Plan for ligations

The overall concentration of vector + insert should be between 1-10 μg/ml for efficient ligation. Vector:Insert molar ratios between 1:1 and 1:10 are recommended (1:3 is typical). If you are unsure of your DNA concentrations, perform multiple ligations with varying ratios.

From <<https://www.neb.com/faqs/1/01/01/how-much-dna-should-be-used-in-a-ligation-using-t4-dna-ligase>>

Average concentration is about 50ng/uL

Planning to ligate 100ng total DNA per sample - the maximum is 150 (for 96 tags) or 300 (for 48 tags) so 100 should work fine and to hit 150 I'd have to repeat more samples

Plan tomorrow's annealing of adapters

In the ligation molarity calculator, you need the frequencies of the two enzymes.

Using grep -c "CUTSITE" on both of the enzymes gives 474597 MSP1 sites - one every 977 base pairs - and 135442 EcoR1 sites - one every 3626 base pairs

491087067/135442=3625.8108

491087067/101911=4818.7837

491087067/517194=949.522

491087067/474597=1034.7454

The SimRad output (the .Rout files in /RADSeq on the DSCR) is fairly consistent with this - 101911 and 517194 - I'll rely on that. Length of the scaffold is about 491087067 (width of the whole lacunosa scaffold in R) or 509492915 (wc in UNIX, presumably including the contig names). Cordatotriloba is actually smaller. So, 4920 and 969.

"2- to 10-fold excess" is a pretty wide range, so this is JUST an estimate

Program PCR machines

Barcodes: Heat to 95◦C for 5 minutes and slowly cool to room temperature.

P2 adapter: Incubate at 97.5°C for 2.5 minutes, and then cool at a rate of not greater than 3°C per minute until the solution reaches a temperature of 21°C. Hold at 4°C.

The Peterson protocol says to anneal stocks in annealing buffer, but the Parchman protocol uses water. I'm going to use water since 1, those are the barcodes I'm using and 2, I am probably not going to need seriously long-term storage on these things.

I want a 40uM stock of my barcodes because that's what the Peterson protocol defaults to and that's easy ("Note that annealed adapters in the molarity calculator are set to dummy values of 4uM (4 pmol/ul) concentration corresponding to the 10-fold dilution of the 40uM annealed adapter stock solution from the annealing reaction described above").

Both the ligation calculator and the NEB calculator, given the same information, say I need ~.3pm of barcodes per sample. I'm using each barcode for 5 samples and then I want enough wiggle room to repeat this, so I need (amount per sample x 5 x 3 = 15x amount per sample). So .3x15=4.5 picomoles per barcode, so let's say I want 5 picomoles of each bar code.

"Mix 1 μL of each oligo in a pair (100 μM stock) with 98 μL of water to make 100 μL of 1 pmole/μL (1 μM) of annealed, double-stranded adaptor stock."

40uL of each oligo +20uL water to make 40pM/uL (40uM).

Except that's way more than I'll need. A quarter of that will be plenty. So 10:10:5. 10 uL of a 100pm/uL stock is 1000pm, and I only need about 5.

Heat to 95◦C for 5 minutes and slowly cool to room temperature. This annealing step only needs to be performed once for a stock solution of adaptor.

Anneal P2 adapters

To create Adapter P1, combine each oligo 1.1 with its complementary oligo 1.2 in a 1:1 ratio in working strength annealing buffer (final buffer concentration 1x) for a total annealed adapter concentration of 40uM/ Purchased oligos are resuspended to an initial concentration of 100uM, use 40ul oligo 1.1, 40ul oligo 1.2, 20ul nuclease-free water). Do the same for oligos 2.1 and 2.2 to create the common adapter P2.

2. In a thermocyler, incubate at 97.5°C for 2.5 minutes, and then cool at a rate of not greater than 3°C per minute until the solution reaches a temperature of 21°C. Hold at 4°C.

I need 1.52pm/sample, so to make enough for 1000 samples I need 1520pm or 1.52nm  
It will be diluted to 100pm/microliter stock solution (I should probably use TE for that!).

Then in the annealing I will set it to 40uM by annealing 40:40:20 adapter:adapter:water.

Write up ligation reaction plans

Per 40uL reaction, I will use:

|  |  |
| --- | --- |
| T4 DNA ligase | 2 µL |
| T4 ligase buffer | 4 µL |
| P2 adapter stock (diluted 1:10 and then 18.94:31.06 from 40µM stock solution) | 1 µL |
| Barcode stock (diluted 1:10 and then 1.97:48.03 from 40µM stock solution) | 2 µL |
| DNA sample | Variable, 100ng |
| Water | Variable, 31-DNA |

I'll actually do the parents and F1 separately in strips - that'll be easier than trying to keep track of them in a plate

Ligate plate 4

|  |  |  |
| --- | --- | --- |
| T4 DNA ligase | 2 µL | 220 |
| T4 ligase buffer | 4 µL | 440 |
| P2 adapter stock (diluted 1:10 and then 18.94:31.06 from 40µM stock solution) | 1 µL | 110 |
| Barcode stock (diluted 1:10 and then 1.97:48.03 from 40µM stock solution) | 2 µL |  |
| DNA sample | Variable, 100ng |  |
| Water | Variable, 31-DNA |  |

Notes

*NEB ligation calculator:* [*http://nebiocalculator.neb.com/#!/ligation*](http://nebiocalculator.neb.com/#!/ligation)

*Peterson ligation calculator:* [*https://docs.google.com/spreadsheets/d/1fcg6mYESNEvi8jfu3T1Bbue-rsVqgeC6RIXjzT9CRrc/edit?hl=en\_US&pref=2&pli=1#gid=0*](https://docs.google.com/spreadsheets/d/1fcg6mYESNEvi8jfu3T1Bbue-rsVqgeC6RIXjzT9CRrc/edit?hl=en_US&pref=2&pli=1#gid=0)