# Standard DNA Cloning

Fanglian He

Carnegie Institution at Stanford

**[Abstract]** This protocol describes general cloning steps from preparation of both vector and insert DNA to the ligation reaction.

## Materials and Reagents

1. Luria-Bertani broth (LB) medium: Bacto-tryptone (BD Biosciences), yeast extract (BD Biosciences)
2. Antibiotics (Sigma-Aldrich/Thermo Fisher Scientific)
3. QIAGEN Plasmid Purification Handbook (QIAGEN)
4. SeaKem® LE Agarose (Cambrex)
5. Plasmid Prep Kit (QIAGEN /Fermentas)
6. PCR Clean-up kit (QIAGEN /Fermentas)
7. Restriction enzymes (New England Biolabs)
8. Alkaline Phosphatase: Calf intestinal alkaline phosphatase (CIP) (New England Biolabs, catalog number: M0290) or Shrimp Alkaline Phosphatase (SAP) (Promega Corporation, catalog number: M8201)
9. Ligase enzyme (New England Biolabs)
10. DNA ladder
11. NaCl
12. LB broth media (see Recipes)
13. Ligation reaction (see Recipes)

## Equipment

1. Nanodrop (Thermo Scientific)

## Procedure

A. Preparing vector DNA for cloning:

Depending on the copy number of the vector plasmid, decide if you need the Mini-prep, Midi-prep, or Maxi-prep kit. If it is a high copy (>10 copies/cell) plasmid, plasmid DNA can be

 

prepared by using the Mini-prep kit. If it is a low copy (<10 copies/cell) plasmid, use the Midi-prep or Maxi-prep kit.

1. Grow *E. coli* cell culture carrying vector plasmid in LB liquid medium with appropriate antibiotics at 37 °C overnight.
2. Follow QIAGEN Plasmid Purification Handbook to obtain DNA. If plasmid DNA does not need to be purified, and to be more economical, plasmid DNA can be extracted without using a plasmid prep kit (See protocol "Plasmid DNA extraction from *E. coli* using alkaline lysis method").
3. Estimate plasmid DNA concentration using one of the following two ways:
   a. Load 2-3 µl plamid DNA and a DNA ladder on a DNA agarose gel and estimate DNA according to the DNA marker.
   b. Easier and more accurate way is to measure DNA using Nanodrop if it is available.
4. Digest 2-5 µg vector DNA using restriction enzymes needed for the insert DNA. To make sure the vector is completely digested, extra enzyme and long incubation may be needed.
5. To reduce the chance of self-ligation, dephosphorylate the 5′ phosphorylated ends of the digested vector with alkaline phosphatase.
   *Note: If the shrimp alkaline phosphatase (SAP) is used, then add 2 µl SAP directly to 100 µl digest solution, incubate at 37 °C for 1 h, then inactivate SAP at 65 °C for 10 min. If the calf intestinal alkaline phosphatase (CIP) is used, then add 5 µl CIP enzyme to 100 µl digestion solution, incubate at 37 °C for 1 h, then inactivate SAP at 65 °C for 30 min.*
6. Perform gel purification of digested vector DNA.

B. Preparing insert DNA for cloning:

1. Obtain insert DNA from digestion of plasmid DNA.
   a. Extract plasmid DNA as described above.
   b. Digest plasmid DNA with appropriate restriction enzymes.
   c. Perform gel purification of insert DNA.
2. Generate insert DNA from PCR product.
   a. Design primers using a free a good quality program online (*e.g.*, http://frodo.wi.mit.edu/primer3/) containing desired cloning sites with several of bases flanking their recognition sequences (http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_olignucleotides.asp).
   b. Amplify insert DNA from a template by PCR, and clean up PCR product by PCR clean-up kit.

 

c. Digest PCR product with the corresponding restriction enzymes. Or, first clone PCR product to pGEM T-easy vector, and then generate insert DNA from the resulting plasmid.
d. Perform gel purification of insert DNA.
e. Estimate DNA concentration.

C. Ligation of insert and vector:
a. Usually (particularly for blunt end ligation), need more insert DNA than vector: 1 mole of vector normally needs 5 or more moles of insert (see protocol "DNA molecular weight calculation").
b. Control ligation: To determine background clones arising from self-ligation of inefficiently phosphatased vector, set a parallel ligation in the absence of insert DNA.

D. Transform 1 µl ligation reaction to competent cell by electroporation or chemical method.

E. Colony PCR to screen for plasmids carrying the correct inserts and then confirm the result by digestion and sequencing of the plasmid.

**Recipes**

1. 1 liter of LB broth media
   10 g Bacto-tryptone
   5 g yeast extract
   10 g NaCl
   Add dd$H_2O$ to get volume 1 L
   Sterilize by autoclaving.
2. Ligation reaction
   X µl DNA vector( ~20 ng)
   Y µl insert (~100-1,000 ng)
   2 µl 10x buffer
   1 µl T4 DNA ligase
   To 20 µl $H_2O$
   --------
   20 µl total

 