

## Designing and creating your gene knockout

### Background

The *radA* gene was identified as a gene, that when mutated, caused cells to become hypersensitive to ionizing radiation. However, why these mutants are hypersensitive or what function the RadA protein has in the cell is not known.

You may wish to look into what is known about this gene function by researching what has been published in the literature. A good place to start your research is by using the PubMed website.

<http://www.ncbi.nlm.nih.gov/pubmed>

Your goal is to construct a deletion of the *radA* gene on the *E. coli* chromosome so that the phenotypes of the mutant can be characterized and compared to wild type cells. Essentially, we will replace the gene on the chromosome with a gene that confers antibiotic resistance. We use an antibiotic resistance gene because it allows us to easily identify cells that are likely to have deleted our gene of interest (they are now resistant to the antibiotic. we can also easily kill off all the cells that did not incorporate the resistance gene).

To begin your construction, you may wish to look at the recombineering method paper, Yu et al. PNAS (2000) 97:5978. This paper describes the rationale of how the recombineering strategy in *E. coli* was devised, how to design primers for deleting genes on the chromosome, and also contains the primer sequences for several antibiotic resistance cassettes that can be used to target and replace your gene of interest (I think it is a good paper, but it is not an easy paper to read).

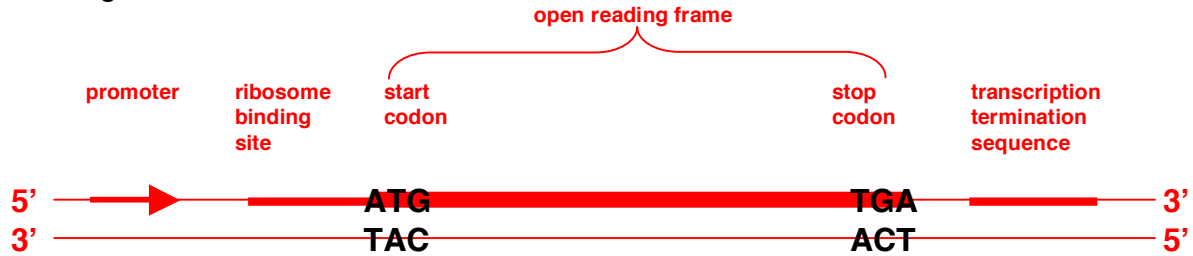
## The recombineering strategy

1) Find the sequence of the gene you wish to delete. At one of the two websites below, enter your gene name in the search box.

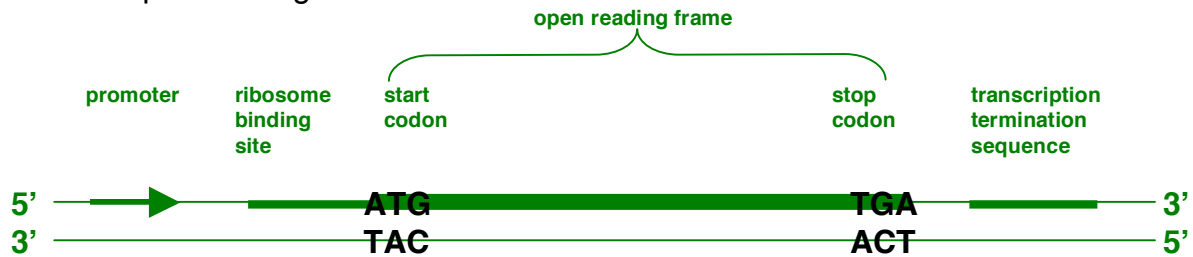
<http://genolist.pasteur.fr/Colibri/>

<http://ecogene.org/>

2) Download or view the sequence of the **gene** you wish to delete on the chromosome, along with the sequences upstream and downstream from the open reading frame.



3) View the sequence of the **antibiotic resistance cassette** you wish to replace the deleted open reading frame with



4) Using these sequences, design PCR primers that will amplify a DNA sequence that is capable of replacing your **gene** on the chromosome with a sequence that expresses the **antibiotic resistance** (if homologous recombination occurs in the right places).

