

The following is what we found works best for making markerless mutations.

1. Amplify the gene of interest along with sufficient flanking DNA (at least 500bp) on each side to mediate homologous recombination. (I found that using Easy A polymerase from Stratagene gives high fidelity and adds 3' A's for easy TOPO TA cloning)
2. Clone the gene into any convenient vector
3. Make your in-frame deletion. We do this by PCRing out from the edges of the gene, in effect PCRing the edges of the gene, the flanking DNA, and the vector without the internal part of the gene. We make the primers to contain a convenient restriction site such that cutting and ligating the ends will result in an in-frame deletion (We found that Herculase from Stratagene works great for PCR of large fragments that require high fidelity)
4. Reclone the gene, now with the in-frame deletion plus flanking DNA, into pCRPrTNeo
5. Transform and select with Neomycin. (For plates we use 500ug/ml neo). This results in a single recombination event and a merodiploid that contains both the wild type and mutant copies of the gene with the vector in between.
6. Streak purify your colony on neo (or just grow up a colony from the transformation in a Neo tube (1 mg/ml neo for liquid)).
7. Pick a colony or inoculate 1 drop into McCas tubes (without neo) (This gives the greatest chance of getting your loop out).
8. Grow up o/n and plate onto McCas / analog plates (8-azahypoxanthine-250 ug/ml). This selects for strains in which a second recombination event has eliminated the vector. (We do serial dilutions of  $10^{-1}$ - $10^{-5}$  along with appropriate controls (LL, Mm900; experience shows that you should get little to no colonies with LL at  $10^{-3}$  on the analog plates, while Mm900 should be a lawn).
9. Pick several putative mutant colonies and inoculate to McCas broth and screen. From screen you then know which colony to streak purify on fresh McCas agar.
10. Perform Southern to verify no vector backbone was left behind and only the mutant copy of the gene is present. You should expect a ratio no greater than 50:50 wild type to mutant.

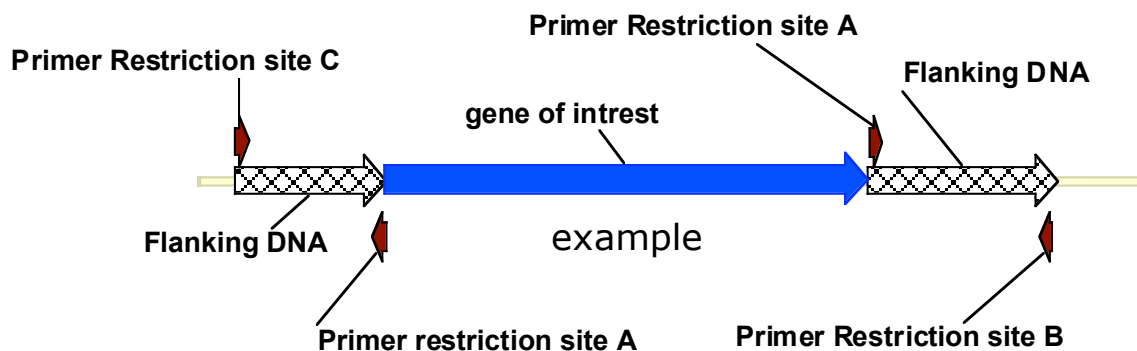
Notes:

- We have found that making in-frame deletions in a standard cloning vector first (outside of pCRPrTNeo) works much better

than trying to go through the Neo cassette with high fidelity enzymes.

- It is imperative you use Casamino acids in place of Yeast extract, otherwise the exogenous purines in yeast extract will preferentially be taken up in place of the analog.
- It is important that you streak purify the final colonies to prevent carry over of the background strain.
- If you have a slow grower (as a result of the knockout) you should expect to have less than a 50:50 ratio of mutants to wild type.
- See addendum for alternative procedure for constructing in-frame deletions.

## Addendum



This is an alternative way to make an inframe deletion (which seems to cut down on steps). Amplify from genomic DNA with appropriate primers (as diagramed above). Digest PCR product with enzyme A. We recommend using *Asc1* for site A (there are no *Asc1* sites in *Methanococcus maripaludis*). PCR using ligation product as a template with primers B and C. Proceed to digesting PCR deletion and pCRPrNeo (with appropriate enzymes B / C) then clone.