-1} \text{NH}_4^+$).

Calculation of the labelling efficiency can be done in a few minutes using a simple thin-layer chromatography technique. An aliquot of the PNK reaction (as little as can be removed from the reaction) is spotted on to the poly-ethylenimine strip, which is then placed in a beaker containing 0.5 mol L^{-1} ammonium hydrogen carbonate. $1 \mu\text{L}$ 5 mmol L^{-1} ATP is also spotted on to the origin to act as a marker, which is visualized as a purple spot under short wavelength (254 nm) UV light. The strip is left in the beaker

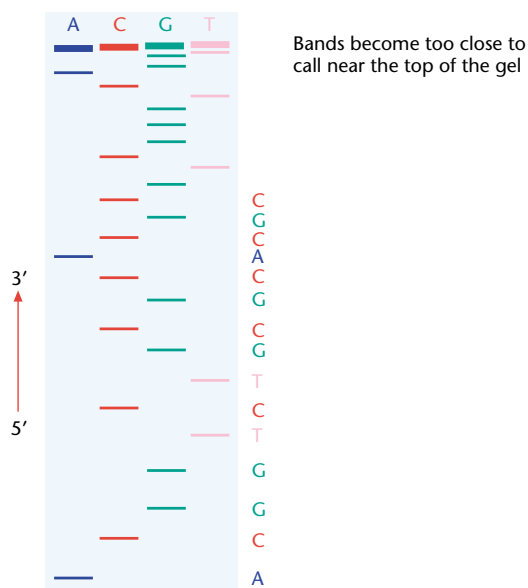


Figure 8 Dideoxy sequencing ladder.

until the solvent front has migrated some 6–8 cm away from the origin. This purple spot indicates where the unincorporated $\gamma\text{-}^{32}\text{P}\text{-rATP}$ has migrated away from the radiolabelled oligonucleotide (which remains at the origin).

Incorporation is calculated by cutting the strip between the ATP spot and the origin. Use a Geiger tube radiation monitor to count each segment. Labelling efficiency is estimated as the number of counts at the origin divided by the total number of counts (i.e. combined counts at the origin and the ATP spot) (Figure 10).

Step 3

3.1

Always use a permanent marker pen that does not easily rub off. Unlabelled sequencing reaction tubes can obviously ruin an entire sequencing experiment.

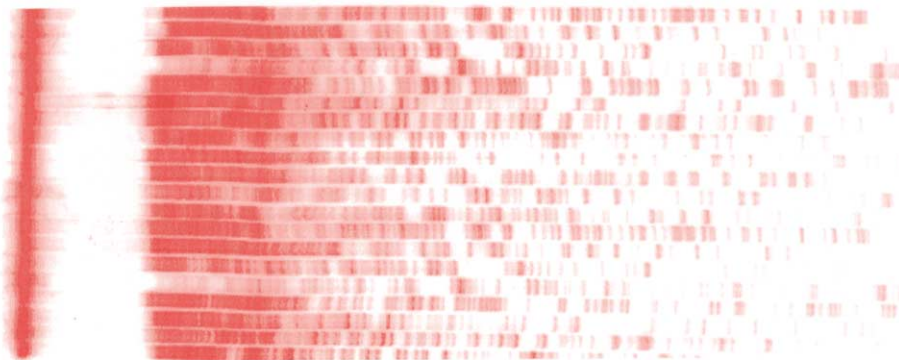


Figure 9 Autoradiograph.

