Procedure for knocking out genes using the lambda red system

Below we describe our method of knocking out genes in Salmonella with the "lambda red" system. We currently use two different systems depending on our needs:

1. The "**Datsenko and Wanner**" system – the lambda red proteins encoded on a temperature sensitive, ampicillin resistant plasmid pKD46. The lambda red proteins are under the control of an arabinose inducible promoter. I have had good luck with this system but the need to grow cells at 30°C may make the Poteete system a better choice for some labs who do not have access to 30°C incubators.
2. The "**Tony Poteete**" system – the lambda red proteins are encoded on an unstable tetracycline-resistant plasmid pTP233. The plasmid is maintained only in the presence of high concentrations of tetracycline (>25 ug/ml). In this system the lambda red proteins are under the control of a lac promoter and can be induced by IPTG. This system does not require that the cells are grown at a special temperature but for some reason I find it is less efficient and yields fewer knockouts after electroporation.

Based on multiple anecdotes we believe the success of a lambda red transformation seems to depend on many factors including:

1. The removal of as much primer as possible from the PCR reaction. Low molecular weight DNA electroporates much more efficiently than higher MW DNA and primers and primer-dimers can compete against the cassette with the lambda red proteins.
2. The removal of salts from the PCR reaction after precipitation. This will allow the electroporation of higher concentrations of DNA without arcing.
3. Following good technique to obtain high competency cells.
4. Remembering to induce the lambda red proteins with either IPTG (Poteete system) or with arabinose (Datsenko system).

Design of knockout oligos

For a source of our drug resistant cassettes we like to use the pKD3 (chloramphenicol cassette) and pKD4 (kanamycin cassette) plasmids. The drug casettes are flanked by FRT sites which allow the cassettes to be removed with the FLIP recombinase at a later step. These plasmids allow you to omit the typical DpnI digestion of the template since the pKD3/4 plasmids require the host to express the lambda-pir protein in order to replicate. They also allow you to amplify both the Km and Cm markers with the same set of primers. Primers should be designed with 40 bp of sequence immediately adjacent to the gene to be knocked out.

For the 5' end of the Cm/Km cassette the primer should be designed as follows:
5'- 40 bp of gene sequence + **gtgtaggctggagctgcttc –3'**
For the 3' end of the Cm/Km cassette the primer should be designed as follows:
5'- 40 bp of sequence + **catatgaatatcctccttag –3' (REMEMBER THE 40bp of 3' sequence must be on the complementary strand to the 5' sequence)**

The PCR is run as you would run any typical PCR. To make large amounts of DNA we **typically run two 100 µl reactions for each knockout** and pool them together after cleanup. The program is as follows.

1 cycle – 95°C 1 min (denaturation)
30 cycles – 95°C 1 min, 45°C 1 min, 72°C 1 min (amplification)
1 cycle – 72°C 5 min (final extention)
hold at 4°C

The chloramphenicol cassette is approximately 1 kb.
The kanamycin cassette is approximately 1.4 kb.

Clean the DNA
You may use any typical method to remove the Taq/salts/primers from the DNA including the Qiagen or Clontech PCR cleanup kits. We find that sometimes the Qiagen kits are not as effective as other kits at removing the PCR primers. For this reason you may have to clean up the DNA twice. Elute in 50 µl the kit's elution buffer.

Concetrate the DNA
We use a butanol extraction protocol to concentrate the DNA. Pool the DNA from the two PCR reactions together to get 200 µl total. Add 1 ml of butanol and vortex for ten seconds. The aqueous phase will enter the butanol phase but the DNA will become insoluble at this point. Spin full speed in a microcentrifuge for 10 minutes. A pellet should be visible at the bottom of the tube. Remove trace salts with one 70% ethanol wash. Dry the DNA and resuspend in 10-20 µl deionized water.

Make the competent cells

The "Datsenko and Wanner" system

1. Grow an overnight culture of the strain containing pKD46 (e.g. TH4702) at **30°C** in LB media containing 100 µg/ml ampicillin.
2. Subculture the overnight culture 1:100 into fresh LB-Ampicillin containing 0.2% arabinose. Grow at **30°C for 3 hours** until the cells are at an OD600 of 0.6 to 0.8.
3. Wash the cells 2 times in an equal volume of cold, deionized water. After the second wash resuspend in 1/250 of the original volume (e.g. 100µl if your starting culture was 25 ml).

4. Add 3 µl of DNA to the cells and electroporate as you normally would for *E. coli*. If you get arcing decrease the amount of DNA. Typically I try three electroporations with 3, 2 and 1 µl of DNA. In many cases there is no correlation between the amount of DNA electroporated and the number of colonies obtained.
5. Immediately add 1 ml SOC or LB media to the cells and incubate 1 hour at 37°C. Plate on kanamycin (50 µg/ml) or chloramphenicol (30 µg/ml) LB plates and incubate at 37°C.

The "Tony Poteete" system

1. Grow an overnight culture of the strain containing pTP233 (e.g. WN053) at **37°C** in LB media containing 25 µg/ml tetracycline.
2. Subculture the overnight culture 1:100 into fresh LB-tet containing 4 mg/ml IPTG (our 200 mg/ml stock is 200x). Grow at **37°C for 3-4 hours** until the cells are at an OD600 of 0.6 to 0.8.
3. Wash the cells 3 times in an equal volume of cold, 10% glycerol. After the third wash resuspend in 1/250 of the original volume (e.g. ≈100µl if your starting culture was 25 ml). *NOTE – IT IS HIGHLY LIKELY THAT THE WATER METHOD OF MAKING ELECTROCOMPETENT CELLS OUTLINED ABOVE WILL WORKJUST AS WELL FOR THE POTEETE SYSTEM BUT I HAVE NOT TESTED IT.*
4. Add 3 µl of DNA to the cells and electroporate as you normally would for *E. coli*. If you get arcing decrease the amount of DNA. Typically I try three electroporations with 3, 2 and 1 µl of DNA. In many cases there is no correlation between the amount of DNA electroporated and the number of colonies obtained.
5. Immediately add 1 ml SOC or LB media to the cells and incubate 1 hour at 37°C. Plate on kanamycin (50 µg/ml) or chloramphenicol (30 µg/ml) LB plates and incubate at 37°C. The pTP233 plasmid will segregate out in the absence of tetracycline.