Lab 5: Restriction Digestion and Size Selection with PEG and Magnetic Beads

Introduction: One way to create physical genetic markers is to sequence parts of the genome. We are going to sequence the entire chloroplast genome because a) it is pretty small and b) Dr. Sisco has some interesting questions about whether the chloroplasts are different in some of the crosses. However, the resistance genes to the fungus are on the chromosomes. Because sequencing the entire nuclear genome is very expensive and huge (hard to analyze), a number of techniques have been developed to create lots of short reads that are scattered evenly (more or less) around all of the chromosomes. The hope is that a number of these will be close to the resistance genes that we are interested in. We are going to carry out the first steps of one of these methods, which is called REST-Seq, which creates the many small fragments that are scattered around, using restriction enzymes to chop up all of the chromosomes. Because the sequencing method can only handle quite small pieces of DNA (200-600bp), then we will use a size-selection method based on precipitating DNA with polyethylene glycol (PEG) onto magnetic beads. The concentration of PEG determines the size-length of DNA we precipitate, and the beads are a handy way to collect the resulting material, remove it from the fragments we don't want, and concentrate it all at the same time.

Restriction Digestion: the genomic DNA can be cut into defined pieces with a restriction endonuclease – an enzyme that cuts the covalent phosphodiesterase backbone of the DNA on both sides to make double-stranded pieces. Usually the recognition site is a palindrome (reads the same forwards and backwards) and the breaking of the covalent bonds is symmetrical around the palindromic sequence, although the ends can end up being blunt, with a 3' overhang or with a 5' overhang. The New England Biolabs site has an encyclopedia of these enzymes, their recognition sites, the types of overhang they produce and the buffers required for good activity. If you have the genome sequence it also has a tool called NEBcutter that will create the fragment profile for you. The frequency of the recognition site in the genome depends on the length of the recognition sequence and its base composition compared to the base composition of the targeted genome. The distribution of the recognition site in a genome is usually not completely random, because sequence distribution itself is not random – telomeres and centromeres (the ends and the middle of chromosomes) tend to have somewhat different sequence composition than the rest of the genome, and coding and non-coding sequences may have somewhat different composition. Using enzymes with different sequence recognition sites can help balance this effect. From any given restriction enzyme digestion of a genome you get a distribution of fragment lengths, some very short and some extremely long, with the man length reflecting the frequency of the recognition site across the genome.

Related genomes tend to have sequences with a similar composition in a similar order, so if you digest two genomes you should get a very similar set (by length) of fragments- where a recognition site has been altered the fragment will disappear from one genome and a new fragment will appear in the other genome. Of course sequence that varies *within* the fragment will be invisible if you only look at fragment lengths (size polymorphisms) so sequencing is needed to fully exploit the amount of variation. We are seeking to exploit the great similarity in fragment length profiles between related genomes to generate a subset of fragments.

Protocol - Step 1, Restriction Digestion

Restriction Digestion

1. Make a stock of your DNA that is 50ng/ul, and make a final volume of 50ul of this stock.
    a. 50*50=2500ng total is needed.
    b. Divide 2500ng by the concentration of your sample.
    c. Put that volume of your sample into a microfuge tube.
    d. Subtract the volume you put in the tube from 50ul, add the resulting volume of TE buffer to the microfuge tube.
    e. Cap, label, vortex, quick-spin.
2. In a 1.5ml tube, combine, then pipette up and down gently 10 times to mix:
    a. Total volume of 50ul contains
        i. 5ul of 10X buffer (NEB CutSmart)
        ii. 40ul of 50ng/ul DNA (2ug genomic DNA)
        iii. 2.5ul TaqαI enzyme (20U/ul) OR MseI (half the class will do one and half the other)
        iv. 2.5ul of H_2O to make up volume
3. Incubate at 65C for 1-3 hours (this can go overnight)
    a. Remove 10ul to a labeled 0.5ml tube and save to run on a 1.5% agarose gel

We use the magnetic beads and their buffer to collect DNA fragments in a particular size range. A small ratio of the beads only collects very large fragments of DNA on the beads, while a pretty high ratio collects the smaller fragments. You are using a 2-step process, first you will discard the really big DNA, then you will collect and save the DNA that is between about 100-600bp in length. We will run these on an agarose gel to see what the results are.

Step 2: Ampure XP bead size selection

1. Add 160ul of molecular-biology grade water to your digested DNA and mix by vortexing for 10 seconds and then doing a quick-spin in a mini-fuge.
2. Add 150ul of Ampure XP beads (make sure they are at room temperature) and mix by pipetting up and down 10 times.
    a. Let sit for 5 min at room temperature
3. Put on magnet and let sit for 2 minutes – your sample is the liquid, not the beads.
    a. Remove the <u>liquid</u> to a new 1.5ml tube.
        i. Discard the beads
4. Add 390 ul of Ampure XP beads to the liquid in the tube and mix by pipetting up and down 10 times.
    a. Let sit at room temperature for 5 minutes
5. Put on a magnetic rack and let sit for 2 minutes – now your sample is on the beads.
    a. Remove the liquid and <u>discard</u>

6. Add 400ul of 70% ethanol to the beads in the tube on the rack (you are rinsing away stuff that is not specifically precipitated on the beads, just trapped)
    a. Turn the tube slowly for 2 minutes, then let beads collect
    b. Remove the liquid and discard
7. Repeat step 6 once more
8. Remove as much liquid as possible, leave the tube open to air dry for 10 minutes
9. Remove tube from magnetic rack
    a. Add 30ul of TE buffer and mix
    b. Let sit for 5 minutes at room temperature
10. Put tube back on the magnetic rack and collect the beads for 2 minutes
    a. Remove the liquid to a labeled tube (very carefully – you don't want any beads). THIS IS YOUR SAMPLE
11. Measure the concentration of the DNA using the Nanodrop spectrophotometer.
12. Run 1 ug of your product on a 1.5% agarose gel, use a 100bp ladder as the size standard (directions below).
13. Store your digested material at 4C in a 0.5ml labeled tube.