**Flow for Allele Replacement Using pKOV**

10 uL

Thawed aliquot of chemically-competent recipient cells.

Incubate at 45°C for 1 minute in a water bath

10 uL

Incubate on ice for 1 – 5 minutes

500 uL of SOC broth

Incubate at **30°C** for 1 hour on side, while shaking at 200 rpm

Transfer cells and DNA to an electroporation cuvette pre-chilled to -20 C

**Day 1: Transformation of Recipient Clone**

5 uL

Thawed aliquot of electrocompetent recipient cells.

Pulse in electroporator set to EC2. Record pulse readings. If get an arc, repeat.

500 uL of SOC broth

Transfer all to 1.5 mL Eppi tube

Incubate at **30°C** for 1 hour on side, while shaking at 200 rpm

Recombinant pKOV with insert containing the mutation of interest flanked by ~500 bp of sequence found to either side of the locus in the recipient genome. May be constructed using either Gibson or traditional restriction enzyme assembly methods.

**Heat Shock Procedure**

**Electroporation Procedure**

All of remaining

10 uL

50 uL

Incubate plates at **30°C** for ~36 – 48 hours

Streak out 4 isolated colonies of transformed recipient to LB + Cam plate and incubate at 30 C for 24 – 48 hours.

**Day 3**

Check LB + Cam plates spread two days ago. Incubate longer if necessary.

**Day 5**

Check LB + Cam plates streaked two days ago. Incubate longer if necessary.

If there are decent-sized colonies, then inoculate one isolated colony from each quadrant to 10 mL of LB + Cam and incubate at 30 C overnight. (Note: You will really only need one of these. We inoculate more than one as a safeguard against lack of growth.)

Choose one of the cultures inoculated yesterday that grew. Discard other cultures. Freeze 2x1 mL aliquots of chosen culture with glycerol in a vial labelled with relevant information and an assigned clone number for records. Dilute the same culture and plate on LB + Cam.

Incubate plates overnight at **43°C**. Seal plates in plastic bags to reduce evaporation. This step is crucial. pKOV has a temperature sensitive replication cycle. The recombinant plasmids will not replicate at this temperature. The only colonies that will grow will be those founded by cells in which the plasmids have integrated into the chromosome by homologous recombination.

**Day 6: Selection for pKOV Integrates**

100 uL

100 uL

100 uL

9.9 mL DT

9.9 mL DT

9.9 mL DT

100 uL

100 uL

100 uL

1 mL

1 mL

Streak 24 colonies total onto new LB + CAM plates. Be sure to include the widest possible variety of colony size and shape. Some integrates tend to yield many successful allele transfers, while others do not. We can’t know for certain which is which beforehand, so it is good to get a good diversity.

Incubate plates at **43°C** in plastic bags overnight.

**Day 7: Selecting and Streaking Your Integrates**

For each streaking: Suspend one colony in 500 uL LB-no-salt broth. Dilute via two DTs made with LB-no-salt (Alternately, you can do the dilution in Eppi tubes or in 96-well plates), and spread 100 uL of the final dilution on LB-no-salt+10% . Incubate plates at 43 C in plastic bags.

This step selects for clones in which pKOV has recombined out of the recipient genome. This selection works because pKOV has a *sacB* gene that converts sucrose to a poison, which prevents the growth of integrates, while the high temperature prevents the plasmid from remaining in the cell outside of the genome because it can’t replicate.

**Day 8: Selection for Loss of Integrated pKOV**

100 uL

100 uL

9.9 mL LBns

9.9 mL LBns

100 uL

From each sucrose plate, choose one colony and streak to LB + Sucrose No Salt. You can choose to streak more colonies, but in general I have found that either most all of the colonies arising from dis-integration of pKOV from one colony suspension have the mutant allele of interest, or almost none do, so looking at multiple colonies can be a waste of time and money. Incubate plates overnight at 43 C.

**Day 9: Subcloning to purify candidate isogenic constructs**



Checking for successful isogenic constructs can be done in various ways depending on the nature of the mutation of interest. If a deletion or an insertion, PCR fragment size polymorphism is fine. For SNPs you can either do Sanger sequencing, which I prefer, or else use mutation-specific PCR primers. The below gives the protocol for PCR fragment size polymorphism and Sanger sequencing approaches.

**Day 10: Checking for isogenic constructs**

Incubate at **37**°**C** while PCR going

Run 5 – 10 uL of each reaction in a 1% agarose gel. Examine gel for bands corresponding to expected insert sizes. For SNPs, purify PCR product using GFX columns and submit for Sanger sequencing to check sequence for presence of desired mutation.

Important note: When you get your sequence results back, be sure to look at the chromatogram for the mutant locus if you find candidate isogenic constructs. Sometimes there is mixed sequence indicating an effective merodiploid rather than a successful isogenic construct with the native sequence fully replaced.

**Continue this screening process until a successful construct with the desired mutation is found. Use culture from 96 well plate for successful isogenic construct to inoculate a new 10 mL LB culture from which you can freeze stock.**

Test primers will vary.

PCR Program:

1. 95°C for 5 minutes
2. 95° C for 1 minute
3. (Varies)°C for 1 minute
4. 72 °C for 30 seconds
5. Go to step 2 33 times
6. 72 °C for 5 minutes
7. 0 C forever

Transfer 1 uL of resuspended colonies from each well to the corresponding well in a 96 well PCR plate, each containing 20 uL of PCR master mix with appropriate primers for testing the presence or absence of the mutation being transfered. Conduct PCR per program below. Include as a positive control a clone known to have the mutation, as a negative control, a clone known to lack it, and a null control into which no template has been added, and a negative control.

Suspend one colony from each streak in 500 uL LB broth in each well of a 96 well plate.

Place mature culture in 4 C incubator for storage.