USING *BAL* 31 EXONUCLEASE TO CONSTRUCT NESTED DELETIONS

SHORT PROTOCOL

1. Completely linearize M13mp::insert RF DNA or equivalent (2 µg for each 150 to 200 bp to be sequenced), cutting at one end of the DNA region to be sequenced. Repeat, cutting at the other end.
2. Phenol extract and ethanol precipitate. Dissolve to 1 µg/µl.
3. Mix 2 µl linearized DNA, 38 µl water, and 5 µl of 10× *Bal* 31 nuclease buffer. Aliquot 9 µl into 5 tubes. Add 1 µl of $^1/_{10}$, $^1/_{20}$, $^1/_{40}$, and $^1/_{80}$ diluted *Bal* 31 nuclease and incubate 30 min at 37°C.
4. Add 1 µl of 200 mM EGTA and heat 5 min at 65°C. Electrophorese in prestained agarose gel.
5. For each 2 µg linearized DNA (1 µg/ml), mix 2 µl DNA, 43 µl water, and 5 µl of 10× *Bal* 31 buffer; preheat at 37°C for 10 min.
6. Add *Bal* 31 to final dilution as determined in step 3 to completely digest DNA in 30 min.
7. Remove 45 µl to tube containing 5 µl of 200 mM EGTA (for each 2 µg DNA) every minute, up to 15 min (gives 150 to 200 bp deletions; adjust time if desired).
8. Repeat steps 5, 6, and 7 with other linearized DNA preparation.
9. Heat *Bal* 31-digested DNA samples 5 min at 65°C. Ethanol precipitate.
10. Dissolve in 20 µl of the appropriate restriction enzyme buffer and digest with second enzyme (used to linearize other portion).
11. Electrophorese on low gelling/melting temperature agarose.
12. Cut out appropriate DNA, add TE buffer to ~400 µl total volume, and heat at 65°C for 10 to 15 min to melt. Phenol extract twice and ethanol precipitate. Dissolve in 30 µl TE buffer.
13. Ligate (overnight, 5°C, 5 to 10 µl final volume, ~200 cohesive end units of T4 DNA ligase or equivalent) 3 to 8 µl DNA with 20 to 40 ng M13 vector prepared to give appropriate ends. Optional: prior to ligation, treat vector with CIP.

    To high-salt digestion of 2 µg mp18 or mp19, add 3 vol TE buffer and 0.2 µl CIP for 20 min at 37°C. Phenol extract and ethanol precipitate. Dissolve in 50 µl TE buffer.

14. Transform MC1061 cells with ≤ $^1/_4$ of each ligation and plate on JM101 or equivalent.
15. Characterize recombinant phage by electrophoresis and prepare selected ones for sequencing by phenol extraction (see support protocol in *UNIT 7.4* for preparing single-stranded template DNA).

