	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	

Title: Gene Deletion Using Lambda Red Recombination			
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Author: Barbara Bowen		Approved by: Martin Wiedmann	

Gene Deletion Using Lambda Red Recombination

FILE NAME: Protocol for Lambda Red Recombinase.doc

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to create a protocol for Salmonella mutants using the Red Recombinase method of Datsenko-Warner, with adjustments as suggested by Chien-che Hung, a postdoc in Craig Altier's lab.

1.2 Scope

This SOP applies to the Food Safety Lab and Milk Quality Improvement Program.

1.3 Definitions

Red Recombinase plasmid: pKD46, in FSL B2-0114 (E.coli)
Template plasmid: pKD4 (also pKD3), in FSL B2-0116 (E. coli)
FLP plasmid: pCP20, in FSL B2-0358 (Salmonella)

1.4 Safety

Salmonella enterica subsp. enterica contains approximately 2600 serovars, some of which can cause serious illness. It is a BSL-2 level organism, and all necessary precautions (personal protective equipment such as lab coat, gloves, and when dealing with concentrated amounts, face shields/eye protection, should be worn; all laboratory BSL-2 regulations must be followed, etc.

SECTION 2 MATERIALS

- **LB media both agar plates and broth, with ampicillin (100 µg/ml) and kanamycin (40 – 50 µ/ml) added, as well as LB plates without antibiotics. Instructions for the preparation of LB are in the Media Room.**

- **SOC media (NEB product no. B9020S)**

- **20% Arabinose, filter sterilized.**
- **10% cold, sterile glycerol.**
- **QiaQuick Post PCR spin kit.**
- **Qiagen miniprep kit.**

SECTION 3 PROCEDURES

3.1. Plasmid DNAs.

(1) Streak out the following on LB plates with their necessary antibiotic added:
FSL B2-0358 (pCP20) on ampicillin plates, at **30°C**. Its host is *Salmonella enterica* (gift of Altier lab), rather than *E. coli*. This is your FLP plasmid. It is temperature sensitive and slow-growing.
FSL B2-0114(BW25113/pKD46) on ampicillin, at **30°C**. This is your Red Recombinase plasmid. It is temperature sensitive.
FSL B2-0116 (BW25141/pKD4) on ampicillin, OR kanamycin, at **37°C**. This is your template plasmid.
(2) Grow overnights from single colonies, at the indicated temperatures, and do plasmid preps following the Qiagen miniprep kit protocol. None of these will give you good yield, so 10 mls rather than 5 is a good starting culture volume.

3.2 Primers

(1) Design primers for the gene of choice. Homology to your gene must be 36 to 50 nucleotides long, flanking the 5' and 3' end of the gene. To the 3' ends of these primer sequences, add the 20 nucleotide sequences specific to pKD4 (see Datsenko and Wanner Figure 3 for a schematic), priming site 1 and priming site 2. The sequences are:

P1: 5' GTGTAGGCTGGAGCTGCTTC 3'
P2: 5' CATATGAATATCCTCCTTAG 3'

These primers flank the FRT and kanamycin gene within the pKD4 plasmid, giving you both the kanamycin resistance gene which will be substituted for the gene you are deleting within your chosen *Salmonella* strain, but also the sequences necessary for the FLP recombinase in pCP20 to eliminate the kanamycin gene afterward.

Your primers will therefore be:

5' Sequence homologous to gene / sequence homologous to pKD4 **3'** →

(2) **Also design primers down/upstream of your gene, to be used for screening PCR.**
(3) Order primers as detailed in SOP 8.1.1.17.

3.3 Make competent Salmonella cells for transforming with pKD46.

(1) Streak out your strain of interest and grow overnight.
(2) Pick a large colony the next morning and use it to inoculate 25 mls LB. Shake at 37°C until OD_{600} = 0.5 – 0.8.
(3) Have on ice: sterile diH2O, sterile 10% glycerol, and have at R.T. SOC Media
Keep everything except the LB broth on ice from here on.

(4) Pellet your culture in the (prechilled) Sorvall floor centrifuge, by centrifuging at 6,500 rpm for 9 minutes at 4°C.
(5) decant supernatant.
(6) resuspend pellet with 20 mls of ice cold H2O.
(7) repeat steps 4 and 5.
(8) resuspend pellet in 10 mls ice cold H2O.
(9) repeat steps 4 and 5
(10) resuspend pellet in 2 mls ice cold H2) and repeat steps 4 and 5.
(11) resuspend pellet in 150 µl of ice cold **10% glycerol**

3.4 Electroporate plasmid into competent cells
(1) Have on ice: cuvettes, plasmid DNA and competent cells.
(2) To cold cuvette add 1 µg plasmid (not to exceed 10 µl or 10% of total volume) and ~ 90µl of competent cells. Negative control: use diH2O instead of plasmid.
(3) Let sit on ice for 15 minutes.
(4) Program electroporator: Preset *E. coli* menu: 1800 volts, cap: 25, res: 200, 1mm cuvette.
(5) Electroporate cells.
(6) Immediately add the SOC media (1 ml).
(7) record results (milliseconds should be ~5)
(8) pipet cells into Eppendorf tube, parafilm the tube closed, tape it horizontally to the rack, and shake it at 70 rpm at **30°C** for 2 hours.

