Easy Subcloning

by Michael Koelle

Subcloning should be easy and fast, and work every time. The following protocols minimize the number of manipulations required to prepare DNA fragments for ligations, thereby both saving time and increasing reliability.

Preparation of DNA fragments for ligation.

1. Restriction digests:

   Always cut a lot of your starting plasmids in a small volume; this will help in the gel purification of your restriction fragments by giving you a high concentration of DNA compared to agarose in your gel slice.. About 1 µg in a 20 µl reaction is good.

   For double digests: you almost never have to digest with one enzyme, adjust the buffer, and digest with the second. Look in the Biolabs catalogue to find a compromise buffer, and save yourself some time.

   Don't use just 1 unit of enzyme and wait an hour: your time is worth too much, and the enzymes are cheap and pure. Use about a 10-fold enzyme excess and digest about 30 min.

2. Playing with the ends.

   Blunting 5' overhangs: Add 1 µl 2 mM all four dNTPs to your 20 µl restriction digest.
   Add 0.5-1 µl Klenow (2-5 units), and incubate at room temp for 30 min.

   Blunting 3' overhangs: Add 1 µl 2 mM all four dNTPs to your 20 µl restriction digest.
   Add 0.5-1 µl T4 DNA polymerase, and incubate at 37° for 5 min. T4 polymerase has a more active 3' to 5' exo activity than Klenow, and so is preferred for this reaction, but Klenow will work.

   Blunting both a 3' and 5' end in the same reaction: using either Klenow alone or T4 polymerase alone or a mixture will work okay.

   Blunting an end, and then cutting with another enzyme to produce another end that is sticky: after blunting the ends produced by the first enzyme, add ~80 µl 0.3 M NaOAC pH 5.2, phenol/chloroform extract, chloroform extract, add 3 volumes EtOH, put at -20° for 20 min., spin 10 minutes, wash with 70% EtOH, dry, and resuspend in the appropriate buffer for the next restriction digest. Then add the second restriction enzyme and proceed. (Note: I've tried just killing the Klenow or T4 polymerase by heating to 70° for 20 minutes, and then adding the second

restriction enzyme. This hasn't given good results, though; I think Klenow in particular is somewhat thermostable).

Phosphatasing to prevent vector reclosures: After the restriction digest, add 1 µl Biolabs calf intestine alkaline phosphatase. Incubate 37° for 1 hr. (In the old days I used Boehringer phosphatase: they sell a high concentration type so that you can put in ~20 units to help phosphatase blunt and 3' overhang ends, which are more resistant to phosphatasing. In the old days we also used to change to an alkaline buffer for phosphatasing, but that appears unnecessary.)

Blunting an end, and then phosphatasing: after blunting, ~kill the Klenow or T4 pol by incubating at 70° for 20 min, and phosphatase as usual. There is a theoretical concern here that the unused free nucleotides will compete with the DNA ends for the phosphatase, but empirically it seems not to matter. If you're worried about this you could EtOH ppt. and resuspend before phosphatasing.

Cloning Taq polymerase PCR products by T/A cloning: Taq polymerase has an efficient terminal transferase activity that adds a single nucleotide to the 3' end of blunt ended duplex DNA. This terminal transferase activity greatly prefers adding A over adding C, G, or T, so most PCR products will have single 3' A overhangs. These can be cloned efficiently into a vector containing single 3' T overhangs on its ends. You could buy kits containing prepared vectors of this structure, but you can easily produce the equivalent yourself. After cutting 1 µg of any vector with a blunt cutter (e.g. Bluescript, using EcoRV) dilute the digest with 1 volume of 1X PCR buffer, add dTTP to 0.1 mM, add 2 U Taq polymerase, and incubate at 72° for 20 minutes. The Taq will add a single T to the 3' ends of the vector DNA. The vector will now self-ligate very inefficiently, and should accept Taq generated PCR products. In practice, however, ligations of such vectors with PCR fragments yield only ~10-50% clones with inserts, so it is best to use blue/white selection to identify colonies with inserts; almost all the white colonies will have an insert.

3. Gel purifying DNA fragments: I generally gel purify all DNA fragments; even if I'm just linearizing a vector I run it out on a gel to purify it away from the enzymes and buffers it is in. Gel purification provides a quick way to purify the DNA, and allows you to check what your putting in to your ligation to make sure it's alright.