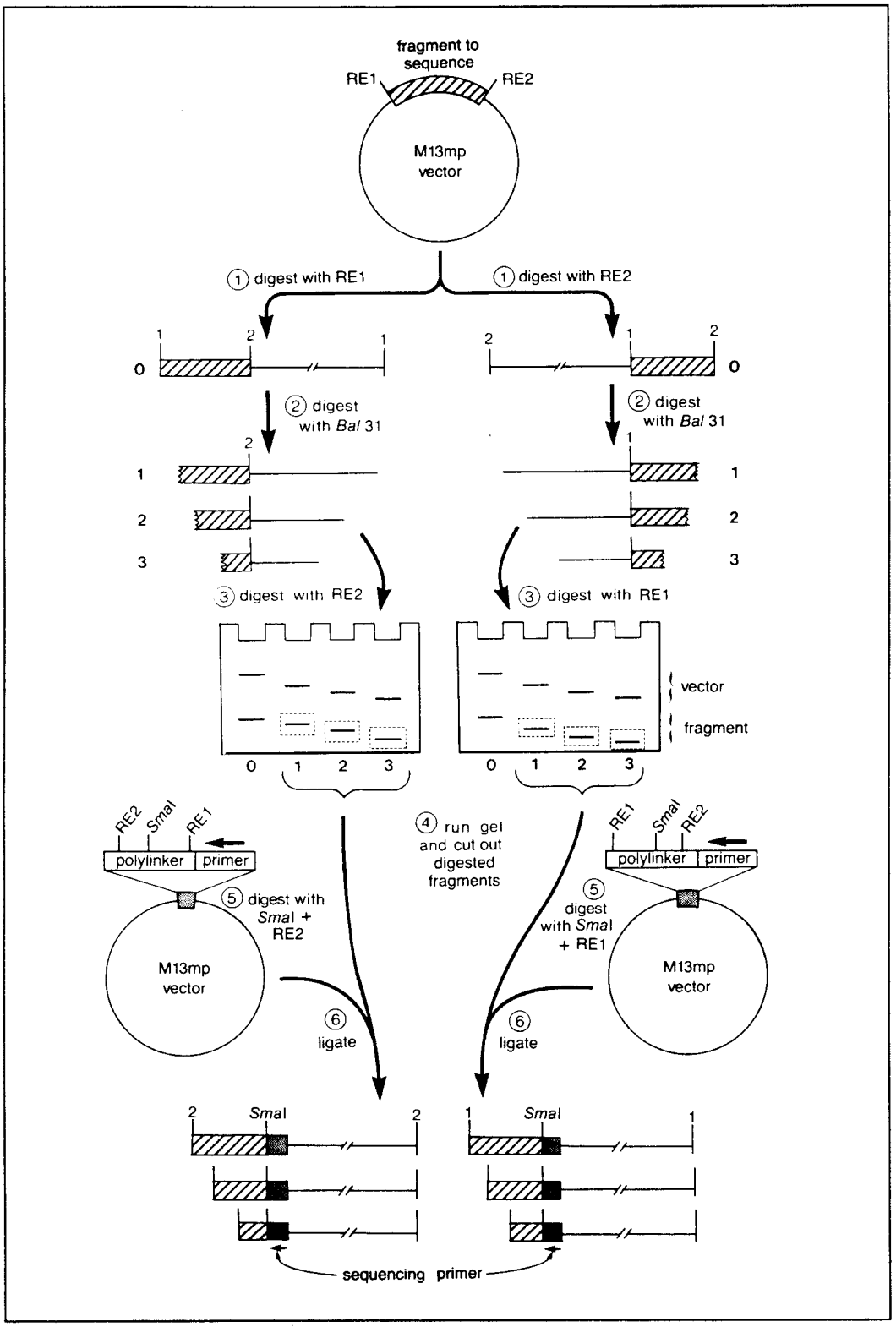

Figure 7.3.3 Strategy for making a nested set of deletions with *Bal* 31 exonuclease. The double-stranded RF form of an M13mp vector containing a fragment to be sequenced is linearized at both ends of the fragment in separate reactions (step 1) and a nested set of deletions is generated with *Bal* 31 exonuclease (step 2). The vector and insert sequences are then separated from each other by restriction endonuclease digestion (step 3) and fractionated by agarose gel electrophoresis (step 4). The digested inserts are eluted from the gel and ligated to a linearized M13mp vector (steps 5 and 6). "RE1" and "RE2" refer to two different restriction endonucleases or their respective recognition sites.

Materials

CsCl/ethidium bromide–purified plasmid DNA (purified twice) containing fragment to be sequenced (5 μg per 150 to 200 bp of insert; *UNIT 1.7*)
Restriction endonucleases and corresponding buffers (*UNIT 3.1*)
Buffered phenol (*UNIT 2.1*)
Ice-cold ethanol
3 M sodium acetate
TE buffer (*APPENDIX 2*)
10× *Bal* 31 nuclease buffer (*UNITS 3.4* and *3.12*)
Bal 31 nuclease (*UNIT 3.12*)
200 mM EGTA
E. coli MC1061 or DH5αF′ made competent for transformation (Table 1.4.5 and *UNIT 1.8*)
1 mg/ml yeast tRNA
10× loading buffer (*UNIT 2.5*)
Low gelling/melting temperature agarose (*UNIT 2.6*)
Ethidium bromide
50× TAE buffer (optional; *APPENDIX 2*)
95% ethanol (optional)
CsCl/ethidium bromide–purified M13mp vector DNA (double-stranded RF; *UNIT 1.7*)
*Sma*I restriction endonuclease (*UNIT 3.1*)
Calf intestine phosphatase (CIP; *UNIT 3.10*)
Overnight cultures of *E. coli* JM101, JM107, JM109 or equivalent in LB or 2× TY medium (Table 1.4.5)
400 U/μl T4 DNA ligase (*UNIT 3.14*)
H top agar
Xgal/IPTG solution (Table 1.4.2)
LB plates (*UNIT 1.1*), prewarmed
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 (*UNITS 2.5* and *2.6*), transforming *E. coli* (*UNIT 1.8*), and preparing single-stranded phage templates (support protocol, *UNIT 7.4*)

