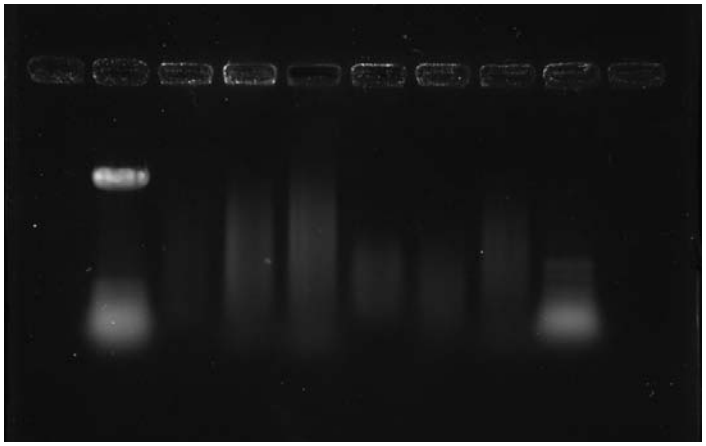
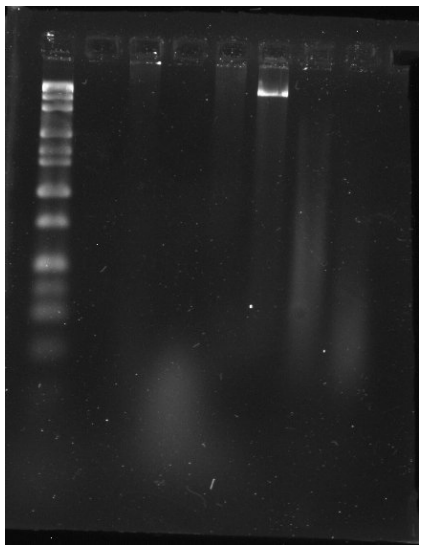
Purpose: Test the adaptor-modified targets for amplification

Introduction: The yields of DNA from the Pippin Prep were extremely small – the DNA peak migrated near 400bp, but we were collecting at 200bp. I tried collecting at 300bp the second time, but the amount of material was still very small. I did still try to amplify, but we need to quantify the library before we continue.

My expectation is that we will have to try some other ways to obtain enough 200bp material.

I obtained some NEB Fragmentase, however, the DNA was completely degraded, so this needs to be refined. The AmPure Beads appear not to bind our DNA effectively, so we will try using Qiagen QiaQuick columns instead.





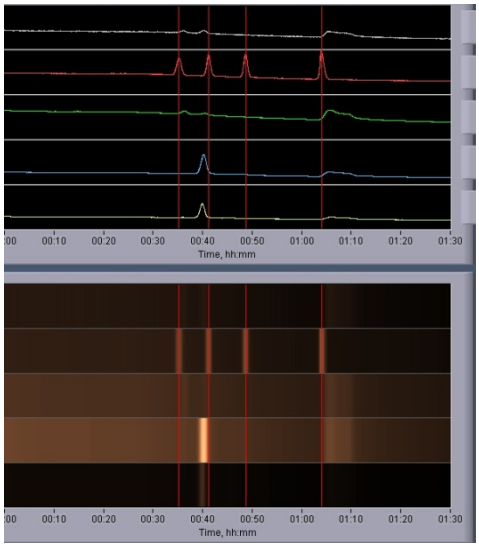
In the first picture I loaded 1ug of sheared DNA from each group and some that Deepthi made - this is the Phaseolus DNA only. This is a 2% agarose gel with a 25bp ladder as markers on the right – the highest marker is 500bp.

