Construction of Nested Deletions for DNA Sequencing

Nested deletions useful for dideoxy DNA sequencing (*UNIT 7.4*) are a set of deletions originating close to a sequencing primer and extending various lengths along the target DNA. Each successively longer deletion brings "new" regions of the target DNA into sequencing range (about 300 base pairs for normal sequencing gels) of the primer site. The *Bal* 31 exonuclease (from *Alteromonas espejiana*) and *E. coli* exonuclease III (exo III) methods for generating a nested set of deletions are described in this unit. *Bal* 31 can also be used to generate nested deletions for chemical sequencing (*UNIT 7.5*) in conjunction with specialized chemical sequencing vectors (*UNIT 7.6*).

We recommend the use of the exo III procedure because it is considerably less time-consuming than the *Bal* 31 procedure. In most cases, it should be possible to identify a cloning vector to carry out the exo III procedure; however, as described below, the exo III procedure is dependent on the presence of suitable restriction sites and therefore cannot be utilized in every case. Under these circumstances, the *Bal* 31 method can be used. Refer to the commentary (background information) for a discussion of the requirements, advantages, and disadvantages of the two procedures.

BASIC PROTOCOL

USING EXONUCLEASE III TO CONSTRUCT UNIDIRECTIONAL DELETIONS

This method, as illustrated in Figure 7.3.1, is based on the enzymatic properties of exo III, which is specific for double-stranded DNA, digests single strands starting at a 3′ end, but cannot initiate digestion at a 3′ overhanging end. The protocol contains the following steps: (1) cloning of target DNA into a polylinker in a suitable plasmid vector or a suitable M13mp replicative form (RF) vector (see strategic planning, below, for a discussion of useful vectors); (2) double digestion of the plasmid with an enzyme that leaves a 5′ overhanging or blunt end and an enzyme that leaves a 3′ overhanging end of four bases (these enzymes must not cut inside the cloned target DNA); (3) exonucleolytic attack with exo III for varying lengths of time to create unidirectional digestion of the target sequence; (4) treatment with a single-stranded nuclease (S1 or mung bean nuclease) to remove the 5′ single strand, and repair of ends with Klenow fragment of *E. coli* DNA polymerase I; and (5) circularization and ligation with T4 DNA ligase and transformation of competent *E. coli* cells.

Materials

CsCl/ethidium bromide–purified plasmid DNA (purified twice) containing target DNA to be sequenced (~5 μg per experiment; *UNIT 1.7*)
Restriction endonucleases and corresponding buffers (*UNIT 3.1*)
Buffered phenol (*UNIT 2.1*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
Ice-cold 100% and 70% ethanol
3 M sodium acetate
1× exonuclease III buffer
Exonuclease III (*UNIT 3.11*)
S1 nuclease buffer
1 U/μl S1 nuclease (*UNIT 3.12*)
S1 nuclease stop buffer
10× loading buffer (*UNIT 2.5A*)
2 U/μl Klenow polymerase (*UNIT 3.5*)

dNTP mix (dATP, dCTP, dGTP, and dTTP at 0.25 mM each; see *UNIT 3.4*)
Exo III ligation buffer
10 mM ATP
1 U/μl T4 DNA ligase (*UNIT 3.14*)
E. coli JM109 (*recA*) or DH5αF′ (*recA;* available from BRL) made competent for transformation (*UNIT 1.8*), if one of the M13mp plasmids is the cloning vector,
or
E. coli DK1 (*recA;* available from BRL) or DH5αF′ (*recA*) made competent for transformation (*UNIT 1.8*), if plasmid vector is used
LB plates containing 100 μg/μl ampicillin (*UNIT 1.1*)
LB medium containing 100 μg/μl ampicillin (*UNIT 1.1*)
H top agar (*UNIT 1.1*)
Xgal/IPTG solution (Table 1.4.2)
2× TY medium (*UNIT 1.1*)
Phage loading buffer
Additional reagents and equipment for phenol extraction and ethanol precipitation (*UNIT 2.1*), agarose gel electrophoresis (*UNIT 2.5*), transforming *E. coli* (*UNIT 1.8*), and plasmid miniprep (*UNIT 1.6*)