Table 3 Hazards associated with this procedure

Acetic acid CH_3COOH	Corrosive. Causes severe burns. Glacial acetic acid is flammable. Harmful if swallowed, inhaled or absorbed through skin. Material extremely destructive of tissues of mucous membranes, upper respiratory tract, eyes and skin. Inhalation may be fatal. Used as a fixative. Solutions are irritant. Use a fume hood and wear face protection and gloves when preparing solutions and at all times when using glacial acetic acid.
Acrylamide $\text{CH}_2\text{CHCONH}_2$	Neurotoxin. May cause cancer and heritable genetic damage. Toxic through skin contact and if swallowed. Danger of serious damage to health by prolonged exposure. Dispense in fume hood and weigh in closed container or balance. Wear protective clothing and gloves and use face protection.
Ammonium persulfate $(\text{NH}_4)_2\text{S}_2\text{O}_8$	Harmful by inhalation and if swallowed. Oxidizing agent.
Bisacrylamide (<i>N,N</i> -methylenebisacrylamide) $\text{C}_7\text{H}_{10}\text{N}_2\text{O}_2$	Harmful. Avoid contact. Wear gloves. Do not breathe dust.
Boric acid H_3BO_3	Harmful by inhalation, in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. Possible Teratogen. Reproductive hazard. In case of contact with eyes, rinse immediately with plenty of water for 15 min and seek medical advice. In case of contact, immediately wash skin with soap and copious amounts of water. If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen. If swallowed, wash out mouth with water provided person is conscious.
Bromphenol blue	Avoid contact and inhalation.
Bromophenol blue	
Chloroform (trichloromethane) CHCl_3	Harmful if swallowed. Irritating to skin. Possible risk of irreversible effects and serious damage to health by prolonged exposure through inhalation and if swallowed. Use in fume hood and wear appropriate gloves. Chloroform should be handled with care, and disposed of by means appropriate for organic solvents.
Dimethyldichlorosilane (Inerton-DMCS) (Inerton DW-DMC) $\text{C}_2\text{H}_6\text{Cl}_2\text{Si}$	Highly flammable. Flash point -4°C . Reacts violently with water. Causes severe burns. Irritating to respiratory system. Use in fume hood. May also be supplied as dilute solution in 1,1,1-trichloroethane.
Dithiothreitol (DTT) $\text{HSCH}_2(\text{CHOH})_2\text{CH}_2\text{SH}$	Harmful in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin.
EDTA (diaminoethanetetraacetic acid; ethylenediaminetetraacetic acid)	Harmful if swallowed. Irritating to eyes, respiratory system and skin.
Electrophoresis	Great care must be exercised when using any electrophoresis equipment, especially high-voltage or constant-current supplies. If possible, always use commercially supplied apparatus that has been designed and built to international electrical safety standards: home-made equipment is always suspect in this regard. Always check that all wiring connections are properly made and any interlocks fitted are secure before switching on the power supply. Always switch off the power supply before disconnecting the apparatus. Arrange the work area to reduce the risk of water or reagents splashing on to the power pack, leads, cables or chambers. Preferably use power supplies fitted

Table 3 Continued

Electrophoresis of radioactive material	with electrical earth leakage detection circuitry and automatic cut-off. Electrophoresis of highly radioactive probes is dangerous. Extreme care is needed. Take local advice concerning the level of radiation protection necessary. See also entries for 'Electrophoresis' and 'Radioisotopes'.
Ethanol (ethyl alcohol) C_2H_5OH Formamide $HCONH_2$	Highly flammable. Flash point $12^{\circ}C$. Use in well-ventilated area away from sources of ignition. Harmful by inhalation, in contact with skin and if swallowed. May cause irritation to skin. May cause birth defects following chronic exposure. Do not breathe vapour. Prepare solutions in fume hood. Wear protective clothing, face protection and gloves. May be fatal if inhaled, swallowed or absorbed through skin. Causes burns. Material extremely destructive of tissues of upper respiratory tract, eyes and skin. Wear protective clothing and gloves and use face protection when using concentrated solutions.
Hydrochloric acid (HCl)	Irritating to eyes, respiratory system and skin.
Magnesium chloride $MgCl_2$ Methanol (Methyl alcohol) CH_3OH	Toxic. Flammable. Flash point $10^{\circ}C$. Used as a fixative and a solvent for stains. Toxic by inhalation and if swallowed. Irritating to eyes, respiratory system and skin. Wear suitable protective clothing, gloves and face protection. Use in well-ventilated area away from sources of ignition.
Radioactive material	Wear gloves and protective shield. Treat radioactivity according to your laboratory rules
Sodium hydroxide NaOH	Causes severe burns. Wear glasses. Eye contact: Rinse immediately with plenty of water for 15 min and seek medical advice. Skin contact: Immediately wash skin with soap and copious amounts of water. Ingestion: If the chemical has been confined to the mouth give large quantities of water as a mouthwash. Ensure the mouthwash is not swallowed. If the chemical has been swallowed, give about 250 mL of water to dilute it in the stomach. In severe cases, obtain medical attention.
Spermidine (N-(3-aminopropyl)-1,4-butanediamine) $NH_2(CH_2)_4NH(CH_2)_3NH_2$ Tris (tris(hydroxymethyl)aminomethane; 2-amino-2-hydroxymethylpropane-1,3-diol) Ultraviolet light sources	Corrosive. Causes burns. Wear suitable protective clothing, gloves and use face protection. Irritating to eyes, respiratory system and skin. Always wear UV goggles or visor. Do not look directly at the light source in transilluminators for unnecessary periods of time even if goggles are being worn. Allow for reflected UV light. Do not expose skin to UV illumination for unnecessary periods of time. If long periods of viewing are necessary, use a UV face visor. Ensure that the eye protection provides adequate UV absorption for the intensity and frequency of UV light being used. Long-wavelength UV is less dangerous than short-wavelength UV.
Xylene cyanol FF	Irritating to eyes, respiratory system and skin. Avoid contact.

