A Rieseberg Lab Production

January 29, 2013

GBS v2

(Pronounced "jibs")

(Genotyping-By-Sequencing)

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Edd Buckler Lab

Refiner:

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Perfectors and Writers:

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Gregory Owens

REQUIRED REAGENTS

Reagent	Amount per 96 well plate
• 96 barcoded adapters (0.4ng/ul)	4.5ul of each
• Common adapters (0.4 ng/ul)	450ul
• Restriction enzyme PstI Hi-Fidelity	50ul
• NEB Buffer 4 (10X)	250ul
• BSA (10ug/ul)	50ul
• NEB T4 DNA Ligase (M0202L) – *NOTE: Do not purchase 'small' size as this is not enough for one plate.*	80ul
• NEB T4 DNA Ligase Buffer (10X)	500ul
• Qiaquick PCR purification kit	4 columns
• Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB, M0531S)	216ul

• GBS PCR primers A and B (12.5ug/ul each)	8 ul each
• Agencourt AMPure XP beads	386ul
• 100% ethanol	5 ml
• 80% ethanol	5 ml
• Qubit 2.0 Fluorometer high-sens buffer and tubes	1 reaction
• Hi-Sens DNA Bioanalyzer chip kit	1 chip per 11 samples
• KAPA Library Quant Kit	variable

Notes before starting:

1. The DNA you intend on using in this protocol must be QCd using both the Nanodrop to check purity and the Qubit to assess quantity. It must be at least 8.5 ng/ul or may be under represented in your data.

2. Previous protocols used 50 ng of DNA per sample while we use 100 ng. Others have had success with 50 ng using CTAB extracted DNA, while we used Qiagen column extracted DNA. It is possible that CTAB extracted DNA works better for GBS. Regardless, more DNA seems to be better

3. Many of these steps can be done using the Aitken EpMotion, they are specified with the robot icon.  See additional instructions on using the robot. If you want to use the robot, speak

to Kristin about it.

PROTOCOL

1.  Plates of barcoded adaptors need to be prepared. To do this, transfer 4.5 uL of each barcoded adaptor from the master plate into a new plate. It is good practice to do a bunch of these at one time to minimize the number of times that the master plate needs to be thawed.

2.  Add 100 ng of DNA into the adaptor plate in a maximum of 11.7 uL water. If less than 11.7 uL is needed to add the full amount of DNA, add water to equal 11.7 uL. This is an ideal step to be done using the EpMotion. See 'Normalizing a Plate with the EpMotion' for detailed instructions.

3.  Create a digestion/ligation master mix and add 13.8 ul to each well (EpMotion works for dispensing master mix).

Reagent	X1 sample	X100 sample
Buffer 4	2.5ul	250ul
PstI Hi-Fi	0.5ul	50ul
BSA	0.5ul	50ul
T4 Ligase	0.8ul	80ul
Ligase Buffer	5ul	500ul
Common Adapter	4.5ul	450ul

4. Incubate in the thermal cycler using the following protocol:

Temperature	Time
37°C	3 hours
22°C	3 hours
65°C	20 minutes
4°C	forever

5.  After thermal cycling, remove 30 uL from each well into a single 2.0 mL tube. Mix the contents and divide equally into two 2.0 mL tubes (Total volume of 2.88 ml). You must do this to accommodate the Qiaquick PCR purification buffer.

6. Clean up the two tubes using the Qiagen Qiaquick PCR purification kit using three columns to further concentrate. Elute in 150 uL TE buffer.

   - ***NOTE: DO NOT FREEZE THIS UNTIL YOU HAVE A COMPLETED LIBRARY. Greg and Kristin found that the PCR amplification does not work well using previously frozen template. We are not sure why this could be.***

   - NOTE: This uses a large amount of PB from the Qiagen PCR purification kit. As a cheaper alternative (pioneered by Allan DeBono) a solution of 4.5M Guanidine isothiocyanate 0.5M Potassium acetate pH 4.6, can be used 1:1 for the DNA binding step instead of PB. If this is used, an additional 80% ethanol wash (for a total of two washes) should be done.)