(OPTIONAL: ↓ 11,000 rpm 2 min. in microfuge, decant super. Resuspend pellet in 100 µl LB.)

(9) Spread all the cells on LB/amp plates.
(10) Incubate plates at given temperature for plasmid. **For red recombinase plasmid, pKD46, this is 30°C.**

3.5 PCR for template (note on polymerases: I have had good luck with Go Taq, but not Taq Gold. It is possible that Q5 will do the best job, but I have not tried it.) For Go Taq, you will have to do a Hot Start, Touch Down PCR of 20 + 20 cycles. In order to get enough amplicon, I recommend doing 6 tubes of 50 µl each. I found using 50 picograms of plasmid to be sufficient for the template. Below is an example of a hotstart, touchdown Go Taq PCR.

Rxn	**Sample**			**Primers**
1 -2	Identical samples			GenedelF and genedelR
3	Negative control			

	uL per reaction	cocktail 7 x rxns	µl per rx Taq mix	Cocktail 7x Taq mix
diH2O	21	147	7.75	54.25
5 X Buffer	10	70	2	14
25 mM MgCl2	3	21	-	-

dNTP (10 mM) Primer F: 10 µM gendelF Primer R: 10 µM gedelR **Polymerase**: Go Taq	1 2 2 See Taq Mix →	7 14 14 See Taq mix →	- - - 0.25	- - - 1.75

Place 39 µl of reaction mix in each tube.

Template: add 1 µl to each tube (plasmid dilution for # 1 – 2, H2O for #3 negative control)

Total volume per reaction thus far: 40 µl; add 10µl each of Taq Mix following procedure below to achieve final volume of 50 µl.

Hot Start Touchdown PCR in Name of Machine	
Initial hold	94°C for 2 min. allow machine to ramp down to 80°C, Press pause. Let temp. stabilize. Aliquot in the 10µl of Taq Mix. Let machine stand 15 seconds. Press "resume" cycling.
20 cycles	94°C 30 sec., 60°C↓50°C 30 sec., 72°C 1 min. 30 sec.
20 cycles	94°C 30 sec., 50°C 30 sec., 72°C 1 min. 30 sec
Final extension	72°C 7 min.
Final hold	4°C

Electrophorese 5 – 7 µl PCR product on an agarose gel to determine results. If good, proceed to clean up.

3.6 Qiagen Post PCR clean up column & Nanodrop reading

Combine all your tubes together over a Qiagen PCR clean up column, following the kit's directions, except at the end, elute with 30 to 35 µl of diH2O after letting it sit on the column 2 to 3 minutes before eluting.

Using an aliquot of the same water as your blank, read your sample in the Nanodrop. This will determine the yield and purity of your PCR amplicon. Remember, it does not matter if you saw primer dimers in the gel, as long as you also had robust bands of the correct size. The Altier lab recommends using 4 to 5 µg of this PCR product (compare to Datsenko and Wanner's 10 to 100 ng!) in your next electroporation, and in my experience this amount of amplicon is necessary (and also allows you to disregard those primer dimers that carry over).

You do not have to gel extract. Do not DpnI digest.

If the yield is good, use this PCR product directly in the next electroporation.

3.7 Make competent cells with Arabinose. This will induce the red recombinase**.**
(1) From your fresh LB/amp plate (or onto a fresh LB/amp plate if you have frozen down at -80°C) your pKD46 transformed *Salmonella*. Incubate overnight at 30°C.
(2) Inoculate 5 mls of LB/amp with a colony and grow with shaking at 30°C overnight.
(3) To 15 mls LB + ampicillin, add 150 µl 20% Arabinose.
(4) Inoculate with 300 µl of the overnight culture.
(5) Shake at 170 rpm at 30°C and take OD600 at regular intervals. It may take as little as 1.5 hours to get to 0.5. Your goal is OD600 of 0.6, but anywhere between 0.5 and 0.80 is acceptable.
(6) Repeat the competent cell protocol above steps 3 through 11.

3.8 Electroporate PCR cassette into competent cells
(1) Have on ice: cuvettes, PCR DNA and competent cells.
(2) To cold cuvette add 1 - 5 µg PCR DNA (not to exceed 15 µl) and ~ 70 µl of competent cells. Negative control: use diH2O instead of plasmid.
(3) Let sit on ice for 15 minutes.
(4) Program electroporator: Preset E. coli menu: 1800 volts, cap: 25, res: 200, 1mm cuvette.
(5) Electroporate cells.
(6) Immediately add the SOC Media, 1 ml.
(7) record results (milliseconds should be ~5)
(8) pipet cells into Eppendorf tube, parafilm the tube closed, tape it horizontally to the rack, and shake it at 70 rpm at 37°C for 2 hours.
(9*)* Centrifuge at 11,000 rpm 2 min. in microfuge, decant super. Resuspend pellet in 100 µl LB.
(10) Spread all the cells on LB/kanamycin plates.
(11) Incubate at 37°C overnight.
(12) If you have more colonies on your sample plates than negative controls, proceed to the following step.

