CHAPTER 3

Troubleshooting in chain-termination DNA sequencing

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1. INTRODUCTION

Although dideoxy sequencing is regarded by many as the method of choice for any large sequencing project, it is often found to be a rather more difficult technique than chemical sequencing to set up afresh in a laboratory. This chapter is intended as a guide to the problems most frequently encountered, and should be consulted in conjunction with the two preceding chapters. It is aimed at the laboratory which has expertise in the elementary aspects of handling nucleic acids, and therefore does not cover the problems associated with routine operations, for which the reader is instead recommended to consult the many molecular biology recipe manuals available (e.g. 1–3). Many of the problems discussed here will be seen as completely trivial to an experienced sequencer—we make no apology for including them, as this chapter is aimed at the beginner for whom even the most trivial problems can appear insurmountable. Problems specifically associated with sequencing of double-stranded DNA are covered in Chapter 4.

2. PROBLEMS ENCOUNTERED IN CLONING

2.1 Generating recombinants for transfection

The first indication that problems are occurring in the cloning of DNA (usually specific restriction fragments, end-repaired fragments produced by sonication or DNase digestion, or material produced by exonucleolytic degradation of larger fragments) into M13 generally arises after the transfection of host cells with supposedly recombinant M13-insert molecules. It is therefore important to include suitable controls in the transfection. The more informative transfections are shown in *Figure 1*, together with the expected and idealized results on plates containing Xgal and IPTG. Of these, transfections (i), (ii) and (vi) are the most important. Deviations from these patterns indicate various problems, and are discussed below, in order of decreasing frequency.

2.1.1 *Transfection (i) gives few white plaques—other results as expected*

This indicates that the vector-insert ligation has failed to take place, although the vector can self-ligate prior to phosphatase treatment, since (iv) gives many blue plaques. One of the most likely causes is that the molar ratio of vector DNA to insert DNA may be suboptimal. This can be identified and rectified by repeating the ligations at a range of vector-insert ratios.

Expt. No.	Vector cut with RE?	Vector phosphatased?	Ligase added?	Insert added?	Expected Blues/Whites	Idealized Blues/Whites
(i)	Yes	Yes	Yes	Yes	Few/Many	None/Many
(ii)	Yes	Yes	Yes	No	Few/Few	None/None
(iii)	Yes	Yes	No	No	Few/V.few	None/None
(iv)	Yes	No	Yes	No	Many/Few	Many/None
(v)	Yes	No	No	No	Few/None	None/None
(vi)	No	No	No	No	V.many/V.few	V.many/None

Figure 1. Transfections useful in M13 cloning. The significance of the reactions is explained in the text.

Another likely cause is that the insert material is unsuitable, either because it contains ligase inhibitors (which may be present as a result of extraction of DNA from gels) or has unsuitable ends (which may be due to inefficient end repairing, or nuclease contamination of the restriction enzymes used to generate it). The presence of ligase inhibitors can be demonstrated by repeating (iv) with insert added, resulting in inhibition of vector self-ligation and a dramatic fall in the number of blue plaques. If this is consistently found to be the problem, a different source of gel material or method of fragment isolation should be employed. Note that certain agarose preparations contain rather efficient inhibitors of ligation that may be extracted from the gel with DNA, probably including free sulphated polysaccharides (1) so this may be a problem if insert material has been isolated using low gelling temperature agarose (unless the latter is known to be of good quality). Other ways of isolating DNA from gels include electrophoresis onto DEAE-cellulose paper (4), NA45 membrane (5), or dialysis membrane (1,6), and electrophoresis into wells cut in the gel (1). Alternatively, further purification of the extracted DNA may be attempted, for example using 'Elu-tips' (3) or Spin-X tubes (see Chapter 1, Section 3.3.15). If the insert material has unsuitable ends, end repairing should be repeated, with altered incubation times or amounts of polymerase if necessary, or alternative restriction enzyme preparations should be tried.

The problem can on occasions be caused by contamination of the phosphatase with nuclease activity, or failure to purify the vector fully away from phosphatase. Provided the phosphatase is from a reputable source and has been used and stored carefully, nuclease contamination is not usually a problem, although there is often significant difference in nuclease activity between batches. Repeated heat inactivation of phosphatase and phenol extraction should remove phosphatase contamination from the vector. The greater the white:blue ratio in (i) than the ratio in (ii), the safer the plaques in (i) are to use.