7. Perform PCR amplification (x 8 reactions). Each reaction will have a total volume of 25 uL. Immediately after the last extension, add additional primers and polymerase and complete one additional cycle (see below). It is convenient to make up the additional primer solution at the same time as the initial PCR solution, and store on ice before use.

Reagent	Volume x1	Volume x 8.15
H_2O	10.5ul	85.6ul
Phusion High-Fidelity PCR Master Mix with HF Buffer	12.5ul	101.9ul
GBS PCR primers A and B	0.5ul each	4.1ul each
Template from step #6	1ul	8.15ul

8. Cycle reactions using the following protocol:

Step	Temperature	Time
1	94°C	3 min
2	94°C	30 sec
3	65°C	30 sec
4	68°C	30 sec
5	Go to #2 17 more times	–
6	68°C	10 min
7	4°C	forever

9. Add additional primers and polymerase to each reaction:

Reagent	Volume x1	Volume x 8.15
Phusion High-Fidelity PCR Master Mix with HF Buffer	1ul	8.15ul
GBS PCR primers A and B	0.5ul each	4.1ul each

10. Cycle reactions using the following protocol:

Step	Temperature	Time
1	94°C	3 min
2	65°C	2 min
3	68°C	12 min
4	4°C	forever

11. Clean and size select your PCR reactions using the Agencourt AMPure XP beads. The magnetic beads work by binding DNA size selectively depending on the concentration of beads used. The DNA can then be eluted into a small volume with relatively little product loss. Before use, AMPure beads should be at room temp (let sit for 30 minutes before use) and well mixed (check for precipitate on the bottom)..

    - OVERVIEW: In the first step, 0.85X volume of beads are added, which selectively bind DNA fragments larger than 350bp. Shorter DNA fragments are removed with the supernatant. The DNA is eluted into water, and in the next step 0.5X beads are added. Here DNA fragments larger than 600 bp are bound by the beads, and elutant (containing 350-600 bp DNA fragments) is retained. A final 1.0X bead step is used to elute the DNA in water. By varying the concentration of beads added, a wider or narrower library can be obtained

12. Pool two 28 uL PCR reactions from the previous step into four wells with 58 uL in each.

13. Add 48 uL (0.85x) AMPure XP beads to each well and pipette 10 times to mix.

14. Incubate for 5 minutes at room temperature.

15. Set plate on magnet and allow solution to clear (approx. 2 minutes).

16. Remove and discard 104 uL from each well. There will be liquid remaining in each well.

17. Add 200 uL of freshly prepared 80% ethanol to each well. Do not remove plate from magnet during the ethanol wash steps.

18. Remove and discard ethanol.

19. Repeat steps 17 and 18 once with 80% ethanol and once with 100% ethanol for a total of three washes.

20. Remove plate from magnet and allow to dry for 3 minutes.

21. Add 27 uL water to each well. Pipette up and down and insure that all magnetic particles are removed from the sides of the well.

22. Incubate for 2 minutes at room temperature.

23. Place plate on magnet and allow solution to clear.

24. Remove 25 uL elute from each well and pool two wells together. Now you will have two wells with 50 uL in each well.

25. Add 25 uL (0.5x) AMPure XP beads to each well and pipette mix each well 10 times.

26. Incubate for 5 minutes at room temperature.

27. Place plate on magnet and let solution clear.

28. THIS STEP IS MOST IMPORTANT: Transfer 72 uL to a new well. Make sure not to transfer any beads.

29. Remove plate from magnet and add 72 uL (1.0x) AMPure XP beads to each well. Pipette mix each well 10 times.

30. Incubate plate for 5 minutes at room temperature.

31. Place plate on magnet and allow solution to clear.

32. Remove and discard 142 uL from each well. The will be liquid remaining in each well.

33. Add 200 uL of freshly prepared 80% ethanol to each well. Do not remove plate from magnet during the ethanol wash steps.

