GENOTYPING BY SEQUENCING (ddRadSeq) PROTOCOL FOR *ANOPHELES*

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ddRAD in *Anopheles* Overview

This is a slightly modified version of the ddRADSeq protocol developed by the Hoekstra Lab at Harvard. It has been optimized to work with Anopheline genomes. It is likely to be useful on other mosquito genomes as well.

The purpose of the protocol is to genotype 576 individual mosquitoes at a consistent, genome-wide set of markers simultaneously in one lane of a single end, 100bp Illumina HiSeq flow cell. Nearly all steps are performed in 96-well plates to speed throughput. The dense genotyping data generated from this protocol is particularly useful for quantitative genetic analysis. It can also be used for population genomic analysis. However, when prepping libraries for population genomic or phylogenomic analysis we only genotype 288 individuals per lane to increase coverage and, thus, obtain more reliable genotype calls. If we have a reference genome we use paired-end reads for population genonmic analysis, if not we use single-end reads. The library prep method is the same for either single end or paired end runs. The basic steps in the protocol, with some explanatory figures, are as follows. If you have additional questions look through the original Hoekstra paper or email Brad White (bwhite@ucr.edu).

Below is a flow chart of the process:

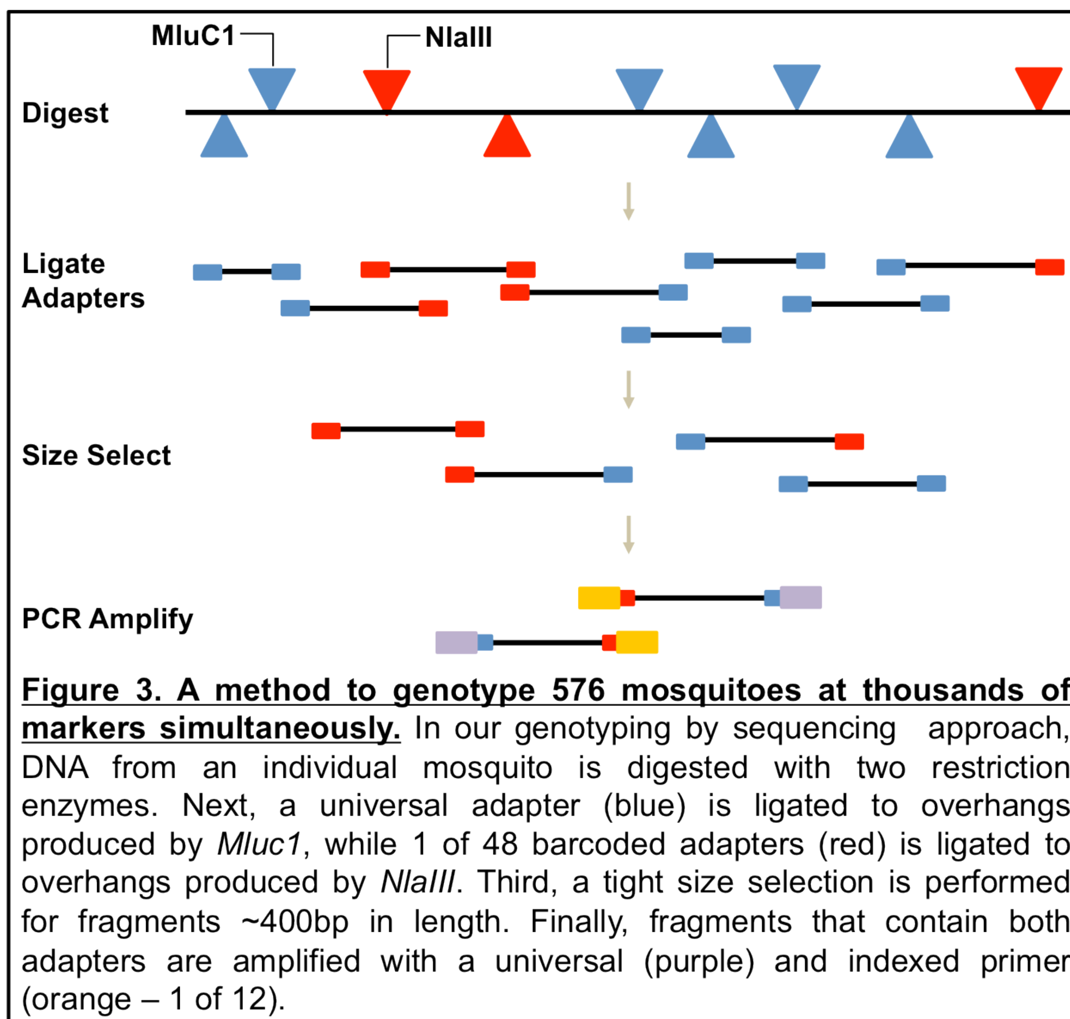


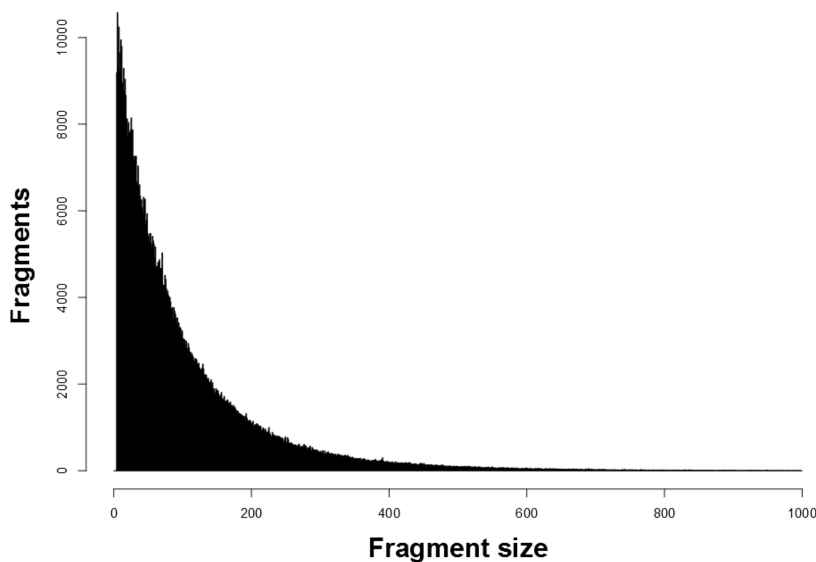
Figure 3. A method to genotype 576 mosquitoes at thousands of markers simultaneously. In our genotyping by sequencing approach, DNA from an individual mosquito is digested with two restriction enzymes. Next, a universal adapter (blue) is ligated to overhangs produced by *Mluc1*, while 1 of 48 barcoded adapters (red) is ligated to overhangs produced by *NlaIII*. Third, a tight size selection is performed for fragments ~400bp in length. Finally, fragments that contain both adapters are amplified with a universal (purple) and indexed primer (orange – 1 of 12).

DNA Extraction

DNA is extracted from individual mosquitoes in 96-well format using a Zymo kit. We do not quantify the DNA yield for each mosquito as we find it to be relatively even across extractions. We simply take $1/7^{th}$ of the DNA extracted from an individual and use it to construct the library. Importantly, we have found that the Zymo kit is not reliable for extracting DNA from older field specimens (especially larvae). In such instances, we recommend using another method.

DNA Digestion

To fragment the DNA, a double RE digest is performed using MluC1 and NlaIII. In *An. gambiae* the fragment distribution after digestion is shown below. To reduce the portion of the genome we are sequencing, we use only fragments centered on 300bp (400bp with adapters).