2.1.2 *Many blue plaques in (i) and (ii)—other results as expected*

This indicates that the vector DNA has not been adequately phosphatased, and therefore self-ligation is taking place. Phosphatase preparations as supplied (e.g. lyophilized or in glycerol) are generally stable at 4°C. Once diluted, storage is possible at −20°C, but not advisable. The white plaques in (i) are safe to use.

2.1.3 *Transfection (i) gives few white plaques, (iv) gives few blue plaques—other results as expected*

This indicates that the ligase has low activity, either because of defective enzyme/buffer or because of inhibitors in the vector DNA. Replacement of the enzyme, buffer and vector DNA should indicate the problem. It is inadvisable to store ligase buffers for periods much greater than a week if they include DTT or ATP. White plaques in (i) are probably safe to use.

2.1.4 *Many blue plaques in (i), (ii), (iii) and (v)—other results as expected*

This indicates that the restriction enzyme has failed to digest the vector, leaving closed circular material which will give blue plaques regardless of subsequent phosphatasing. Digestion of vector should be checked by running an agarose gel, but note that quantities of undigested DNA too small to be readily visualized in a gel can give a very high background of blue plaques, because transfection with undigested vector is much more efficient than digestion, religation and transformation. The white plaques in (i) are safe to use.

2.1.5 *No plaques at all*

This suggests a very low transfection efficiency. See Section 2.2.

2.1.6 *Transfection (ii) gives many white plaques—possibly also (iv)*

This indicates that DNA other than the desired insert is being ligated into the vector. It can be caused by contamination of vector, ligase, phosphatase or buffers with low levels of nuclease (fragmenting some vector DNA, which is then ligated into intact vector) or (less likely) DNA. Systematic replacement of each of these components should indicate which is at fault. White plaques in (i) are NOT safe to use.

2.1.7 *Many white plaques in (vi), and probably others*

This indicates contamination of the vector strain (see Section 2.2.2). A blue plaque picked from (vi) may be safe to grow up more vector DNA. White plaques in (i) are NOT safe to use.

2.1.8 *No blue plaques at all, but many white ones*

This suggests that the IPTG or Xgal have been omitted or become inactive, or that the colour has simply failed to develop. This can be caused by incubation at too high a temperature. Leaving plates out on the bench or at 4°C for a few hours may enhance blue colour. The wrong host strain may have been used (see Section 2.2). White plaques in (i) are probably unsafe to use.

2.1.9 *Very few plaques in (i) − (v); (vi) as expected*

This is likely to be caused by severe nuclease contamination. Integrity of DNA can be assessed by gel electrophoresis (although this may not reveal low levels of exonucleolytic activity). Demonstration of endonucleolytic activity may also be possible by incubation

of uncut DNA with buffers or ligase, followed by transformation, when a significant reduction in the number of blue plaques would be expected. Any white plaques in (i) may not be safe to use.

2.2 Problems in transfection

2.2.1 *Standard microbiological technique*

Some of the problems encountered here are 'trivial' ones associated with pouring suitable lawns and so on. The most common ones are:

(i) *Large clear areas up to 1 cm in diameter.* These are usually caused by condensation, generally resulting from inadequate drying of plates before pouring lawns. Plates should also be incubated with the agar side uppermost (ensuring that the soft agar has set before inverting the plates). Clear areas may be plaques caused by contaminating bacteriophages, many of which will give much larger and clearer plaques than M13. Suspect media should be autoclaved and discarded, and scrupulous attention paid to sterilizing equipment and media before use.

(ii) *Streaking of plaques.* Instead of being circular, plaques have a 'comet-shaped' appearance. This is also usually due to inadequate drying of plates.

(iii) *Mottling of lawns.* The lawn has a very uneven appearance, which makes it impossible to distinguish plaques. This is usually caused by partial solidification of the top agar before pouring.

2.2.2 *Transfection*

(i) *A very thin lawn, with no plaques visible.* This usually indicates more or less confluent lysis. It is unlikely that all the transfections outlined above will lead to confluent lysis, and a more probable explanation is that the media or the host culture used are contaminated with another phage, not necessarily M13. Fresh media should be made up, and a new host culture set up. Contaminated media can be identified if necessary by spotting a few microlitres of suspect material on a freshly seeded lawn, allowing to dry by leaving the plate open near a lit bunsen burner and then incubating.

Another cause of a very thin lawn can be top agar which is too hot. If the vessel containing the molten top agar cannot be comfortably held in an unprotected hand, the agar is almost certainly too hot, and will kill most of the cells before plating. Top agar should remain molten in a 42°C incubator or waterbath, at which temperature it should be quite safe to use.