34. Remove and discard ethanol.

35. Repeat steps 33 and 34 once with 80% ethanol and once with 100% ethanol for a total of three washes.

36. Remove plate from magnet and allow to dry for 3 minutes.

37. Add 17 uL water to each well. Pipette mix to get all magnetic particles back into solution.

38. Incubate for 2 minutes at room temperature.

39. Place plate on magnet and allow solution to clear.

40. Transfer 15 uL from each well to a single PCR tube. You will have 30 uL of library in total.

41. Proceed to QC your completed library. Purchase Greg and Kristin coffee for making such a fabulous protocol.

42. Perform QC using the Bioanalyzer to assess fragment size range, the Qubit to assess concentration of library and qPCR to check that adaptors have been correctly ligated. Only sequence your library if it passes all three QC tests! Ideally, the Qubit, Bioanalyzer and qPCR should give the same concentration readings (corrected for size of insert), although qPCR is most pertinent for sequencing. The quality required for sequencing in Biodiversity are:

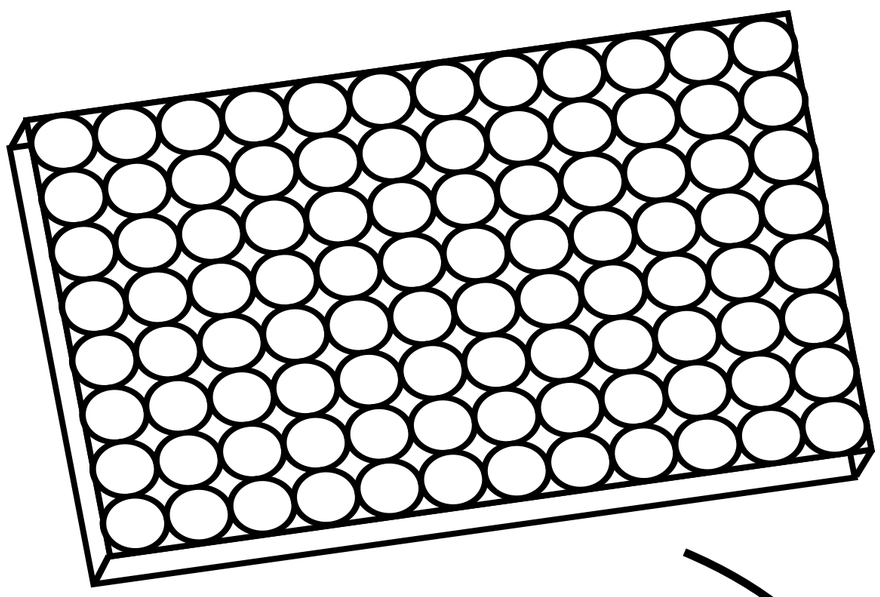
    - qPCR: Concentration should be greater than 2nM. Remember to take into account the average size of your library
    - Qubit: Greater than 0.5 ng/ul of total double stranded DNA.
    - Bioanalyzer: There should be a broad peak from 300 to 700 bp wide. It should be relatively smooth. Peak height is not necessarily quantitative but as a rough estimate your peak should be great than 50 FU in height. If your library has a size range that is too high, or primer dimers (approximately 140bp), both can be fixed by additional bead purification with only minimal loss of library concentration.

    As the Qubit is the cheapest and easiest test, generally you should start with that and not continue if your Qubit result is much lower than the requirement.

