PCR-product mediated Knockouts a la Datsenko and Wanner (PNAS, 2000)

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PCR

Use pKD3 (Cm) or pKD4 (Kan) if you would like to knockout a gene with minimal polarity effects on downstream genes.

Use pKD13 (Kan), pKD32 (Cm), or pKD81 (Kan from Tn903) to knockout entire operons, single genes, or any other situation in which polarity shouldn't matter.

1. Do PCR. I use 50 ul reactions
2. Check PCR product on gel
3. MinElute purify the reaction(s), eluting in 10 ul ddH_2O pH 7.0-8.5 (our water is around pH 6)

   Alternatively, you can do 1-3 PCR reactions and combine them in a single QIAQuick PCR cleanup. Speedvac to concentrate if necessary/desired. Lately, I've just been doing just 1 PCR reaction and doing a standard Qiaquick. I electroporate 2-5 ul of this, and it works fine.

Electroporation

Because this technique demands electroporation and linear DNA recombination in a single step, the yield of colonies is generally low. I often see 1-2 colonies from my transformations, but I've also recovered as many as 150. My belief is that one of the most important things is to get the competent cells as dense as you can without causing the electroporation to arc.

1. Grow cells 5-50 ml 2XYT + 100 ug/ml Amp LT2/pKD46 (I've also successfully made knockouts in TR7065/pKD46 (aka LB5010/pKD46) and 14028S/pKD46, but these strains are not as good for P22 production) – 4-6 hours 30 - 32 degrees C. 10 ml is generally sufficient for doing one knockout.
2. Induce the Red recombinase with 10 mM L-arabinose for 1 hour (inducing for the entire time works fine, too).
3. Harvest in small rotor floor centrifuge, 10 min 8k rpm
4. Resuspend pellet in 1 ml cold ddH_2O (I find that my transformations work fine without any glycerol), spin 30 sec in high-speed Eppendorf microfuge (14k rpm in Eppendorf 5417C)
5. Wash 4 more times as in step 4. (Note: I do these quick washes for speed and convenience; you can certainly use a standard washing protocol in a big centrifuge, but this way, I can get all of my washes done in 5 minutes).
6. Resuspend in 40-250 ul cold ddH_2O. Generally, I find that about 1.5X – 2X the pellet volume results in good density (high, but not enough to cause arcing with cells alone).
7. Electroporate (I use 2 mm gap cuvettes, 2.5 kV), recover at 37 degrees C in 1 ml 2XYT. I recover in the cuvette without agitation for convenience. Recovery up to 3 hours may be worthwhile, but after that I've never seen any increase in yield of clones.
8. Plate on LB with antibiotic, incubate at 37 degrees C

Additional Notes

1) While 1 mM L-arabinose seems to work fine for *E. coli*, 10 mM seems to work better for *Salmonella* in my hands.
2) It is important to titrate your drug concentrations. I had low efficiencies making Cm(R) strains, but I now know that it was because I was simply using too high a concentration. I went from 30 ug/ml down to 8 ug/ml and the efficiency is similar to making Kan(R) knockouts.
3) I don't think that the length of arabinose induction is critical, because I've obtained mutants inducing for the entire growth of the culture or for just 1 h before harvesting. I tend to do it for just 1 h before harvesting because I have the vague notion that I get more recombinants this way, but I've never tested this rigorously.