(ii) *An apparently normal lawn, with no plaques visible.* It is unlikely that the cells will fail to become competent, so before discarding such plates, check that there are indeed no plaques. If the lawn was seeded too thickly (i.e. too many cells used for each transformation) then the plaques may be too small to be readily distinguished without careful examination. Tiny plaques may also rise from the insertion of too large a piece of DNA, slowing down phage replication and also increasing the selective advantage of any deletion mutants that arise. Furthermore, deletion mutants may themselves give small plaques.

It seems that a lack of plaques can also be caused by use of top agar which is too hot (albeit not hot enough to kill the cells as stated above). Possibly this causes some

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2.2.3.

2.2.3 *Strain genotyp*
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3 PREPARATION
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High or low growth

physiological shock, which inhibits the growth of phage in the host.

The effect can also be caused by a loss of the F′ plasmid from the host. This results in a loss of the sex pilus, which is required for the phage to infect cells. Continued subculturing of the host strain in a rich broth or on rich plates is the commonest cause of this, as there is consequently no selection for retention of the plasmid. Cultures of the host strain should therefore be set up by inoculating broth (usually YT) with a single colony from a minimal plate (which therefore lacks proline). The ability to synthesize proline depends on genes on the F′ plasmid for all hosts used in sequencing work, so selection for proline prototrophy ensures the presence of the plasmid. Genotypes of the two strains most commonly used and a recipe for F′ selection are given in Section 2.2.3.

2.2.3 *Strain genotypes and proline selection*

JM101 (7) K12, Δ(*lac pro*) *supE thi* 1F′*tra*D36*pro*A$^+$B$^+$ *lac*I^q,ZΔM15
TG1 (8) K12, Δ(*lac pro*)*supE thi*-1*hsd*D5 F′*tra*D36*pro*A$^+$B$^+$ *lac*I^q,ZΔM15
Minimal medium: 2% agar in distilled water, autoclave in 300-ml quantities, add 100 ml of minimal salts to 300 ml of molten agar, carbon source (e.g. glucose) to 0.4% w/v, thiamine to 1 mg l^{-1}. Minimal salts: K_2HPO_4 28 g, KH_2PO_4 8 g, $(NH_4)_2SO_4$ 4 g, $(Na)_3$citrate 1 g, $MgSO_4$ 0.4 g, distilled water to 1 litre, autoclave in 100 ml quantities.

3. PREPARATION OF SINGLE-STRANDED DNA

Most of the problems associated with this set of operations do not become apparent until the sequencing gels themselves have been run and the autoradiographs developed. A few points may be worth making, however. Generally, the pellet produced by NaCl/PEG precipitation of the M13 phage should be fairly readily visible, and about the size of a pinhead. If the pellet is much larger, it is possible that the cultures were incubated for too long during growth of the phage, or that the cell density of the starting culture was too great. This is likely to lead to degradation of the DNA, (as a consequence of cell lysis) and therefore a high level of artefact bands in the sequencing gels—see Section 4.1.3 and *Figure 2*.

A number of factors may cause the pellet to be very small, or even invisible. The most obvious is a failure to inoculate the culture with sufficient viable phage. This may happen if the plate containing the plaques has been kept for too long before using them to prepare single-stranded DNA. The 'life expectancy' of plaques on a plate is variable, but ideally one should aim to use them within 24 h of their generation. If they are kept for longer, storage in a refrigerator is necessary. Deletion of the insert (see Sections 2.2.2 and 4.1.2) is also more likely the longer the plaques are kept.

Low yield may also be due to having too low a cell density at the time of inoculation with phage, or using an unsuitable host strain. Aside from the possibility of contamination with strains that do not support growth of M13, this problem may again be caused by loss of the F′ plasmid, resulting in fewer cells in the culture being infected by phage and thus giving a lower titre. This problem can be circumvented by use of colonies picked off a selection plate which does not contain proline (see Section 2.2.2 and 2.2.3).

High or low growth temperatures may also inhibit phage growth, so the incubator

Figure 2. Effect of growth conditions on ssDNA template preparation. **Lanes 1–4**, T,C,G,A tracks of template isolated from cells grown for twice the normal length of time (10 h), after infection of an *Escherichia coli* culture of four times the usual cell density. **Lanes 5–8**: T,C,G,A tracks of template isolated from *E.coli* cells after infection of a culture of four times the usual cell density, and grown for 5 h post infection. **Lanes 9–12**, T,C,G,A tracks of template isolated from *E.coli* cells grown for twice the normal length of time after infection.