Strategic planning

Figure 7.3.1 gives an overview of the experimental strategy for constructing a nested set of unidirectional deletions with exo III. Between the target DNA and the sequencing primer site there must be a recognition site for a restriction enzyme that generates a 5′ overhanging end or a blunt end; between that restriction site and primer site there must be a recognition site for a restriction enzyme that generates a 3′ overhanging end of four base pairs to protect from exo III digestion. A thio nucleotide analog incorporated in the end adjacent to the primer site provides an alternative means of protection (see support protocol in this unit).

If the target DNA will be sequenced using double-stranded templates, it may be possible to construct deletions from both ends of the fragment if both sequencing priming sites and suitable restriction sites are present on both sides of the target fragment (see next paragraph). This will allow both strands of the target DNA to be sequenced. On the other hand, if the target fragment will be sequenced using single-stranded M13 templates, in order to sequence both stands of the fragment, it will be necessary to clone the target DNA in both orientations with respect to the primer sequencing site.

Choice of vector depends on the method that will be used for sequencing. For dideoxy sequencing using single-stranded DNA templates we recommend M13mp18 or M13mp19 (see *UNIT 7.4*). For "double-stranded" dideoxy sequencing (Chen and Seeburg, 1985), high-copy-number vectors containing multiple cloning sites in the polylinker should be used (e.g., pUC18/pUC19; pSPT18/pSPT19; pGEM; Bluescript M13). The Bluescript M13 vector has several convenient cloning sites absent from the pUC series of vectors (e.g., *Bst*XI, *Not*I, *Spe*I, *Sac*II, *Dra*II, *Apa*I). pSPT18, pSPT19, and pGEM have polylinkers corresponding to those in pUC18 and pUC19. Bluescript M13 and pSTP18/19 have two phage promoters each (T3 and T7; SP6 and T7, respectively), which serve as convenient priming sites for DNA sequencing reactions. The presence of multiple cloning sites as well as two priming sites in these latter vectors allows unidirectional deletions to be made from either end of an insert, so that both strands of the insert DNA can be sequenced. Table 7.3.1 lists the restriction enzyme recognition sites (and recommended suppliers) of the vectors mentioned above.

This protocol is written for constructing deletions in a ~2.0-kb fragment. However, we have successfully constructed nested sets of deletions in fragments as long as 7.0 kb,

and it is likely that even longer insert fragments could be used. Scale up the protocol proportionately for longer fragments.

Table 7.3.1 Restriction Enzyme Recognition Sites in the Polylinker Regions of Selected Vectors and the Types of Ends Created[a]

Bluescript M13		**pUC18/19**	
*Sac*I	3′	*Eco*RI	5′
*Bst*XI	3′	*Sac*I	3′
*Sac*II[b]	3′	*Kpn*I	3′
*Eag*I	5′	*Sma*I	blunt
*Not*I	5′	*Bam*HI	5′
*Xba*I	5′	*Xba*I	5′
*Spe*I	5′	*Sal*I	5′
*Bam*HI	5′	*Pst*I	3′
*Sma*I	blunt	*Sph*I	3′
*Pst*I	3′	*Hin*dIII	5′
*Eco*RI	5′		
*Eco*RV	blunt	**pSPT18/19** and **pGEM**	
*Hin*dIII	5′	have the same polylinker	
*Cla*I	5′	as pUC18/19	
*Sal*I	5′		
*Acc*I	5′		
*Xho*I	5′		
*Dra*II	5′		
*Apa*I	3′		
*Kpn*I	3′		

[a]Vectors are available from the following suppliers: Stratagene (Bluescript M13); Pharmacia (pUC18/19); Boehringer Mannheim (pSTP18/19); and Promega Biotec (pGEM).
[b]*Sac*II generates a 2-bp overhang which is not sufficient to protect against exo III digestion.