Adapter Ligation

The ddRADSeq protocol utilizes a combinatorial multiplexing approach to uniquely identify all 576 individuals in a single lane. Twelve 'sub-libraries' that each contains 48 individual mosquitoes are prepared. Each of the 48 mosquitoes in a 'sub-library' has a unique in-line barcode. This barcode is part of the adapter and is ligated to the NlaIII cut site. Additionally, a universal adapter is ligated to the MluC1 cut sites. Only fragments that have NlaIII and MluC1 cut sites will be sequenced. Adapter sequences are given in an excel spreadsheet exactly as they should be ordered from IDT. Note that modifications are present.

Size Selection

After the adapters have been ligated automated size selection is conducted with a target of 400bp. Size selection can be done manually but results in more variation between 'sub-libraries'.

PCR amplification

Each of the twelve 'sub-libraries' is amplified with a distinct, indexed primer that has a unique 6bp index sequence that is read in a separate 7-cycle reaction when sequencing is performed. This combinatorial

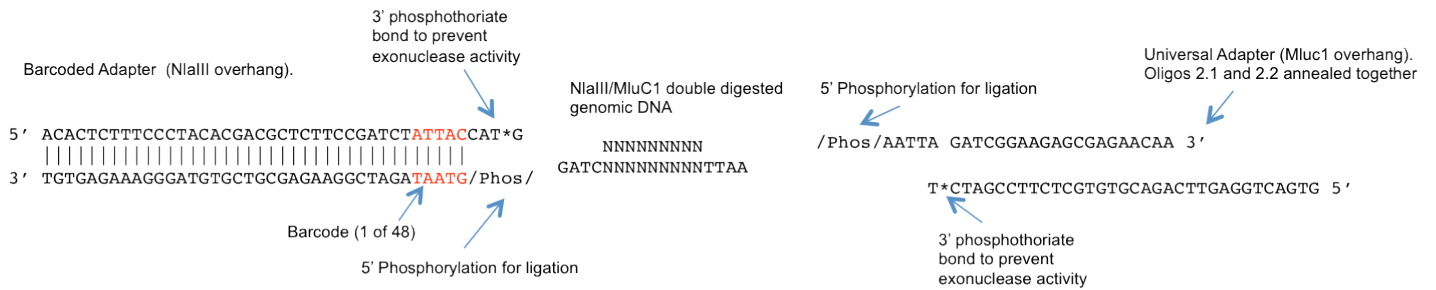
multiplexing allows 576 individuals (12 indexed primers x 48 inline barcodes) to be pooled in a single library. Reads are first sorted by index and then by barcode. Using this system >98% of reads can be uniquely assigned to an individual mosquito. Primers are given in an excel spreadsheet exactly as they should ordered from IDT. Note that modifications are present.

Bioinformatics

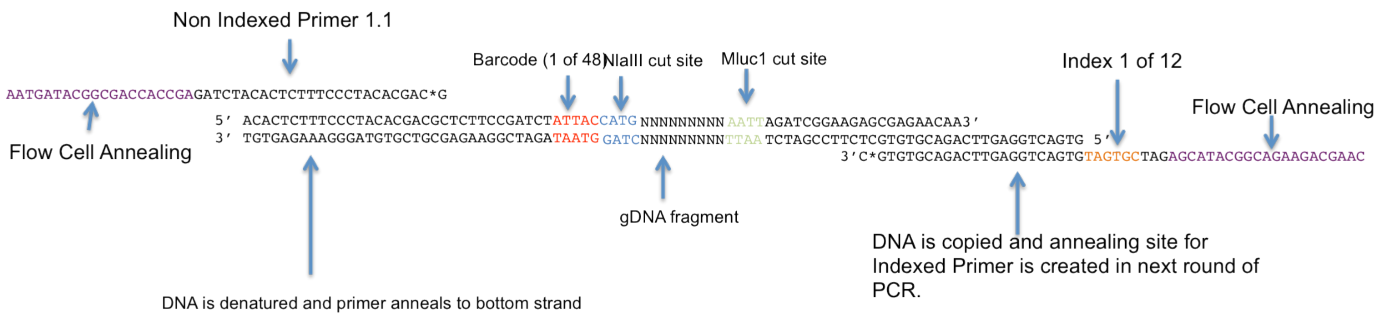
We have developed a pipeline for analyzing ddRAD data generated from *An. gambiae* experimental crosses. The pipeline assigns reads to individuals, maps these reads to the reference genome (BWA), calls genotypes for a group of individuals (GATK), generates a VCF, and finally reconstructs ancestry based on posterior probabilities. We expect to make this pipeline publicly available in the next few months.

