Robertson Laboratory
Modified Protocol for BAC Recombineering
(adapted from http://recombineering.ncifcrf.gov/reagent_request.asp)

I – Amplifying Selection Cassette for Electroporation

1. Basic set-up (for amplifying template for transformation)

Sterile water	:	35uL
10X PCR Buffer (Mg)	:	5uL
10X dNTPS	:	5uL
20uM Primer 1	:	1uL
20uM Primer 2	:	1uL
Taq Polymerase	:	1uL
50ng template	:	2uL
TOTAL	:	50uL

Alternatively, when using PCR Buffer without Mg^{2+}, set up the amplification as follows:

Sterile water	:	34uL
10X PCR Buffer	:	5uL
10X dNTPS	:	5uL
Mg^{2+} (final []=2mM)	:	1uL
20uM Primer 1	:	1uL
20uM Primer 2	:	1uL
Taq Polymerase	:	1uL
50ng template	:	2uL
TOTAL	:	50uL

2. PCR Cycle set-up

94^0C	:	3:00 minutes
94^0C	:	1:00 minute
56^0C	:	1:00 minute
72^0C	:	2:00 minutes
Repeat cycle 35 times		
72^0C	:	10:00 minutes
4^0C	:	infinite

3. Reactions are prepared for four amplifications to yield enough DNA for electroporation

II – Gel Purification of Amplified Insert

1. Pool individual 50uL reactions into a sterile 1.7mL Eppendorf tube
2. Digest the residual template plasmid (pL452) with DpnI enzyme at 37C for 1 hour; use 0.5uL of enzyme per 50uL of PCR product
3. Mix digested amplicon with DNA loading dye and run on a 0.6% agarose gel
4. Cut out DNA from agarose slab and transfer gel fragments to a spin-x filter
5. Flash freeze the tube containing gel fragments for <20 minutes at -80C

6. Centrifuge for 20 minutes at 13000RPM (RT)
7. Aliquot resulting DNA solution into two sterile 1.7mL Eppendorf tubes
8. Add 1/10 volume of 3M sodium acetate and 2.5X total volume of cold absolute ethanol; mix well by inverting tubes
9. Flash freeze for <20 minutes at -80C
10. Pellet precipitated DNA by centrifugation at 15000 RPM for 15 minutes (4C)
11. Aspirate supernatant, wash resulting pellet with 100uL of 70% ethanol, and recover pellet by centrifugation at 15000RPM for 3 minutes (RT)
12. Aspirate supernatant and dry pellet by inverting tube and letting stand for 5-10 minutes
13. Resuspend resulting DNA pellet in 10uL of ddH_2O
14. Completely dissolve DNA by incubating at 37C for 30 minutes

III – Preparing Electrocompetent Cells

1. Inoculate a colony from EL350-BAC36 plate in 3mL of sterile LB-chloramphenicol media (12.5ug/mL concentration of antibiotic); grow overnight by continuous shaking (250RPM) at 30-32C
2. The following day, inoculate 500uL of overnight seeding culture in a 50mL baffled flask with 25mL sterile LB-chloramphenicol media
3. Incubate with continuous shaking (250RPM) at 30-32C until OD_{600} reaches 0.5
4. When OD_{600} reaches 0.5, aliquot 10mL of the inoculated culture into a fresh 50mL baffled flask
    - At this point, there will be two 50mL flasks with approximately 10mL of culture each
5. Incubate one flask with continuous shaking at 30-32C; incubate the remaining flask with continuous shaking at 42C; label each flask accordingly
    - IMPORTANT: both cultures should be simultaneously incubated in the indicated temperatures for EXACTLY 15 MINUTES!!!
6. After the 15 minute incubation in the indicated temperatures, chill both cultures on ice for 10-15 minutes
7. Transfer each batch of cells to sterile, DNA-free 30mL Nalgene centrifuge tubes
8. Spin at 4100RPM for 5 minutes at 4C
9. Aspirate the supernatant (be careful as the resulting pellet may be loose); resuspend the cell pellet in 1mL of ice-cold sterile water
10. Completely resuspend cells in 9mL of ice cold sterile water
11. Pellet cells once again at 4100RPM for 5 minutes at 4C
12. Repeat steps 7-8 at least twice for a total of three washes
13. Aspirate the supernatant and gently resuspend the cell pellet in the residual amount of water left
14. Gently pipette out the cell suspension and transfer to a pre-cooled Eppendorf tube
15. Pellet the cells at 3000RPM for 5 minutes at 4C, aspirate the residual supernatant and completely resuspend the cells in 50uL of cold sterile water
16. Keep cells on ice (warming them up to room temperature dramatically decreases the transformation efficiency).

IV – Transformation of Competent Cells with DNA

NOTES:

- DNA used for electroporation is ideally a purified PCR-amplified product with a secondary selection cassette
- For our studies of generating KSHV deletion mutants, we amplify our secondary selection cassette that likewise contains *loxp* sites from the pL452 plasmid gifted from the NCI (http://recombineering.ncifcrf.gov/Plasmid.asp)

1. Transfer 25uL of the freshly prepared electrocompetent cells to a pre-cooled 0.1cm electroporation cuvette
2. Pipette in 1-5uL (1uL is sufficient from 200uL total PCR product) of the DNA to be transformed and mix well by tapping the bottom of the cuvette
3. Transform the DNA into the cells by electroporation at 1.75kV
4. Immediately add 1mL of LB to the cuvette
5. Completely resuspend the cells by gentle pipetting
6. Transfer newly-transformed cells to an Eppendorf tube and incubate in a shaking 32C water bath at 250RPM for an hour
7. Plate the transformed cells on the appropriate double selection plate and incubate at 32C until single colonies form
8. Inoculate single colonies in 10mL LB and perform small-scale DNA isolation for initial characterization of resulting DNA

V – Floxing the selection cassette (through the transient expression of the arabinose-inducible *Cre* gene

1. Inoculate a single colony of the transformed EL350-BAC36 (from part II of this protocol) in 3mL of LB with the appropriate antibiotics
2. Grow cells overnight (at least 12 hours) by shaking (250RPM) at 32C
3. The next day, inoculate 1mL of overnight culture into 10mL of LB and grow by shaking (250RPM) at 32C until the OD_{600} reaches 0.5
4. Add 100uL of 10% L(+) arabinose (SIGMA A-3256) to the culture and let induction go for one hour
5. Prepare ten-fold dilution samples of parent culture and grow on the appropriate selection plate overnight at 32C

   NOTES:

   - At this point, the secondary selection cassette should have been "floxed" out by the action of the *Cre* enzyme; therefore, the resulting cells should be plated on agar with the original selection antibiotic ONLY. As a negative control, plate 100uL of the parent culture in a double selection plate; there should be no growth on the negative control plate
   - I normally get single colonies that are ideal for inoculation from the 10^{-4} and 10^{-5} dilutions
6. Inoculate resulting single colonies in 10mL of LB with appropriate antibiotic, grow overnight by shaking (250RPM) at 32C
7. Perform small-scale DNA isolation to characterize DNA of deletion mutants