should be checked. Good aeration of the cultures is also important for phage infection and growth, so they should be shaken at 200–250 r.p.m. during growth. Even if the phage pellet is scarcely visible at all, however, it is worth continuing with the DNA preparation, although it may be helpful to dissolve the DNA produced at the end in half the usual volume of buffer, to avoid having a very dilute solution for sequencing

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(which would then require prolonged autoradiography). Note that, even with a normal sized phage pellet, one should not expect to see a *nucleic acid* pellet at the end of the preparation, although some 'ghosting' may be apparent at the bottom of the centrifuge tube. A clearly visible pellet usually indicates contamination of the DNA, most likely with salt (i.e. the sodium acetate used in precipitation) or chromosomal DNA, but possibly also PEG or phenol, and will usually result in poor quality sequence (see Section 4.1.3). It may be possible to rescue this by redissolving and then reprecipitating the DNA before sequencing, but it is sensible to try sequencing with the DNA first to determine the seriousness of the problem.

4. PROBLEMS ENCOUNTERED WITH SEQUENCING REACTIONS AND GELS

After preparing the single-stranded DNA, the next stage at which problems are likely to manifest themselves is when the autoradiograph of the sequencing gel has been developed, and the sequence is found to be too faint, the bands are too diffuse, or there are bands in more than one track at the same level. Some of these problems cannot be easily rectified, and allowance must then be made in reading the gel. Guidance on this is given in Section 5. Many problems can be fairly easily solved however, and these are discussed below, with photographs of specimen gels illustrating some of them. Defects are grouped according to whether they are caused by deficiencies in template, priming, sequencing reactions or electrophoresis. Note however that different deficiencies can give rise to the same overall appearance.

4.1 Poor quality template

This is probably one of the most common areas of difficulty, and one can often expect one's very first template preparations to be of rather low quality, without any one particular fault predominanting. With practice, a rapid improvement is usually seen. It should also be mentioned that the quality of template needed is often determined by the DNA polymerase used. Some preparations will give perfectly adequate sequence from even quite low quality DNA, which might give totally unreadable results with other enzyme preparations. In our experience, 'Sequenase' (Chapter 2, Section 3.3) is less affected by poor quality DNA preparations.

4.1.1 *Sequence of good quality but very faint*

This is likely to be due simply to a low yield in the DNA preparation (see Section 3). A longer exposure in autoradiography may be sufficient to compensate, but this will slow down a sequencing project of any size.

Contamination of DNA pellets with salt causes a dramatic inhibition of polymerase, resulting in very faint gels. The sequence often also has artefact bands (see below).

4.1.2 *No sequence visible at all*

This can be due to a complete failure of the phage to grow (in which case no phage pellet would be seen) — see previous paragraph and Section 3. Alternatively, deletion of part of the phage genome may generate mutants which give white plaques on Xgal

plates, but to whose DNA the primer cannot anneal. Such deletion mutants may often outgrow the other phage. They are more likely to be a problem on plates which have been stored for some time after transfection.

4.1.3 *Sequence shows a high occurrence of artefact bands (i.e. bands at the same level in more than one track)*

Contamination with salt and/or PEG are often blamed for generating artefact bands, and also making bands more diffuse. In our hands, PEG at least is not usually a major problem (indeed deliberately adding PEG to the DNA preparations had no significant effect), but this may be a reflection of the enzyme preparation used (see Section 4.3.1). Increasing the amount of enzyme added may help to reduce the number of artefact bands. Note that a high salt concentration will allow a less stringent annealing between primer and template. This may be particularly problematic if the template is contaminated with chromosomal DNA.

Artefact bands can also be caused by nicking of the template DNA during preparation, often as a result of incubating the cultures for too long before harvesting the phage, or having too high a cell density at the start of the incubation (*Figure 2*). It is probable that some cell lysis occurs, liberating nucleases which attack the DNA. RNA fragments may also be released, and act as random primers in sequencing.

Occasionally, artefact bands throughout a sequence may be generated as a result of careless picking of plaques for phage growth, resulting in picking a mixture of two different phage. This will obviously result in the superposition of two sequences (not necessarily of equal intensity) throughout, but should be found only in isolated DNA preparations within one batch. It is more likely to be a problem when plates have not been properly dried and condensation spreads phage particles over the surface of the lawn, when the plaque density is very high, or when plates have been stored for several days before using the plaques, since phage particles can diffuse through the soft agar.

