Cloning Tips

For many applications, conventional restriction cloning is still the best method. A few simple tricks will help to ensure that your cloning goes smoothly.

General Tricks

1. First and foremost, be careful at each step of a procedure. This approach saves time in the long run.
2. Make sure the DNA is very clean. Start with about 2 μg of DNA when preparing a vector or excising a fragment to be inserted.
3. After each enzymatic reaction, purify the DNA with a spin-column. Elute the DNA in 40 - 45 μl of 10 mM Tris (pH 8.5), and then perform the next reaction. (There is no need to use a spin-column before purifying a DNA fragment with a gel.)
4. To maximize efficiency, minimize the number of steps in a procedure. For example, it works better to clone a blunt-ended fragment into a blunt vector site—such as a SmaI site—than into a site that has been blunted with Klenow or T4 DNA polymerase.
5. When in doubt, purify a DNA fragment with a gel. Cleaner starting material will yield a better outcome.

Preparing the Vector

The most common problem with restriction cloning is that the starting vector is recovered after the procedure. This problem has two causes: incomplete digestion of the vector, and re-ligation of the cut vector with itself. The tricks described below will minimize these effects.

1. Ensure that digestion of the vector goes to completion. Use an excess of restriction enzyme (~20 U for 2 μg DNA), and digest for ~4 hr. If the vector needs to be cut with two enzymes that have different optimal reaction buffers, perform the digests sequentially, with a spin-column purification in between.
2. After digesting the vector (and blunting, if appropriate), treat with phosphatase: 1 hr at 37°C for 5' overhangs, or 1 hr at 50°C if the DNA has any blunt ends or 3' overhangs.
3. Remove the phosphatase with a spin-column. Gel purification of the vector is usually unnecessary, because phosphatase treatment will inactivate any extra DNA fragments that were generated.

With the vector, ensure that the digestion and phosphatase reactions go to completion. Vortex the mixtures thoroughly and repeatedly, and do not allow any droplets of liquid to escape enzyme treatment. It is a good idea to spin briefly after vortexing to ensure that no droplets are left on the side of the tube.

Preparing the Inserted Fragment

1. For preparation of an inserted fragment, incomplete digestion is not a serious concern because partial recovery of the fragment is acceptable. You can use relatively short digestion times, and for a double digest, you can use a reaction buffer that is suboptimal for one or both enzymes.
2. Purify the inserted fragment with a gel. In addition to removing unwanted or incompletely digested DNA, this procedure removes enzymes and small molecules. **When visualizing DNA bands with a transilluminator, do <u>not</u> use short wavelength UV light of 312 nm or below because the DNA will be severely damaged. Use 360-365 nm UV light instead.**
3. If the inserted fragment was generated by PCR, purify it with a gel or spin-column before doing any restriction digests. If you plan to clone a blunt-ended PCR fragment (generated with a polymerase such as Pfu) into a phosphatase-treated vector, ensure that your PCR primers have 5'-phosphates.

Ligation and Transformation

1. With a phosphatase-treated vector, perform a control ligation in which the inserted fragment is omitted. This control ligation should yield very few transformants because only circular DNA molecules transform *E. coli* efficiently, and re-circularization of the vector cannot occur without ligation to a phosphorylated fragment.
2. If the DNA has only sticky ends, ligate at room temperature for ~1 hr. If the DNA has any blunt ends, ligate at room temperature for ~4 hr and use high-concentration T4 ligase.

Minipreps and Diagnostic Digests

1. If you obtain many more transformants for the ligation with the inserted fragment than for the control ligation, your cloning almost certainly worked, and you can usually get away with making just 3-4 minipreps.
2. When performing a diagnostic restriction digest of your minipreps, always include the starting vector as a reference standard.