GBS Protocol Diagram

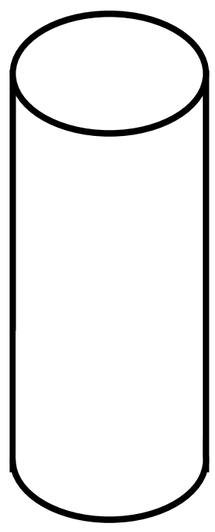
By Kristin Nurkowski and
Gregory Owens

DNA Plate



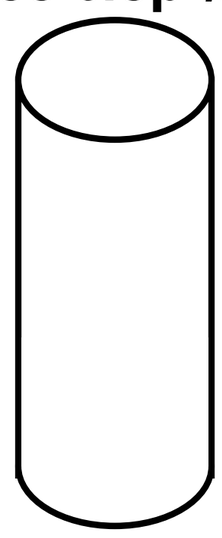
100 ng DNA per well (Max 11.7 ul)

dH2O



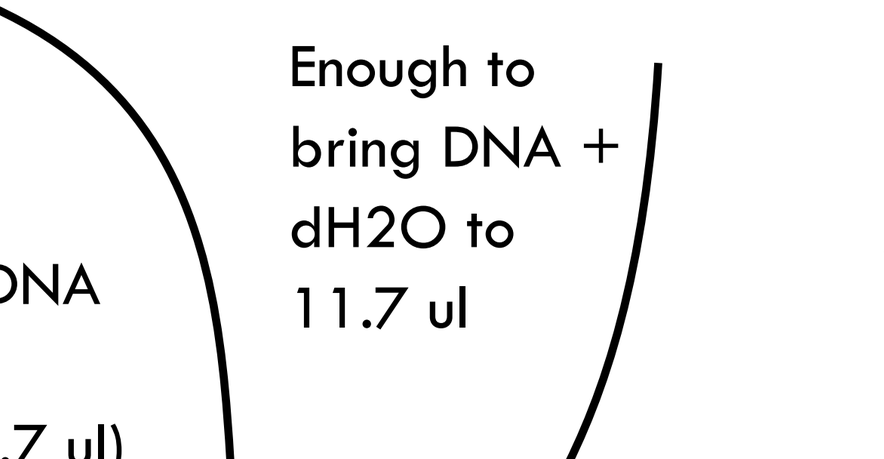