A. Use a low melt agarose gel, run in fresh 1X TAE in a decently clean gel box. Use Seaplaque agarose from FMC. For most purposes, use a 0.8% gel. If you need good resolution under 500 bp, you can go up to 2%. Nusieve agarose from FMC also works for cloning, and can resolve fragments down to 50 bp or less. Post-staining the gel wastes time, so run the gel with ethidium in it. Include 1 µl of 10 mg/ml ethidium bromide per 50 ml of gel. You don't need to add EtBr to the running buffer unless you are running the gel very long and are looking at small fragments (in which case the EtBr will run out of the bottom of the gel and screw

you up unless you add it to the running buffer). I load 1 µg of DNA in a 3.5 mm wide well.

B. Don't waste your time by running the gel slowly. Use about 5 volts per cm, i.e. 100 V for a minigel box, 150 V for a medium gel box.

C. When the gel is done, place it on a clean piece of saran wrap on a long wave (365 nm) UV box and take a picture. Make sure to use long wave UV and minimize the exposure to UV to avoid damaging the DNA. Cut the desired bands out of the gel with a clean scalpel. Turn the gel slice on its side and trim off the extra agarose. Place the gel slice in an eppendorf tube.

D. Spin the gel slice down to the bottom of the tube, and place in a 70° tempblock to melt. Pipette the melted agarose up/down to get a uniform mixture. Total volume of the gel slice should be ~40 µl. Can freeze the gel slice to store.

Ligation

Add 2 µl 10X ligase buffer to a tube (the commercial NEB stuff is fine). Add 2 µl water. Melt your gel slices in a 70° water bath. Add 10 µl of your insert gel slice, 5 µl of your vector gel slice. Wait ~30 sec for mixture to cool, add 1 µl of T4 DNA ligase, mix by pipetting up/down, and immediately place on ice to cool. Always do a no insert control, in which you replace the insert gel slice with 10 µl water. Incubate the ligation overnight at 16°, or at room temp. for ~3 hours.

10X Ligase buffer

0.5 M Tris pH 7.8

0.1 M MgCl2

50 mM DTT

2 mM EDTA

4 mM rATP

Transformation

A. Melt the ligation mix in a 70° tempblock. Add 80 µl 0.1 M Tris, pH 7.4, and place on ice to cool. Add 100 µl competent cells, and transform using the protocol in this manual. There is also a protocol for quick plasmid minipreps, and large scale preps.

------ AIM: From Koelle transformation protocol

Transformation

6. Thaw cells at room temp (or by holding between fingers), and put on ice. Use 100 µl/transformation. Add DNA and incubate on ice 30'. It is best to add <100ng DNA in <40 µl. **However, ligations performed in 20 µl low melt agarose can be transformed by melting at 70°, adding 80 µl 0.1M Tris pH 7.5, chilling on ice, and adding this to 100 µl cells.** (emphasis mine –AIM)

7. Heat shock at 42°, 90", return to ice for 1'-2'.

8. Add 1 ml LB, incubate 1 hr at 37°.

9. Plate on L plates containing 50-100µg/ml carbenicillin. (Note: I prefer carbenicillin to the more widely used ampicillin. Carb. is much more stable; plates and media containing carb. can be kept indefinitely, whereas ampicillin is slowly hydrolysized so that solutions and plates can only be used for a couple of weeks after they are made. On the other hand, carb. is 15X more expensive.)

