Generating Libraries for Whole Genome Sequencing

by Robyn Tanny July 2014

This protocol uses reagents purchased from Illumina, Qiagen and Life Technologies:

Company	Kit	Catalog Number
Illumina	Nextera DNA Sample Preparation Kit	FC-121-1030
Illumina	Nextera Index Kit (24 indexes, 96 samples)	FC-121-1011
Illumina	TruSeq Dual Index Sequencing Primer Kit, Paired End	PE-121-1003
Qiagen	DNAEasy Blood and Tissue Kit	69506
Qiagen	MinElute PCR Purification Kit	28006
Life Technologies	Qubit dsDNA BR Assay Kit	Q32850
Life Technologies	Qubit dsDNA HS Assay Kit	Q32851
Life Technologies	Qubit Assay Tubes	Q32856

Generate DNA Samples

1. Isolate DNA according to the “Nematode DNA Isolation Using Qiagen DNAEasy Kit (cat# 69506)”.

2. Determine which samples will be amplified using which index primers.
   If you are indexing 24 libraries at once and plan to pool all 24 libraries, you don’t need to consider which libraries have which indexes. However, if you plan on pooling fewer than 24 libraries, you need to ensure that you combine the appropriate indexes. See Illimuna’s guide on “Nextera Low Plex Pooling Guidelines”.

3. Quantitate your samples using Qubit Broad Range dsDNA kit. Follow the protocol provided by Qubit.
   - Label enough Assay Tubes for your samples, standard 1 and standard 2.
   - Prepare the Qubit Working Solution by diluting the Qubit BR reagent 1:200 in Qubit buffer. Prepare 200 µl of Working Solution for each standard and sample.
   - For each standard, add 190 µl of of Working Solution to the appropriate tubes.
   - For each sample, add 198 µl of Working Solution to the appropriate tubes.
   - Add 10 µl of each standard to the appropriate tube.
   - Add 2 µl of each sample to the appropriate tube.
   - Briefly vortex all tubes.
   - Incubate the tubes for 2 minutes at room temperature.
   - Insert tubes in the Qubit Fluorometer to take readings.

4.Dilute each of your DNA samples to 2.5 ng/µl.

The concentration of your DNA will dictate the average length of molecule after the tagmentation process. Lower sample concentration will result in smaller-sized libraries and higher sample concentration will result in larger-sized libraries.

Tagmentation Reaction

5.Remove necessary tagmentation reagents (Tagmentation DNA Buffer, Tagment DNA enzyme and DNA samples) from -20°C storage and thaw on ice.

Make sure all reagents are mixed by gentle pipetting or gentle inversion followed by microcentrifugation.

6. In a 96-well plate, add 20 µl of each genomic DNA sample to each well.

7. Add 25 µl of Tagmentation DNA Buffer to wells containing genomic DNA. **Change tips between samples**.

Calculate the total volume of Tagmentation DNA Buffer for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the 96-well plate.

8. Add 5 µl of Tagment DNA Enzyme to the wells containing genomic DNA. **Change tips between samples**.

9. Using a multichannel pipette, gently pipette up and down to mix. **Change tips between samples**.

10. Cover the 96-well plate with an appropriate seal (plastic or foil).

11. Place the 96-well plate in a thermocycler and run the following program:

- have heated lid on
- 55°C for 5 minutes
- hold at 10°C.

Clean-Up Tagmentation Reaction

12. Add 5 volumes of Buffer PB to each reaction.

The tagmentation reaction is 50 µl, so you need to add 250 µl of Buffer PB. Note: The final volume of 300 µl comes close to the maximum volume capacity of a 0.2 ml 96-well plate. **Be careful when pipetting!**

13. Place a labeled MinElute column in a provided 2 ml collection tube in a suitable rack.

14. To bind DNA, apply the sample to the labeled MinElute column and centrifuge for 1 min.

15. Discard flow-through. Place the MinElute column back into the same tube.

16. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min.

17. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

18. Place the MinElute column in a clean, labeled 1.5 ml microcentrifuge tube.

19.To elute DNA, add **11 µl** Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA. The average eluate volume is 9 µl from 10 µl elution buffer volume.

