Transposon-mediated Mutagenesis

Step 1 - Amplify ORF from MG1655

1.0 ul	genomic DNA (30ng/ul)
5.0 ul	gene specific primer mix
4.0 ul	2.5 mM dNTP
5.0 ul	10x Turbo Pfu buffer
0.7 ul	Turbo Pfu
34.3 ul	sterile distilled H_2O
50 ul	

95°C	95°C	55°C	72°C	72°C	4°C
1min	15sec	15sec	4min	5min	forever
		25 cycles			

Step 2 - Transposition reaction

6 ul	ORF PCR product (~100-200ng)
2 ul	Tn (Transposon, 200ng = 0.2pM)
1 ul	10x Tn reaction buffer (Epicentre)
1 ul	Tnase (Transposase; Epicentre)
10 ul	

Vortex, spin down
37°C for 2hr
Add 1 ul stop solution, 10min 70°C
Purify by passing through G50 column

Step 3 – Transformation

MG1655 cells harboring the pKD46 plasmid (Datsenko and Wanner, 2000, PNAS 97(12):6640-5) are induced with arabinose to activate expression of the lRed genes and prepared for electroporation.

1. Mix following on ice:

    4.4 ul transposition reaction
    40 ul MG1655 w/pKD46 electrocompetent cells

2. Transfer entire volume (~45ul) to chilled cubette (0.1cm gap), kept on ice

3. Electroporate with the following settings (for BioRad Pulse Controller)

    Low Range 200
    High Range 500
    Capacitance (uF): -25
    Total Volts: 1.8kV

4. Resuspend cells with 1ml LB and transfer contents to a 48-well growth block

5. Outgrow at 37°C for 1hr

6. Spread entire outgrowth on a corresponding labeled LB + Amp(100ug/ml)/Kan (50ug/ml) plate in a hood and air dry.

7. Grow at 300 (to maintain pKD46). **(1- 2 days)**

Step 4- Picking

Day 1

1. Streak 2 colonies to single colonies on LB + Amp(100ug/ml)/Kan (50ug/ml) plates. These will be the first colonies to verify.

2. Pick 3 additional colonies into separate 96-well flat bottom plates containing 200ul Freezing media + Amp(100ug/ml)/Kan(50ug/ml) maintaining original well location.

3. Grow 300 overnight.

Day 2:

1. Grow 1 colony from each streak plate in a 96-well block with each well containing 1ml Freezing media + Amp(100mg/ml)/Kan(50mg/ml). Grow 300 overnight.

2. Freeze overnights of the other 3 plates.

Step 5– Verify mutation by Culture PCR

1. Prepare sample

    Mix culture thoroughly.
    Transfer 20ul of culture into a 96-well plate and dilute with 80ul H2O Mix thoroughly

2. PCR Reaction.

5 ul	diluted culture
4 ul	gene specific primer mix (same as in Step 1)
2.5 ul	ExTaq premix
16 ul	H_2O
50 ul	

95°C	94°C	55°C	72°C	72°C	4°C
5min	30sec	15 sec	4min	5min	forever
		30cycles			

3. Run 7 ul on 1 % test gel in 0.5x TAE to check.

4. Analyze gel results; mutants will be ~1.2kb larger than original gene length.

Step 6 - Confirm mutations by sequencing

1. EXOSAP clean up

    - Transfer 10ul of PCR product for all genes with mutant sized fragments into a separate PCR plate
    - Add 4ul ExoSAP-IT (USB) to each reaction
    - Spin briefly

- React @ 37°C for 30min, then 15min@ 80°C

2. Sequence

    1. Add a mix of the following to each ExoSAP reaction:

        1ul of primer (KAN-2 FP-1 @ 10uM -Epicentre)
        2ul Big Dye dilution Buffer (Promega)
        3ul Big Dye

    2. Reaction conditions: (10sec@96C; 5sec@50C; 4min@60C) for 25cycles.

    3. Purify through a G50 column

    4. Dry plate and run on sequencer

3. Make tube stocks of confirmed mutants

Step 7 - Curing the temperature sensitive pKD46 plasmid

1. Streak confirmed mutants on a LB + Kan plate (from the above stock). Grow at 43° overnight (non-permissive temperature for pKD46 replication).

2. With a single colony inoculate the following:

    1. Growth block well containing 1ml Freezing media + Kan(50ug/ml)/well .
    2. LB+Kan agar in a 96 well plate
    3. LB+Amp agar in a second 96-well plate

3. Grow overnight at 37°C. Cured cells will grow on Kan but not on Amp.

4. Confirm by PCR and sequencing as above.