Preliminary steps

1. Clone the DNA fragment to be sequenced (insert DNA) in the polylinker of an appropriate M13mp or plasmid vector (see Fig. 7.3.1).

   If an M13mp vector is used in conjunction with single-stranded sequencing templates, it will be necessary to clone the target DNA in both orientations with respect to the sequencing primer site in order to sequence both strands of the target DNA. For double-stranded sequencing, it may not be necessary to clone the fragment in both orientations if appropriate restriction sites as well as DNA sequencing priming sites are present on both sides of the insert (see Fig. 7.3.1).

   See UNIT 7.1 for information about cloning in M13mp RF vectors and UNIT 3.16 for information about subcloning DNA fragments.

2. Prepare CsCl-purified DNA of the recombinant plasmids constructed in step 1. Purify the DNA in two consecutive CsCl gradients.

   It is important that the DNA be supercoiled; nicks in the vector DNA may cause degradation in the exonucleolytic step. See UNIT 1.7 for information about obtaining supercoiled DNA.

DAY ONE

Linearize M13mp::insert RF DNA or plasmid::insert DNA

3. Completely linearize 5 μg M13mp::insert RF DNA or plasmid::insert DNA by double digestion with restriction enzymes that leave a 3′ overhang adjacent to primer site and a 5′ overhang or blunt end adjacent to the insert DNA (see Fig. 7.3.1).