Single-stranded DNA preparations should be kept frozen, when they are generally quite stable. Repeated freeze thawing should not usually be necessary, as any one clone should not need to be sequenced more than a few times at most. Although a few cycles of freeze-thawing do not usually lead to marked deterioration of sequence quality (notably the appearance of artefact bands combined with a general reduction in band intensity), this should be avoided as far as possible. If it is necessary to make fresh template from a single-stranded DNA stock, this can be done by transfection in the usual way, even though the DNA is single-stranded. Generally 1 μl of a 100 $\times$ diluted stock of sequencing template gives a suitable number of plaques on the lawn. Note that a small proportion of molecules in the stock may contain deletions or other rearrangements, so it is advisable to work up several plaques from the retransfected lawn.

4.2 Priming

4.2.1 *Sequence of good quality but faint*

This can be caused by using too low a concentration of primer, or by carrying out the annealing at much too low or high a temperature (*Figure 3*). Repeated freeze-thawing of the primer will bring about its degradation, which will also make the sequence rather faint. In addition, non-specific annealing of primer fragments may increase the

Figure 3. Effect of primer
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Figure 3. Effect of primer concentration on sequencing reactions. **Lanes 1–4**, T,C,G,A tracks of template using 15 picomoles of primer per track. **Lanes 5–8**, T,C,G,A, tracks of template using 0.05 picomoles of primer per track. **Lanes 9–12**, T,C,G,A tracks of template using 0.005 picomoles of primer per track. For **lanes 1–12**, primer was annealed to template at 55–60°C. **Lanes 13–16**, T,C,G,A tracks of 0.05 picomoles of primer per track annealed to template at 20°C.

background of artefact bands. Stock solutions of primer should be kept in small aliquots at −20°C (or below) to avoid excessive freezing and thawing.

4.2.2 *Sequence has many artefact bands, but is not faint (may be abnormally strong)*

This can be caused by having too high a concentration of primer. The artefact bands generated often have a rather uneven spacing, by comparison with 'normal' artefact

bands. A titration of various primer concentrations with representative template DNA preparations will usualy indicate the optimal quantity to use, and should normally be carried out when starting a new batch of primer. See *Figure 3*.

4.3 Sequencing reactions

4.3.1 *Sequence has many artefact bands*

Assuming the quality of the template is good, the most likely cause of this is the polymerase preparation used. As mentioned earlier, some polymerase preparations appear more tolerant of suboptimal conditions than others, and increasing the concentration of polymerase may be helpful. Anything which is likely to decrease the activity of the polymerase should be avoided. Factors which are important here include keeping the polymerase stock at −20°C (but not at −80°C, when the repeated freeze-thawing needed will rapidly denature the enzyme), avoiding diluting the enzyme until just before it is to be added to the reaction mixes (regardless of whether the diluted enzyme is kept on ice before addition), and the temperature at which the reactions are carried out (although we see little difference between reactions carried out at room temperature and those carried out at 37°C, except when templates have a high degree of secondary structure). Of particular importance, however, seems to be the quality of dithiothreitol added to the sequencing reactions. This is especially so when (as is now usual) sequencing is carried out with $[\alpha\text{-}^{35}S]$dATP rather than $[\alpha\text{-}^{32}P]$dATP. DTT solutions are not very stable, even at −20°C, a fact which is exacerbated by repeated freeze-thawing. Deterioration of DTT can result in the appearance of artefact bands and reduction of intensity of genuine bands in the gel, especially pronounced in regions nearer the top of the gel. Different tracks may show this to greater extents—very often the C track is one of the first to be affected. Although it is not necessary to make up a fresh DTT solution from solid every day, it is wise to do so each week, and divide it into aliquots to avoid freezing and thawing. Solutions of DTT have a characteristic smell, and any diminution of this smell usually indicates deterioration of the solution, which should be discarded.

Artefact bands restricted to one track can be due to an unsuitable nucleotide mix (see below). This track will usually be significantly fainter than the others. Cross-contamination of mixes will also, obviously, lead to artefact bands.

4.3.2 *Uneven distribution of radioactivity throughout the gel*

It is sometimes the case that the bands towards the bottom of the gel are much more intense then those further up, so that it may be possible to read the sequence over only a small part of the gel. This usually indicates that there is an imbalance in the molar ratios of deoxy- and dideoxynucleotides, so that most chain-termination is taking place early on. It is a simple matter to run a series of reactions with varying ratios to find the optimum. Often this problem is restricted to one track (see *Figure 4*). A severe disturbance of the ratio can lead to the generation of artefact bands. Efficient sequencing of regions with markedly abnormal base composition may require compensating adjustments to the nucleotide mixes.