Strategic planning

Refer to Figure 7.3.3 for an overview of the experimental strategy that can be employed for constructing a nested set of deletions with *Bal* 31 exonuclease. Prior to generating the nested set of deletions, the DNA fragment to be sequenced is cloned in an M13mp vector (e.g., M13mp18 or M13mp19; see *UNITS 7.1, 1.14,* and *1.15*) or in a high-copy-number plasmid vector (e.g., pUC13, Bluescript M13, or pSPT18/pSPT19; see Table 7.3.1).

Choice of vector depends on the method that will be used to sequence the nested set of deletions. See strategic planning in the exo III protocol, above, for a description of various vectors and the factors involved in their selection. In addition to the vectors mentioned there, pSP64CS and pSP65CS (*UNIT 7.6*) can be used if the nested set of deletions will be sequenced by the chemical method (*UNIT 7.5*).

The following factors are important in selecting the M13mp double-stranded replicating form (RF) or high-copy-number plasmid to be subjected to *Bal* 31 digestion:

- The vector and insert must differ sufficiently in size so that they can be readily separated by agarose gel electrophoresis. For example, if the vector is one of the pUC series plasmids which are ~2.7 kb, the insert should be ≤2.3 kb or ≥3.2 kb.

  NOTE: *Inserts larger than 2.5 kb should not be constructed in M13mp vectors*

because large inserts are frequently unstable and delete spontaneously, although it has been reported that the recA⁻ M13mp host, JM109, may stably propagate larger inserts (Yanisch-Perron et al., 1985).

- The M13mp RF or plasmid must be constructed such that the region to be sequenced is flanked by *different* unique restriction sites. Moreover, the restriction sites flanking the insert must be compatible for cloning into one of the M13mp vectors if the ultimate goal is to generate single-stranded templates for dideoxy sequencing (see UNIT 7.4).
- The major advantage of using M13mp vectors is that they are generally considerably larger than the region to be sequenced and can be readily separated from the insert by gel electrophoresis.
- For each attempt to construct a nested set of deletions, 5 μg of plasmid DNA is required per 150 to 200 bp of DNA to be sequenced. Therefore, a high-copy-number vector is useful.

Preliminary steps

1. Clone the fragment to be sequenced in an appropriate pair of M13mp vectors such that the insert is cloned in both orientations with respect to the M13mp DNA sequencing priming site (refer to UNIT 7.1 and to Fig. 1.14.2 for information about the use of M13mp vectors in dideoxy sequencing; and to UNIT 1.15 for a protocol for cloning into M13mp vectors).

   It is necessary to clone the target DNA in both orientations so that sequence can be obtained from both ends of the undeleted fragment. However, it is only necessary to prepare CsCl-purified DNA for one of the orientations (see Fig. 7.3.3).

2. Optional: Clone the fragment to be sequenced in an appropriate high-copy-number vector such as pUC13, Bluescript M13, pSPT18/19, or pSP64CS/pSP65CS.

3. Prepare 5 μg of CsCl-purified DNA for each 150 bp to be sequenced (i.e., 50 μg for a 1.5-kb fragment; this is a 2-fold excess to allow for repetition if necessary) of one of the two M13mp RFs constructed in step 1 or the plasmid constructed in step 2.

   We recommend that the DNA be purified in two consecutive CsCl gradients because RNA will interfere with digestion by Bal 31 exonuclease. Refer to UNIT 1.7 for appropriate protocol.