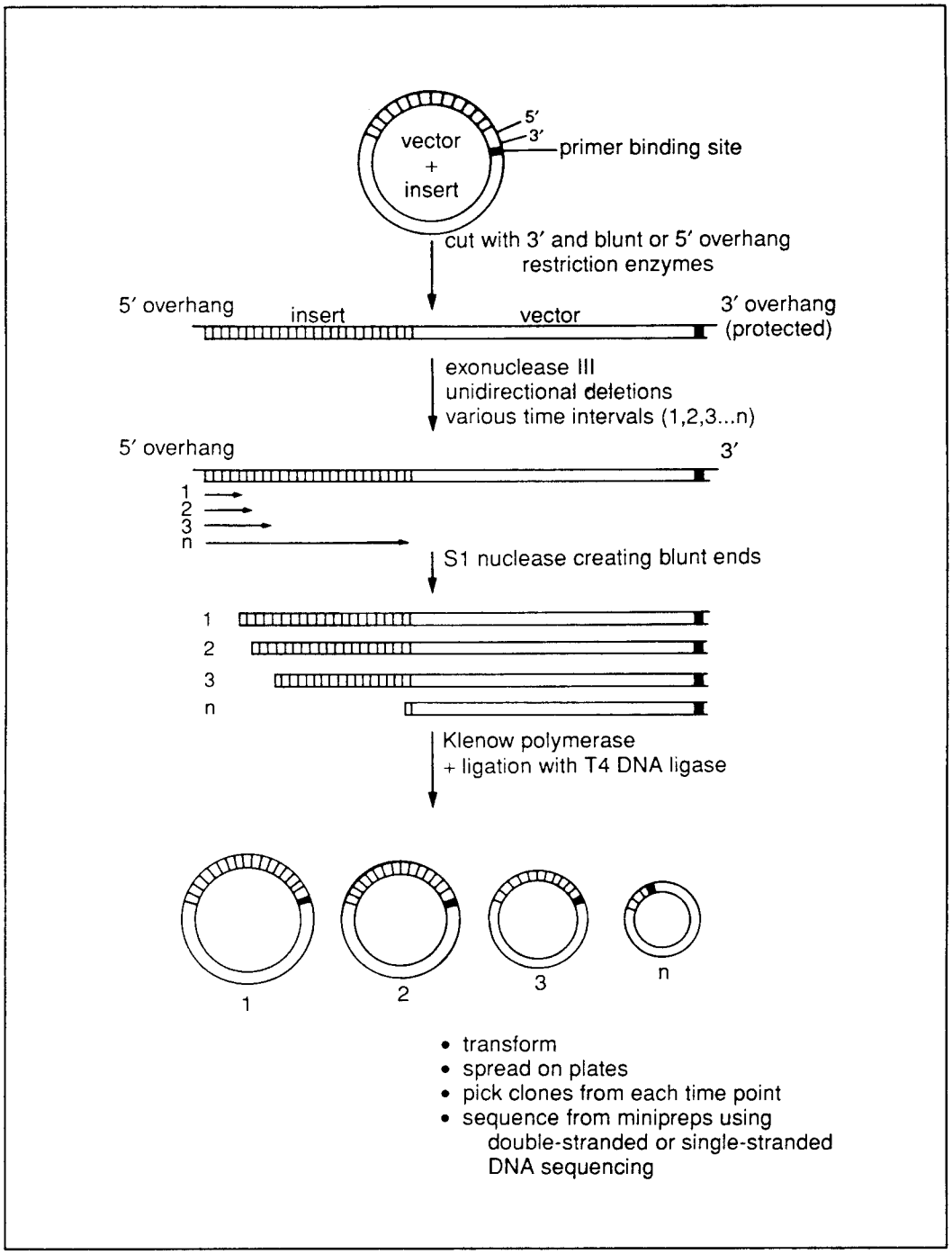

Figure 7.3.1 Construction of unidirectional deletions using exonuclease III nuclease. See commentary (background information) for a full description of events.

The 5′ or blunt restriction site must be positioned between the 3′ restriction site and the insert. Verify that digestion is complete by running a small aliquot on agarose minigel (UNIT 2.5).

4. Extract with buffered phenol, extract with phenol/chloroform/isoamyl alcohol, add 1/10 vol 3 M sodium acetate, and precipitate with ethanol (see *UNIT 2.1*). Wash the pellet in cold 70% ethanol and dry it.
5. Dissolve the dried pellet in TE buffer to give a final concentration of 0.2 μg/μl.

Create a nested set of unidirectional deletions

The extent of digestion of exo III is regulated by the reaction temperature and the time of incubation. Rates of 250 bp/min at 37°C and 120 bp/min at 30°C were obtained using the conditions outlined in the following steps.

6. Mix 12.5 µl (2.5 µg) of the linearized DNA (from step 5) with 5.0 µl of 5× exonuclease III buffer and 7.5 µl water to give a final volume of 25 µl.
7. Incubate 2 min at 37°C. Add 150 U exo III per pmol susceptible 3′ ends.

   Conversion factor: 1 µg of 1 kb DNA = 3 pmol 3′ ends.
8. Remove 3-µl aliquots at 1-min intervals to individual microcentrifuge tubes (giving a total of eight samples) and place immediately on dry ice for 5 min. Add 3 µl water and inactivate the exo III by incubating 10 min at 70°C. Put the samples on ice.
9. Add 15 µl S1 nuclease buffer and 4 µl (4 U) S1 nuclease to each sample and incubate 20 min at room temperature.
10. Stop the S1 nuclease reaction (by a pH-shift) by adding 5 µl S1 nuclease stop buffer to each sample.

Subject deleted DNA samples to electrophoresis

11. Add 2 µl of 10× loading buffer to 8-µl aliquots of each sample and subject to electrophoresis on a 1% agarose gel, including ethidium bromide in the gel and buffer. Also run a lane of molecular weight markers (e.g., bacteriophage λ DNA, cut with *Hin*dIII) to determine the actual rate of exonuclease degradation. Decide which aliquots are worth saving.

    The photograph of an agarose gel in Figure 7.3.2 demonstrates the extent of deletions created on a Bluescript vector with a 3-kb insert starting with 10 µg DNA.