To get homologous recombination to occur in your recombineering strain of bacteria, you need ~40 bp of homology between the chromosome and your PCR. To replace the open reading frame of a gene on the chromosome, this means that you want to use the 40bp upstream and downstream of the open reading frame in the 5' end of your primers (be careful to use the proper polarity on each strand!).

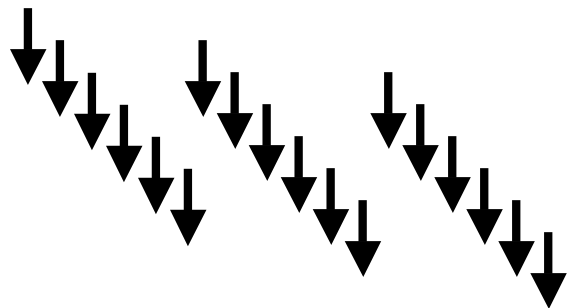
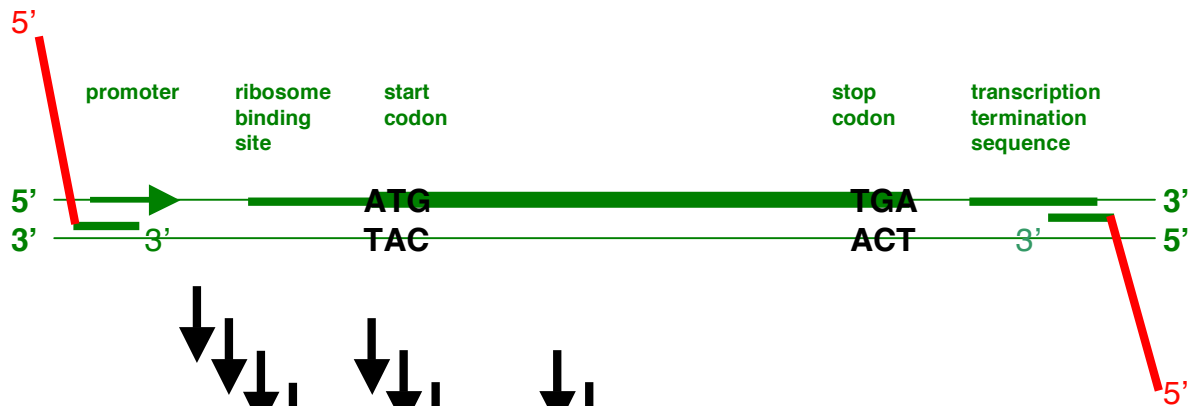
The 3' ends of the primers are homologous to the upstream region and downstream region of the antibiotic resistance cassette and should include the promoter region, ribosome binding site, and often the transcriptional terminator. These regions are often not directly identified in the databases, and candidate promoters and terminators are usually confirmed experimentally. In this case, we have provided the primer sequences upstream and downstream for the chloramphenicol resistance cassette from the plasmid pKD3.

**Upstream primer**

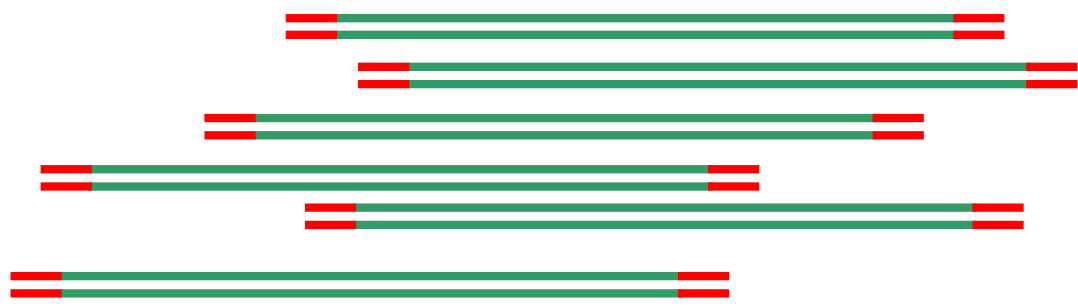
5' -USE THE DIRECT CHROMOSOME SEQUENCE AS DOWNLOADED GTGTAGGCTGGAGCTGCTTC-3'