B. Choice of host strain for transformation: Strains generally used for cloning have mutations that suppress recombination, the idea being that this helps avoid getting rearranged clones. In practice, unless your plasmid contains a repeated sequence, the frequency of such rearrangements is so low as to not be worth worrying about. The rec- mutations, however, make the E. coli strains sick, and as a consequence your transformants grow up slowly. Therefore, I recommend using a healthy E. coli strain for routine cloning. The strain TG1 has the following genotype: supE hsd 5thi (lacproAB) F ' [traD36 proAB+ lacI9 lacZ M15]. TG1 colonies grow up in ~8 hr. The colonies can be picked and grown up to 2 ml cultures for minipreps in 5 hours. Really tiny 8 hour colonies are most easily picked under a dissecting scope using a straight platinum wire. Because it grows so fast, you can often save a day by using TG1 rather than a less healthy strain. TG1 can be used for blue/white selection with pUC an bluescript vectors: when growing the strain up to make competent cells, start with a colony from a minimal plate (this selects for cells carrying the F ' episome, which carries the necessary lacZ construct). It takes longer than 8 hours for the blue color to develop, (~11 hours), so you have to wait a little longer to pick the colonies if you're using this. Try looking at the plates against black and white backgrounds to help see the color when it is just starting to develop. Bluescript SK and pUC vectors give good color development. Bluescript KS takes much longer to develop the blue color, so don't use this vector unless you have to. For DH5 alpha plated in top agar, using blue/white selection with BS SK, get tiny colonies at 12 hours, pickable colonies at 14 hours. At 14 hours, the blue color is just barely visible.

   To use ordinary amp plates with an IPTG/XGAL overlay for blue/white selection:

1. My old method: Take the amp plates out of the cold room to warm up to room temp. during the transformation. Melt some L soft agar in the microwave, and aliquot 3 ml into sterile glass tubes, and place in a 50° temp.block. To plate a transformation mix: just before use, add 40 µl 24 mg/ml IPTG, 40 µl 20 mg/ml Xgal in DMF, 10 µl 20 mg/ml amp (or carb) (store all these stocks separately at -20°; you can mix the stocks together immediately before use to make a premix) to the 3 ml of top agar, add the transformation mix, quickly vortex and pour onto the amp plate. Colonies take longer to grow up embedded in top agar than on an ordinary plate. Some colonies will burst up to the surface and become much larger than the others. After the colonies are evident, you can put the plates at 4° and the blue color will continue developing.

2. My new method, adapted from Ewa Davison. Make up 2% X-gal in DMF, and 24 mg/ml IPTG in water, as in method #1. Mix these two solutions 1:1, and spread 100 µl of the mixture on an ordinary Amp plate. Let the plate sit at least one hour to allow the liquid to absorb into the agar. Then plate the transformation mix as per usual. The IPTG/Xgal that has diffused into the plate will turn the appropriate colonies blue.

Commentary

A. Don't be a wimp about multi-step constructs. The turnaround time on clones can be as little as 24 hours with the above protocols. Make liberal use of partial digests and triple ligations to help cut down on the number of steps required; these work just fine.

B. People who complain that blunt end ligations or triple ligations don't work well are generally inexperienced cloners who are trying to place the blame that really lies with other mistakes they made in the procedure; in my hands these ligations work ~100% of the time. People also complain that doing the ligation in the agarose has a lower efficiency than you would get if you purified the DNA fragments first (e.g. by ß-agarase digestion and ethanol precipitation, or using glass bead purification). While this may be true in theory, in practice I get my clone ~100% of the time doing the ligations in agarose (sample size = several hundred clonings), so it seems pointless to increase the efficiency any more. The purification procedures can take several hours, and carry the risk of catastrophic failure: you could lose the DNA pellet and not realize it's missing until your cloning fails. I found that when teaching novices to clone, they have the highest success rate when using a procedure that minimizes the number of steps they might screw up.

C. Partial digests: Set up 8 tubes containing 2 µg plasmid in 20 µl restriction buffer. Add 8 units enzyme to the first tube. Tranfer 10 µl from the first tube to the second, mix, transfer 10 µl from the second tube to the third, mix, and so on. Finally transfer 10 µl from the last tube to the first one. Incubate all the tubes at 37° for 1 hour, and heat kill the enzyme at 70° for 10 min. Because the patterns

from partial digests can be complex, it is often necessary to run quite a long agarose gel to resolve all the products. Be careful, because two of the bands that show up will be supercoiled and nicked plasmid; don't confuse these with linear fragments. Also, if your fragment runs near the supercoiled or nicked plasmid and gets contaminated with even a small amount of either of these, you will get back your starting clone at a high frequency from the transformation. To control for this, do a no ligase control ligation, and see if it gives you colonies.

D. Triple ligations work great as long as each of the three joints being made has a different type of end, so that the orientation of the ligations are forced and only one type of triple ligation product is possible.

Miscellaneous

The enzyme Sma1 doesn't always leave a blunt end as advertised. Therefore, be careful about using this site when trying to preserve an open reading frame.