Purpose: Polish Sheared DNA, add adaptors, size select, seal nicks and Amplify (rev 2)

Introduction: We are going to take the sheared DNA we made and polish the ends, add adaptors, size select and perform pre-amplification. We are going to concentrate on the Phaseolus DNA samples – the one that has the best characteristics when we are done with all of the processing will be the one that we sequence. The DNA that we sheared for 7 15-minute cycles got vaporized, so we are going to use the 60-minute sheared material.

We will use the Ion Fragment Library Kit. The kit will end-repair the DNA, then provide adaptors and the enzymes that ligate them, we will perform size selection and then carry out nick-translation of the modified DNA prior to amplifying it.

Protocols

1. Phenotype check: observe/record whether your soy and/or phaseolus is blooming, if they have not already. Record the colors and date (there is clearly a wide range in time to blooming with these varieties).

2. Remove your ethanol-precipitated sheared DNA from the freezer. For the time being you can process both the soy and Phaseolus so you have a balance tube.
    a. Centrifuge for 30 minutes at 13,000 rpm.
    b. Remove the ethanol
    c. Allow the sample to air dry for 5 minutes
    d. Add 30ul or Low-TE ('L-TE' tube) buffer and pipette gently up and down to resuspend the sheared DNA.
3. If you cannot find the sheared DNA, we have repeated the process with your Phaseolus samples. Please collect the tube and perform a Nanodrop UV reading (in duplicate) to estimate the concentration. The goal is to have 500- 600ng of DNA to carry into step 4.
4. ER-reactions: Into a *labeled* 0.5 ml Lo-Bind Tube place the following reagents
    a. 30 ul of DNA (only the Phaseolus DNA) containing 500-600ng of DNA (you can use as little as 300ng)
    b. 49 ul of nuclease-free water ('NF-H2O' tube) – or whatever volume is left if you need additional volume for your DNA.
    c. 20 ul of 5X end-repair buffer combined with 1 ul of End-repair enzyme cocktail (tube on ice labeled 'ERB')
    d. Your total volume should be 100ul – check that this is true.
    e. Pipette gently up and down 3-4 times, then quick-spin.
    f. Incubate a room temperature for 20 minutes.
5. APB step: Purify the product using Agencourt Ampure beads (tube labeled 'APB' You will need it several times so don't lose it!). For the size range we want, we will use a 1.8-fold ratio of beads

to DNA (or 180ul of beads). You will also need 1 ml of freshly made 70% ethanol and 1ml of Low-TE buffer.

a. To your sample tube add 180 ul of beads
b. Place on the GenieVortexer that is set at 8-10rpm for 10 minutes
c. Place the tube in the magnetic rack. Once the solution clears remove the solvent and discard it (the tube stays on the rack while you are doing this).
d. Add 500ul of 70% ethanol, incubate for 30 sec, slowly rotating the tube (on the magnetic stand). Remove the ethanol and discard.
e. Repeat step 4d. Remove as much of the solvent as possible.
f. Leave the cap open and let the sample air-dry for 5 minutes.
g. Add 30 ul of low-TE buffer. Close the tube, vortex for 10 seconds, pipette up and donw several times (don't lose beads in the pipette tip, pipette very slowly and carefully!).
h. Cap the tube and Vortex a second time, then quick-spin to collect the liquid.
i. Place the tube back in the magnetic rack and wait until the solution clears.
j. KEEP THIS SOLUTION!!! Remove the solution to a fresh *labeled *0.5 ml Lo-Bind tube.
k. Note: the DNA in this solution can be stored at -20C at this point.

6. AD – Adaptor Ligation – in this step the A and P1 adaptors are added to your target DNA.
    a. Use the Nanodrop UV to determine how much DNA you have – you want 300ng in ~25ul (we can use as little as 50ng).
    b. Collect the 'AD' PCR tube – it contains 10ul of 10X Ligase buffer, 25ul of adaptors and 1.5 ul of DNA ligase, or 36.5ul in total.
    c. To the AD tube, add 50-300ng of your DNA (you can use as little as 50ng, if that is your entire yield).
    d. Add sufficient water to make the total volume 100 ul (If the DNA sample is in 25 ul and the reaction solution is 36.5 ul then you will add 38.5 ul of water). Check the final volume.
    e. Pipette gently up and down 4-5 times, quick-spin.
    f. Let incubate at room temperature (RT) for 30 minutes.
7. APB step: Purify the product using Agencourt Ampure beads (tube labeled 'APB' - you will need it several times so don't lose it!). For the size range we want, we will use a 1.8-fold ratio of beads to DNA (or 180ul of beads). You will also need 1 ml of freshly made 70% ethanol and 1ml of Low-TE buffer.
    a. Transfer you sample to a 0.5ml Lo-Bind tube.
    b. To your sample tube add 180 ul of beads
    c. Place on the GenieVortexer that is set at 8-10rpm for 10 minutes
    d. Place the tube in the magnetic rack. Once the solution clears remove the solvent and discard it (the tube stays on the rack while you are doing this).
    e. Add 500ul of 70% ethanol, incubate for 30 sec, slowly rotating the tube (on the magnetic stand). Remove the ethanol and discard.
    f. Repeat step 4d. Remove as much of the solvent as possible.
    g. Leave the cap open and let the sample air-dry for 5 minutes.

h. Add 30 ul of low-TE buffer. Close the tube, vortex for 10 seconds, pipette up and donw several times (don't lose beads in the pipette tip, pipette very slowly and carefully!).
i. Cap the tube and Vortex a second time, then quick-spin to collect the liquid.
j. Place the tube back in the magnetic rack and wait until the solution clears.
k. KEEP THIS SOLUTION!!! Remove the solution to a fresh *labeled *0.5 ml Lo-Bind tube.
l. Note: the DNA in this solution can be stored at -20C at this point.

