General scheme for cloning in plasmids

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This protocol may be use for inserting a DNA fragment into a plasmid vector.

Keywords: cloning insert ligation vector

Expand

1. **Fragment prep.** - the fragment to be cloned must be obtained and cleaned-up (semi-purification). Fragment preparations typically involve a restriction enzyme digestion to remove the desired fragment from its source. Two typical restrictions digests are outlined below:
(A) Preparative digest - start with 25-100 µg DNA containing the fragment of interest. The amount of enzyme added is dependent upon the amount of DNA used. Generally, for every 1 µg of DNA present, 1.5 U of enzyme are added (i.e., if you have 50 µg of DNA to be digested, you would add 1.5 x 50 or 75 U of enzyme). A typical digest might be:

50 µl DNA @ [1 µg/µl] --------> we need 75 U of each enzyme
30.5 µl H2O
10 µl 10X buffer H
4 µl EcoRI @ 20 U/µl ---> 75U/(20 U/µl) ~ 4 µl needed
5.5 µl BamHI @ 14 U/µl ---> 75 U/(14U/µl) = 5.4 µl needed
100 µl Vt

Note: Since the buffer is initially 10X concentrated, the amount added to the reaction mixture is 1/10 the total volume of the solution. Also note, the sum of the volumes of enzymes to be added should not exceed 1/10 the total reaction volume due to the presence of 50% glycerol in the enzymes. If the sum of enzyme volumes had exceeded 10 µl, the total reaction volume would have been increased. The reaction mix is incubated at 37°C for 1-2 hr.

(B) Analytical digest - Typically, these digest conditions are followed when digesting less than 5 µg DNA or when one wishes to completely digest all DNA present in ~1 hr time. Here, 0.5 µl enzyme is added per 1 µg DNA.

1 µl DNA @ [1µg/µl]
7.5 µl H2O
1 µl 10X buffer H
0.5 µl Sal I
10 µl Vt

Digest 1 hr @ 37°C.
Following the digest, the DNA fragment is generally gel-purified. The digest mixture is loaded into an agarose gel and electrophoresed until the desired fragment band is well separated from any neighboring fragment bands. A number of techniques may be used to retrieve the desired fragment. Once retrieved, the fragment is further purified via butanol extraction or phenol/$CHCl_3$ extraction (depending on the method of retrieval) and ethanol precipitation. The fragment can now be resuspended, its concentration determined, and stored at -20°C for further use.

2. **Vector prep.** - fragments are ligated into a circular plasmid which serves as a vehicle to shuttle the DNA fragment into a bacteria for amplification. These circular plasmid vectors are equipped with restriction enzyme sites into which our linear fragment can be cloned. First, however, the vector must be cut at sites complementary to the restriction enzyme sites on the ends of our DNA fragment. An analytical digest is used to prepare the vector. Generally, 2 µg of vector DNA is digested. Any unused cut vector DNA can be stored at -20°C. An example vector digest is outlined below.

2 µl pEMBL18 @ [1 µg/µl]
14 µl H_2O
2 µl 10X buffer H
1 µl BamHI
1 µl Sal I
20 µl Vt

Incubate 1 hr at 37°C. Dilute a 1 µl aliquot of the vector prep to 10 µl with H_2O. Run in a 1% agarose gel to determine if all vector is cut. If not, add more enzyme, adjusting buffer and volume accordingly if needed, and digest longer. Check cutting again. Once all vector DNA has been cut/linearized, extract 1X with an equal volume of phenol (5 min spin); extract 1X with $CHCl_3$ (equal volume - 1 min spin). Ethanol precipitate with 1/40 volume of 4 M NaCl and 2 volumes of ethanol. Place at -70°C for 15-30 min to precipitate (5-10 min on dry ice ; O/N at -20°C). Spin 10-15 min at 4°C. Pull off all liquid; dry at 37°C briefly. Resuspend in TE to a final concentration of ~100 ng/µl. Store at -20°C until use.

3. **Ligation** - once the vector and insert DNAs have been prepared, the two can be ligated together. Remember, <u>like goes to like</u>, vector and fragment ends must be complementary to stick together. Typically, 100 ng of vector is used in the ligation reaction with ~3-10X excess of insert DNA to push the reaction in favor of ligation of insert with vector. One must determine the amount of insert needed to yield a 1:1 molar ratio of vector to insert. Example calculations follow.

(1) pEMBL ~4000 bp = 1 mole vector
insert ~1750 bp = 1 mole insert

We typically add 100 ng of vector, therefore:
1 mole vector : 1 mole insert
100 ng/4000 bp = x ng/1750 bp
x = 43.75 ng insert needed

The insert is at 1 µg/µl conc. = 1000 ng/µl.

We, therefore, need to add:
insert volume = 43.75 ng/[1000ng/µl] = ~0.045 µl insert
so with 10X excess, we would add 0.45 µl of insert.

Our protocol will be as follows:
1 µl pEMBL18 @ [100 ng/µl]
0.45 µl insert DNA frag. @ [1 µg/µl]
2.55 µl H2O
0.5 µl 10X ligase buffer
<u>0.5 µl T4 ligase enzyme</u>
5 µl Vt
The reaction is incubated 4 hr to O/N at 4°C.

(2) vector = BS+ ~3000 bp = 1 mole
insert ~600 bp = 1 mole insert
[insert] = 1.95 µg/µl = 1950 ng/µl

1 mole vector : 1 mole insert
100 ng/3000 bp = x ng/600 bp
x = 20 ng insert needed

insert volume = 20 ng/(1950 ng/µl) = 0.01 µl insert

A 10x excess is 0.1 µl insert which is difficult/impossible to pipette accurately. Here, one could further dilute the insert; however, I would add 0.5 µl DNA.

(3) vector = pPLRCAT ~4530 bp = 1 mole
insert ~1400 bp = 1 mole
[insert] = 140 ng/µl

1 mole vector : 1 mole insert
100 ng/4530 bp = x ng/1400 bp
x = 31 ng insert needed

insert volume = 31 ng/(140 ng/µl) = 0.22 µl insert

Protocol:

1 µl vector DNA
2.2 µl insert DNA
0.8 µl H2O
0.5 µl 10X ligase buffer
0.5 µl T4 ligase
5 µl Vt

4. **Transformation** - once the ligation reaction is complete, the mixture is ready to be transformed into competent bacteria for amplification. See "Transformation Protocol".