CLONING IN PHOSPHATASE-TREATED COSMID VECTORS

The basic steps of this procedure are shown in Figure 3.5.

Preparation of Vector DNA

1. Digest 20 μg of closed circular pJB8 DNA, prepared according to the methods given in Chapter 1, with a two- to threefold excess of *Bam*HI for 1 hour. Remove an aliquot (0.3 μg) and analyze the extent of digestion by electrophoresis through a 0.8% agarose gel, using as a marker 0.3 μg of undigested pJB8 DNA. If digestion is incomplete, add more restriction enzyme and continue the incubation.

2. When digestion is complete, extract the sample with phenol:chloroform and precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge, and redissolve it in 180 μl of 10 mM Tris · Cl (pH 8.3). Remove a 4-μl aliquot (~0.5 μg) and store at −20°C.

3. To the remainder of the sample, add 20 μl of 10 × CIP dephosphorylation buffer and 0.25 unit of calf intestinal alkaline phosphatase (CIP). Incubate the reaction for 30 minutes at 37°C.

0.4 µl 500 mM EDTA
1.0 ~~0.5~~ µl 20% SDS
0.1 µl 20 mg/ml proK
to 40 µl digest.

tion buffer

3)

1.2 µl 500 mM EDTA
3 µl 20% SDS
0.3 µl 20 mg/ml proteinase K stock
to 120 µl digest.

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reaction
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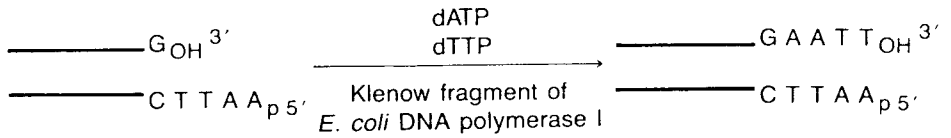
5. Cool the reaction to room temperatu
once with phenol:chloroform. Add 0.. - acetate (pH 7.0). Mix well, and add 2 volumes of ethanol. Mix well, and store at 0°C for 15 minutes. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol at 4°C and recentrifuge.

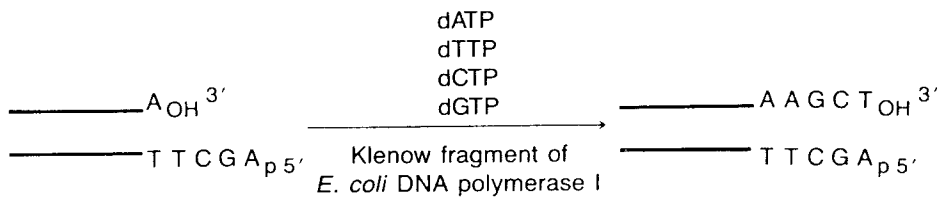
6. Redissolve the DNA in 20 μl of TE (pH 7.6) and carry out a test ligation as described below to determine the effectiveness of the phosphatase treatment.

FILLING RECESSED 3′ TERMINI

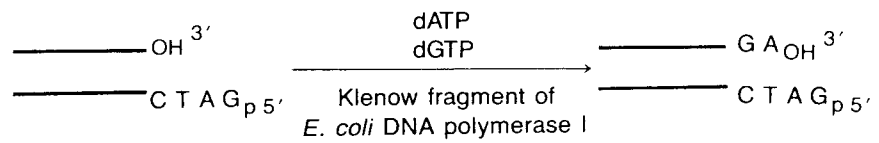
Recessed 3′ termini can be filled by the polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I in the presence of the appropriate dNTPs. Which of the four dNTPs are added to the reaction depends on (1) the sequence of the protuding 5′ termini at the end(s) of the DNA and (2) whether partial or complete filling is required. For example, to fill recessed 3′ termini created by cleavage of DNA by *Eco*RI, only dATP and dTTP need to be present in the reaction:



On the other hand, all four dNTPs are required to fill recessed termini created by *Hin*dIII:



Partial filling of recessed termini created by *Sau*3AI requires the presence of dATP and dGTP. The newly created, shorter protruding terminus is complementary to a partially filled terminus created by cleavage with *Xho*I (Zabarovsky and Allikmets 1986) (see Chapter 5):



1. In a 20-μl reaction, digest 0.2–5 μg of DNA with the appropriate restriction enzyme(s).

2. When digestion is complete, add 1 μl of a solution containing each of the desired dNTPs at a concentration of 1 mM.

3. Add 1 unit of the Klenow fragment of *E. coli* DNA polymerase I for each microgram of DNA in the reaction. Incubate the reaction for 15 minutes at room temperature.

   The Klenow fragment of *E. coli* DNA polymerase I works well in virtually all buffers used for digestion of DNA with restriction enzymes. There is no need to purify the DNA prior to filling recessed 3′ termini created by restriction enzymes.

   DNA fragments that have been purified by gel electrophoresis before filling of recessed 3′ termini should be redissolved in TE (pH 7.6). $MgCl_2$ should then be added to a final concentration of 5 mM before the appropriate dNTPs and the Klenow fragment of *E. coli* DNA polymerase I are added.

4. Inactivate the Klenow fragment of *E. coli* DNA polymerase I and the restriction enzyme(s) present in the reaction by one of the following methods:

   - Heat to 75°C for 10 minutes.

     Not all restriction enzymes are completely inactivated by this procedure. Check the manufacturer's specifications that are supplied with the enzyme.

   - Add an equal volume of TE (pH 7.6) and extract the solution with phenol:chloroform. Collect the DNA by precipitation with 2 volumes of ethanol.

Notes

i. If desired, the DNA can be separated from unincorporated dNTPs by chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E). This is not necessary when the filled fragment of DNA is to be used in ligation reactions. Bacteriophage T4 DNA ligase is not inhibited by the presence of dNTPs and works adequately in virtually all buffers used for digestion of DNA with restriction enzymes.

ii. For a full description of the method used to fill recessed 3′ termini of genomic DNA in preparation for cloning, see Chapter 9, page 9.29.