Nucleotide-level Schematic of GBS

In the figure below, DNA has been digested with MluC1 and NlaIII. Annealed adapters are being ligated onto the digested DNA fragments.



In the figure below, ligated DNA fragments are being PCR amplified.



96-well Plate DNA Extraction using 'Zymo-96 quick-gDNA'

1. Place mosquito into a 1.5ml centrifuge tube.
2. Add 4 stainless steel balls and 500µl of Genomic Lysis Buffer into each tube.
    a. http://www.amazon.com/gp/product/B000FMULKC/
3. To lyse cells: Grind up to 24 sample simultaneously via automated vortex for 5-10 minutes.
    a. http://www.usascientific.com/vortex-genie-2.aspx
    b. http://www.usascientific.com/vertical-24x1.5-ml-tube-holder.aspx
4. Centrifuge lysates to pellet un-dissolved mosquito carcass
5. To bind DNA: Transfer supernatants from centrifuge tube to Silicon-A Plate. Try to avoid transferring mosquito bits as they can clog the column.
6. Place Silicon-A Plate onto collection plate
7. Centrifuge for 5 minutes at 3,000xg.
8. Pre-Wash: Add 150µl DNA Pre-Wash Buffer to each well.
9. Centrifuge for 5 minutes at 3,000xg.
10. Discard the liquid in the collection plate, but do not dispose of the collection plate.
11. Wash 1: Add 200µl g-DNA Wash Buffer to each well.
12. Centrifuge for 5 minutes at 3,000xg.
13. Wash 2: Again, add 200µl g-DNA Wash Buffer to each well.
14. Centrifuge for 5 minutes at 3,000xg.
15. Discard collection plate and transfer Silicon-A Plate onto a 96-well PCR Plate.
16. Elute DNA: Add 35µl of DNA Elution Buffer to each well and incubate at room temperature for 5-15 minutes.
17. Centrifuge for 5 minutes at 3,000xg.
18. Discard Silicon-A plate and cover PCR plate with Silicon sealing mat to prevent evaporation during long-term storage.
    a. VWR 10011-130

Digest DNA

1. Transfer 5ul of DNA from stock DNA plate into a new 96 well PCR plate.
2. Prepare digestion master mix. We generally digest 6 plates (1 full library) at a time and prepare the master mix in a 15ml falcon tube.
3. Reagent table:

Reagent	1x	600x
10x NEB CutSmart Buffer	2	1200
NEB MluC1 (10U/ul)	0.3	180
NEB NlaIII (10U/ul)	0.2	120
ddH20	12.5	7500
DNA	5	-------

4. Add 15ul master mix to each well.
5. Incubate at 37C for 3 hours in thermocycler or incubator.
6. After incubation, immediately move onto next step or place samples in fridge to prevent star activity. Do no heat kill the reactions!

Prepare Homemade Ampure XP

Ampure XP is extraordinarily expensive. Using homemade Ampure XP is ~1/50th the cost of commercial product. This protocol is taken directly from Brian Faircloth (UCLA).