Enough to bring DNA + dH2O to 11.7 ul

Digestion-Ligation Master Mix
See step #3



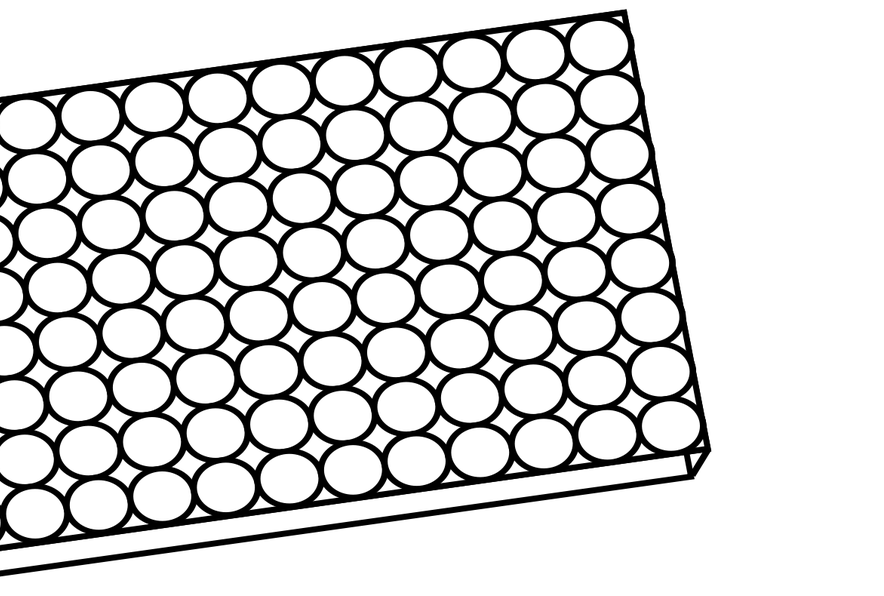
13.8 ul per well

Adapter plate

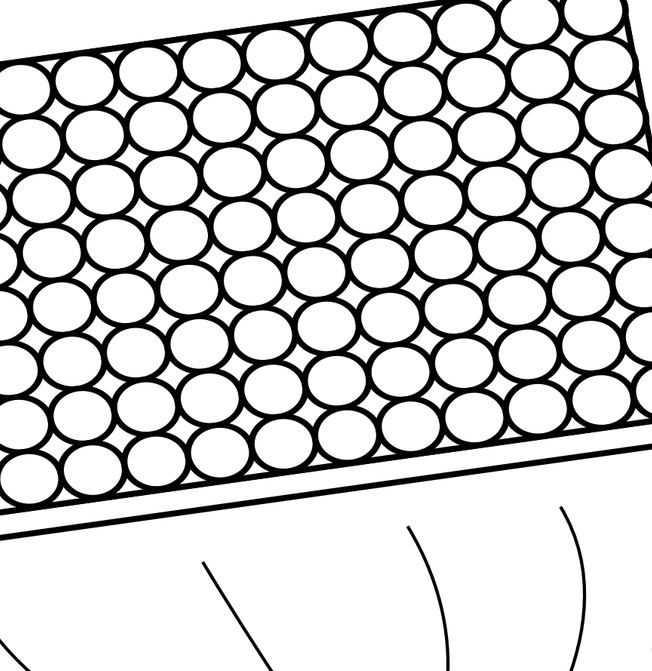


4.5 ul of specific adapter per well