If an apparent imbalance in ratios appears, when previously there had been no problem, then it is likely that one of the components of the reaction mixes is deteriorating. Although

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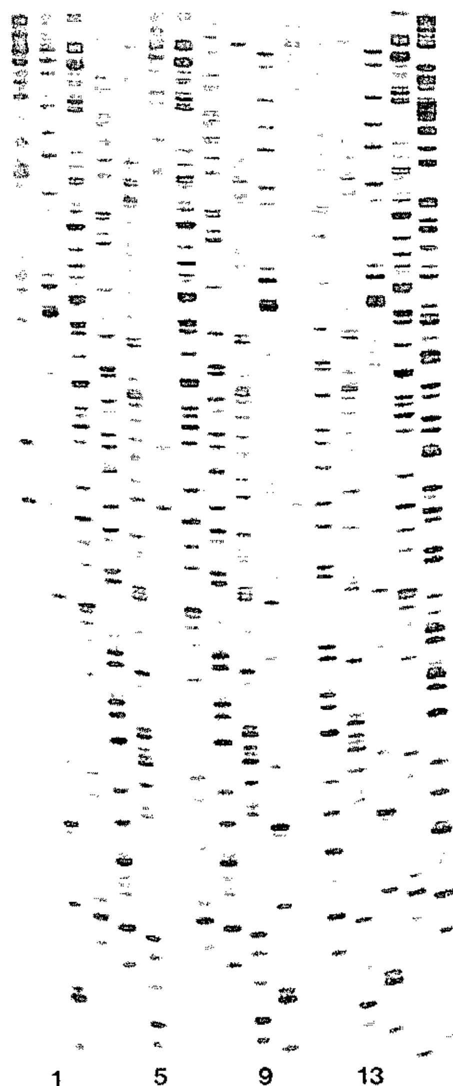

Figure 4. Effect of nucleotide mixes on sequencing reactions. **Lanes 1–4**, T,C,G,A tracks with twice usual amounts of dT in T nucleotide mix. **Lanes 5–8**, T,C,G,A tracks with twice usual amounts of dC in C nucleotide mix. **Lanes 9–12**, T,C,G,A tracks with twice usual amounts of dG in G nucleotide mix. **Lanes 13–16**, T,C,G,A tracks with usual amounts of nucleotides in all four mixes (for composition of nucleotide mixes see Chapter 2, *Table 2*).

the effect of this is likely to be greatest in one track, it can be expected to reduce the quality of other tracks too. When beginning sequencing for the first time it is wise to buy fresh stocks of nucleotides, aliquot the stock solutions and avoid freeze-thawing. Very often a nucleotide solution which has been found perfectly suitable for other biochemical purposes will be found to be unsuitable for sequencing purposes.

4.3.3 *Progressive overall reduction in band intensity with time*

Although the half life of the ^{35}S nucleus is some three months, it should be borne in mind that the chemical stability of $[\alpha$-^{35}S$]$dATP is notably less, and can be significantly reduced, as might be expected, by freeze-thawing repeatedly.

4.4 Electrophoresis

Problems encountered here usually arise as a result of incorrect preparation and loading of gel or samples, rather than in the gel electrophoresis itself. The resolution obtained with very thin gels (0.2 mm) is particularly sensitive to the nature of the sample, and the suppliers' protocols should be consulted carefully.

4.4.1 *Formation of bubbles while pouring the gel*

Pouring the thin gels used for sequencing requires some practice. To help avoid bubbles, ensure that the plates are very clean and grease-free, and run the gel solution between the two plates continuously. Try to avoid pausing, or interrupting the flow. Most X-ray film is thinner than most gel spacers, so if bubbles do form, it may be possible to dislodge them with a long strip of X-ray film inserted between the plates.

4.4.2 *Dark specks on the autoradiograph, often with thin lines extending downwards*

This is caused by dust on the gel plates prior to pouring the gel. Some types of paper towel used for drying plates leave a great deal of dust. See *Figure 5*.

4.4.3 *Disintegration of sample wells*

This is caused by a failure of polymerization and results in part of the well being washed away when the comb is removed and buffer added. It may be due to insufficient ammonium persulphate or TEMED, not leaving the gel for long enough to polymerise, or air getting to the wells. If the latter is the case, wrapping the top of the gel in Saran Wrap while polymerization is occurring may help. Degassing the gel mix may also help, as dissolved oxygen inhibits polymerization.

4.4.4 *Bands on gel very fuzzy, or even not discernible*

This is a common problem, and may have several causes. It can result from not leaving the gel for long enough for polymerization to be completed, and this should be suspected if the wells do not form properly. The time needed will depend on the exact amounts of ammonium persulphate and TEMED used as well as ambient temperature, but as a rough guide, the gel should be left for at least 30 min after pouring before use. Degassing the gel mix may help.