8. Size selection is now carried out using the Pippin Prep. We will select for the 180-210 bp range using the 2% agarose gel cassette and 30ul of the ligated DNA. We can only run 4 samples at a time, so we will repeat the process with one groups sample once we see how today goes.
    a. Inspect the device to make sure you understand how the cassette is loaded and what the interface looks like.
        i. Select your protocol – Main Screen has 'Protocol' then Protocol Editor screen.
        ii. Select New, then Cassette Type, then Ref Lane to identify the marker you will use
        iii. For your minimum size range enter a value for 'BP Target', or if you are accepting a wider range you can use the 'BP Start' and 'BP End' fields.
        iv. Label the sample to go into each lane.
        v. Save as, then Return to the Run screen.
    b. Prepare the cassette
        i. Wearing gloves, remove from foil
        ii. Inspect columns and do not use if there is a clear break.
        iii. Check the bottom of the cassette in the optical region of the gel columns to make sure there are no bubbles.
        iv. With the sample well side DOWN, tilt the cassette to dislodge bubbles – tap if necessary.
        v. Place the cassette on the optical tray, holding firmly in place, peel back the adhesive seals.
        vi. Close the lid (it slides forward)
        vii. In the Run Screen, select Manual Mode (from Protocol Name) and press Test – you are checking to see if the cassette PASSes. If it does you can load your samples – if a separation lane is out of range do not use it. If an elution lane is out of range, remove the buffer and add a fresh 40ul aliquot and re-test.
        viii. Slide back the lid.
    c. Load samples
        i. Remove 40ul of buffer from each well
        ii. Load 40ul of DNA marker in the reference lane
        iii. Add 10 ul of the loading buffer ('PLB' tube) to your sample.
        iv. Load 40ul of sample in each of the other 4 designated wells
    d. Close the lid
        i. Select the Protocol, and press START.
    e. Hit RUN - Electrophoresis should take 70-80 minutes – one of you MUST return to capture the eluant.

f. When complete, remove all samples from the elution wells (they will be in Tris-TAPS buffer in ~40ul)
g. Remove the eluant to a fresh *labeled* 0.5ml nuclease-free sample tube.
h. Check the volume and adjust to 40ul with nuclease-free water.

9. NT-PCR step: next you will carry out the nick-translation step of the Ion Fragment Library kit immediately followed by a PCR amplification, which allows us to verify that the adaptors did ligate to your samples. This step will only take about 20 minutes.
    a. Collect a PCR tube containing 100ul of Platinum PCR SuperMix High Fidelity solution that also contains 5 ul of primer mix.
    b. Add 20ul of your size-selected sample.
    c. Gently pipette up and down and then quick-spin it.
    d. Place in the thermocycler. The profile is as follows:
        i. 72C 20min (nick-translation part)
        ii. 95C 5 min First melting step
        iii. 9 cycles of
            1. 95C 15 sec
            2. 58C 15 sec
            3. 72C 60 sec
        iv. 4C HOLD
10. Repeat the Agencourt Ampure Bead purification, except use 225ul of beads, since you have 125ul of sample. When it is done spinning, remove the solution, avoiding the pellet, which should be slightly up on the side where the hinge is.
    a. To your sample tube add 225 ul of beads
    b. Place on the GenieVortexer that is set at 8-10rpm for 10 minutes
    c. Place the tube in the magnetic rack. Once the solution clears remove the solvent and discard it (the tube stays on the rack while you are doing this).
    d. Add 500ul of 70% ethanol, incubate for 30 sec, slowly rotating the tube (on the magnetic stand). Remove the ethanol and discard.
    e. Repeat step 4d. Remove as much of the solvent as possible.
    f. Leave the cap open and let the sample air-dry for 5 minutes.
    g. Add 20 ul of low-TE buffer. Close the tube, vortex for 10 seconds, pipette up and donw several times (don't lose beads in the pipette tip, pipette very slowly and carefully!).
    h. Cap the tube and Vortex a second time, then quick-spin to collect the liquid.
    i. Place the tube back in the magnetic rack and wait until the solution clears.
    j. KEEP THIS SOLUTION!!! Remove the solution to a fresh *labeled * 0.5 ml Lo-Bind tube.
    k. Repeat steps g-j with a fresh 20ul aliquot of TE buffer – combine it with the first batch.
    l. Note: the DNA in this solution can be stored at -20C at this point.
11. Next time we will quantify the libraries, before we proceed to emPCR and sequencing.