For introduction of point mutations, deletions, and short insertions into the *E. coli* genome. Your mutation/alternate sequence is introduced from ssDNA or dsDNA using homologous recombination facilitated by the λ-Red system. The wild-type sequence is selected against using the sgRNA/Cas9 system, resulting in enrichment of cells containing your desired mutation.

You will need:

* pKDsgRNA-XXX plasmid as template, SpecR and temperature sensitive
* primers to clone your protospacer into the pKDsgRNA plasmid using CPEC or Gibson cloning
* oligo / dsDNA with desired mutations / modifications
* pCas9CR4 plasmid, CmR

\*\* Note: can do steps 1-3 and 4 in parallel.

1. Design primers for cloning your protospacer (target) into the pKDsgRNA plasmid. The protospacer must precede a PAM site of NGG on either strand. The diagram below demonstrates the primer design for targeting a PAM on either strand.
	1. Add this sequence to the 3’ end of the forward primer - gttttagagctagaaatagcaag
	2. Add this sequence to the 3’ end of the reverse primer - gtgctcagtatctctatcactga



1. Clone your specific target into the pKD plasmid. Do this by two PCR reactions to amplify the 7 kb plasmid in two parts – 3 kb and 4 kb, then CPEC the two parts together.
	1. To amplify part 1: use your designed F primer with this primer: tttataacctccttagagctcga
	2. To amplify part 2: use your designed R primer with this primer: ccaattgtccatattgcatca
	3. DpnI digest and gel purify. Then set up the CPEC reaction. Transform into DH5alpha or other cloning strain. Plate on Spec (50 ug L-1) and grow at 30C. Should see colonies after ~ 20 hours.
2. Can verify correct cloning by sequencing. See end of protocol.
3. Transform desired host strain with the Cas9 plasmid, grow on Cm at 37C.
4. Grow the host strain with the Cas9 plasmid at 37C + Cm, and transform with the new pKD plasmid which contains your protospacer. (Do not attempt to do a double transformation with the pKD plasmid and Cas9).
5. After the recovery step at 30C, plate on Spec/Cm/+aTC and on Spec/Cm – no aTC. aTC induces Cas9 and so should see a lot fewer colonies on that plate if everything worked properly.
6. Take 1-3 big colonies from the no aTc plate and grow up in Spec/Cm at 30C. At OD 0.4 – 0.5, add arabinose (1.2% final concentration), to induce λ-Red recombinase. Incubate for an additional 15 minutes.
7. Then, make the cells electrocompetent. Transform with 200-1000 ng of dsDNA or 1-10 µM ssDNA. If using a gBlock we typically PCR amplify in 50 µl, purify, and use half the amount for the transformation.
8. At this point, it would be useful to also include a control DNA transformation. This can be any sequence of similar length and serves to account for cell death during electroporation.
9. Recover at 30C, then plate on Spec/Cm/+aTC. Plate only ~ 1ul or do serial dilutions. Expect to see a lot more 2-500-fold more colonies on the experimental plate.
10. **Verify correct mutation by PCR / sequencing / restriction digest, etc.**
11. To cure plasmids: first cure the pKDsgRNA plasmid. Grow up in liquid culture at 37C for 4-24 hours, then streak out, and incubate at 37C. Check for Spec sensitivity.
12. Then, transform with pKD-kill plasmid, which is SpecR and temperature sensitive.
13. Grow up, induce with aTC in liquid culture. Plate on Spec + aTC at 30C.
14. Patch individual colonies on Cm + / Cm- at 37C. Passage one more time to make sure that the pKD plasmid has been cured.

Primers to verify protospacer in pKD plasmid: ~ 1kb product; Ta= 50C with OneTaq

pKD\_Seq5 = CAGTGAATGGGGGTAAATGG

sgRNA\_R = GCCTGCAGTCTAGACTCGAG

Submit for sequencing with sgRNA A primer = AGCTTTCGCTAAGGATGATTT