PJB's hints for sub-cloning:

1) Prior to any ligation reaction, you should always run one gel in which purified insert and cut backbone are run side by side, preferably beside a known amount of cut DNA. You can then use this to estimate how much backbone and insert to use in your ligation reaction. In general, a large excess of insert over backbone works well, but you should try two different ratios for every ligation (I used to use ~1:10 backbone/insert and ~1:50 backbone/insert).

2) For every ligation reaction that is transformed into bacteria, you **must** always transform a known amount of closed circular DNA so you can calculate the transformation efficiency of the competent cells you are using. Even if someone told you the efficiency, or you calculated it on your last ligation, this is an essential control for **every** transformation to make sure that the cells are still competent, the electroporator is working as expected, you are still doing the transformations correctly, etc. You should get a standard plasmid of known concentration to use for every experiment (Bluescript is a nice control because it will turn blue on xgal/IPTG plates). If you assume the efficiency of the cells is between 10^7 and 10^8/µg of DNA, then you need to dilute the plasmid to a concentration of 0.1 ng/µl. Then transform 1 µl (0.1 ng) and plate both high (90% of the transformation) and low (10% of the transformation). If the low plate has 100 colonies, your transformation efficiency was 10^2/0.01ng, which translates to 10^7/µg. I never had problems storing transformation control DNA at 0.1 ng/µl in the freezer, so you don't have to dilute it each time you do a transformation.

3) Every transformation of a ligation reaction should also include controls for trouble-shooting the ligation reaction itself. For example, transformation of a ligation of backbone with no insert will allow you to evaluate if your backbone is circularizing when it shouldn't; a transformation of backbone plus insert, no ligase, will tell you if there are closed circular plasmids in either your backbone or insert preps.

4) Something in agarose can inhibit ligation reactions. Therefore it is best to keep gel purifications to a minimum: e.g. gel purify the insert (because you have to), but it shouldn't be necessary to gel purify the backbone. Always use low melt agarose for gel purifications (even if you're not going to recover the insert by melting the gel slice)-- the regular agarose supposedly has more of the "something" that inhibits ligase. Since low melt agarose is "sloppier" than regular agarose, you will probably have to run a 1.5% or 2.0% gel for the gel separation.

5) Something in phenol can inhibit ligation reactions. If you need to get rid of a restriction enzyme, check to see if you can heat kill it (back of most molecular biology catalogs includes a list of which enzymes are heat inactivated and what conditions to use). Then you don't need to do a phenol extraction to remove the enzyme.

6) Isolating insert:

A) It is best to run the reaction on a long gel (**not a mini-gel**) to get maximal separation of the insert from the cut backbone and any residual uncut backbone. Even if you can't see it on a gel, there can still be some closed circular DNA that didn't cut. When you run a mini-gel, you run the risk of isolating uncut vector along with your insert. Since closed circular DNA transforms bacteria so efficiently, you might end up with your parental plasmid back again. In the past week, two people have shown me gels that demonstrate this problem -- so it really does happen even when you've gel purified your insert. To prevent it, you can try cutting with another enzyme in addition to the ones that liberate your insert. This enzyme should cut the backbone, but not your insert.

You can treat the purified insert with this enzyme, or you can add this enzyme during the reaction to get the insert.

B) UV radiation can damage DNA. It is best not to spend a lot of time admiring your gel when it is on the UV box. I recommend that you refrain from taking a picture before you cut out the band. Just turn on the UV light for long enough to cut the band out, then take a picture of the gel after the band was cut out, which will show you approximately where it was on the gel. Since there should ALWAYS be molecular weight markers on your gel, you should be able to verify that the band you cut out was the right molecular weight.

C) However you choose to isolate your insert is up to you. However, the final step will probably be an ethanol precipitation. You should always use tRNA as a carrier, since you can easily lose small amounts of DNA in a precipitation. ~20µg of tRNA works well. tRNA needs to be prepared as described in the cloning manual to make sure it is free of DNAse.

7) Preparation of backbone:
I have never gel purified a cut backbone, although some people have success doing this. What I found easy, and which worked, was to cut a known amount of backbone (e.g. 5 µg in 50 µl). If you are doing a double digest, cut with one enzyme first (the less reliable one), save an aliquot of the reaction for a gel, then cut with both enzymes and save an aliquot after you are done with the reaction. Now run a gel of the aliquots of the cut backbone, and make sure that the digest after the first enzyme looks just like the digest after both enzymes. If the enzymes you are working with can be heat-killed, then heat-kill them, otherwise you will have to phenol extract to remove them. I never worried about the little piece that is liberated when preparing a backbone cut with two enzymes, but if you are worried, you can ethanol precipitate (the backbone will precipitate much more efficiently than the little piece). If you resuspend your cut backbone in 50 µl, you now have backbone at a known concentration, which is 100 ng/µl. This should be enough for many ligations (I usually used only a few ng's of backbone per ligation). I have saved cut backbone for years in the freezer, and it would still work for ligations. So once you get a backbone cut that you will use again (e.g. PBJ5-GS cut with Xho and Not), save it for future use.

8) If you see satellite colonies around bigger colonies on Amp plates, you probably added the Amp to the agar when it was too hot. Wait until it cools to ~60˚C before adding drugs. If blue/white selection doesn't work, it might be for the same reason. For blue/white selection in general: I have NEVER gotten it to work well by spreading x-gal and IPTG on plates. The only way I've ever seen a clear cut color difference is by adding xgal and IPTG directly to the agar before pouring the plates. Here's a recipe that works for xgal/IPTG/amp plates:

1 liter 2XYT with agar
Cool to 60˚ C
Add 1 ml stock amp (100 mg/ml)
5 ml xgal (20 mg/ml in DMF -- store in the dark by wrapping container with tin foil)
1 ml IPTG (1.0 M)

Happy cloning!