Recombineering protocol #2

Manipulation of cloned DNA with the bacteriophage lambda Red system – conditional knock-out vectors

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This procedure is adapted from Dr. E-Chiang Lee's protocol [1]

 phage RED system has recently been "re-discovered" for manipulating cloned DNA in *E.coli* [1-12]. Red system offers a number of advantages over other recombination systems in the bacteria. First, it is extremely efficient. Secondly, there is no noticeable increase of spontaneous mutation rates when RED proteins are transiently expressed. Thirdly, very short homology sequence is required (45bp) so a targeting vector for DNA modification can be easily obtained from PCR. Fourthly, single strand DNA oligos are better substrates for recombination with RED system. Therefore generating point mutations can be achieved with DNA oligos. Moreover when single strand oligos are used, the only known recombination protein required is the beta-protein. Principles of RED system can be found in some recent papers. Neal will have an extensive review on this technology in the coming issue of NATURE REVIEW GENETICS. I have used RED system for making conditional knockout constructs, retrieving genomic fragments from BACs, making point mutation in a BAC with an oligo. Here I describe my experience of using this system for making a conditional KO construct**.**

E-Chiang in Neal and Nancy's lab, Daiguan in Dr. Court's lab constructed a number of DH10B derived *E.coli* strains that contain a defective prophage. I use two strains, EL250 and EL350. In both strains, *P*L operon encoding *gam* and the red recombination genes, *exo* and *bet*, is under tight control of the temperature sensitive repressor (*cI857*). EL250 has a *Flpe* gene under the tight control of *AraC* and P_{BAD}; while EL350 has a *Cre* gene under the same promoter. Upon addition of arabinose in the medium, expression of *Cre* and *Flpe* will be induced in the both strains. These strains are useful for removing F*lox*ed *Neo* or *FRTNeoFRT*.

One requirement for using RED system is that one needs to have the genomic sequence of a gene, at least the region that needs to be manipulated. Generally, 15-20kb sequence would be sufficient for making a conditional KO construct.

If one starts with a BAC, then the BAC needs to be characterized to make sure it has all the genomic regions you are interested in.

Some BAC vectors have *lox*P or mutant *lox* sites that need to be removed. This can be easily done using a targeting vector to replace the *lox* sites with selection makers, such as *Kan*, *Bsd*, *Amp*, *Tet*. I have a construct from E-Chiang that specifically deletes the *lox*P site on pBeloBAC11.

Transform BAC or plasmid DNA into the recombinant strains

Pick up a single colony of EL350 and inoculate to 5ml LB, grow at 32C O/N (16-20 hrs, always grow these recombination strains at 32C).

Next day, collect cells by centrifuging at 5000 rpm at 0C for 6 mins using an Oak Ridge tube. Resuspend cell pellet with 888ul (a chinese lucky number) ice-cold water. Transfer cells to a 1.5ml eppendorf tube (on ice) and centrifuge using bench-top centrifuge for exact 20 seconds at Max speed. Put tube on ice. Aspirate water. Be careful not to touch cell pellet. Resuspend cell pellet with another 888ul ice-cold water. Centrifuge again.

Repeat the process three times (wash the cell pellet three times with cold-water).

Finally, resuspend cell pellet in 50 ul ice-cold water, Pipett 50 ul cell suspension to an electroporation curvette (0.1 cM gap). Add 2ul BAC DNA (100ng), or plasmid DNA (1.0ng). Electroporation is performed with a Biorad GenePulser electroporator under the following condition: 1.75 kV, 25uF with pulse controller set at 200ohms. Time constant is usually around 4.0. Add 1.0ml LB or SOC to each curvette and incubate at 32C for 1 hour. Plate cells.

Pick up four to six colonies and check BAC integrity by cutting with several restriction enzymes and compare restriction patterns with the original BAC.

Making targeting vector to introduce the first loxP site

1. Using PCR. Design two 70-80mer primers. Each primer has about 20 bases homology to a F*lox*ed Neo plasmid (such as PL400), and 50 or more bases that are homologous to the region you are going to target. Do several PCR reactions to make sure you obtain enough PCR fragments (I use the ROCHE Biochemical High Fidelity PCR Kit, Cat# 1-732-641). PCR fragment can be purified with gene-clean, Qiagene kit, or Spin-column (Sigma 5-6500). PCR fragments should be dissolved in water at 100-200ng/ul. Use 1ul or 2ul for electroporation.
2. Using a cloned targeting vector. One can extend each homology arm to increase targeting efficiency. I find it is more efficient when longer homology is used. You make left and right arm using PCR (Four primers with appropriate cutting site on each primer). Multiple-way ligation with the left, right arm, f*lox*ed Neo and pSK+ will give you the targeting vector. Excise the fragment for targeting with NotI/SalI.
3. Prepare competent cells that are induced to express RED recombination proteins. For making conditional targeting vectors that contains *lox*P sites, I use strain EL350. EL350 has *exo*, *beta*, and *gam* genes under *P*L control (*cI857*, temperature sensitive). Additionally, Expression of Cre recombinase gene is under the tight control of *Ara* operon. *Cre* expression is induced in 0.1% arabinose.

Pick up a single colony of EL350 to 5ml LB, grow at 32C O/N (16-20 hrs, always grow these recombination strains at 32C).