4. If single-stranded dideoxy sequencing will be carried out, prepare at least 2 μg of CsCl-purified RF DNA for each of the M13mp vectors that will be used for preparing the sequencing templates (UNIT 1.7). Alternatively, prepare at least 2 μg CsCl-purified plasmid DNA for each of the vectors that will be used for preparing double-stranded sequencing templates or fragments for chemical sequencing.

Linearize M13mp::insert RF DNA or plasmid::insert DNA (Fig. 7.3.3, step 1) *DAY ONE*

5. Completely linearize 2 μg of M13mp::insert RF DNA or plasmid::insert DNA for each 150 bp to be sequenced (plus an additional 2 μg; i.e., 22 μg for a 1.5-kb fragment) using a restriction endonuclease that cuts at one end of region to be sequenced. Verify that digestion went to completion by running a small aliquot on an agarose minigel (UNIT 2.5).

   The maximum amount of DNA that should be linearized is 28 to 30 μg, i.e., sufficient to construct deletions in a 2.0-kb fragment. This step exposes one end of the fragment to be sequenced so that it can subsequently be digested with Bal 31.

6. Using a separate aliquot of M13mp::insert RF DNA or plasmid::insert DNA, linearize the same amount of DNA as above using a second restriction endonu-

clease which cuts at the other end of the region to be sequenced. Verify that digestion went to completion by running an agarose minigel.

Constructing deletions from each end of the fragment allows both strands of the insert DNA to be sequenced.

7. Clean up the two restriction digests from above by extracting with buffered phenol; add 1/10 vol 3 M sodium acetate and precipitate with ethanol (see UNIT 2.1). Dissolve the washed DNA pellets in TE buffer at a final concentration of 1 μg/μl. Store DNA samples at 4°C.

Determine the sensitivity of each batch of linear M13mp::insert RF DNA or plasmid::insert DNA to digestion using Bal 31 nuclease

This is necessary because DNA preparations can contain impurities affecting the rate of *Bal* 31 digestion and because *Bal* 31 may lose activity during storage.

8. Mix 2 μl linearized DNA, 38 μl water, and 5 μl of 10× *Bal* 31 nuclease buffer. Aliquot 9 μl into 5 microcentrifuge tubes and add 1 μl of 1/10, 1/20, 1/40, 1/80 *Bal* 31 nuclease diluted in *Bal* 31 buffer.

9. Repeat with the second linearized DNA.

10. Incubate each reaction 30 min at 37°C, then stop the reactions by adding 1 μl of 200 mM EGTA to each and heating 5 min at 65°C.

EGTA chelates Ca^{++} required by Bal 31.

11. Analyze the samples by agarose gel electrophoresis (a minigel is acceptable; see UNIT 2.5) to determine the dilution of *Bal* 31 nuclease which completely digests the DNA in the 30-min incubation.

DNA samples typically require 0.03 to 0.10 U Bal 31 nuclease per μg linearized DNA. As the digestion proceeds, the DNA bands first decrease uniformly in size, but gradually become increasingly diffuse before becoming completely digested. Electrophoresing in the presence of ethidium bromide saves time (UNIT 2.5).

DAY TWO

12. Prepare *E. coli* MC1061 or DH5αF′ cells competent for transformation and store at −70°C, as described in UNIT 1.8. These will be used on day 4. (See step 29 below and step 19 of the exo III protocol for a discussion of the appropriate strain to use.)

Create a nested set of deletions (Fig. 7.3.3, step 2)

Perform *Bal* 31 digestions on the two linearized M13mp::insert DNA or plasmid::insert DNA preparations (from step 5). Use 2 μg DNA of each for each 150 bp to be sequenced. Use the amount of *Bal* 31 nuclease previously determined (steps 8 to 11) to completely digest the DNA. The protocol assumes that 1-min time points will be taken.

