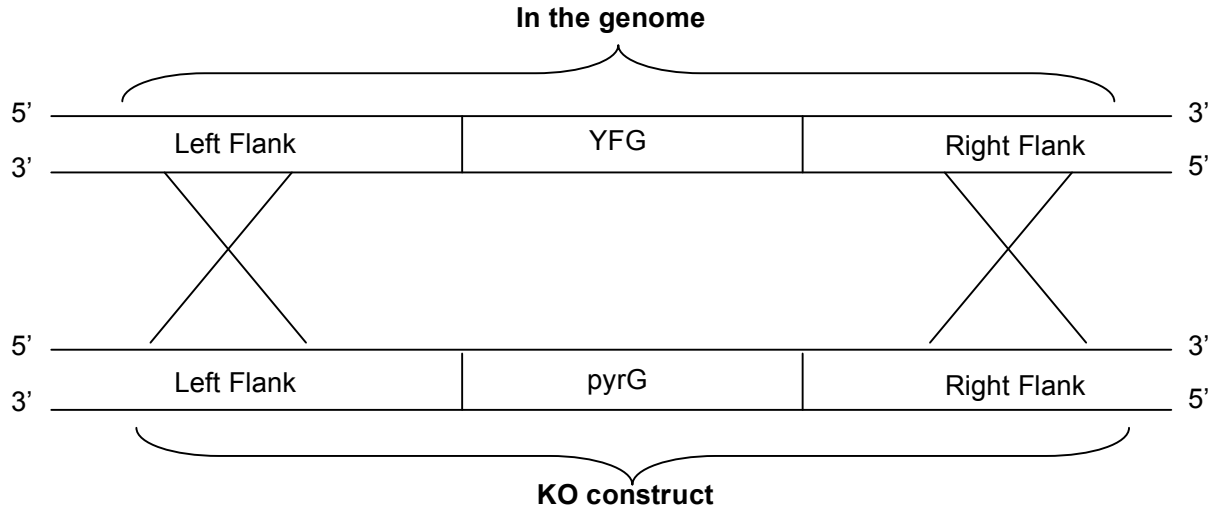
Creating A Gene Replacement Construct

***Note: There are actually several ways to create a gene replacement construct. These include standard PCR/cloning/cutting pasting technique, overlap PCR, and split-marker recombination.**

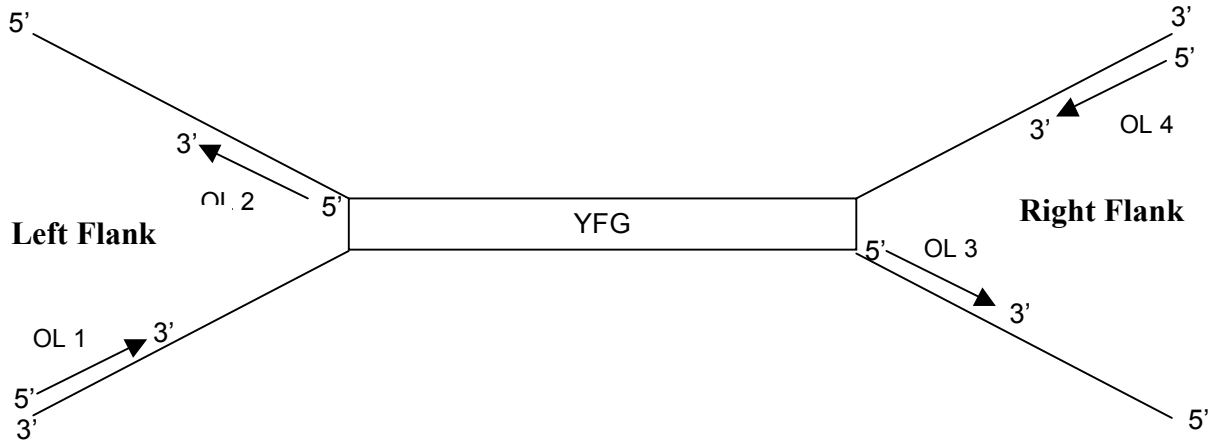
I. General Scheme—Designing KO Construct

YFG = Your Favorite Gene



- So homologous sequence for flanks in KO construct comes from fungal genomic DNA. Use PCR to get left and right flank using fungal genomic DNA as template and clone into a plasmid with pyrG. Genomic sequence comes for *A. fumigatus* can be obtained from the CADRE database http://www.cadre-genomes.org.uk/. **In *A. fumigatus*, we have to use at least 1 KB of flanking homologous sequence for each flank!**

II. Designing Primers - Schematic



OL 1 = 5' Primer Left Flank
OL 2 = 3' Primer Left Flank
OL 3 = 5' Primer Right Flank
OL 4 = 3' Primer Right Flank

- 5' Primer= Sequence comes from "Top" or 5'→3' strand (also called "Forward")
- 3' Primer = Sequence comes from "Bottom" or 3'→5' strand (also called "Reverse")
- Primers can be picked using programs such as Primer 3 or IDT Primer Quest (preferred)

III. Designing KO Construct, - The Details

1. Obtain *genomic* DNA sequence of YFG. Include 2 kb of flanking sequence on each side of the gene. This sequence can be obtained from *Aspergillus* CADRE database (http://www.cadre-genomes.org.uk/).

2. Import sequence to **DNADynamo.**

3. In **DNADynamo**, use the **Features** to define the open reading frame (ATG start codon to whatever Stop Codon) of YFG. Now, you can select 1-1.2 kb of left and right flanking sequence.

4. Check for compatible restriction enzyme sites in your flanks. Ideally, you want restriction enzyme sites to be *absent* from *both* flanks. For example, if you are using pyrG (uracil) as your marker, your selectable marker will be in plasmid pJW24. Use the map of pyrG to look at the restriction enzymes that are available for cloning. Remember you want to keep the flanks in the correct orientation! You may be able to move the flanking sequence around to make the enzymes work. Ideally, you want to include 20 or so base pairs of YFG's ORF, but this is not 100% critical.

5. Select enzymes (keep orientation) you will use in cloning your left and right flanks into pJW24 and jot down their cut sequences. Enzyme sequences can be found at www.neb.com.

6. Once you have identified which enzymes will work within a given sequence range, you can select 1-1.2 kb of left flank sequence to design primers, copy to clipboard.

7. Go to www.idtdna.com → **Sci Tools → Primer Quest**.

8. Paste sequence into box → select **Standard**
   - Default settings are fine *except* **Standard Product Design**
   - Change **Product Size Range** to be 1000-1200
   - Calculate
   - Select primer set

This will return 5 sets of primers that you can use to amplify your left flank sequence. Select a primer set that will given you at least a 1 KB product but no more then 1.2 KB if possible.

9. Now, manually add the appropriate restriction enzyme sites to 5' end of each primer, adding 2 base pairs at the very beginning before the RE site that do not bind to the 3' end of the primer → Add these primers to your order using the nomenclature: **'OL1-Enzyme-YFG,' 'OL2-Enzyme-YFG'**

10. Repeat steps 6-9 for right flank, primers **OL3 and OL4.**

11. Finally, go back to **DNADynamo** and select a 5' (Forward) primer labeled as YFG KOLF and a 3' (Reverse) primer labeled as YFG KORT. These primers occur *outside* your flanking region, typically 100-200 bp upstream of OL 1 and downstream of OL 4. They will then be used with primers PYRGKOLF and PYRGKORT that are already created to assess whether homologous recombination occurred at YFG creating a gene replacement mutant.