Next day, inoculate1.0ml of O/N culture to 20ml LB, 32C for 2-3 hrs or OD600=0.5. Transfer 10ml cells to a new flask and shake in 42C water bath for 15 mins.

Immediately after 42C induction, put the flask into wet ice. Shake the flask to make sure temperature drops as fast as possible. Leave the flask in wet ice for another

10-20mins. Transfer cells to a 30ml glass centrifuge tube. Spin at 5000rpm at 0C for 6 mins (do not forget to use the rubber adapters for glass tubes).

Resuspend cell pellet with 888ul ice-cold water. Transfer cells to a 1.5ml eppendorf tube (on ice) and centrifuge using bench-top centrifuge for exactly 20 seconds at Max speed. Aspirate water, be careful not to touch cell pellet. Resuspend cell pellet with another 888ul ice-cold water. Centrifuge again.

Repeat washing process three times (wash cell pellet three times with cold-water).

Finally, resuspend cell pellet in 50ul ice-cold water and pipett 50ul cell suspension to an electroporation curvette. Add 2ul purified PCR or plasmid fragment. Electroporation is performed with a Biorad GenePulser electroporator as described previously.

Add 1ml SOC or LB medium to the curvette and incubate at 32C for one hour (this is very important for selection makers such as Kan, Cat). Plate 100ul cells to one plate with appropriate slection antibiotics and the rest to a second plate.

Drug selection concentration:Kana, Cat (chloramphenical): 12.5 ug/ml for BAC, 25ug/ml for multiple copy plasmids; Bsd,: 75ug/ml for both BAC and multiple copy plasmids; Amp: 25ug/ml for BAC and 100ug/ml for multiple copy plasmids

If one uses a multiple copy plasmid to make a KO vector, the basic procedure is the same. One difference is that when you obtain the targeted colonies with F*lox*ed *Neo*, most if not all of them are mixed population (you will see from restriction digestion). One targeted plasmid in a cell with 50 copies of non-targeted plasmid will make this cell resistant to Kana. I re-transform the mixed plasmid to EL350 cells (prepare EL350 cells, use1.0ng per electroporation) and select on Kan plates. All of the non-targeted plasmid will be eliminated.

I find it is easier to manipulate DNA (making KO construct) in plasmid than using BAC. If you have detailed restriction map or better, sequence of the region interested, you can easily clone the appropriate fragment into pBluescript and start from there.

Pop-out the Floxed Neo

Targeted plasmid or BAC should be examined closely with restriction digestion to make sure no rearrangements. Random rearrangements have rarely been observed. To pop out the targeted F*lox*Neo, one needs to transiently express Cre recombinase.

If you use BAC, inoculate a single colony of the targeted cells to 5ml LB. 32C O/N. Next day, inoculate1.0ml of O/N culture to a 10ml LB, 32C for 2-3 hrs or OD600=0.5. Add 100ul 10% L(+) arabinose (sigma A-3256) to the culture (final concentration, 0.1%) and let Cre induction go for one hour. Plate diluted culture to both Cat (on BAC backbone) and Kan plates. I normally get single colonies from 10^{-4} to 10^{-6} dilutions. You should see some colonies on the Cat plate but nothing on the Kan plate. Pick up several colonies from Cat plate and make BAC DNA to check precise excision and BAC integrity.

If one uses plasmid, one can simply transform the targeted plasmid to electro-competent cells that Cre recombinase is already induced. Inducing Cre expression in cells that already have multiple-copy plasmid will lose all the plasmid.

Pick up a single colony of EL350 to 5ml LB, grow at 32C O/N (16-20 hrs, always grow these recombination strains at 32C).

Next day, inoculate1.0ml of O/N culture to 10ml LB, 32C for 2-3 hrs or OD600=0.5. Add 100ul 10% arabinose to the culture and shake at 32C for one hour. Collect cells by centrifuging at 5000 rpm at 0C for 6 mins using an Oak Ridge tube. Resuspend cell pellet with 888ul ice-cold water. Transfer cells to a1.5ml eppendorf tube (on ice) and centrifuge using bench-top centrifuge for exact 20 seconds at Max speed. Take out the tube and put on ice. Aspirate water, be careful not to touch cell pellet. Resuspend cell pellet with another 888ul ice-cold water. Centrifuge again. Repeat the process three times (wash the cell pellet three times with cold-water). Resuspend cells in 50ul cold water. Perform electroporation with the targeted plasmid (1.0ng) and plate diluted cells to both Amp and Kan plates. I normally get single colonies from 10^{-4} to 10^{-6} dilutions. You should see some colonies on the Amp plate but nothing on the Kan plate. Pick up several colonies from Amp plate and make plasmid preparations to check correct excision as well as plasmid integrity.

Introducing the Second loxP site

You should follow the same procedure to target the F*loxNeo* to the desired region. This time you do not have to pop-out the F*lox*Neo that will be used for G418 selection in ES cells. If you have used plasmid for DNA manipulation, you can easily clone other cassettes (such as TK) to make your final targeting vectors. If you have modified BAC to construct your targeting vector, you need to retrieve the targeting vector from BAC. Detailed retrieving protocol have been published [1,11]. One thing I noticed is that you need to have longer homology arms for efficient retrieving. 45bp homology on each side usually gives me low colony number and bizarre products. However it works for many people

Finally, you need to sequence the genomic regions that have been modified to make sure only desired mutations introduced.

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