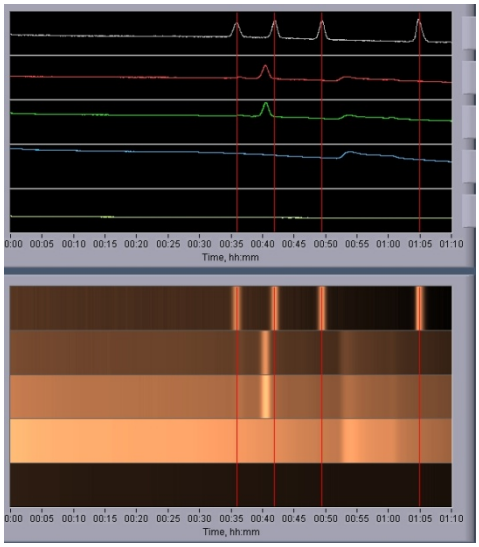
It the second image, I loaded the Hi-Lo marker, which goes from 50bp to 10kbp. The first 4 lanes are material from my first NEB Fragmentase digestion followed by the AmPure bead purification. Clearly the material was lost somewhere. The 5th lane is intact DNA, the 6th is from a 30 min Fragmentase digestion and the final lane is once again sheared. The shearing looks like it should be in the 300bp size range, but does not seem to get smaller. The Fragmentase reaction did not proceed long enough.

For the Pippin Prep samples, here are the NanoDrop values. The gel from last Monday had 3 samples, they were recovered in ~25ul, of which 20 went into the ligase-PCR reaction. The first 3 we collected at the 200bp mark. Because the main peak seemd to be at 300 bp on that gel, I collected the second batch at 300bp (but in that case the peak looked to be closer to 400bp, very frustrating).

S1 50ng/ul (recovered in ~25ul)
S2 48ng/ul “”
S3 43 ng/ul “”
JS 31 ng/ul (but recovered in 40ul, so probably about the same yield)
AL 33 ng/ul “”
DC 31 ng/ul “”

So we should have put about 600-1000ng into the ligase-PCR reactions. This may have been enough to carry forward, IF the material was adaptor modified.





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. The library quantification protocol from Kapa Biosystems is attached. Briefly
    a. Dilute the library you have been given 1:500 with ‘DB’ (Dilution Buffer).
    b. We will share a qPCR plate.
    c. Each of you will set up one of the DNA standards as well as your library.
    d. Each reaction is to be set up in triplicate.
    e. Collect the MM tube – it should have 40ul of solution in it.
    f. In the optical PCR plate, add 6ul of the MM to each of 6 wells.
    g. To each of the wells add 4ul of the library or 4ul of the DNA standard.
    h. Note where your wells are located.
    i. The plate is sealed with optical film or caps.
    j. The plate is centrifuged briefly to make sure all of the solution is on the bottom.
    k. The plate is placed in the BioRad ICycler.
    l. The cycling profile is
        i. 95C 5 min

ii. 35 cycles of
    1. 95C 30 sec
    2. 65C 45 sec

m. Using the guidelines provided determine whether your library passed the criteria to continue on to emulsion PCR (step 10).

3. Improving the library size: alternative approaches (pick one of these two):
    a. A time-course of digestion on full-length gDNA using NEB fragmentase.
        i. To 5 ug of DNA in 10ul of TE buffer
        ii. Add 4ul of 5X Fragmentase Buffer
        iii. Add 3 ul of NF-H_2Ograde water and 2ul of BSA
        iv. Mix by pipetting gently up and down
        v. Add 1ul of Fragmentases and mix by pipetting again.
        vi. Incubate at 37C.
        vii. After 20 minutes remove 4ul to ice, return the tube to the heating block, and remove successive 4ul aliquots at 5 minute intervals.
        viii. Pour a 2% agarose gel in 1X TBE
        ix. Run the samples on the gel. Use the Amersco marker. Run at 50V for ~90 minutes.
        x. The goal is to determine what level of incubation gives the best digestion.
        xi. Stain with SYBR Green I.
    b. A time-course of digestion of sheared DNA using NEB fragmentase.
        i. To 5ug of *sheared* DNA in 25ul of TE buffer
        ii. Add 8ul of 5X Fragmentase Buffer
        iii. Add 2 ul of NF-H_2Ograde water and 4ul of BSA
        iv. Mix by pipetting gently up and down
        v. Add 1ul of Fragmentases and mix by pipetting again.
        vi. Incubate at 37C.
        vii. After 20 minutes remove 8ul to ice, return the tube to the heating block, and remove successive 8ul aliquots at 5 minute intervals.
        viii. Pour a 2% agarose gel in 1X TBE
        ix. Run the samples on the gel. Use the Amresco marker. Run at 50V for ~90 minutes.
        x. The goal is to determine what level of incubation gives the best digestion.
        xi. Stain with SYBR Green I.
4. Alternative purification method – repeat the sizing scheme above that gave the highest yield closest to 200bp – start with 5ug of DNA. Test 1ug of that DNA on an agarose gel to make sure the method worked the same way the second time. Since the AmPure beads do not seem to be effective, we will use a combination of ethanol precipitation and Qiagen PCR columns for removing nucleotides and enzymes.
    a. Size-select the fragmented DNA using a Qiaquick PCR purification column
    b. Add 5 volumes of Buffer PBI to 1 volume of the DNA sample. Mix.
    c. Place a pink QiaQuick column in a 2 ml collection tube