1. Mix Sera-mag SpeedBeads and transfer 1ml to a 1.5ml centrifuge tube.
    a. http://www.thermoscientific.com/en/product/sera-mag-magnetic-speedbeads-carboxylate-modified-particles.html
2. Place Speed Beads on magnetic rack until beads are separated.
    a. e.g. https://www.neb.com/products/s1506-6-tube-magnetic-separation-rack
3. Remove supernatant.
4. Add 1ml TE (pH 7.5-8.0) to beads, remove from magnet, mix by pipetting up and down, return to magnet.
5. Remove supernatant.
6. Add 1ml TE to beads, remove from magnet, mix by pipetting up and down, return to magnet.
7. Remove supernatant.
8. Add 1ml TE to beads, remove from magnet, mix by pipetting up and down, but do NOT place back on magnet.
9. Add 9g PEG-8000 to a new 50ml conical tube.
    a. http://www.amresco-inc.com/POLYETHYLENE-GLYCOL-8000-0159.cmsx
10. Add 2.92g NaCl conical
11. Add 500ul 1M Tris-HCl to conical
12. Add 100ul 0.5M EDTA to conical.
13. Fill conical to ~49 ml using ddH20.
14. Mix conical for ~5 min until PEG goes into solution.
15. Add 25ul Tween 20 to conical and mix.
16. Add SpeedBead and TE solution from step 8 to conical and mix.
17. Fill conical to 50ml mark with ddH20.
18. If you want, you can test Homemade Ampure XP against commercial Ampure XP. However, we do not perform this step as we find the homemade Ampure to be extremely reliable.

Clean Digested DNA

1. Add 30ul Homemade Ampure into each well.
2. To bind DNA to beads: cover plate, briefly vortex to mix, and then quick spin contents to bottom of well.
3. Place on 96-well magnet for ~5 minutes.
    a. e.g. http://www.lifetechnologies.com/order/catalog/product/AM10027
4. Remove supernatant.
5. Wash 1: Add 150ul 75% EtOH to each well and let sit for 1 min. Still on magnet
6. Remove EtOH.
7. Wash 2: Add 150ul 75% EtOH to each well and let sit for 1 min. Still on magnet
8. Remove EtOH. Be sure to use 10ul tip to remove residual EtOH at bottom of wells.
9. To ensure most EtOH is removed dry plate at room temp for ~10 minutes. Still on magnet
10. Remove plate from magnet. Elute DNA from beads by adding 30ul ddH20. Vortex to resuspend beads in water. If too dry, not all beads will go into solution. This does not appear to effect DNA recovery.
11. Place plate back on magnet to separate beads from DNA solution. Wait ~5 min and transfer supernatant to a clean 96-well plate. Small amounts of bead carry over do not effect downstream reactions.

Prepare Adapters

Barcoded Adapters

Barcoded adapters are ligated to the overhang produced by NlaIII. When sequenced, the first nucleotides will be the 5 bp barcode and then the NlaIII cut site. This protocol utilizes 48 different barcoded adapters. Adapters are double stranded and you need to order the top and bottom oligos separately and then anneal them together. A spreadsheet is included in the protocol that details exactly what should be ordered from IDT.

1. Resuspend each oligo in ddH20 to a concentration of 500pmol/ul
2. In a new 96 well plate set up the following reactions to anneal the two oligos together and create 100ul of barcoded adapter at 50pmol/ul
3. Reagent 1x 50x

Reagent	1x	50x
10x Annealing Buffer	10ul	500ul
Top Oligo	10ul	--------
Bottom Oligo	10ul	--------
ddH20	70ul	3500ul
	100ul	80ul each well

4. After setting up reactions, anneal in a thermocycler under the following conditions:
   97.5C for 2.5 min
   95C for 1 min
   Decrease temp 1° per cycle for 74 cycles
   Hold at 4C

5. Split the annealed adapters across four plates to avoid repeated freeze thaw. Only use one plate at a time. Store in fridge if being used often.
6. Before adding barcoded adapters to the DNA samples they need to be diluted.
7. In a new 96 well plate, combine 1.2ul barcoded adapters and 28.8ul H20. The working concentration is ~1.9 pmol/ul barcoded adapter, which means that for every NlaIII sticky ends there will be ~8 barcoded adapter molecules.