3.9 Screen colonies by PCR for correct insert
(1) Pick colonies with sterile toothpicks and streak on LB/kan plates; at the same time use them to make dirty lysates in 100 µl of diH2O.
(2) Using any polymerase you please, and the temperatures required by your primers, do PCR using lysates as template. You will use one vector specific primer (P1 or P2) and one external genome primer (which you designed up or downstream of your gene in section 3.2).
(3) Run a gel on the PCR amplicons to visualize your results.
(4) If they are the correct size to be the kanamycinR gene substitution for your gene, proceed to the next step.

3.10 Grow the substreaks and make competent cells and transform with the FLP recombinase plasmid pCP20
(1) Follow the competent cell procedure from **3.3**

(2) Follow the Transformation procedure **3.4** except for step **(2),** add 20 to 30 ng of pCP20 plasmid, including growing at 30°C on LB/amp plates.
(3) If you get colonies, test them by substreaking them simultaneously, on LB/kanamycin plates, then on LB/amp plates, i.e., take a sterile toothpick to scoop part of a colony, touch it on the amp plate, then on the kanamycin plate. Go back with another sterile stick or loop and finish the streak. Incubate at 30°C.
(4) If there is growth on the amp plates, and no growth on the kanamycin plates, you have successfully eliminated the kanamycin resistance gene.

3.11 Cure the ts pcp20 plasmid from your mutant
(1)Streak colonies from the LB/amp sub plates onto LB/plain plates.
(2) Incubate at 42°C overnight.
(3) substreak from these plates onto LB/amp plates to test for loss of pCP20 simultaneously with testing by PCR with external gene primers to prove the loss of both your knockout gene and the kanamycinR gene. To do this, from the LB plain plate incubated at 42°C, pick a colony with a sterile toothpick. Touch the toothpick to LB/amp plate, plain plate, and then swoosh toothpick in 100µl of diH2O for lysate. Continue plate streaks with fresh sterile stick or loop and incubate plates at 30°C for LB/amp and 37°C for plain.
(4) Do PCR with your two external primers (up and downstream) using whatever polymerase you like and the recommended temperature for your primers. Use the dirty lysate made in (3). Run the amplicons on an agarose gel.
(5) If the LB/amp plates show no growth, and the PCR amplicons are the correct size, proceed to the next step.

3.12 Send the PCR product for sequencing
(1) Use ExoSAP treatment to clean up your PCR product before submitting for sequencing. Refer to SOP 8.1.1.19.
(2) Submit ExoSAP-treated PCR product to Biotech using the instructions outlined in SOP 8.1.1.19.
(3) When they come back correct, celebrate, set up overnights, and name, freeze down, and put in Pathogen tracker your mutant(s).

SECTION 4 TROUBLESHOOTING

Poor yield with Plasmid preps. This is more or less to be expected. These plasmids are low copy number. Use more broth and grow a larger culture. Do a midiprep if you have to.

Difficulty transforming Salmonella with pKD46. Some *Salmonella* serotypes require methylated DNA. Try transforming the *Salmonella* strain with pKD46 that has been passed through a *Salmonella* serotype (*S.* Javiana works well).

Poor recovery of Salmonella with kanamycin resistance gene: Recover cells with SOC (purchased from NEB) after electroporation. Warm media to room temperature (NOT 37°C).

Difficulty getting the PCR cassette to transform your isolate:
Make sure you're using fresh culture for the competent cells, from a freshly streaked plate. Use PCR product that has just been amplified and purified; do not let it sit around before use. Use more! Up to 5 µg is good. As always, check that your reagents are not old.

The Altier Lab makes *Salmonella* mutants using the Datsenko-Wanner procedure routinely. They're a good resource.

SECTION 5 REFERENCES

One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products by Datsenko, KA and Wanner, BL. p.6640-6645 PNAS June 6, 2000 vol. 97 no. 12

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	Unknown	Barbara Bowen	Original SOP
Version 2	03/29/2017	Rachel Cheng	Updated the rotor and centrifuge information in section 3.3 on pg. 6. Changed the media used to recover the cells after electroporation to SOC media (section 3.4 pg. 7). Updated the number of PCR reactions for section 3.5 pg. 7. Added two sections to the troubleshooting (section 4, pg. 10)
Version 3	04/24/2020	Maureen Gunderson and Rachel Cheng	- Changed media from LB (-NaCl + 0.2% glucose) to SOC media in section 2, 3.3 step 3, and section 3.8 step 6. - Updated SOP to new standard lab template. - Updated the SOP information for ExoSAP and submission of PCR products for sequencing to Cornell BRC.