Protocol for Sequencing BAC subclones

1. Day 1
    a. Prepare Media and agar plates needed for the next week of steps.
        i. 1 sleeve of LB agar plates with Chloramphenicol (12.5mg/ml), X-Gal (20mg/ml) and IPTG (200mg/ml). May only need 1 plate depending on number of BACs being cloned.
        ii. 2 sleeves of LB agar plates with Kanamycin (10mg/ml), X-Gal (20mg/ml) and IPTG (200mg/ml).
        iii. 5ml LB media tubes (1 per BAC)
        iv. 200ml LB media flasks (1 per BAC)
        v. Solutions not included in Qiagen Large Construct Kit
        vi. Solutions not included in Invitrogen Topo Shotgun Subcloning Kit
        vii. Woo's Freezing Broth
        viii. Terrific Broth
        ix. Solution I, Solution II and Solution III (prepare morning of use)
2. Day 2
    a. Pull 1ul of needed BAC from library plate and put into 50-100ul ddH20.
    b. Streak 10-15ul of BAC onto LB agar plates with Chloramphenicol (12.5mg/ml), X-Gal (20mg/ml) and IPTG (200mg/ml).
    c. Let plate grow at 37°C overnight (approximately 18 hours) and then place in 4°C for a couple hours to allow for blue/white selection.
3. Day 3
    a. In the morning, start a 5ml culture using 5ml LB media with 5ul Chloramphenicol. Pick a single white colony off the plate, using a pipette tip, and eject the tip into the LB media.
    b. Allow this to grow for 6-8 hours at 37°C 280rpm.
    c. Transfer 500ul of 5ml culture into 100-500ml LB media with 100-500ul Chloramphenicol. Allow this to grow for 18 hours at 37°C 280rpm.
4. Day 4
    a. Perform DNA extraction using the Qiagen Large Construct Kit, following the kit protocol.
    b. Determine DNA concentration via the nanodrop.
    c. May also want to determine DNA concentration by digesting the BAC with NotI and running the digest out on a CHEF gel.
5. Day 5
    a. Prepare the DNA for subcloning following the Invitrogen TOPO Shotgun Subcloning Kit protocol. **
        i. Shear DNA to approximately 3kb fragments.
        ii. Purify Sheared DNA (not needed but may be helpful)
        iii. Blunt-End repair DNA
        iv. Dephosphorylate DNA
        v. TOPO cloning of DNA
        vi. Spread cloned cells onto LB agar plates with Kanamycin (10mg/ml), X-Gal (20mg/ml) and IPTG (200mg/ml). I spread 75ul of cells onto 1 plate and let grow overnight to make sure transformation worked, then the next day spread 100ul onto 4 plates.
        vii. Let plates dry in hood and then place in 37°C incubator for 18 hours.

    **These steps may take more than 1 day, I usually Shear, Purify, Blunt-End Repair and start Dephosphorylation on the first day (to step 4) then finish Dephosphorylation and clone on the second day.