Universal Adapters

The universal adapter is ligated to the overhand produced by MluC1. It does not have a barcode and the same adapter is ligated to all of the samples in a library. Adapters are double stranded and you need to order the top and bottom oligos separately and then anneal them together.

1. Resuspend the top and bottom universal adapter in ddH2O to 500pmol/ul
2. In a strip of eight PCR tubes set up the following reactions to create 400ul (8 rxns x 50ul/rxn) of universal adapter at 50pmol/ul.

Reagent	1x	10x
10x Annealing Buffer	5ul	50ul
Top Oligo	5ul	50
Bottom Oligo	5ul	50
ddH20	85ul	850ul
	100ul	100ul each well

3. After setting up reactions, anneal in a thermocycler under the following conditions:
   97.5C for 2.5 min
   95C for 1 min
   Decrease temp 1° per cycle for 74 cycles
   Hold at 4C
4. These adapters are added directly to the ligation master mix and do not need to be diluted further. The final concentration in any ligation reaction represents a 8-fold excess of universal adapter molecules to MluC1 sticky ends.

<u>10x Annealing Buffer Recipe</u>

-10mM Tris-HCl (pH 7.5-8)

-50mM NaCl

-10mM EDTA

Ligate Adapters

1. Prepare barcoded adapters according to "Prepare Adapters" protocol
2. Add 2ul of Barcoded Adapters to the appropriate well.
3. Prepare Ligation Master Mix in 15ml Falcon tube
4.

Reagent	1x	600x
NEB 10x T4 DNA Ligase Buffer	4	2400
NEB T4 DNA Ligase (400U/ul)	0.2	120
Universal Adapter (50pmol/ul)	0.1672	100
ddH20	3.6328	2180
DNA	32	-------
	40	

5. Add 8ul master mix to each well.
6. Ligate in a thermocycler using the following conditions:
   22 for 2 hrs
   65 for 9 min
   65 for 1.5 min
   Decrease temp 2° per cycle for 22 cycles
   Hold at 4
7. Place in fridge until proceeding to next step.

Pool and Clean each 'sub-library'

1. Pull together the 48 individuals that compose a 'sub-library' into a single Falcon tube
2. Add 2880ul Homemade Ampure to tube, mix, and let sit at room temperature to bind DNA beads.
3. Place tubes on magnet until beads are pelleted and supernatant is clear (~5 min).
    a. e.g. http://www.lifetechnologies.com/order/catalog/product/12301D#
4. Remove and discard supernantant. Still on magnet.
5. Wash 1: Add enough 75% EtOH to cover pellet. Let sit for 2 min. Still on magnet.
6. Remove EtOH.
7. Wash 2: Add enough 75% EtOH to cover pellet. Let sit for 2 min. Still on magnet.
8. Remove EtOH. Be sure to remove residual EtOH at bottom of the tube.
9. To ensure most EtOH dry tube at room temp for ~10 minutes. Still on magnet.
10. Remove tubes from magnet and elute DNA from beads by adding 300ul ddH20. Pipet up and down to fully resuspend beads.
11. Place tubes back on magnet. Wait ~5 min until full separation and transfer supernatant to clean 1.5ml centrifuge tube.
12. Each sub-library needs to be cleaned a second time so that the entire volume can be size selected in a single well on the Pippin Prep.
13. Add 450ul Homemade Ampure to tube, mix, and let sit at room temperature to bind DNA beads.
14. Place on magnet for ~2 minutes.
15. Remove supernatant.
16. Wash 1: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
17. Remove EtOH.
18. Wash 2: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
19. Remove EtOH. Be sure to use 10ul tip to remove residual EtOH at bottom of tube.
20. To ensure most EtOH is removed dry tubes at room temp for ~10 minutes. Still on magnet
21. Remove tubes from magnet and elute DNA from beads by adding 30ul ddH20. Pipet up and down to resuspend beads in water.
22. Place tubes back on magnet to separate beads from DNA solution. Wait ~5 min and transfer supernatant to clean 1.5ml centrifuge tubes. Small amounts of bead carry over do not effect downstream reactions.

