CLONING FROM LOW MELT AGAROSE

A quick method for cloning fragments with the following ends (see Note 1):

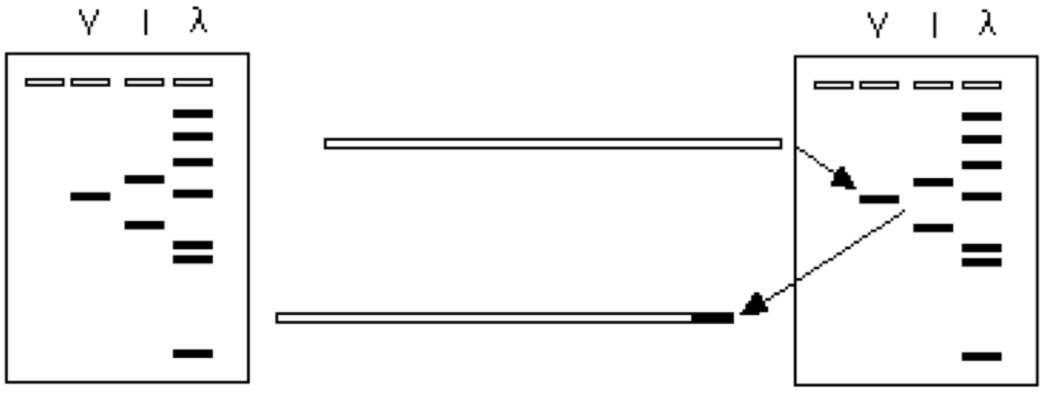
1) A double cut fragment being cloned into a double cut vector if the two restriction sites are non-cohesive. This is the method we use for most of our cloning experiments. Sticky to blunt also works.
2) A single cut fragment being cloned into a single cut vector if the vector has been properly dephosphorylated. Dephosphorylation is not essential, but will greatly reduce screening needed to identify insert clones (unless some screen or selection is available- e.g. blue/white). We recommend using NEB antiarctic phosphatase which can easily be inactivated at 65°C.
3) A small blunt fragment, e.g PCR product, being cloned into a dephosphorylated blunt vector.

1. Digest vector and insert with enzyme(s), and dephosphorylate the vector if appropreate. PCR products should be cleaned up to remove the Taq polymerase before being digested. We recommend running out the inserts in the gel even if they represent the only product in the digest (Note 2).

2. Pour a low-melt agarose (Note 3). TAE mini-gel with ethidium bromide in the gel and the buffer. Since low-melt agarose gels are fragile take care to leave enough clearance between comb and gel box such that removing the comb does not rip out the bottom of the well. We pour these gels in the cold room to help the gel solidify. We also use as low an agarose percentage as possible. We typically use 0.8% agarose for almost any size fragment except those under 300 bp.

3) Load the wells with enough of the digest to have nicely visible bands of vector and insert (i.e ~50-100 ng of each; see Note 11). Run the gel at 50-100 volts until vector and insert bands are well seperated from other bands of the digests. The gel can be run in the cold room if you have difficulty in handling the gel.

4) Place the gel under a trans-illuminator and puncture the gel with a 100 µl glass micro-pipette and remove as large a fraction of the vector band and insert bands as possible (Note 4). Blow the gel slice into an eppendorf tube, and repeat the procedure for the insert band. Minimizing DNA damage is key to success of the protocol. We use a medium wavelength (300 nm) trans-illuminator and keep our gels on a thin glass plate (a 5cm x 8 cm slide; see Note 11) to reduce low wave length UV transmission. Alternatively, use a long wavelength type (360 nm) trans-illuminator, or place a plexiglas sheet between the UV box and the gel to protect the DNA from being damaged during this process. Our rule of thumb is if you can see it, you can clone it. Typically the punch will be about 8-10 ul.



5. Place vector and inserts at 65º C for 2-3 minutes to melt.

6. Set up ligations in the following order at room temperature (Note 5)

2.5 µl 10X NEB ligation ligation buffer (Note 6)
14-20 µl H20
1-4 µl melted vector band (20-50 ng)- pipette up and down to mix well
1-4 µl melted insert band (20-50 ng)- pipette up and down to mix well
0.5 µl NEB T4 DNA Ligase (400,000 units /µl)- pipette up and down to mix well

It is important that the ligations be set up before the gel hardens, so that the vector insert and ligase are evenly distributed in the gel. Ligate 1-2 hrs at 15°C for most ligations. I sometimes do ligations at 15ºC overnight for blunt end ligations and this seems to work well.

