5

Restriction Digestion and Ligation of DNA

Restriction enzyme digestion is performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. The optimal sodium chloride concentration in the reaction varies for different enzymes, and a set of three-standard buffers containing three concentrations of sodium chloride are prepared and used when necessary. Typical digestions include a unit of enzyme per microgram of starting DNA, and one restriction enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. These reactions usually are incubated for 1–3 hours, to ensure complete digestion, at the optimal temperature for enzyme activity, typically at 37°C.

DNA ligations are performed by incubating DNA fragments with appropriately digested cloning vector in the presence of ligation buffer containing ATP and T4 DNA ligase. For random shotgun cloning, sonicated or nebulized fragments, in which the fine mist created by forcing a DNA solution through a small hole in the nebulizer unit is collected. The size of the fragments obtained by nebulization is determined chiefly by the speed at which the DNA solution passes through the hole, altering the pressure of the gas blowing through the nebulizer, the viscosity of the solution, and the temperature. Nebulization is easy, quick, and requires only small amounts of DNA (0.5–5 µg). The resulting DNA fragments are distributed over a narrow range of sizes (700–1330 bp). It requires ligation of DNA before nebulization and end-repair afterward, are ligated either to *Sma* I linearized, dephosphorylated double-stranded M13 replicative form or pUC vector by incubation at 4°C overnight. A practical range of concentrations is determined based on the amount of initial DNA and several different ligations, each with an amount of insert DNA within that range, are used to determine the appropriate insert to vector ratio for the ligation reaction. In addition, several control ligations are performed to test the efficiency of the blunt-ending process, the ligation reaction, and the quality of the vector. These usually include parallel ligations in the absence of insert DNA to determine the background clones arising from self-ligation of inefficiently phosphatased vector. Parallel ligations are also performed with a known blunt-ended insert or insert library, typically an *Alu* I digest of a cosmid, to ensure that the blunt-ended ligation reaction would yield sufficient insert containing clones, independent of the repair process.

5.1 RESTRICTION DIGESTION OF DNA

Protocol

1. Prepare the restriction digestion reaction mixture by adding the following reagents in the order listed to a microcentrifuge tube:

DNA (1 µg)	=	10 µL
10X restriction enzyme assay buffer	=	2 µL
Restriction enzyme (1–10 units)	=	1 µL
Sterile ddH_20	=	7 µL
Total restriction volume	=	**20 µL**

Note

(i) If desired, more than one enzyme can be included in the digest if both enzymes are active in the same buffer and the same incubation temperature.

(ii) The volume of the reaction depends on the amount and size of the DNA being digested. Larger DNAs should be digested in larger total volumes (between 50–100 µL).

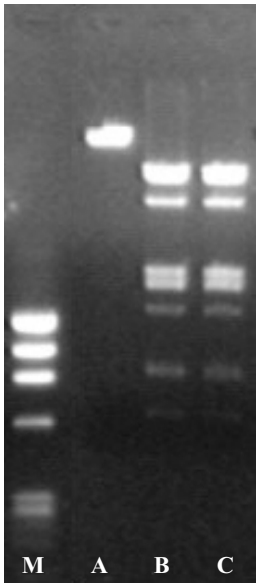
(iii) The supplier's catalogue should be referred to the chart of enzyme activity in a range of salt concentrations to choose the appropriate assay buffer (10X High, 10X Medium, or 10X Low salt buffers, or 10X *Sma* I buffer for *Sma* I digestions).

2. Gently mix the restriction digestion reaction mixture by pipetting up and down and incubate the reaction at the appropriate temperature (typically at 37°C) for 1–3 hours.
3. After incubation for 1–3 hours inactivate the enzyme(s) by heating at 65°C for 10 minutes or by phenol extraction. Prior to use for subsequent dephosphorylation or ligation, an aliquot of the digestion should be assayed by agarose gel electrophoresis along with non-digested DNA and a size marker, for conformation of the digestion (Figure 5.1).

5.2 PURIFICATION OF RESTRICTED DNA FRAGMENTS

Protocol

1. Dilute the restricted DNA to 500 µL with TE buffer.
2. Extract once with equal volume of equilibrated phenol (add equal volume of phenol, mix and centrifuge at 10,000 rpm for 10 minutes at 4°C. Transfer the upper phase into another sterile tube).
3. Add equal volume of chloroform: isoamyl alcohol (24:1) and extract as in previous step.
4. Take the upper phase and add sodium acetate (pH 4.6) to a final concentration of 0.3 M. Mix well and add four volume of ice cold 100% ethanol.
5. Mix well and incubate for 1 hour at –20°C and centrifuge at 10,000 rpm for 10 minutes at 10°C.
6. Decant the supernatant and wash the DNA pellet with 70% ethanol.
7. Air dry the final DNA pellet and resuspend it in 10 µL of sterile double distilled water for subsequent ligation reaction or 10 µL of TE buffer for storage.