Size-select each 'sub-library'

1. The entire 30ul of each 'sub-library' will be loaded onto the Blue Pippin/Pippin Prep for size selection.
2. Turn Blue Pippin on.
   a. http://www.sagescience.com/
3. Set protocol to perform narrow size selection with a target size of 400bp.
4. Select 'internal standards'
5. Calibrate with calibration panel.
6. Place calibration panel over the led lights, close pippin prep and press calibrate, make sure screen states "calibration ok"
7. Open new 1.5% gel cassette, make sure all buffer chambers are full with only a few bubbles (if not fill with electrophoresis buffer). Make sure the bottom of the gel is not coming away from the plastic cassette (no gel bubbles). Gel bubbles on the top do not cause issues.
   a. Sage Science Product #BDF1510 for Blue Pippin (2% gel can be used in place)
8. Tip the gel cassette so the elution wells are above the loading wells, tap to remove bubbles next to the elution wells.
9. Place cassette in blue pippin (loading wells on the left) so that the led lights shine through the gel closer to the elution wells.
10. Remove plastic covering from loading wells and elution wells.
11. Fill loading wells to the top with electrophoresis buffer.
12. Remove all buffer from elution wells and replace with 40ul electrophoresis buffer. Cover with plastic provided and rub with the thumb until a tight seal is formed.
13. Close pippin prep and click test. If test is passed move to step 12. If test is failed, make sure loading wells are full and elution wells are properly sealed. If test still fails, use a different gel.
14. Remove 40ul from loading wells
15. For each 'sub-library', mix 30ul of sample and add 10ul of R2 marker (make sure it is not expired). Vortex and spin down.
16. Load samples. Be sure to type the name of your samples in the screen to keep track of what they are and which well they are in.
17. Close blue pippin and press start. Press start again when it asks if you have calibrated.
18. Come back in 30 minutes, the blue pippin should have stopped running. Make sure the protocol progression has stopped moving forward and open the machine.
19. Remove the plastic covering the elution wells (there may be some condensation, don't worry about it). Using a p200 set at 40ul remove as much of the eluate as you can and put in your pre-marked 1.5ml centrifuge tube.
20. Remove cassette and close blue pippin before you leave.

Amplify and clean each 'sub-library'

1. The size-selected eluate will be used as template for PCR amplification of each sub-library. Amplification also adds an index onto each 'sub-library' so they can be pooled and run on a single flow cell lane. Each of the 12 'sub-libraries' should be amplified with a different indexed primer. Each library should be amplified in 8 different rxns to avoid PCR bias.
2. Set up eight PCR reactions (25ul) for each sub-library with the following master mix.

Reagent	1x	10x
5x QF Buffer	5ul	50ul
DNTPs (10mM)	1ul	10ul
Indexed Primer (10pmol/ul)	2ul	20ul
Universal Primer (10pmol/ul)	2ul	20ul
Q5 polymerase (2U/ul)	0.125ul	1.25ul
Template DNA	4ul	------
ddH20	10.875ul	108.75
	25ul	

