<u>ET BAC Recombination Protocol</u> (modified from Stewart lab)

*pBADαβγ was obtained from the Stewart lab and is Amp resistant.
** the plasmid containing the GFP-Kan fusion was obtained from the Raible lab (pCRII EGFP frt Kan frt)
*** Stewart lab ET-recombination references:

1) Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res. 1999 Mar 15;27(6):1555-7.
2) A new logic for DNA engineering using recombination in Escherichia coli. Nat Genet. 1998 Oct;20(2):123-8.

OLIGOS

Oligos were designed using a 48 bp region of homology to the gene of interest.

<u>GFP oligo:</u> The forward oligo included promoter + the first 10 codons of the gene (including the ATG). The 3' end of the oligo contained 22 bp of GFP sequence starting with ATG. (GFP sequence used: 5'-ATG GTG AGC AAG GGC GAG GAG C-3')

<u>Kan oligo:</u> The reverse oligo included a 48 bp region of homology to the gene of interest that was approximately 1000bp downstream of the ATG (when possible) + 19 bp of the Kan sequence.(Kan sequence used: 5'-CTT TGC CAC GGA ACG GTC T-3')

OLIGO Purification

1) Order oligos at the 50nmol scale from Qiagen (w/o HPLC purification) and resuspend at 80µM in sterile dH_20
2) Use 100µL of oligo solution and add 12µL 3M NaOAc (pH 7.5)
3) Add 120µL phenol::$CHCl_3$
4) Vortex for 30 seconds and spin at 14000 rpm/3 minutes
5) Transfer aqueous to new tube and add 360µL EtOH
6) Mix and place at -80ºC/10 minutes
7) Spin for 5 minutes and wash in 70% EtOH
8) Spin for 5 minutes and decant supernatant
9) Vacuum dry pellet and resuspend in 100µL dH_20

PCR of GFP-Kan fusion (best done with EcoRI gel purified GFP-Kan fragment)

10x Taq Buffer	5
50mM $MgCl_2$	3.75
10mM dNTP's	1.25
oligo 1:(80µM)	1.5
oligo 2: (80µM)	1.5
GFP-Kan gel pure (1:10)	0.5
dH_20	35.5
Taq	1
	50

30 cycles at 60°C annealing and 1.5 minute extension
Purify using Qiagen purification kit but elute with 2x 50µL

Add 12µL 3M NaOAc and 360µL EtOH and place at -80°C/10 min.
Spin at max. speed for 10 minutes at 4°C
Decant and add 600µL 70% EtOH and spin for 5 minutes
Decant and resuspend in 5µL dH_20 (~0.5 µg/µL)

Transformation of BAC line with pBADαβγ vector

1) grow 1mL culture O/N from single colony
2) dilute 1:100 (0.7mL into 70mL)
3) grow until OD_{600} = 0.5 and spin 70mL at 5K rpm / 10 min / 4°C

(do all subsequent steps in cold room and keep cells on ice at all times!)

4) decant and resuspend in 6mL ice cold 10% glycerol
5) split between 3-2mL tubes and spin in microfuge at 7K rpm / 4 min
6) repeat three times resuspending in 2mL 10% glycerol each wash
7) remove supernatant and spin one more time at 7K rpm / 4 min
8) remove supernatant and resuspend remaining volume in 40µL 10% glycerol
9) each tube should contain approximately 50µL
10) immediately use cells or flash freeze in liquid N_2 store at -80°C
11) use electroporation protocol below but with 1µL of 1:10 dilution pBADαβγ (and recover cells for only 1 hour)

Making of electrocompetent cells with BAC;pBAD lines (streamlined by Bruce Draper)

12) grow 1mL culture O/N from single colony
13) dilute 1:100 (0.7mL into 70mL) (NON-glucose LB!)
14) grow until OD_{600} = 0.15 and add 0.7mL 10% arabinose
15) incubate at 37°C for another 60 minutes and check OD_{600} (should be 0.3-0.4)
16) spin 70mL at 5K rpm / 10 min / 4°C

(do all subsequent steps in cold room and keep cells on ice at all times!)

17) decant and resuspend in 6mL ice cold 10% glycerol
18) split between 3-2mL tubes and spin in microfuge at 7K rpm / 4 min
19) repeat three times resuspending in 2mL 10% glycerol each wash
20) remove supernatant and spin one more time at 7K rpm / 4 min
21) remove supernatant and resuspend remaining volume in 40µL 10% glycerol
22) each tube should contain approximately 50µL
23) immediately use cells or flash freeze in liquid N_2 store at -80°C

Transformation of BAC;pBAD cells with GFP-Kan fragment for recombination

1) add 4-5µL of GFP-Kan PCR fragment to 50µL electrocompetent cells
2) incubate on ice for 1 minute and transfer to an ice-cold, 1mm cuvette
3) electroporate at 600Ω, 1350V, 10µF
4) immediately add 1mL LB and transfer to a 1.5mL tube
5) incubate (shaking) at 37°C for 1.5 hour
6) plate all cells (200µL per plate) on Chlor (10 µg/mL), Kan (12.5 µg/mL), Carb (50 µg/mL)
7) incubate at 37°C O/N and check for recombinants by cfu PCR and sequencing