TA Subcloning of PCR Products

I. CONSTRUCTION OF T-VECTOR

1. suspend 10ug pUC Vector in:

   - 4.0ul 10X reaction buffer
   - 2.0ul (20U) SmaI/EcoRV
   - X ul ddH2O to a total vol. of 40ul

Incubate at 30 (not 37) degrees for 1 hour. This is easier if done in a 0.4ml tube in a thermal cycler.

2.Heat to 70 degrees for 15 min. to kill the enzyme

3.Bring to 100ul w/ water (add 60ul).

4.Extract w/ phenol, phenol/chloroform and then chloroform.

5.add 9ul 3M sodium acetate.

6.ppt. in ETOH, wash with 70% ETOH (be careful with the pellet!).

7.Dry in spin vac at room temp (do not use heater!).

II. T-TAILING THE VECTOR

At this point, it is assumed that there has been 80% recovery of the cut plasmid DNA.

1.Resuspend the plasmid DNA in 63ul water (conc approx. 130ng/ul)
2.To the resuspended plasmid add:

   - 10ul 10X PCR buffer (standard cetus stuff, no MgCl)
   - 20ul 10mM dTTP [2mM final]
   - 6ul 25mM MgCl2 [1.5mM final]
   - 1ul Taq polymerase (Cetus amplitaq 5U/ul)
   - _____
   - 100ul total volume.

3.Incubate for 3 hours at 70 degrees C.
4.Extract with Phenol, Phenol/chloroform, chloroform.
5.Extract twice with ether (so I'm paranoid!)
6.add 75ul 2M **ammonium** acetate (assuming 75ul recovery from extractions).

7.Add 150ul isopropanol. Spin 20mins in microfuge at full speed at 4 degrees.
8.Wash with 70% ETOH 9 Dry pellet in spin vac and store at -20 degrees until use.

III.TREATMENT THE PCR PRODUCTS

1.Add an equal volume of chloroform (*NO* IAA) to the PCR reaction and spin 1-2 minutes in microfuge at RT.
2.Remove the oil which is now on the ****BOTTOM***.
3.Spin again for two minutes and remove the last little bit of oil from the bottom. You will know when you have gotten it all when you see the interface in the pipette tip. It is important that all the oil be removed otherwise subsequent procedures will be very difficult.
4.Add 100ul 4M ammonium acetate, vortex, and then add 200ul isopropanol.
5.Centrifuge 20min at 4 degrees, wash in 70% ETOH.
6.Dry in speed vac.
7.Resuspend the DNA in 8-10ul TE, add loading buffer and load onto a 4% Nusieve (TAE) agarose gel. Run until the desired band is well separated. The more DNA in the band, the easier it is to subclone.
8.Cut out the band. Minimize the exposure of the gel (and you!) to short wave UV

IV.LIGATION OF PCR PRODUCTS TO T-VECTOR

1.Heat the gel containing the PCR fragment to 65C for 10 minutes, place in a 37C water bath or block and add to a separate tube (also at 37C):
    - 10 ul gel
    - 4ul 5X ligase buffer (commercial buffer that comes with BRL T4 ligase)
    - 4ul water
    - 1ul vector (25-50ng)
    - 1ul ligase
    - Incubate at 12C overnight.
2.Heat the mixture to 68 degrees for 5 minutes and add 100ul water.
3.Extract with phenol, phenol/chloroform, and chloroform. These steps are to remove the agarose.
4.Add 10ul 3M NaAcetate and precipitate with ethanol.
5.Wash the pellet in 70% ETOH, dry in the speed-vac. Resuspend in 5ul of water just prior to transformation.

*Transformation - We usually use electroporation into XL-1 blue cells. You need cells that can achieve at least 1 x 10^7 transformants per ug of DNA if a CaCl based protocol is used.

*Storage: The T-vector should be stored at -20C at all times. When stored in dry form, the T-overhangs will last longer (I don't know how long yet). In solution, it lasts at least a couple of weeks at - 20C.

*Enzymes - The batch of SmaI that is used is particularly critical. Some are contaminated with an endonuclease that removes a few bases from the cloning site. The batch of smaI should be checked before it is used to cut vector for cloning purposes. If bluescript is used, EcoRV can be substituted for sma I.

Shanghai ShineGene Molecular Bio-tech Co.,Ltd.

Add：Floor 2 ,Building A,328# Wuhe Road, Shanghai 200233 China

Tel：+86-21-54460832

Fax:+86-21-54460831

E-mail:master@shinegene.org.cn

Website：www.synthesisgene.com