Thermocycler for 6.5 hours
See step #4

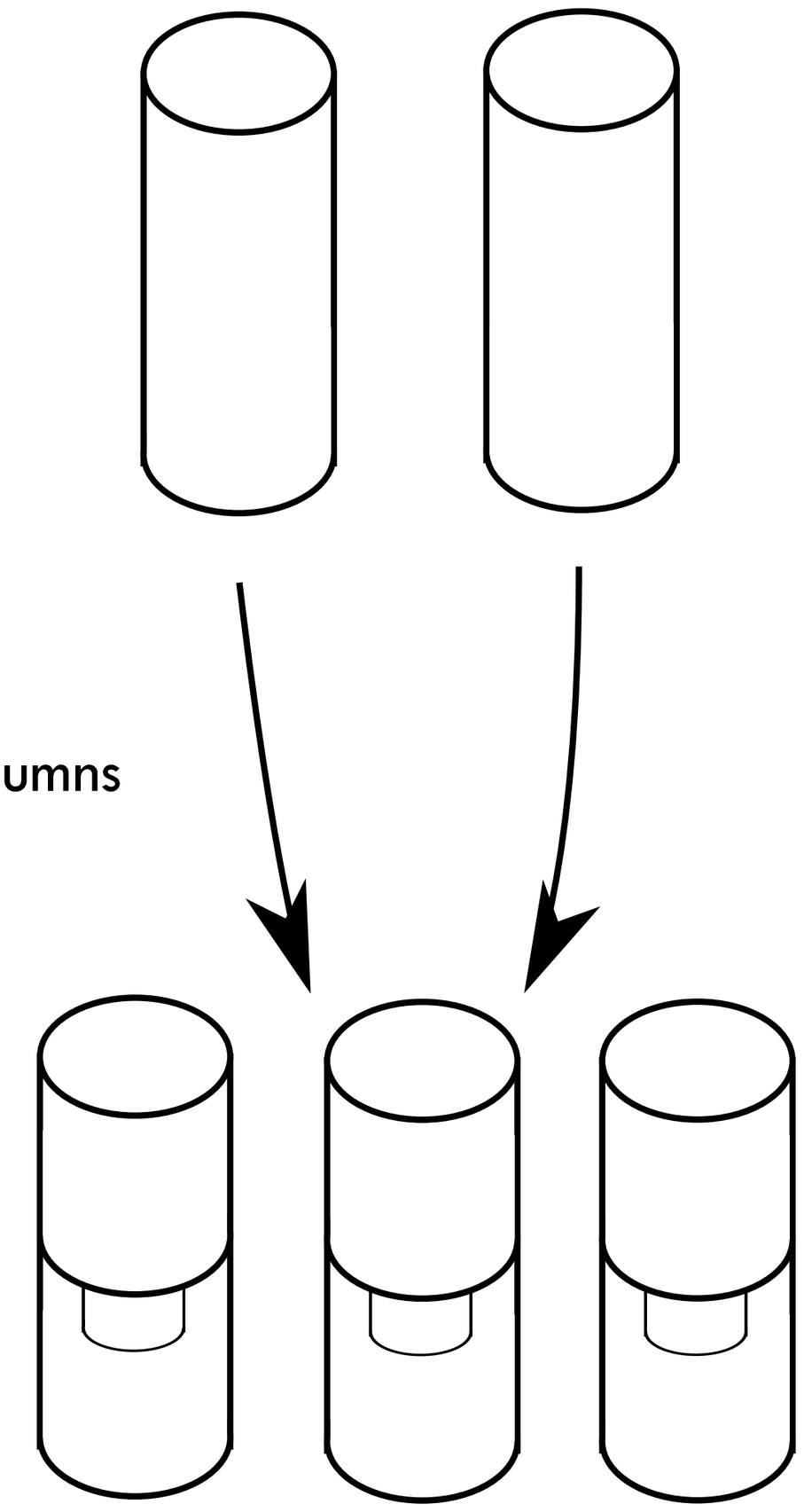


Remove 30ul from each well and pool

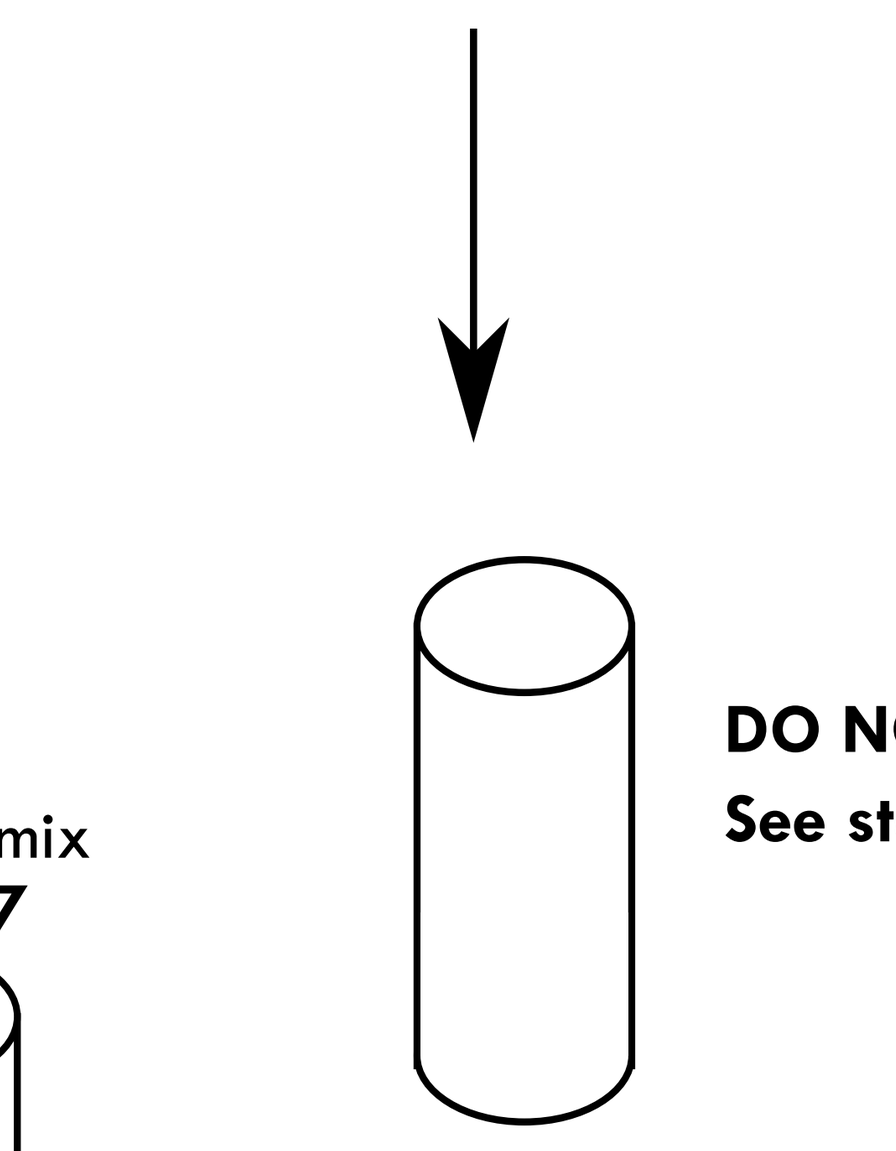


Divide into 2 tubes with 1440 ul each

Qiagen PCR