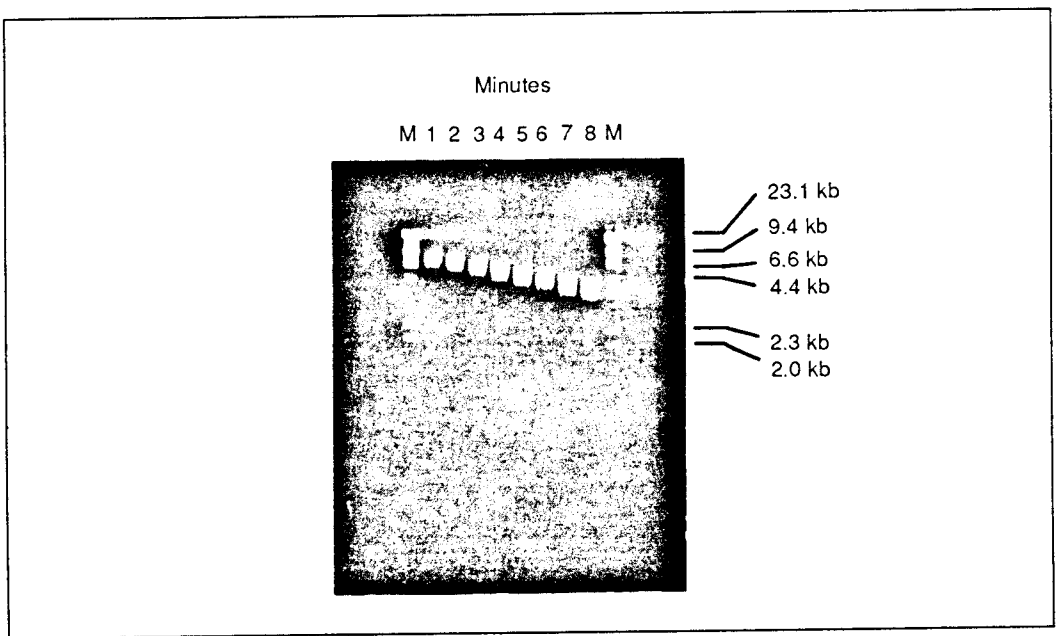

Figure 7.3.2 Ethidium bromide–stained agarose gel depicting exo III digestion. Bluescript vector (10 µg with a 3-kb insert) was cut with *Xba*I, which leaves a 3′ overhanging end, and with *Bst*XI, which leaves a 5′ overhanging end. The vector was then subjected to exo III digestion for eight different time intervals, followed by S1 nuclease digestion. Approximately 1 µg of each DNA sample was loaded on the gel. The DNA fragments in the lanes labeled "M" are molecular weight markers.

Create blunt ends for ligation

12. Add to each remaining 22 µl of the selected samples 1 µl (2 U) Klenow polymerase. Incubate ~2 min at 37°C. Then add 1 µl dNTP mix and continue incubation at 37°C for 10 min to create blunt ends.

Recircularize the deleted molecules by ligation

13. Add 20 µl exo III ligation buffer, 3 µl of 10 mM ATP, 14 µl water, and 1 µl (1 U) T4 DNA ligase. Incubate 5 hr at room temperature or overnight at 15°C.

If you are using a plasmid vector (including M13mp vectors) and are planning to carry out dideoxy sequencing with double-stranded plasmid template, refer to steps 14 to 18. If you are using an M13mp vector and are planning to carry out dideoxy sequencing with single-stranded templates, proceed to step 19.

Transform ligated DNAs into competent E. coli

DAY TWO (DOUBLE-STRANDED TEMPLATE)

14. Transform 100 µl of competent cells (*E. coli* DK1 or DH5αF′) with 10 µl of each ligation reaction and proceed according to the transformation protocol (*UNIT 1.8*). Spread 1/5 of each of the transformation cultures on LB plates containing ampicillin (100 µg/µl), and incubate overnight at 37°C.

    Any competent E. Coli recA strain can be used for transformation. About 2000 colonies are expected for each sample.

Characterize deleted plasmid clones

DAY THREE (DOUBLE-STRANDED TEMPLATE)

15. Pick 2 to 4 colonies from each plate and inoculate 5 ml LB medium containing ampicillin (100 µg/µl) and grow overnight. Subject 1.5 ml of each overnight culture to one of the miniprep procedures described in *UNIT 1.6*. Make a frozen glycerol stock with 1 ml of each culture (see *UNIT 1.3*) and store the remainder of each overnight culture at 4°C for DNA sequencing template preparation.