PCR Set-Up

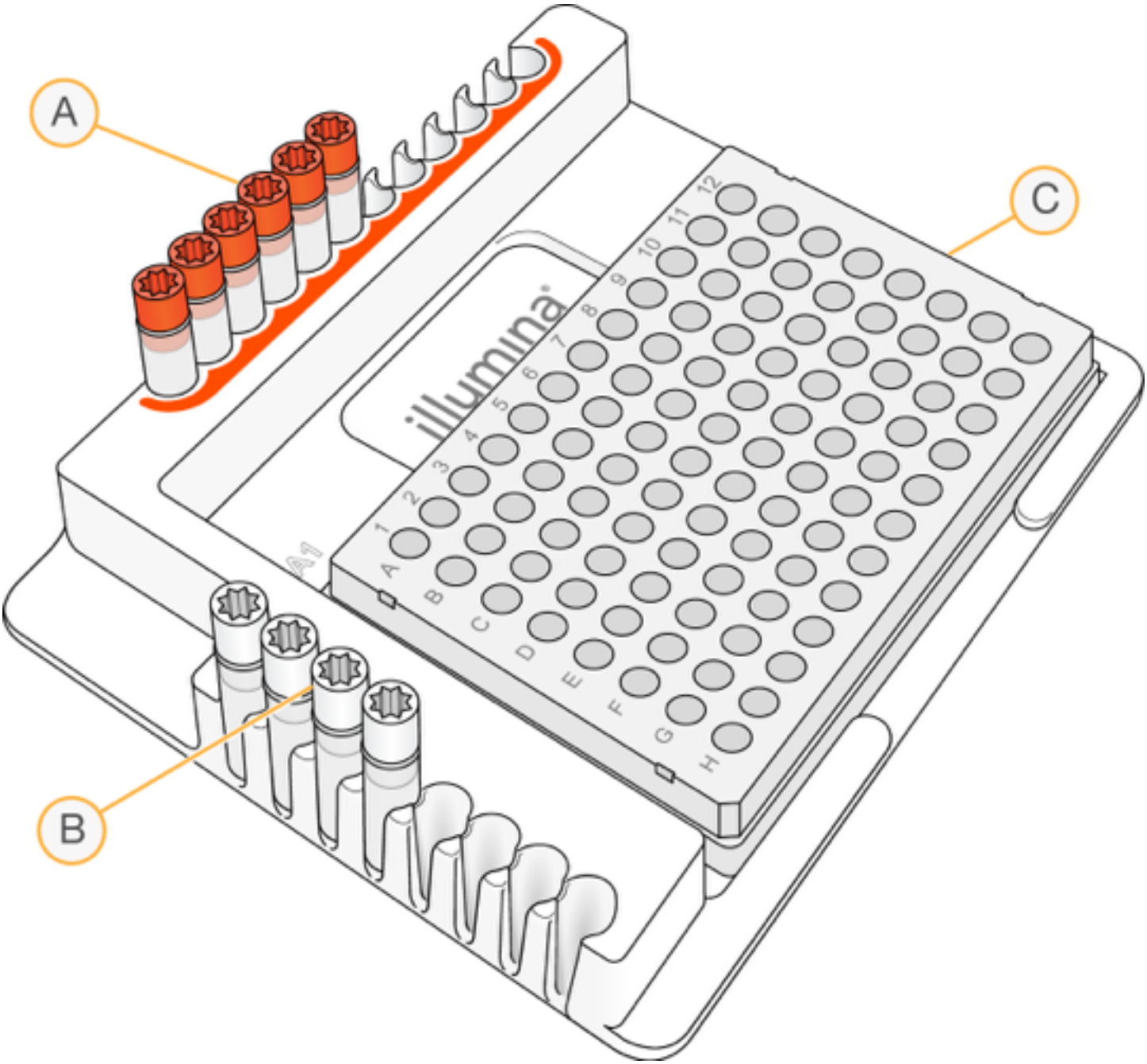
20. If the full set of 24/96 libraries is to be prepared for pooling and sequencing proceed to the next step. If less than a full set of libraries is pooled for sequencing, ensure that the correct index 1 (i7) and index 2 (i5) primers have been selected. See the *Dual Indexing and Low Plexity Pooling Guidelines* section at the end of this protocol, and use the Illumina Experiment Manager to verify that the correct index primers have been selected.

21. Remove the Nextera PCR Master Mix, the PCR Primer Cocktail and the index primers from the freezer and thaw at room temperature.

22. After all reagents are completely thawed, gently invert each tube 3–5 times to mix and briefly centrifuge the tubes in a microcentrifuge. Use 1.7 ml Eppendorf tubes as adapters for the microcentrifuge.

23. For 24 libraries arrange the index primers in the TruSeq Index Plate Fixture using the following arrangement:

a. Arrange index 1 (i7) primers (orange caps) in order horizontally, so N701 is in column 1 and N706 is in column 6.
b. Arrange index 2 (i5) primers (white caps) in order vertically, so N501(or N517) is in row A and N504 is in row D.
c. Record their positions on the Lab Tracking Form, if you are using.