Table 4 Primer labelling

Primer length	ng primer to equal 10 pmol
20mer	67
24mer	80
30mer	100

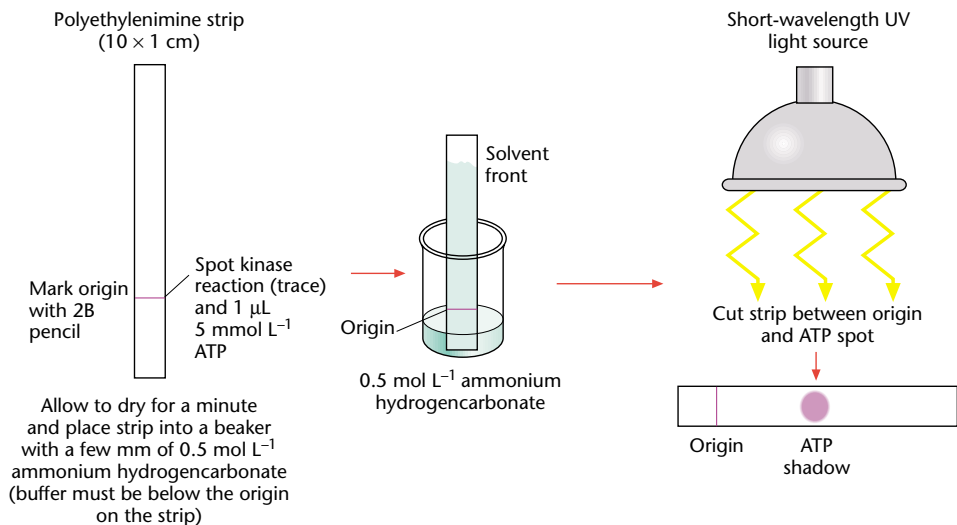


Figure 10 Thin layer chromatography to check 5'-radiolabelling efficiency.

3.2

Do not pipette the solution vigorously as this can lead to radioactive aerosols contaminating the barrel of the micropipette.

3.3

Preheating the thermal cycler results in more efficient and rapid denaturation, thereby reducing the number of misprimed sequences being extended.

Step 4

4.1

Always use a permanent marker pen that does not easily rub off. Unlabelled sequencing reaction tubes can ruin an entire sequencing experiment.

4.2

Do not pipette the solution vigorously as this can lead to radioactive aerosols contaminating the barrel of the micropipette.

4.3

Preheating the thermal cycler results in more efficient and rapid denaturation, thereby reducing the number of misprimed sequences being extended.

Step 7

7.1

It is possible to obtain further sequence information by multiple loadings of the sequencing gel. Load the first set of sequencing reactions on one side of the gel and electrophorese until the bromophenol blue marker has migrated to the bottom of the gel. A second set of the same reactions can then be loaded on to the other side of the gel and fractionated until the second bromophenol blue tracker dye is near the bottom of the gel.

