**Gene Deletions Using the pKOV\_unstuff System**

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Overview: This protocol is modified from one received from the Kerr lab at UW. Briefly, the process follows the following steps:

1. The deletion construct is produced using a cross-over PCR technique that amplifies and joins DNA 5’ and 3’ of the deletion target, leaving a 21 bp scar where the target used to be.
2. This PCR product is then cloned into TOPO. Then, it is extracted by restriction digest and gel-purified.
3. The fragment is cloned into a complimentary site in pKOV\_unstuff, which contains a gene for chloramphenicol resistance as well as the *sacB* gene. This plasmid has a temperature-sensitive origin of replication and therefore its host must be grown at 30C.
4. Then, the plasmid is extracted from this strain and used to transform the target *E. coli* strain. Chloramphenicol resistant colonies are resuspended in salt-free LB and then diluted onto salt-free LB plates containing sucrose. Only cells that have excised the plasmid (and hence *sacB*) will grow. These mutants will then only have one or the other allele: either the deletion or the wild-type version. The sucrose-resistant colonies are purified and then screened by PCR or phenotype for the desired deletion. Roughly 50% of them should have the deletion.

**STEP 1: Cross-over PCR**

**Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: Application to open reading frame characterization. Journal of Bacteriology **179:**6228-6237.

In this procedure you will perform 2 initial PCRs to amplify DNA on either side of the region you want to delete. The products of an initial round of PCR will have “tails” extending into the targeted region that are complementary to each other. You will then do a second round of PCR using the 2 initial products together (annealing to each other by their tails) as the template, yielding a single product that is identical to the native chromosome, only replacing the targeted region with a short “scar” sequence formed by the tails.

1. Design primers to amplify 200-1000 bp immediately 5’ and 3’ of the area you want to delete. Make sure the Tm’s of both sets are similar.
2. Add the sequence gattcgaggagcgatagagct to the 5’ end of the Reverse primer for the 5’ fragment.
3. Add the complimentary sequence agctctatcgctcctcgaatc to the 5’ end of the Forward primer for the 3’ fragment.
4. In two separate 25 μL reactions, amplify the 5’ region and the 3’ region using the appropriate melting temperature and annealing time (1 min/kb). In these first reactions, use 600 μM of the outside primer (the ones without the tails) and 60 μM of the inside primer (the ones with the tails).
5. Check the products on a gel. If necessary, gel purify the appropriate band.
6. Do a single secondary 25 μL PCR using the outer primers (the ones without the tails) and 1 μL each of the products from the initial PCRs. This time use 600 μM of each primer.
7. Check on a gel. Often, the secondary reaction gives several bands. If so, gel purify.

**STEP 2: TOPO cloning**

The purpose of this step is largely convenience. It allows the no-fuss preservation and amplification of the cross-over PCR product and makes the next ligation step cookie-cutter by using compatible MCS restriction sites on the TOPO and pKOV\_unstuff vectors. In principle, you could skip it by adding appropriate restriction sites to your outside primers. However, I’ve had little (no) luck with that and prefer to shell out the $10-20 for a TOPO reaction. It’s a lot cheaper than the time you’ll spend figuring out why your ligation didn’t work.

1. In a PCR tube mix 2 μL of your freshly extracted final PCR product, 1 μL salt solution, 2.5 μL nuclease-free H2O, and 0.5 μL TOPO vector.
2. Let stand at room temp for 5 min.
3. Meanwhile thaw 1 tube of TOP10 cells (provided with the TOPO kit) on ice.
4. Add 2 μL of the TOPO reaction to the TOP10 cells and mix by swirling (don’t pipet up and down or vortex).
5. Incubate on ice for 10 min
6. Heat shock in a 42C water bath for 30 sec then return to ice
7. Add 200 μL room-temp SOC medium and incubate horizontally, shaking at 200 rpm, at 37C for 1 h.
8. Plate entire mixture on LB + kanamycin spread with IPTG and X-Gal: 25 μL, 75 μL, 200 μL.
9. Incubate at 37C overnight. The next day pick white colonies into LB+kan liquid and grow overnight. Also pick 1 blue colony.
10. Perform a confirmatory PCR on these cultures using 1 μL of the culture as the template. Use the M13 primers that come with the TOPO kit; annealing temp 55C, extension time determined by the size of your product. Use an initial 10 min step at 95C to lyse the cells.
11. Check on a gel. Run your secondary PCR product from Step 1 as a positive control. The M13 product should be about 100bp larger. The blue colony should give a ~100 bp product and is another positive control to make sure the PCR worked.
12. Pick one of your positive cultures and make a glycerol stock, then proceed with step 3.

**STEP 3: Digest and ligation**

In this step you will digest both your TOPO vector and pKOV\_unstuff with complimentary enzymes, gel purify both, and then ligate the pCR product into pKOV. Lastly, you will clone the ligate into *E. coli*. This digestion scheme assumes there are no BamHI or SmaI sites in your PCR product; if there are, you must redesign this process.

1. Grow up a pKOV-containing host overnight at 30C.
2. Perform a miniprep on 5 mL of culture of the TOPO clone containing your insert. Also do a miniprep of 5 mL of pKOV. Elute each in 50 μL volume.
3. Add 5.8 μL NEB buffer #4 to each tube.
4. Add 1 μL each NEB BamHI-HF and EcoRV-HF enzyme to the TOPO tube. Incubate at 37C overnight.
5. Add 1 μL each NEB BamHI-HF and SmaI enzyme to the pKOV tube. Incubate at room temperature overnight.
6. Run entire digests (might need to use more than one lane) on a 0.8% low-melting-point agarose gel at low voltage until the bromophenol blue band is near the gel’s edge. Extract the appropriate bands as precisely as possible.
7. Quantify DNA yield from gel extractions on the NanoDrop.
8. You want a 3:1 insert:vector molar ratio for the ligation reaction. Use the following formulas to calculate how much plasmid and insert to use in the ligation reaction:

$$y=\frac{vector size \left(bp\right) (5700 for pKOV\\_unstuff)}{insert size \left(bp\right)}$$

$$vector \left(ng\right)=\frac{250y}{y+3}$$

$$insert \left(ng\right)=250-vector (ng)$$

1. In a PCR tube mix insert, vector, and water to make 17 μL. Add 2 μL T4 DNA ligase buffer (make sure it stinks – the dithiothreitol is unstable and the reaction won’t work if you can’t smell it). Add 1 μL T4 DNA ligase.
2. Incubate in a thermocycler at 16C overnight.
3. Meanwhile grow up a 10 mL overnight culture of E. coli DH5α or other suitable *recA*-deficient cloning strain.
4. The next day, spin down the whole DH5α culture and resuspend in 100 μL ice-cold 0.1M CaCl2. Add 5 μL of the ligation mixture and incubate on ice for 5 minutes.
5. Heat shock in a 42C water bath for 1 min. Immediately put back on ice.
6. Add 400 μL room-temp SOC medium and shake horizontally at 200 rpm at 30C for 1 h.
7. Plate entire mixture on LB+chloramphenicol plates: 25 μL, 75 μL, 200 μL x 2.
8. Incubate overnight at 30C.
9. Screen colonies by PCR as described above for TOPO, but use pKOV-specific primers. Make glycerol stock of positive culture.

**STEP 4: Transformation of target E. coli strain and sucrose selection.**

See Allele transfer protocol.