Cloning protocols/tips by Astrid

PCR:

Primer design: I normally make the complementary part of my primers 18 bp long and make sure that there are enough bases behind a restriction site (NEB 249) so you can do a direct restriction on the PCR product.

Set up the following reaction mixture and mix well:

UP demi	16.5 ul
NTP's (2 mM)	2.5 ul
10x reaction buffer	2.5 ul
3'primer (25 uM)	1.0 ul
5'primer (25 uM)	1.0 ul
DNA template (0.1 ug/ul)	1.0 ul
Pfu polymerase (2.5 U/ul)	0.5 ul
	---------- +
	25.0 ul

I mostly do 3 PCR cycles at a temperature that is 5º C lower than the annealing temperature of the complementary part of the primer. Then I do 12 cycles at the annealing temperature.

Typical run: 1' 94º C / 1' 55º C / 1' 72º C } 3 cycles; 1' 94º C / 1' 60º C / 1' 72º C } 12 cycles then 7' at 72º C

Check the PCR product on a low melting point agarose (LMP) gel (1%) make sure you also run a marker on the gel. If you have the right product extract it from the gel. If the product is very little, set up a new PCR reaction and do 22 cycles instead of 12 (at the higher annealing temperature). If you don't have a product you can try using Vent or Taq polymerase, they are less picky but the chance for mutations is higher.

Extracting DNA from gel:

(use the Promega Wizard miniprep kit)

- cut the band with the DNA out of the gel
- melt the gel 5-10' at 70 **ºC**
- add 1 ml purification resin and mix
- put the resin in the column
- wash with 2 ml 80% isopropanol
- spin 2' at 14000 rpm
- air dry 5'
- add 50 ul UP demi and incubate 2'
- spin 30 seconds at 14000 rpm

Restriction of PCR product:

If you had a bright DNA band after PCR you can do a cut on 20 ul PCR product. If you had a faint band, speed vac the 50 ul you got from the gel extraction and use 20 ul concentrated product.

- do the restriction in a total 50 ul mix
- choose the buffer that is good for both enzymes you are going to use and add 5 ul
- add 1 ul of each enzyme
- incubate at 37° C (or other temperature) for 1 hour or longer
- heat inactivate 20' at 65° C (if the enzymes are heat inactivatable)

Restriction of vector:

- cut 5-10 ug of vector in a 50 ul mix for 1.5 hours using the first enzyme
- run the DNA on a 1% LMP agarose gel
- cut out the (cut) DNA band and gel extract
- take 25 ul and cut this with the second enzyme in a 50 ul mix for 1.5 hours save the other 25 ul
- run the DNA on a 1% LMP agarose gel again and cut out the DNA band

Ligation

* mix the following things together: 1 ul cut vector
5 ul insert
1 ul Mg++ (20x)
5 ul H2O

do a negative control (only vector)

- mix
- incubate 5' at 37° C
- add 2 ul 10x ATP/DTT (10mM/100mM)
- add 1 ul T4 DNA ligase
- incubate 2 hours at room temperature then ON at 16° C

Transformation:

Use electrocompetent cells with an efficiency of at least 10e7

- taw the competent cells on ice
- add 1 ul ligation mix
- electroporate at 25 uF, 200 Ω, 2.5 Kvolt
- add 1 ml of SOC media and incubate 1 hour in the shaker at 37° C
- spin cells down 6 sec at 14000 rpm
- take ~ 800 ul SOC out and resuspend the bacteria in the left over media
- plate cells on a agarose plate containing the right antibiotic
- let grow ON in 37° C incubator