Step 8

8.1

Some researchers prefer to use freshly made 25% ammonium persulfate. This solution can break down over time so that it is no longer efficient in gel polymerization. However, correctly stored, this solution can remain active

over several months. Store the ammonium persulfate at 4°C and keep it at this temperature. If the gels are taking longer than usual to set, prepare another sample of this catalyst.

Step 10

10.1

The layout 'ACGT' for each set of reactions is used so that it is possible to turn the autoradiograph over and read the complementary strand (i.e. the T is now on the left side of the set of four reactions and is read as 'A'. The next lane was G which is read as 'C' and so on. Note that the polarity of the inverted complementary sequence is now 3' to 5' from the bottom of the gel towards the top (Figure 11).

Troubleshooting

1

Cross-banding evident. Are bands occurring in the same position in all lanes?

YES. Go to 12.

NO. Go to 2.

2

The most common cause of problems with DNA sequencing, especially once the various reagents have been used successfully with control templates and primers is the quality of the template. This can be summed up by the old proverb: Garbage in, garbage out.

Weak bands or no bands detected on the autoradiograph. Are end-labelled primers being used?

YES. Go to 3.

NO. Go to 9.

3

Has the labelling efficiency been confirmed to be satisfactory (at least greater than 50%)?

YES. Go to 4.

NO. Go to 5.

4

Has the quality of the template been confirmed by gel electrophoresis?

YES. Go to 7.

NO. Go to 8.

Flip the autoradiograph

Complementary sequence

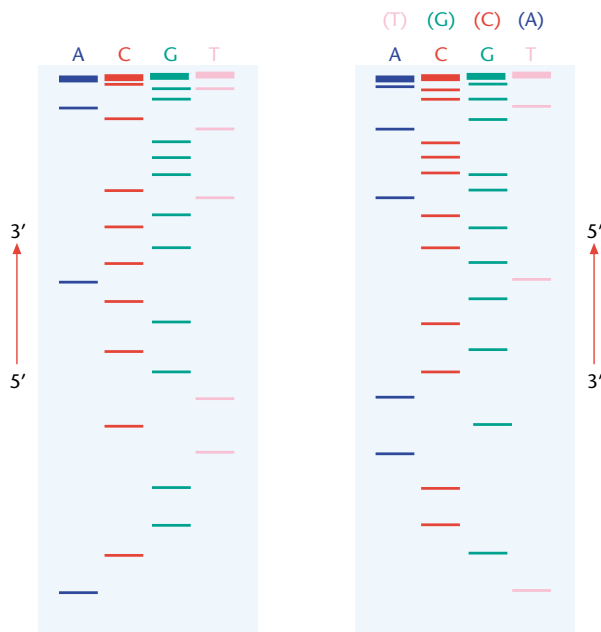


Figure 11 Reading the sequence of either strand from a sequencing ladder. The layout ACGT for each set of reactions is used so that it is possible to turn the autoradiograph over and read the complementary strand. The T is now on the left side of the inverted set of four reactions and is read as A. The next lane was G, which is read as C, and so on. Note that the polarity of the complementary sequence is now 3' at the bottom to 5' at the gel origin.

5

Check primer quality and labelling efficiency as described in Hints and Tips for Step 2.

Is the labelling efficiency of the sequencing primer now satisfactory (at least greater than 50%)?

YES. Go to 4.

NO. Go to 6.

6

Has labelling been successful with a known positive control primer?

YES. Go to 7.

NO. Go to 15.

7

Is the annealing temperature likely to be too high for the sequencing primer? (A 20mer with 50% G/C should anneal to templates at 60°C.)

YES. Go to 16.

NO. Go to 8.

8

Check the amount and purity of the DNA templates on an agarose gel. Make sure there is only a single band. If multiple bands are present it is necessary to prepare fresh PURE template. Use the recommended amount of template. If possible, check the A_{260}/A_{280} ratio and make sure it is between 1.8 and 2.0.

Has the control template and primer (supplied with the kit) generated a satisfactory sequencing ladder?