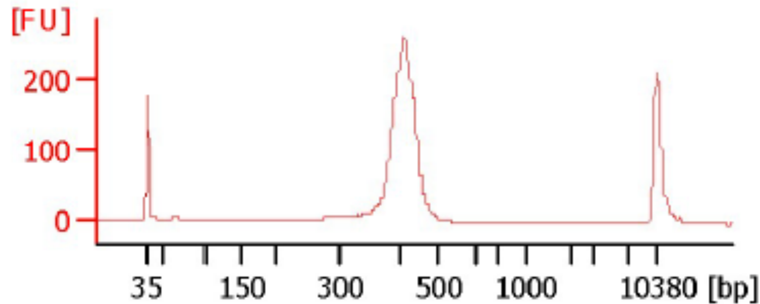
3. All eight reactions for 12 'sub-libraries' can be run in a single 96 well plate.
4. PCR conditions should be as follows:
   98C for 1 min
   10 cycles of:
   "98C for 8 sec
   68C for 20 sec
   72C for 20 sec"
   Final extension at 72C for 2 min
   Hold at 4C
5. For each sub-library, pool the eight PCR rxns and clean.
6. Add 300ul Homemade Ampure to tube, mix, and let sit at room temperature to bind DNA beads.
7. Place on magnet for ~2 minutes.
8. Remove supernatant.
9. Wash 1: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
10. Remove EtOH.
11. Wash 2: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
12. Remove EtOH. Be sure to use 10ul tip to remove residual EtOH at bottom of tube.
13. To ensure most EtOH is removed dry tubes at room temp for ~10 minutes. Still on magnet
14. Remove tubes from magnet and elute DNA from beads by adding 30ul ddH20. Pipet up and down to resuspend beads in water.
15. Place tubes back on magnet to separate beads from DNA solution. Wait ~5 min and transfer supernatant to a clean 96-well plate. Small amounts of bead carry over do not effect downstream reactions.

Quality Check and pool 'sub-libraries' together

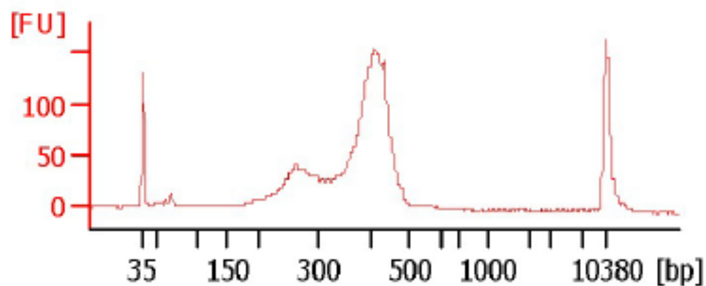
1. To determine quality and concentration, submit each 'sub-library' for Bioanalyzer analysis.
2. Pool equal molar amounts of each of the twelve sub-libraries into a single tube.
3. Measure volume of pooled libraries and add 1.5x volume Homemade Ampure to tube, mix, and let sit at room temperature to bind DNA beads.
4. Place on magnet for ~2 minutes.
5. Remove supernatant.
6. Wash 1: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
7. Remove EtOH.
8. Wash 2: Add 500ul 75% EtOH to each well and let sit for 1 min. Still on magnet
9. Remove EtOH. Be sure to use 10ul tip to remove residual EtOH at bottom of tube.
10. To ensure most EtOH is removed dry tubes at room temp for ~10 minutes. Still on magnet
11. Remove tubes from magnet and elute DNA from beads by adding 30ul ddH20. Pipet up and down to resuspend beads in water.
12. Place tubes back on magnet to separate beads from DNA solution. Wait ~5 min and transfer supernatant to a clean 1.5ml tube. Ensure not bead carry over into final library.
13. Submit library for sequencing.

Bioanalyzer Trace Analysis

Below is an example of what the bioanalyzer trace should look like for each 'sub-library' and the final pooled library.



Below is an example of a bioanalyzer trace for a 'sub-library' that has been 'over-amplified.' Libraries that look like this should not be sequenced as the vast majority of reads will come from the first hump at ~250bp.



Below is an example of a bioanalyzer trace for a 'sub-library,' which has excess primer at ~60bp. This excess primer will be removed during the final ampure step when 'sub-libraries' are pooled; it is not a concern.