M: 1 μg ϕ X 174/HaeIII Markers
A: Undigested DNA;
B: *BpuAm1*
C: *Sac* 1

Figure 5.1 Agarose gel electrophoresis of restriction fragments produced by cleavage of Ad2 phage DNA.

Buffer

(i) **3 M Sodium acetate (pH 4.6)**
(Dissolve sodium acetate salt in less volume of distilled water and adjust the pH to 4.6 with glacial acetic acid and finally make up the volume).

5.3 DNA LIGATION

Protocol

1. Combine the following reagents in a microcentrifuge tube and incubate overnight at 12–16°C:

Digested insert DNA fragments	=	4 μL (50 ng/mL)
Digested cloning vector (with same restriction enzymes)	=	2 μL (10 ng/μL)
10X ligation buffer	=	1 μL
T4 DNA ligase	=	1 μL (10 U/μL)
Sterile ddH_2O	=	2 μL
Total volume	=	**10 μL**

2. Include control ligation reactions with no insert DNA and with a known blunt-ended insert (such as *Alu* I digested cosmid) as controls.

3. Transform the ligated DNA in to *E. coli* either by competent cell transformation or by electroporation.
4. Score the efficiency of ligation.

REFERENCES

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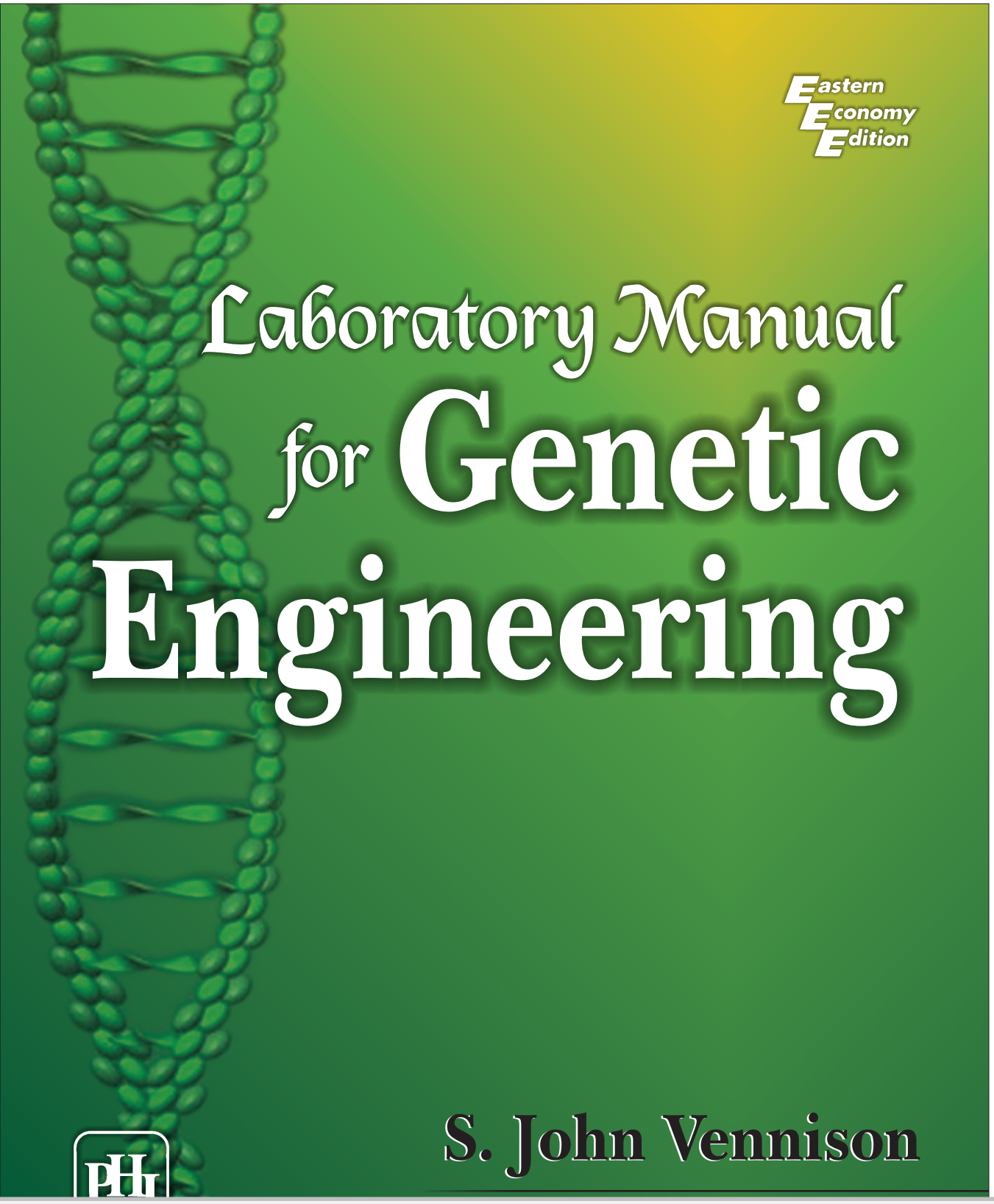

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