

## 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 $\mu$ g)	=	10 $\mu$ L
10X restriction enzyme assay buffer	=	2 $\mu$ L
Restriction enzyme (1–10 units)	=	1 $\mu$ L
Sterile ddH <sub>2</sub> O	=	7 $\mu$ L
<b>Total restriction volume</b>	=	<b><u>20 <math>\mu</math>L</u></b>

### Note

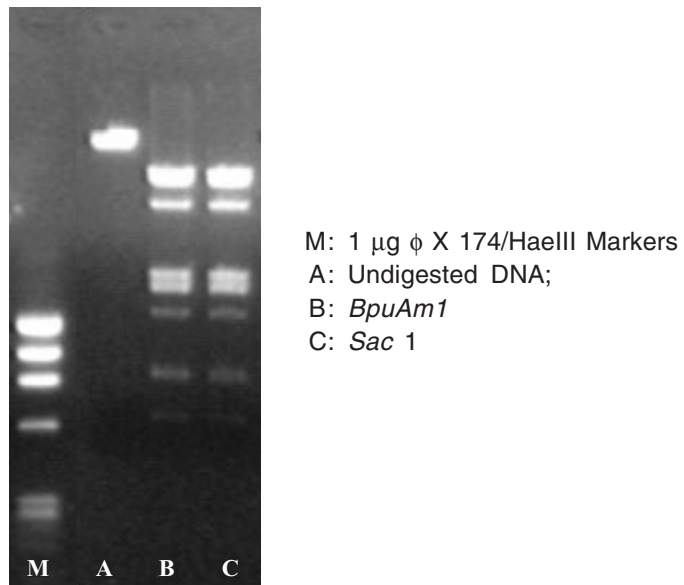
- (i) If desired, more than one enzyme can be included in the digest if both enzymes are active in the same buffer and the same incubation temperature.
  - (ii) The volume of the reaction depends on the amount and size of the DNA being digested. Larger DNAs should be digested in larger total volumes (between 50–100  $\mu$ L).
  - (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 confirmation of the digestion (Figure 5.1).

## 5.2 PURIFICATION OF RESTRICTED DNA FRAGMENTS

### Protocol

1. Dilute the restricted DNA to 500  $\mu$ L 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  $\mu$ L of sterile double distilled water for subsequent ligation reaction or 10  $\mu$ L of TE buffer for storage.

00002



**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 <sub>2</sub> O	=	2 µL
<b>Total volume</b>	=	<b><u>10 µL</u></b>

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

- Ausubel, F.M., et al. (1994–2000), *Current Protocols in Molecular Biology*, vol. 1, John Wiley & Sons, Inc., Brooklyn, New York.
- Sambrook, J. and D.W. Russel (2001), *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press.



**Eastern  
Economy  
Edition**

Laboratory Manual  
for **Genetic  
Engineering**

**S. John Vennison**



**DOCKET  
ALARM**

Find authenticated court documents without watermarks at [docketalarm.com](http://docketalarm.com).

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.