**Downstream primer**

5' -USE THE INVERSE COMPLEMENT CHROMOSOMAL SEQUENCE CATATGAATATCCTCCTTA-3'



So after 40 cycles of PCR, you hopefully have a tube full of DNA fragments that look like this



5. Once you have your primers and plasmid template, you need to Assemble the PCR reactions, which can be done as follows using a 2X ExTaq premix from TaKaRa. The premix contains buffer at a 2fold concentration, Taq polymerase, and dNTPs: Mix these ingredients together in a microtube on ICE.

10.5 $\mu$ l nuclease-free H<sub>2</sub>O  
12.5 $\mu$ l 2x ExTaq mix  
1 $\mu$ l forward primer  
1 $\mu$ l reverse primer

Now touch the tip of your pipette to a bacterial colony that contains the plasmid pKD3 and use it to gently mix your reagents together (a few cells containing the plasmid will mix with your solution).

6. Set up PCR as follows:

Denaturation	94C	3 min
Cycle (35 total)	94C	30 sec
	50C	30 sec
	72C	2:30 min
Final extension	72C	3 min

7. Electrophorese your PCR reaction in a 0.5% TAE agarose gel to determine if a product of the correct size was amplified. You are looking for an ~ 1kb fragment of DNA.

To purify the fragment, use a straw to punch it out of the gel, place it on a bed of polyester pillow stuffing, and spin the tube(s) at 9,000 rpm for 6 min. A good portion of the DNA will spin out of the gel with the liquid.

8. Preparation of electrocompetent cells (This step has been done for you)

a) Use 100 $\mu$ l of a freshly grown overnight culture of strain DY329 (W3110 *Dlac169 nadA::Tn10 gal490 lcl857 D(cro-bioA)*) to inoculate a 100 ml culture in LBthy media. Grow at 30C with aeration to an OD<sub>600</sub>=0.6.

b) Thermoinduce the *gam bet* and *exo* genes in these cells by incubating the culture in a 42C shaking water bath for exactly 15 min. Then place cells on slushy ice for 10 min.

c) Pellet the cells by centrifuging at 2,800 rpm for 10 min, 4C. Resuspend the cell pellet in 2 ml ice cold 10% glycerol. Divide cells into 10-Eppendorf tubes and bring each tube's volume up to 1 ml with ice cold 10% glycerol. Pellet cells by centrifuging at 14,000 rpm for 25 sec, 4C.

d) Wash cell pellets in each tube three times in 1 ml 10% glycerol.

e) Resuspend each cell pellet in 100 $\mu$ l 10% glycerol. Store competent cells at -80C.

9. Transform your recombineering strain with your PCR fragment

a) Allow the frozen competent cells to thaw on ice. Mix 40l of DY329 electrocompetent cells with 3l of purified PCR product.

b) Transferred the mixture to a prechilled cuvette and electroporate at 1.8 kV, 25 mF, 200 ohms. A reasonable time constant for these conditions is 4-5 ms.

c) Within 10 seconds of electroporation, add 1 ml of SOC (at room temperature) to the transformed culture. Pipet the contents of cuvette 2-3 times and transfer to a culture tube.

d) Allow cells to recover at 30C for 3 h, before plating 2-100l aliquots onto LBthy plates containing 20µg/ml chloramphenicol. The remaining culture can also be grown overnight, and plated in the morning.

e) Colonies that grow under chloramphenicol selection are cultured in LBthy media containing 20µg/ml chloramphenicol at 30C.

At this point, if the recombination event occurred correctly, your recombineering strain should have a deletion on its chromosome for your **gene**.

If both regions of homology on the PCR fragment undergo recombination events with their homologous sequences on the chromosome, should replace the open reading frame of your **gene** of interest with the **antibiotic resistance cassette**.