13. For each 2 μg linearized DNA (1 μg/ml), mix 2 μl DNA, 43 μl water, and 5 μl of 10× *Bal* 31 nuclease buffer.

14. Preheat diluted DNA by incubating 10 min at 37°C.

15. For each of the two linearized DNA preparations, set aside a 1.5-ml microcentrifuge tube for each 1-min time point (i.e., one tube for each 2 μg DNA to be subjected to *Bal* 31 digestion). Add 5 μl of 200 mM EGTA to each tube and label tubes to indicate the linearized species and the *Bal* 31 digestion time.

16. Add *Bal* 31 nuclease of the determined amount to one of the two tubes of linearized DNA, vortex gently, and incubate at 37°C. Transfer 45 μl to the appropriately labeled tube from step 15 containing 200 mM EGTA every minute and place tubes at room temperature. Repeat with the other tube of DNA.

Usually 0.03 to 0.10 U Bal 31 nuclease per μg linearized DNA is the desired amount. The digestion described generates 150- to 200-bp deletions; adjust the time interval if desired.

We do not recommend taking time points for longer than 12 to 15 min. At longer digestion times, the DNA bands that form following electrophoresis (see step 23 of this protocol and Fig. 7.3.4) become too diffuse to be eluted efficiently from an agarose gel.

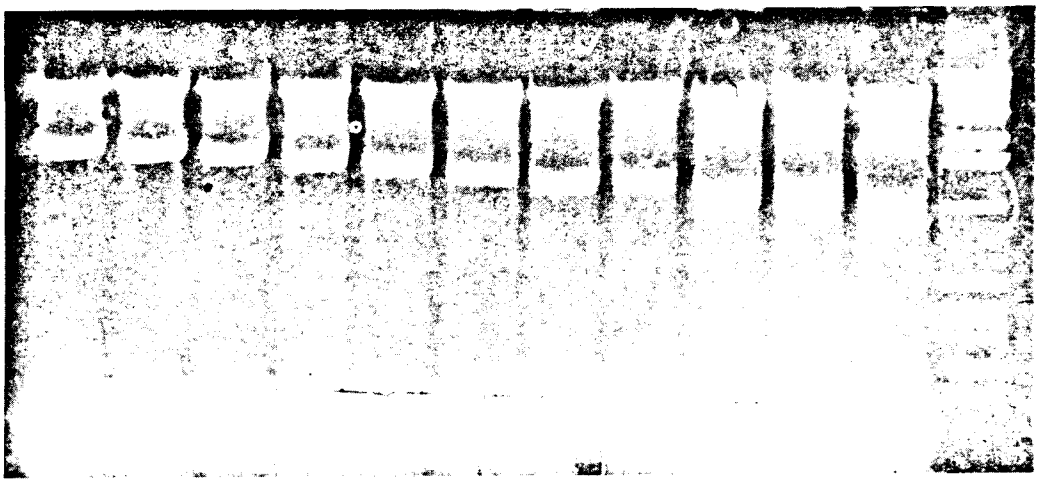

Figure 7.3.4 Ethidium bromide–stained agarose gel depicting successive digestion of a linearized plasmid with *Bal* 31 exonuclease. Following *Bal* 31 digestion for increasing amounts of time (left to right), the plasmid was digested with a restriction enzyme which separated the vector from the DNA fragment of interest.

17. After all *Bal* 31 digestions have been collected, incubate them 5 min at 65°C.

Separate digested insert and vector sequences (Fig. 7.3.3, step 3)

18. Add 2 μg of 1 mg/ml yeast tRNA to each tube and ethanol precipitate the samples using 3 M sodium acetate. Dissolve the washed pellets in 20 μl of the appropriate restriction enzyme buffer for the next step.

19. Digest each of the DNA samples with the restriction enzyme whose recognition site was protected from *Bal* 31 digestion (i.e., reverse the enzymes used for linearization).

Subject digested DNA samples to electrophoresis (Fig. 7.3.3, step 4)

20. Add 2 μl of 10× loading buffer to each digested DNA sample and subject to electrophoresis on low gelling/melting temperature agarose (*UNIT 2.6*), including ethidium bromide in the gel and buffers. Also run a lane of molecular weight markers spanning the size of the fragment to be sequenced.

    This gel can be run overnight at low voltage. If an M13mp vector has been used, run a 1% gel and allow Bromphenol Blue dye to migrate ~4 cm. This gives good separation between vector and insert.