24. Label a new 96-well microplate.

25. Using a multichannel pipette, add 5 µl of index 2 primers (white caps) to each column of the NAP1 plate. Changing tips between columns is required to avoid cross-contamination.
26.Using a multichannel pipette, add 5 µl of index 1 primers (orange caps) to each row of the NAP1 plate. ***Tips must be changed after each row to avoid index cross- contamination.***

27. To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps provided in the kit.

28. To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps provided in the kit. Remove all the index primer tubes from the working area.

29. Add 15 µl of Nextera PCR Master Mix to each well containing index primers in your 96-well plate. Change tips between samples.

Calculate the total volume of Nextera PCR Master Mix for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the 96-well plate.

30. Add 5 µl of PCR Primer Cocktail to each well containing index primers and Master Mix in your 96-well plate. Change tips between samples.

Calculate the total volume of PCR Primer Cocktail for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the 96-well plate.

31. Add 10 µl of your cleaned-up tagmentation reaction to the appropriate well in the 96-well plate.

Be very sure to add each sample to the appropriate combination of primers!

32. Add 10 µl of dH_2O to each well. Change tips between samples.

Calculate the total volume of dH_2O for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the 96-well plate.

Gently pipette up and down to mix the samples.

33. Cover the plate with a foil and seal well with a roller.

34. Perform PCR using the following program on a thermocycler:

- Have heated lid **on**.
- 72°C for 3 minutes
- 98°C for 30 seconds
- 5 cycles of:
    - 98°C for 10 seconds
    - 63°C for 30 seconds
    - 72°C for 3 minutes
- Hold at 10°C

Pool and Purify Samples

35. Determine the concentration of each sample using the Qubit HS dsDNA kit. Follow the protocol

- Label enough Assay Tubes for your samples, standard 1 and standard 2.
- Prepare the Qubit Working Solution by diluting the Qubit HS reagent 1:200 in Qubit buffer. Prepare 200 µl of Working Solution for each standard and sample.
- For each standard, add 190 µl of of Working Solution to the appropriate tubes.
- For each sample, add 198 µl of Working Solution to the appropriate tubes.
- Add 10 µl of each standard to the appropriate tube.
- Add 2 µl of each sample to the appropriate tube.
- Briefly vortex all tubes.
- Incubate the tubes for 2 minutes at room temperature.
- Insert tubes in the Qubit Fluorometer to take readings.

36. Combine the appropriate samples so that each sample contributes 100 ng of DNA.

37. Add the appropriate amount of 6X DNA dye. Make sure to use dye without bromophenol blue as this dye might obscure your product on the gel.

38. Electrophorese the samples on a 2% agarose gel.

You will probably have to run each pooled library in two lanes because there is too much volume. Also, make sure to leave an empty lane between different pooled libraries if you are electrophoresing multiple pooled libraries at the same time.

39. Excise the DNA that ranges from 450 bp - 600 bp.
    The size you excise depends on the machine on which you sequence your samples. For the HiSeq2500, we excise bands from 450 bp - 600 bp. For the HiSeqX10, we would excise bands around 350 bp.

    If you are excising bands from different pooled libraries, make sure to either use a new razor blade for the different libraries or spray your razor blade with 95% ethanol and wipe it off between different libraries.

40. Weigh the gel slice in a clear microcentrifuge tube. Add 3 volumes of Buffer QG to 1 volume of gel (e.g. - add 300 µl of Buffer QG to each 100 mg of gel).

41. Incubate at 50°C for 10 minutes or until the gel slice has completely dissolved.

42. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.

43. Place a labeled MinElute column in a provided 2 ml collection tube in a suitable rack.

44. To bind DNA, apply the sample to the labeled MinElute column and centrifuge for 1 min.

45. Discard flow-through. Place the MinElute column back into the same tube.

46. Add 500 µl of Buffer QG to the spin column and centrifuge for 1 min.

47. Discard flow-through. Place the MinElute column back into the same tube.

48. To wash, add 750 µl Buffer PE to the MinElute column, incubate at room temperature for 2 min and centrifuge for 1 min.

49. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.
    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

50. Place the MinElute column in a clean, labeled 1.5 ml microcentrifuge tube.

51. To elute DNA, add **11 µl** Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.
    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA. The average eluate volume is 9 µl from 10 µl elution buffer volume.

52. Quantitate your pooled libraries using the Qubit HS kit. (See above for directions).

53. Determine molarity on this site: http://molbiol.edu.ru/eng/scripts/01_07.html.