A frequent cause is the use of inadequate gel materials. The highest grades of reagents should be used (e.g. 'Electran' acrylamide), and filtering and deionizing carried out. Warming of the gel mix to dissolve the urea is not advised, and should certainly be very gentle indeed (no more than about 40°C). Excessive heating may reduce resolution later or even bring about spontaneous polymerization. Gel mixes should be stored at 4°C. Storage of mixes (especially those for buffer gradient gels) for more than a month may also lead to deterioration.

Figure 5. Effect of dirty
note dark specks/streak

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the readable seque
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Excessive heating
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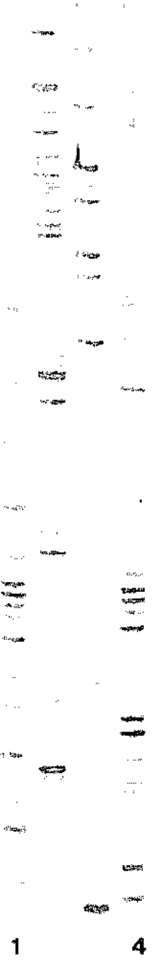

Figure 5. Effect of dirty gel plates on appearance of sequencing gel. **Lanes 1 – 4**: T,C,G,A tracks of template; note dark specks/streaks particularly visible here in the G track

Gel running buffers should also be made up reasonably frequently (storage of 10 × TBE for more than a few days causes a precipitation). Some sequencers find that a deterioration of buffer quality causes a localized fuzziness, towards the top of the readable sequence, rather than throughout the gel. When carrying out prolonged gel runs, it is wise to circulate the buffer or change it half-way through the run.

Excessive heating of sample before loading can cause a combination of effects. Bands may be fuzzy, but they may also be faint, and the gel may also show a high level of

Figure 6. Effect of overheating sequence reactions before loading. **Lanes 1–4**, T,C,G,A tracks of reactions loaded after 10 min of incubation at 100°C.

artefact bands and a darkish background in the tracks (see *Figure 6*).

Before loading the samples into the wells, it is important to flush out any urea that has diffused out of the gel. Significant amounts of urea can diffuse out in quite a short time, so this should be done immediately prior to loading. It can be done by filling a Pasteur pipette or a syringe fitted with a Gilson tip with buffer and squirting the contents into the well. Failure to do this results in fuzzy and rather uneven bands (see *Figure 7*). Loading too much sample in the well will also cause fuzzy bands. As a general

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4.4.5 *'Smiling' in g*
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Figure 7. Effect of poor gel loading on appearance of sequencing gel. **Lanes 1–4**; T,C,G,A tracks showing effect of presence of excessive urea in gel wells. **Lanes 5–8**; T,C,G,A tracks showing effect of loading too large a volume of sample in gel wells prior to electrophoresis.

rule, this will happen if the well is loaded more than about half as deep as it is wide. Samples should be loaded as rapidly as possible and the power switched on immediately after loading is complete, to prevent reannealing of the DNA in the wells.

4.4.5 *'Smiling' in gels*

This is the name given to changes in mobility of oligonucleotides across the width of the gel, so that they run faster in the middle than at the edges. This means that bands

which should be at the same level would form a 'U' across the gel. It is caused by variation in temperature across the gel, and can usually be avoided by clamping a metal sheet (2 mm thick aluminium is ideal) to the exposed gel plate, although some workers find this results in less sharp bands. Thermostatted plates are also available (at a price) for some sequencing gel apparatuses. Smiling can usually be allowed for in gel reading without much difficulty but may be a problem in automated sequencing.

4.4.6 *Difficulty in drying gels down for autoradiography*

This can be an indication that the urea has not been sufficiently leached from the gel during fixation for autoradiography. It may also render the bands diffuse. Fixing for 15 min is usually sufficient. Note that close contact between the gel and the film is needed during autoradiography, or the bands will become fuzzy and very faint.

5. SEQUENCE-DEPENDENT PROBLEMS

These are manifested (especially when using Klenow polymerase) as localized regions of sequence which are difficult to read easily. Certain rules can be applied and are set out in more detail in ref. 9 (and see Chapter 2, Section 3.3), but in general it is advisable to ensure that the same region is sequenced on the complementary strand, when the same problem will usually not arise. Sequencing on the complementary strand is of course necessary in any case.

5.1 Variations in band intensity

(i) *A bands*. In a run of As the bottom band is frequently the strongest.

(ii) *C bands*. Where two or more Cs are adjacent, the lowest is generally much weaker than the next one, so that the former may be scarcely visible. Individual C bands may also be very faint, less so if preceded by a G.