    Because electrophoresis buffer and chambers can develop nuclease contamination, when fragments will be isolated from gels for subsequent cloning, it is desirable to use autoclaved 50× TAE for preparing buffers and casting gels. It is also helpful to rinse chambers and gel formers with 95% ethanol prior to electrophoresis.

Prepare M13mp vectors for cloning the nested Bal 31-digested fragments (Fig. 7.3.3, step 5; alternatively, prepare plasmid vectors, if single-stranded dideoxy sequencing is not going to be used.)

These will not be used until day 3; they can be prepared anytime in advance.

21. The M13mp RF vector DNA(s) are first cut with *Sma*I nuclease to create a blunt end. Alternatively, DNA polymerase (see p. 3.5.8) or single-stranded nuclease (p. 3.12.3) can be used to convert a sticky end to a blunt end. This blunt end will be ligated to the blunt ends created by *Bal* 31 digestion. One-half of the blunt-ended vector (or one of the two M13mp vectors if two are used) is then cut with the enzyme that was originally used to clone the fragment to be sequenced and that was protected from *Bal* 31 digestion. The remaining blunt-ended vector is digested with the second enzyme used in the original cloning.

    Choose M13mp vector(s) such that the Bal 31-digested fragments can be cloned with the blunt ends created by the Bal 31 digestion adjacent to the sequencing priming site in the vector.

    Depending on the enzymes used for the original cloning of the fragment to be sequenced, it may be necessary to use two complementary M13mp vectors. This will be the case if the two enzyme sites are on opposite sides of the restriction-enzyme site used to provide a blunt end. A typical protocol follows:

    a. For each 10 µg of DNA that will be subjected to *Bal* 31 digestion, digest 1 µg of mp18 and/or mp19 with *Sma*I.

        It is important to determine the minimal amount of SmaI needed to linearize the vector; too much SmaI can damage ends and prevent subsequent ligation. Usually, ~2 U SmaI per microgram of mp18 or mp19 DNA for 60 min at 37°C is sufficient.

    b. Extract with buffered phenol and precipitate with ethanol.

        If the DNA preparations are really clean, it may be necessary to add 1 to 2 µg yeast tRNA before precipitation to see the pellets.

    c. In two separate digestions, cut the *Sma*I-linearized M13mp RF DNAs with each of the restriction enzymes used to linearize the M13mp::insert or plasmid::insert DNAs. Use the minimum amount of enzyme necessary. If desired, remove phosphate groups by adding ≤0.2 µl CIP (~5 U) for the last 20 min of the digestion with the chosen restriction endonuclease.

        The following restriction buffers work for this procedure: 10 mM Tris·Cl (pH 7.6), 10 mM $MgCl_2$, and 10 to 150 mM NaCl. A vast excess of phosphatase is used, allowing the use of a suboptimal buffer. Phosphatase treatment sometimes results in a nonfunctional vector. If dephosphorylation is omitted, transform with less DNA in step 30.

        The result should be two M13mp vectors, each with a blunt end adjacent to the DNA sequencing priming site and each with a sticky end corresponding to the restriction site flanking the insert DNA protected from Bal 31 digestion.

    d. Extract with buffered phenol, precipitate with ethanol, and dissolve in 50 µl TE buffer. Stored at –70°C, such vectors last months.

DAY THREE

22. Inoculate *E. coli* JM101, JM107, JM109, DH5αF′, or equivalent into 5 ml LB or 2× TY medium and grow at 37°C overnight. This culture is required on day 4 as plating bacteria for plaquing recombinant M13mp phage (refer to step 29 below and to step 19 in the exo III protocol).

Elute Bal 31-digested fragments from gel

23. The digested fragments should appear as increasingly diffuse bands with increased digestion time (see Fig. 7.3.4).

24. Using a longwave UV lamp and the molecular weight markers as a guide (and minimizing exposure to UV light), cut out a series of DNA fragments (usually the entire band of digested insert DNA) differing in molecular weight by 150 to 200 bp from each lane (Fig. 7.3.4) and place in individual microcentrifuge tubes.