d. Apply the sample to the column, place in a centrifuge (balance!) and spin at 13,000 for 60 sec
e. Discard the flow-through (your sample is on the column)
f. Place the Qiaquick column back in the collection tube
g. Wash with 750ul of Buffer PE
h. Centrifuge at 13,000 rpm for 60 sec
i. Discard the flow through.
j. Place the column back in the same tube and spin for another 60 seconds
k. Place the column on a 1.5 ml clean microfuge tube
l. Add 50 ul of Buffer EB (10mM Tris pH 8.5) to the column. Let stand 60 seconds
m. Centrifuge at 13,000 rpm for 60 seconds – in this case the solution in the tube is your sample – label it and keep it
n. Discard the column
o. Check the concentration of the sample in the Nanodrop.
p. Run 0.5ug on a 2% agarose gel.

5. End-Repair 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.
        i. Use a Qiagen PCR column to purify the sample – as above
        ii. Check the concentration on a Nanodrop.
6. Repeat the Adaptor Ligation as before AD –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. Run the Pippen Prep if you have at least 3ug of sample - collect the sample around 200bp.
8. Put 20ul of the Pippin Prep eulan this into the Nick-translation-PCR mixture.

9. Repeat the qPCR test for library quantification.
10. Emulsion PCR – this is the step in which the template molecules get attached to the Ion Sphere Particles (ISPs). The goal is to attach one per sphere, then make a mini-reactor in an oil bubble, so that all of the products in the sphere come only from the single template – this is essentially clonal PCR. This is why the library quantification is so important.
    a. Make the correct dilution of your library.
    b. We will use the OneTouch instrument, which performs the emPCR and then purifies away the oil. After the emPCR is complete there is a step in which the template is made single-stranded.

Dilution Buffer for qPCR

10mM Tris pH 8
0.05% Tween 20.

5X Fragmentase Buffer
100mM Trish, pH 7.5
50mM MgCl2
250mM NaCl
0.75% Triton X-100
We also have 1mg/ml BSA, which is added to the reaction to make 100ug/ml (1:10 dilution).
Inactivation occurs by heating to 65C for 15 minutes in the presence of 50mM DTT.

Qiaquick PCR purification columns

1. Add 5 volumes of Buffer PBI to 1 volume of the DNA sample. Mix. Make sure the sample stays yellowish (otherwise the pH will be wrong).
2. Place a pink QiaQuick column in a 2 ml collection tube
3. Apply the sample to the column, place in a centrifuge (balance!) and spin at 13,000 for 60 sec
4. Discard the flow-through (your sample is on the column)
   place the Qiaquick column back in the collection tube
5. Wash with 750ul of Buffer PE
6. Centrifuge at 13,000 rpm for 60 sec
7. Discard the flow through.
8. Place the column back in the same tube and spin for another 60 seconds
9. Place the column on a 1.5 ml clean microfuge tube
10. Add 50 ul of Buffer EB (10mM Tris pH 8.5) to the column. Let stand 60 seconds
11. Centrifuge at 13,000 rpm for 60 seconds – in this case the solution in the tube is your sample – label it and keep it
12. Discard the column
13. Check the concentration of the sample in the Nanodrop.
14. Run 0.5ug on a 2% agarose gel.