16. Add 2 µl of 10× loading buffer and 10 µl water to each 2.5 µl of the plasmid DNA from step 15 and run on a 1% agarose gel. As size markers, also load 0.1 µg each of the undeleted plasmid and parent vector on the gel. You should be able to discern a ladder of deletions from full insert size to ~300 bp.

Prepare double-stranded plasmid templates for DNA sequencing

DAY FOUR (DOUBLE-STRANDED TEMPLATE)

17. Choose a set of clones (from step 16) that differ from each other by intervals of 250 to 300 bp, spanning the complete region of the target DNA. Prepare plasmid DNA from the overnight cultures stored at 4°C (step 15) suitable as template for dideoxy sequencing (alternate protocol).

18. Subject the plasmid DNA of the deleted clones to nucleotide sequencing analysis, employing the double-stranded template sequencing technique (alternate protocol).

    You can also first characterize the clones by T-track analysis, in which each template is used in a dideoxy-T sequencing reaction. The sequencing pattern will allow you to determine if there are overlapping regions in the chosen clones.

Follow steps 19 to 35 if you are using an M13mp vector and are planning to carry out dideoxy sequencing using single-stranded templates.

Transform ligated DNAs into competent E. coli

DAY TWO (SINGLE-STRANDED TEMPLATE)

19. Transform 100 µl of competent JM109 or DH5αF′ *E. coli* cells with 10 µl of each reaction mix (from step 13) and proceed according to transformation protocol (*UNIT 1.8*). Also transform with 0.5 ng of the M13mp RF vector DNA and 0.5 ng of the M13mp::insert RF DNA that carries the nondeleted fragment.

    E. coli DH5αF′ can be maintained on rich media without loss of the F′ factor, is supressor plus, restriction minus, modification plus, recombination deficient (recA1), and exhibits a higher transformation efficiency than JM109.

20. Following the heat shock step in the transformation protocol, add 0.2 ml of an overnight culture of *E. coli* JM109 or DH5αF′, pipet contents into 3 ml H top agar containing 60 µl Xgal/IPTG solution, mix, and pour on LB plates. Incubate overnight at 37°C.

    Also transfect with the M13mp RF vector used to clone the target DNA fragment and the M13mp::insert RF recombinant plasmid carrying the undeleted fragment.

    About 2000 plaques are expected for each sample.

DAY THREE (SINGLE-STRANDED TEMPLATE)

Characterize deleted phages

21. Dilute an overnight of *E. coli* JM109 or DH5αF′ (or equivalent) 100-fold with 2× TY medium and dispense 1.5-ml aliquots in loosely capped 18 × 150-mm test tubes, one tube for each M13 phage to be tested or sequenced (including tubes for the M13mp vector and M13mp::insert as controls).

22. Pick 2 to 4 M13 plaques from each transfection plate (i.e., 2 to 4 per exonuclease time point) and inoculate one M13 plaque per 1.5 ml DH5αF′ culture using sterile Pasteur pipets, making sure that a plug of agar is transferred.

    Following sequencing, you may find that particular deletion intervals are missing. For this reason, it is useful to be able to examine additional phage from a particular time point. Since phage diffuse within the agar, we advise picking several additional colorless plaques from each time point into individual wells in a 96-well microtiter plate containing 200 µl of 2× TY medium plus 15% glycerol, growing them at 37°C for 6 hr, and freezing at –20°C for future use, if necessary.

23. Grow the cells 5 to 6 hr at 37°C (preferably on a tube roller; see *UNIT 1.2*).

24. Transfer cells to 1.5-ml microcentrifuge tubes; spin 5 min in microcentrifuge.

25. Transfer supernatants to fresh microcentrifuge tubes and store at 4°C.

    If further small-scale manipulations with double-stranded DNA are desired, harvest cells in addition to the supernatants and isolate RF DNA by the alkaline lysis method described in UNIT 1.6.