25. Add TE buffer to ~400 µl total volume for each tube, incubate at 65°C *until all the agarose melts* (about 5 to 10 min), and extract with 1 ml buffered phenol by vortexing at low speed for 30 sec.

    When phenol-extracting agarose, it is important to obtain sufficient mixing for equilibration between the phenol and water. Do not use phenol/chloroform or phenol/chloroform/isoamyl alcohol; they will not extract efficiently.

26. Repeat the phenol extraction on the aqueous phase combined with its milky interface which, after the second extraction, will become much less abundant.

27. Finally, precipitate with ethanol. Add 2 µg of 1 mg/ml yeast tRNA, if desired. Dissolve the washed pellets in 30 µl TE buffer and store at 5°C.

    Low gelling/melting temperature agarose (UNIT 2.6) is very reliable for isolating fragments for subsequent ligations. It is sometimes possible to save time by diluting melted slices to <0.1% agarose and ligating without extraction and precipitation of the DNA.

Ligate eluted Bal 31-digested fragments to M13mp (or plasmid) vector (Fig. 7.3.3, step 6)

28. Ligate (see UNIT 3.14) 3 to 8 µl of each DNA sample eluted from the agarose gel with 20 to 40 ng of the appropriately prepared M13 vector using ~0.5 µl T4 DNA ligase (~200 cohesive end units or equivalent; see UNIT 3.14). Incubate overnight at 5°C.

    The amount of Bal 31-digested DNA used in this ligation should be chosen according to the recovery of the DNA from the agarose gel. Three microliters would be used for >90% recovery, etc. A convenient concentration of M13 vector DNA is 20 µg/ml; therefore, 1 to 2 µl would be added to the Bal 31-digested DNA. Adjust the final volume of the ligation reaction to 10 µl. A large amount of ligase is required for the blunt-end ligation.

Transform ligated DNAs into E. coli MC1061 (see UNIT 1.8)

DAY FOUR

Strain MC1061 helps the efficiency because of its high transformation frequency, but cannot be used for M13mp7, 8, 9, 10, and 11 because amber mutations in these vectors are not suppressed by MC1061. *E. coli* JM101, JM107, JM109, DH5αF′, or an equivalent strain may be used for these vectors (refer to step 19 in the exo III protocol).

29. Mix each of the ligation reactions with 40 µl of competent cells.

    If the vector was not treated with phosphatase, many more plaques—predominantly blue—will result. Consequently, under these circumstances, transform with ≤1/4 of each ligation reaction.

30. Also transform with 0.5 ng of the M13mp RF vectors used to clone the *Bal* 31-deleted fragments and the two M13mp::insert RF (or plasmid::insert) recombinant plasmids carrying the undeleted fragment in both orientations.

    Expect nearly a lawn of M13 plaques with the RF control DNA.

Controls: Transform with M13mp vector (Fig. 7.3.3, step 6) cut with SmaI and additional enzyme and either untreated or treated with ligase. This controls for the amount of undigested M13mp vector molecules that will form blue plaques.

31. Following the heat shock step in the transformation protocol, add 0.2 ml of an overnight culture of *E. coli* JM101, JM107, JM109, DH5αF′, or equivalent, pipet contents into 3 ml H top agar containing 60 μl Xgal/IPTG solution, mix, and pour on LB plates.

32. After the top agar hardens, incubate plates upside down overnight at 37°C.

    Prior to pouring top agar, it is essential to prewarm LB plates to at least room temperature to retard setting of the top agar. It is essential to incubate plates at 37°C for plaques to form.

DAY FIVE

Characterize the recombinant phage

If there are many colorless (not blue) plaques, two plaques for each time point and two plaques from each of the control plates are usually sufficient.

Each transformation should result in up to 100 or more colorless plaques. Depending on the fidelity of vector preparation, these will be accompanied by zero to many blue plaques. If only a few colorless plaques are present, they may not be the desired recombinants. The presence of inserts detected by electrophoresis will resolve the issue, as will "C testing." Refer to steps 21 to 26 in the exo III protocol.

DAY SIX

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

Refer to steps 27 to 30 in the exo III protocol and to the support protocol in *UNIT 7.4* for preparing single-stranded template DNA.