7. To move the clone into E. coli, I use standard low temp growth competent cells (DH5α at competency of 10^8 cfu/µl of pUC18). Place 50-200 µl of competent E. coli into a cold ependorf tube. Melt the ligation mix at 65° C for 5 minutes and add a 1-5 µl aliquot of the ligation mix to the competent cells and vortex quickly. Incubate 20-40 minutes on ice (note 8), heat shock 40 seconds at 42º C, and return to ice 2-10 minutes (note 10). Add 1 ml SOC (note 7), and incubate 1 hr at 37ºC shaking lightly (note 9). I usually plate the 100 ul and keep the rest in the fridge in case I need additional colonies. Alternatively, one can plate the whole transformation after spinning down the cells in a low speed centrifuge at 2000 rpm for 5 minutes.

8. Typically, for a double sticky cloning (e.g. inserting a 2kb cDNA fragment into a 10 kb vector), we will get hundreds to thousands of colonies on our vector plus insert experimental sample, and less than 5% as many colonies on our vector only control if the double digest went to completion.

9. Minipreps of 4 colonies should in most all cases be sufficient to get your clone (note 12)

Notes

1. This method may be less efficient for very large blunt inserts and inserting into re-ligatible vectors because the agar reduces diffusion.
2. We used to avoid gel purify vector unless this was required, but we have found that gel purifying the vector often reduces background.
3. We have had great and lasting success using Sea Plaque low melt agarose. I personally would not use a substitute. We use 12 ml gels poured on 5cm x 8 cm slides using surface tension. For a gel like this, ~3 ng is a visible amount of DNA and 100 ng a very bright band.
4. Punching the bands takes a little practice. Try punching out marker lanes until you get the hang of it.
5. Control ligations could include vector alone or insert alone ligations.
6. We used to make our own ligation buffer [660 mM Tris pH 7.5, 66 mM MgCl2, 5 mM ATP (added fresh)]. NEB ligation buffer works great, but I would aliquot it into 100 µl aliquots so the buffer doesn't go through too many freeze thaw cycles.
7. Add at least 5 volumes of media. We used to rescue with LB, but we have found using SOC increases transformation efficiency by at least 10X with our comp cells. They must like the sugar. SOC per liter :20g Bacto Tryptone, 5g Bacto Yeast Extract, 2ml 5M NaCl, 2.5ml of 1M KCl. Sterilize and supplement with 10ml of 1M sterile MgCl2,10ml of 1M sterile MgSO4, 20ml of sterile 1M glucose. We make 5ml aliquots and freeze to reduce odds of contamination.
8. Expect a 2-fold loss in transformation efficiency for every 10 minutes you shorten this step according to NEB.
9. Expect a 2-fold loss in transformation efficiency for every 15 minutes you shorten this step according to NEB.
10. We found a dramatic increase in transformation efficiency (~10 fold) by increasing the length of the cold quenching to 10 minutes.

11. We recently realized that low iron glass slides provide for much better visualization of DNA in gels on a glass slide that traditional high iron glass. You can tell the difference between the two because the low iron glass will look white in cross section, and the high iron glass will look green in cross section.
12. If you do not get your construct, analysis of the structure of clones that do appear can be diagnostic of the problem.

--- If one gets lots of the parent vector, typically, the digests did not go to completion, and either single cut or uncut vector yield these colonies.

---If the colonies represent the parent of the insert, then the digestion of the insert did not go to completion, and the insert is contaminated with uncut or single cut vector that religated.

--- If the resulting fragments represent your vector with the two sticky end forced together having lost the spacer region between the restriction sites, then it is likely that your insert is of low quality. Sometimes restriction sites on the ends of PCR products won't digest well, or the PCR product is contaminated with primer dimers that contain the restriction site at high concentration, and are soaking up the restriction enzyme.

--- If the products you get have no relationship to the vector or the insert the problem is likely contamination of one of the reagents with plasmid DNA.

--- Finally, if you get no colonies that you are probably damaging your DNA during the UV punching process.

Updated 7-27-2015