(iii) *G bands*. G may be weak in the sequence TG.

5.2 Artefact bands

As well as the non-specific artefacts already discussed, a few sequence-specific artefacts are sometimes (but not always) seen.

(i) *TGCC*. This sequence may cause an artefact band in the C track at or between the levels of the T and G bands.

(ii) *GCA*. Here there may be an artefactual T or C at the level of the A band.

5.3 Compressions

These are probably caused by G:C hairpins forming localized secondary structure in the DNA, persisting even under the conditions of electrophoresis. This secondary structure causes oligonucleotides to behave as though they were shorter than is actually the case, and thus migrate faster. It is diagnosed by bands running very close together, sometimes superimposed, usually with a gap or increased band spacing in the region above. There are three solutions to this problem. One is to make the conditions under which the gel is run more denaturing, which can be done by running at higher power (although this may also decrease resolution of the gel, and can cause plates to crack, particularly if they are chipped or scratched) or including formamide in the gel to a

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5.4 **Pile-ups**

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complementa
Carrying out
('Taq' polym

6. KEY

6.1 **Plaque g**

Insufficient w

Too many wh

Plaques too s

Confluent pla

Mottled lawn

No plaques

final concentration of 25–50%. The use of high formamide concentrations may lead to a higher background in the gel, however. Another solution is to sequence the complementary strand, when the position of the compression will usually have shifted a few bases. The third is to use ITP instead of GTP, as I.C base pairs are weaker than G.C ones, and secondary structures will therefore be less stable. For protocols for this, see Chapter 2, Section 3.2.1. Secondary structure may also lead to termination of synthesis by the polymerase, and may lead to compressions or pile-ups. Running the sequencing reactions at a higher temperature may alleviate this problem.

5.4. Pile-ups

These are also known as 'walls'. They are diagnosed as strong stops in all four tracks, often at two or more consecutive positions, and are generated during the sequence reactions rather than electrophoresis. Their occurrence may be dependent on the quality of template and enzyme, probably due to the presence of salt. Sequencing the complementary strand usually resolves the problem. (See also the previous section.) Carrying out the reactions at higher temperatures, using a thermostable polymerase ('Taq' polymerase, Cetus) may help (see Chapter 2, Section 3.4).

6. KEY

6.1 Plaque generation

Insufficient white plaques	Incorrect insert/vector ratio (2.1.1) Poor insert material (2.1.1) Incorrect phosphatasing (2.1.1, 2.1.2) Ligase/buffer faulty (2.1.3) Inefficient vector digestion (2.1.4) Nuclease contamination (2.1.1, 2.1.9)
Too many whites	Contamination of vector, buffers (2.1.6) No Xgal/IPTG or failure of colour (2.1.8) Contamination of host (2.2.2, 2.1.7)
Plaques too small	Lawn seeded too heavily (2.2.2) Insert too large/deleted (2.2.2)
Confluent plaques/very thin lawn	Wet plates (2.2.1) Contamination of host (2.2.2, 2.2.3) Cells overheated (2.2.2)
Mottled lawn	Agar too cool (2.2.1)
No plaques	Cells not competent (2.2.2) Pili lost/strain contaminated (2.2.2) Nuclease contamination (2.1.9)

6.2 Sequencing reactions, gels

Problem	Possible causes
Artefact bands in all tracks	Dirty template (4.1.3) Poor quality polymerase (4.3.1) Excessive freeze thawing (4.1.3, 4.2.1) Cells grown too long/too heavily seeded (3, 4.1.3) Excess primer (4.2.2)
Artefact bands in one track	Imbalanced nucleotide mix (4.3.2) DTT old (especially C track) (4.3.1)
Duplicate bands	Mixed template (4.1.3) Cross-contamination of mixes (4.3.1)
Bands faint/absent	Deletion in phage (4.1.2) Low yield of DNA (3, 4.1.1, 4.1.2) Poor nucleotide mixes/radioisotope (4.3.2, 4.3.3) Faulty primer (4.2.1) Freeze-thawing of template (4.1.3)
Bands fuzzy	Urea not washed out of wells (4.4.4) Too much sample loaded (4.4.4) Poor quality/stale electrophoresis reagents (4.4.4) Incomplete polymerization of gel (4.4.3) Gel not processed correctly before autoradiography (4.4.6)
Background dark, bands fuzzy	Samples overheated (4.4.4) Plates dirty (4.4.2)
Gel difficult to read, though bands distinct	Smiling (4.4.5) Compression (5.3) Pile-up (5.4)

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