YES. Go to **17**.

NO. Go to **18**.

9

When using direct incorporation, is the radioisotope likely to be too old? (^{32}P should preferably be used within 3 weeks, ^{35}S can be used up to 2 months if stored at -70°C .)

YES. Go to **19**.

NO. Go to **10**.

10

Has the quality of the template been checked on a gel?

YES. Go to **11**.

NO. Go to **4**.

11

Has the quality of the sequencing primer been checked?

YES. Go to **8**.

NO. Go to **7**.

12

Is the cross-banding at specific places?

YES. Go to **20**.

NO. Go to **13**.

13

Is the cross-banding occurring throughout the gel?

YES. Go to **21**.

NO. Go to **14**.

14

Inhibition of DNA synthesis due to secondary structures. Use a longer primer or with a greater G + C content so that the extension temperature can be raised to 75°C .

The bands on the sequencing gel are fuzzy and difficult to resolve. Are the bands fuzzy throughout the gel?

YES. Go to **22**.

NO. Go to **23**.

15

Problem must lie with one of the reagents used in the labelling reaction. Repeat the labelling with fresh enzyme, buffer and label.

16

Lower the annealing temperature of the sequencing reactions to 50°C .

17

Recheck quality of the DNA template on an agarose gel. Make sure that the absorbance is due to the template and not contaminating RNA/DNA.

18

Problems with the sequencing kit. Check expiry date on reagents. Use another kit.

19

Use fresh radioisotope.

20

Mixed sequencing templates so the cross-banding is due to superimposed ladders. The single bands are occurring where that particular base is present in both templates (this can happen once in every four bases by chance).

21

Dirty template DNA. If using plasmid DNA, contaminating RNA or DNA can act as a primer which will generate many random transcripts. Alternately, check the purity of the sequencing primer in case there are significant amounts of failed sequences that are compromising the reaction.

22

Gel problems, either poor quality acrylamide or run too hot. Use commercially available pre-mixed acrylamide solution. Run the gel at a lower temperature.

23

Poor contact of the film with the gel. Avoid wrinkles when drying the gel. Bands in the sequencing ladder are not of an even intensity but weak at either the bottom of the gel or fading out towards the top of the gel.

Alamar Exhibit 1028

Ratio of ddNTP to dNTP is not properly 'balanced' for DNA sequencing

Too much ddATP to dATP will result in early termination of DNA transcripts at 'A's (bands at the bottom of the gel and fading out prematurely). If using a commercial preparation, it is necessary to obtain a fresh kit. If using 'in-house' stop reactions, prepare a new 'A stop' with lower ddATP concentration (try 50% to 75% of the original suggested concentration).

Too much dATP to ddATP will result in late termination of DNA transcripts at 'A's so that only long transcripts are seen with no or weak bands at the bottom of the gel. If using a commercial preparation, it is necessary to obtain a fresh kit. If using 'in-house' stop reactions, prepare a new 'A stop' with a higher ddATP concentration (try 25% to 50% of the original suggested concentration).

There are missing bands or compressed bands occurring at specific and repeatable positions

Abnormal migration of bands due to secondary structures in the transcripts. This may be overcome in one of the following manners:

- Increase the temperature of the gel electrophoresis.
- Include 40% formamide in the sequencing gel.
- Sequence the complementary strand.

References

- Cammeron-Mills V (1988) Modified T7 DNA polymerase versus Klenow DNA polymerase. The capacity of DNA polymerase to read through stretches in template strands. *Comments (United States Biocorp)* **14**: 8.
- Innis MA, Myambo KB, Gelfand DH and Brow M-AD (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proceedings of the National Academy of Sciences of the USA* **85**: 9436–9440.
- Lee J-S (1991) Alternative dideoxy sequencing of double-stranded DNA by cyclic reactions using *Taq* polymerase. *DNA* **10**: 67–73.
- Maxam AM and Gilbert W (1977) A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the USA* **74**: 560–564.
- Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain termination inhibitors. *Proceedings of the National Academy of Sciences of the USA* **74**: 5463–5467.