purification using 3 columns

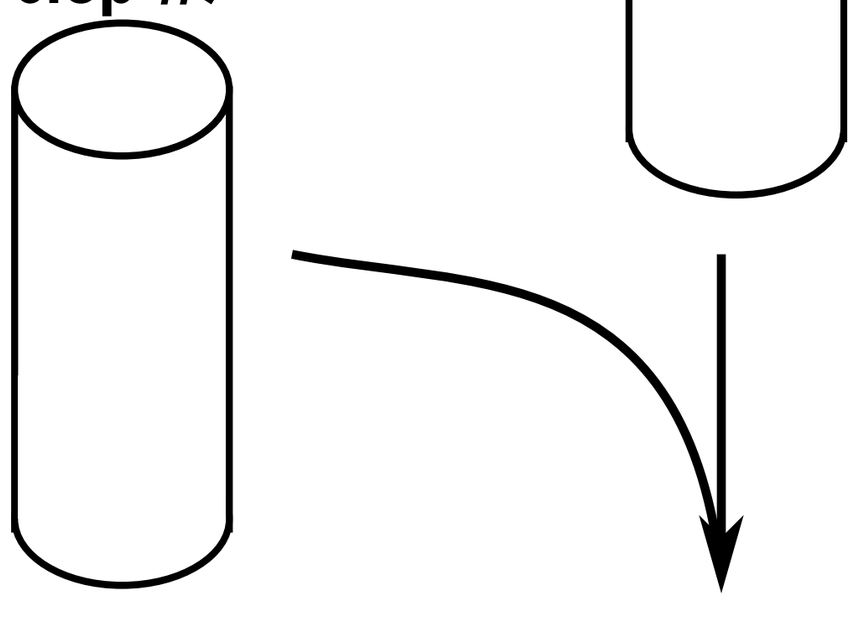


Elute in 150 ul
TE



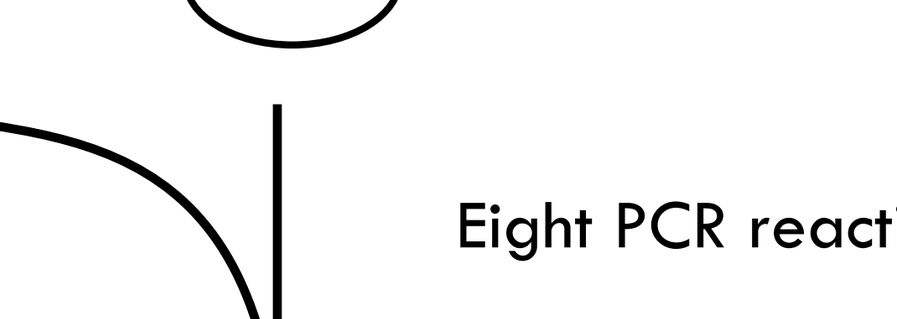
DO NOT FREEZE THIS
See step #6

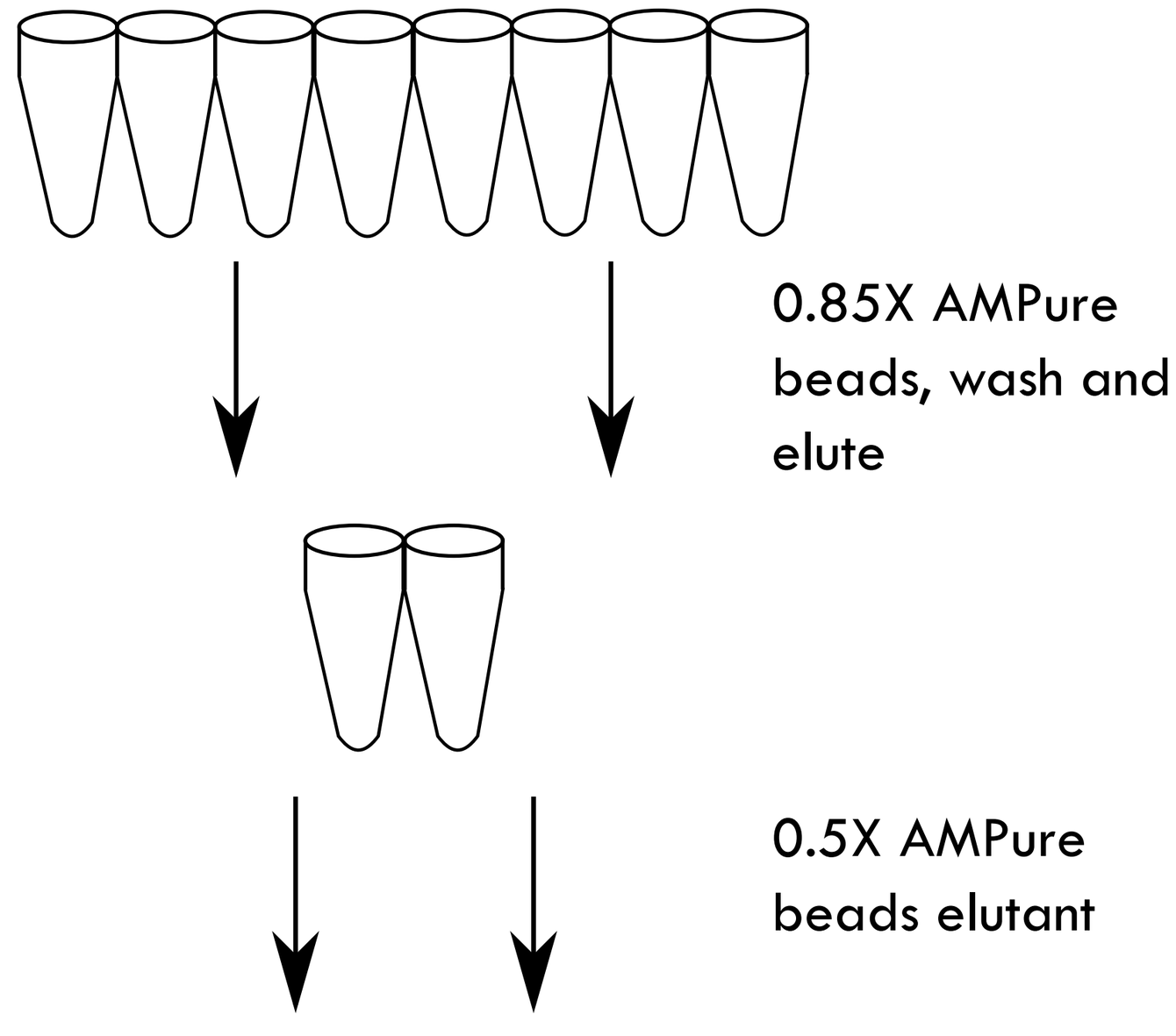
PCR master mix
See step #7



Eight PCR reactions

Thermocycling **See step #8**





0.85X AMPure beads, wash and elute

0.5X AMPure beads elutant

1.0X AMPure beads, wash and elute in 30 ul total

Completed Library

Quality Control:
-Qubit
-qPCR
-Bioanalyzer