6. Day 6
    a. Prepare 384-well plates for colonies by adding 70ul Woo's Freezing Broth with Kanamycin (10mg/ml) to each well. If using a lot of plates, use Q-Fill to load.
    b. Pick white colonies from the LB agar plates containing subcloned DNA into the 384-well plate, using sterile toothpicks. Put one colony per well.
    c. Allow plate to grow for 18-22 hours at 37°C.
7. Day 7
    a. Fill 4-96 deep well blocks with 300ul Terrific Broth and Kanamycin (10mg/ml) into each well.
    b. Replicate the 384-well plate into the blocks using the 96 long-pin replicator.
        i. Fill the sand with 95% Ethanol.
        ii. Push replicator into sand a couple times to help clean the pins.
        iii. Flame replicator with ethanol burner and allow to cool on bench.
        iv. Place replicator into block to further cool then transfer to 384-well plate placing the upper left pin into well A1.
        v. Swish replicator in plate a few times to collect cells and return to 96-well block and swish to release cells.
        vi. Push replicator into the sand and repeat steps ii-v for all 4 blocks replicating the 384-well plate into the 4-96 well blocks (A1, A2, B1, B2).
    c. Cover the blocks with 2 layers of AeraSeal and place in the 37°C shaking incubator for 18 hours at 280rpm.
    d. Cover the 384-well plate with Foil Seal and place into –80°C for future use.
8. Day 8
    a. Perform 96 well High Copy Plasmid Preps
        i. Pellet the cells by centrifuging in refrigerated bench-top centrifuge at 3000rpm for 15 min, 4°C.
        ii. While centrifuging, label 1 Uniplate and 1 Unifilter for each block.
        iii. Pour off supernatant and place blocks upside to dry on paper towels.
        iv. Add 150ul of cold Solution I to each well, Cover with tape and vortex to re-suspend pellets then remove tape. The culture should only be in Solution I for 10 minutes.
        v. Add 150ul of Solution II to each well. Cover with tape and mix by inverting 3-4 times then remove tape. The culture should only be in Solution II for 5 minutes.
        vi. Add 150ul of cold Solution III to each well. Cover with tape and mix by inverting 3-4 times.
        vii. Place block on ice for at least 10 minutes.
        viii. Add 270ul of isopropanol (0.6 volumes) to each well of the Uniplate.
        ix. Place Unifilter on Uniplate and add 450ul of each sample into the Unifilter. ***Make sure blocks are all in the same direction***
        x. Centrifuge at 3200 rpm for 30 minutes 4°C.
        xi. Throw out Unifilters, pour off supernatant and let drain upside down.
        xii. Add 270ul of 70% Ethanol to each well.
        xiii. Centrifuge at 3200 rpm for 10 minutes 4°C.
        xiv. Pour off supernatant and allow to drain upside down. Place Uniplates in hood to dry completely for approximately 10 minutes.
        xv. Add 100ul ddH2O to each well and shake Uniplate slightly to resuspend pellet.
        xvi. Cover with Thermoseal and place in –20C until further use.
9. Day 9
    a. Set-up cycle sequencing reactions using ABI Big Dye v3.1 protocol.
        i. Allow Subclone samples to thaw in Uniplate. Label half-skirted PCR plates with BAC name, Uniplate name and primer for sequencing.

ii. Add 1ul sample from Uniplate into half-skirted PCR plate.
iii. Mix cocktail of sequencing reagents (10ul total volume per well). Make 100 reactions per plate.

1. 3ul ddH2O	300ul in cocktail
2. 3ul 5x Sequencing Buffer	300ul
3. 2ul Forward or Reverse M13 primer (1.6pmol/ul)	200ul
4. 1ul ABI Big Dye Ready Reaction Mix	100ul

iv. Aliquot 9ul per well, tap plate to mix then quick centrifuge to make sure all contents are at the bottom of plate.
v. Place plate into Thermal Cycler and run on subclone sequence program.
    1. Denature at 96°C for 1 minute
    2. Denature at 96°C for 10 seconds
    3. Anneal at 50°C for 5 seconds
    4. Extend at 60°C for 4 minutes
    5. Repeat steps 2-4 24 times
    6. Place at 4°C forever, until plates are removed.

10. Day 10
    a. Purify cycle sequencing using a EDTA/Ethanol precipitation protocol.
        i. Add 5ul of 125mM EDTA to each well. Tap plate to mix.
        ii. Add 60ul of 100% Ethanol to each well. Cover with seal and invert plate to mix.
        iii. Let plate sit at Room Temperature for 15 minutes to precipitate DNA.
        iv. Centrifuge plate at 3200xg for 30 minutes, 4°C.
        v. Remove plates and discard supernatant.
        vi. Add 60ul of 70% Ethanol to each well.
        vii. Centrifuge at 1650xg for 15 minutes, 4°C.
        viii. Remove plates, discard supernatant and invert plates on paper towels.
        ix. Centrifuge inverted plates at 185xg for 1 min to remove ethanol.
        x. Dry samples in hood for 10 minutes then resuspend pellets with 10ul Formamide.
        xi. Cover with Septa Seal and place into Sequencer holders. If these are not available, wrap plates in Aluminum foil and place in 4°C.
    b. Enter sequence input into Excel Spreadsheet
    c. Put the Sequence input files into Greg's personal file folder→Sequencer3730→Sequencer input→Lori→create folder with date created.
    d. Give plates to Greg to sequence.