Protocols for ET recombination (orginal protocol by F. Stewart at http://www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/stewart/ETprotocols.html).
(Modified by Alex Nechiporuk, March 2009)

Oligo design

* The 5' end (the homology arm) - choose 42 or more (I usually choose about 50) nts for the homology arms from the target DNA sequence simply according to where you want to insert the PCR fragment. I just order my primers de-salted, but if you are extra paranoid get them purified. Original protocol requires primer purification, I skip that step altogether and go to PCR.

33 ul dH2O
5 ul 10 x PCR reaction buffer
5 ul 2.5 mM dNTP (10 x)
1.5 ul upper oligo
1.5 ul lower oligo
2 ul template
0.5 ul Taq polymerase (5 U/ul)

* Annealing temperature is important, usually 60oC - 62oC is optimal. Purify the PCR products by Qiagen column and elute with 2x 45 ul dH2O.

* Add 10x Dpn 1 buffer and 2 ul Dpn 1 (NEB) and digest (to eliminate template DNA) for 1 hour.

* Extract with Phenol:CHCl3 once and once with chlorophorm; add NaAc, pH=5.2 to a final concentration 0.3M and precipitate with ethanol; redissolve in 5 ul dH2O (from 50 ul original PCR products, about 0.3 ug/ul).

Getting pBADαβγ plasmid into your BAC clone

- Transform your BAC host with the ET expression plasmid (pBADαβγ) using electroporation procedure described below.
- Plate on 12.5 ug/ml chl and 100 ug/ml amp (if using pBADαβγ that is amp resistant).
- Pick 3-6 single colonies and grow in 5 ml LB medium overnight.
- Extract using alkaline lyses and run on a gel against pBADαβγ plasmid to check for plasmid integrity.

Making electrocompetent cells from your BAC culture (with or without pBADαβγ)

* Transfer 0.7ml into 70 ml of LB medium (without glucose!) and grow them at 37oC with shaking.

* Prepare 10% glycerol with dH2O, and cool down on ice for at least 3 hours before using.

* When the cells reach OD600 = 0.1-0,15, add 0.7ml 10% L-arabinose to induce ET protein expression (need to do this step only when BAC contains pBADαβγ).

* After a further 45-60 minutes, the cells should be at OD600 of 0.3-0.4. Harvest.

* make sure the centrifuge and SA600 or SS34 (Sorvall) rotor is very cold by centrifuging for 10 min, 0°C at 4,000 rpm (I use regular 50 ml conical tubes with 600TC Sorvall rotor; Beckman has an analogous rotor for 50 ml conical tubes).

* Spin 35 mls cells for 10 min at 7,000 rpm at 0°C. Put the other 35 mls on ice.

* Pour away the supernatant, add the second 35 mls and respin.

* Pour away supernatant, put tube on ice, resuspend cells in 5 ml ice cold 10% glycerol with an ice cold 5ml pipette. Add a further 25 mls and centrigue.

* Repeat the above step twice.

* Pour away supernatant and immediately dry the tube out with Kleenex tissue takning care not to touch the pellet.

* Resuspend the cells in the remaining liquid (you should have a little more than 100ul final resuspended volume).

* Transfer 50 ul of cells into each pre-cooled eppendorf tube and freeze in liquid N2 or use immediately.

All the steps should be done as cold as possible, always on ice! The glass pipettes should be cooled once or twice by pipetting cold 10% glycerol up and down before pipetting cells (I found that glass pipettes work much better than plastic).

Transformation (same conditions for both pBADαβγ and PCR product)

* (If cuvettes were reused) Wash cuvettes at least 10 times with dH2O, precool them on ice for least 5 min.

* Thaw competent cells on ice and add 1 ul of PCR product (should be at least 0.1ug, 0.3 ug works better).

* Electroporate the cells at 2.3 kV (Bio-Rad Gene Pulser), 25 uF with Pulse controller set to 200 ohms)

* Add 1 ml of LB medium and transfer back into the eppendorf tube.

* Incubate at 37oC for 1 to 1.5 hours with shaking.

* Plate 100ul on chl/amp/kan (12.5/100/12.5 ug/ml) plates, spin the rest, resuspend in 100ul and plate that.

Genotyping

- Colonies may have an uneven appearance and that is usually a good indicator that reaction worked.
- Pick a single colony into 50 ul of LB containing chl/kan and use 2 ul of that in 20 ul PCR genotyping (no need to grow). I usually genotype between 24 and 48 colonies.
- Store the cultures at 4°C.
- Once a positive clone is identified (I've had a variable luck with >95% of all clones being positive or as little as 5%), pick into 100 ml of LB with chl/kan for midiprep. Without the amp bugs will rapidly loose pBADαβγ plasmid.

BAC midiprep

- I use Clontech AX 100 columns, they provide a protocol for extracting low-copy plasmids. The typical yield is 25-50 ug from 100 ml of culture.
- At the end, resuspend the pellet in 50 ul of TE^{-4} (Tris HCl, pH=8.0 and **<u>0.1</u>** mM EDTA).

- Desalt the prep as follows: float Millipore 0.025 um membrane (cat#VSWP04700) shiny side up in 60 mm Petri dish filled about half-way with TE^{-4}. Carefully pipet BAC prep on top of the membrane (will stay in a single drop), cover and let float for at least 2 hours. Collect with a pipet.
- Inject 20-120 ng/ul of supercoiled BAC diluted in water to check for transient expression.
- Rip the rewards…

References:

Zhang Y, Buchholz F, Muyrers JP, Stewart AF. A new logic for DNA engineering using recombination in Escherichia coli. Nat Genet. 1998 Oct;20(2):123-8.

Muyrers JP, Zhang Y, Testa G, Stewart AF. Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res. 1999 Mar 15;27(6):1555-7.