26. Place 20 µl of each supernatant into 4 µl of phage loading buffer and load on a 1% agarose gel. Also load M13mp vector(s) and M13mp::inserts (undeleted) supernatants as size markers. You should be able to discern a ladder of deletions from full insert size to about 300 bp.

    Optional test for detecting small inserts: C testing entails mixing 10 µl of a phage supernatant carrying a presumptive insert with 10 µl of the supernatant from the M13 control carrying the full-length, undigested opposite strand. Add 4 µl phage loading buffer and incubate 30 min at 65°C prior to electrophoresis. Annealing between the phages causes significant retardation in the gel compared to the single-stranded circular forms.

    C testing can also be used to determine the orientation of an insert.

DAY FOUR (SINGLE-STRANDED TEMPLATE)

Prepare single-stranded phage templates for DNA sequencing, choosing phage that contain appropriate deletions from previous step

27. Choose clones from step 26 that differ from each other by intervals of 250 to 300 bp, spanning the complete insert DNA.

28. Respin the stored phage supernatants from step 25 (there will be some cell growth, even at 4°C) and transfer supernatants to fresh 1.5-ml microcentrifuge tubes.

    The presence of chromosomal DNA will interfere with sequencing reactions.

29. Proceed immediately to step 5 of the support protocol in *UNIT 7.4* for preparing single-stranded template DNA for DNA sequencing.

30. Subject the prepared single-stranded DNA templates to dideoxy sequencing as described in UNIT 7.4. Also perform sequencing reactions on the vector containing the undeleted fragment.

    You can also first characterize the chosen clones by T-track analysis in which each template is used in a dideoxy-T sequencing reaction. The T patterns obtained on the sequencing gels will allow you to determine if there are overlapping regions in the chosen clones.

PROTECTION OF DNA FROM EXONUCLEASE III DIGESTION USING [αS]dNTPs

SUPPORT PROTOCOL

Creating nested deletions for DNA sequencing using exonuclease III requires that the end of the molecule adjacent to the sequencing primer site be protected from digestion. This is typically accomplished by using restriction endonucleases that provide four-base, 3′ overhanging ends. Incorporating a thio nucleotide analog, [αS]dNTP, adjacent to the primer site provides an alternative means of protecting against exo III digestion (Putney et al., 1981). Restriction sites adjacent to the primer site which leave a 5′ overhang can be filled in with Klenow polymerase using the appropriate thio nucleotide analog to cap the 3′ end. This increases flexibility in designing a deletion strategy, as any restriction site that leaves a 5′ overhang can be appropriately modified to provide the necessary protection from digestion by exo III (Ozkaynak and Putney, 1987).

Additional Materials

5 mM dNTPs (UNIT 3.4)
5 mM [αS]dNTPs (Pharmacia)

1. In a 20-µl reaction mixture, completely linearize 5 µg M13mp::insert RF DNA or plasmid::insert DNA by digesting with a restriction enzyme that leaves a 5′ overhanging end adjacent to the sequencing primer site.

    Verify that the digestion is complete by running a small aliquot on an agarose minigel (UNIT 2.5).

2. Add 1 µl of each of the appropriate 5 mM [αS]dNTPs and 5 mM dNTPs, and 1 U Klenow fragment (UNIT 3.5). Incubate 30 min at room temperature.

    The [αS]dNTPs and dNTPs required depend upon the sequence of the 5′ overhang left by the restriction endonuclease. Adequate protection from exo III digestion will be obtained for both partially and completely filled-in restriction sites, providing that the 3′-most nucleotide added by Klenow polymerase contains the thio group.

    Ozkaynak and Putney (1987) used higher concentrations of [αS]dNTPs; however we have found that the above concentration will provide adequate incorporation.

3. Stop the Klenow polymerase reaction by incubating 10 min at 75°C. Digest the DNA with a second restriction endonuclease that generates a 5′ overhanging or blunt end adjacent to the insert DNA.

    Be sure to adjust the salt concentration of the reaction mix to that appropriate for the second enzyme (see UNIT 3.1).

4. Proceed with step 4 of the basic protocol for constructing nested deletions with exo III.