CLONING FROM BEGINNING TO END

DIGEST:

1. Digest 10–20ug DNA to isolate either the desired insert or vector.

2. After digesting, sometimes it is necessary to dephosphorylate the ends of the vector so as to prevent religation before the insert can ligate to vector. If this is necessary:

VECTOR DEPHOSPHORYLATION (AP/CIP):

a. After digesting (it is best to do this before running on the gel and Gene Cleaning so no re-ligation can occur during these steps), precipitate the DNA with EtOH (2X the original volume) and NaOAc (10% of original volume).

b. Place at 4 degrees for 10', then spin at 4 degrees at 13,000 rpm for 20'.

c. Once you have a pellet, resuspend with dW

d. Add alkaline phosphatase with buffer (see below). 1 ug DNA requires a 10 ul reaction (scale up as desired).

- Reaction should end up: 1 unit alkaline phosphatase (AP or "CIP")/ 1 ug DNA
- Dilute 10X buffer to 1X
- Bring up to volume with dW
- Example: For 20 ug pellet - resuspend in 70 ul dW
- Add 20 ul AP (1unit/ul)
- Add 10 ul 10X buffer (so 1X)

5. Add dye (15% Ficoll, 0.25% Bromo Phenol Blue, 0.25% Xylene) at 1:10 to make digests heavy enough to sink into gel wells.

6. Run the digests out on a gel made with "Seakem" brand agarose (it is higher quality than technology grade) at 80–100V, and look at the bands under UV light. Cut out appropriate bands and Gene Clean them (SEE BIO 101 gene clean kit for instructions).

7. If it is necessary to make the ends of the cut insert/vector "blunt ended" for the ligation, now is the time to do it. For this, T4 polymerase is used in combination with the necessary nucleotides (ATGC) to fill in the "sticky ends" with base pairs. The protocol is as follows.

T4 POLYMERASE REACTION (BLUNT ENDING):

1. For 800 ng of a gene cleaned fragments, The reaction is:
   * T4 polymerase
   * 10X buffer
   * BSA
   * GATC
   * Distilled water to a final volume of 20 ul

2. Allow reaction to proceed for 20' in a 16 degree water bath.

3. Bring volume up to 100 ul with distilled water and phenol chloroform extract.
    - Add equal volume of phenol chloroform, vortex and spin for 5' at 4 degrees. Transfer supernatant to new tube.

4. Add 10 ul 3M NaOAc. Vortex.

5. Add 220 ul 100% EtOH. Vortex.

6. Place on ice 10' and spin @ 13,000 fro 15' @ 4 degrees.

7. Discard supernatant and resuspend pellet in 10 ul TE pH 8.0.

LIGATION:

1. Once you have the Gene Cleaned DNA, put together the ligation as follows.

2. Combine the DNA so that you have a ratio of insert to vector at 3:1. The rest of the reaction consists of T4 Ligase, 10X Ligase Buffer, and distilled water. A typical recipe for one reaction is:
    - 10X Ligase Buffer 1 ul
    - T4 Ligase 0.5 ul
    - Vector 1 ul
    - Insert 3 ul
    - Distilled water 4.5 ul (or bring to 10 ul final volume)
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3. Mix reactions and put in a 16 degree water bath overnight. It is good to include a reaction mix with vector alone to make sure you have something other than religated vector once you transform and plate them.

TRANSFORMATION AND PLATING: See Transformation Protocol
MINIPREP and DIAGNOSTIC DIGEST: to see which clones worked and can be maxiprepped then used for transfections.