Genome Skimming Library Preparation

Adapted from manufacturer's protocol and Glenn *et al* 2019
Updated 11-Mar-2024 by Katie Murphy

BACKGROUND:

This library construction kit and protocol is the same that you could use for general whole genome sequencing (WGS) library preparation. However, genome skimming will often have success with lower input and/or degraded gDNA compared to WGS where you intend to get entire nuclear genomes.

Please note that this protocol utilizes the NEBNext Ultra II FS DNA Library Prep Kit, which includes enzymatic fragmentation. We have chosen to use enzymatic fragmentation over sonication because it limits sample handling and speeds up library handling. However, this method may not be ideal for other applications of WGS; for example, if your ultimate goal is actually a full nuclear genome rather than genome skimming, you should weigh the pros and cons of different fragmentation methods.

Because our protocol includes fragmentation, end repair, and dA-tailing all in a single step, you ***cannot*** just omit the fragmentation step if you wish to skip fragmentation due to old/degraded samples. If you wish to use the same NEB FS kit as below but skip fragmentation for some of your samples, you will need to purchase a separate end repair and dA-tailing module (NEB # E7546S or E7546L); you can then treat your samples with this following manufacturer's protocol, and then begin this protocol from step 2.1 afterwards. Alternatively if you wish to skip fragmentation for **all** of your samples, you could use NEBNext Ultra II DNA Library Prep Kit (NEB # E7645S or E7645L) and follow manufacturer's protocol for End Prep (Step 1); then use this protocol for Step 2 onwards.

HELPFUL HINTS:

- This protocol uses half reaction volumes compared to the manufacturer's protocol.
- Input DNA can range from 100 pg to 500 ng. However, it's best to have >> 1 ng, as the more DNA you have, the better your DNA diversity and coverage should generally be.
- We recommend using a double-sided bead cleanup in Step 3 for size selection. In cases of very low input, you can use a single 0.9X cleanup instead, but this will retain many smaller fragments.
- Input DNA can be in 1X TE, 0.1X TE, or 10 mM Tris. Recommended pH is 8.0, but 7.5 is acceptable.
- The total volume of input DNA must be 13 µL. If using less than this, bring the volume up to 13 µL using 1X TE, 0.1X TE, or 10 mM Tris.

MATERIALS:

- NEBNext Ultra II FS DNA Library Prep Kit for Illumina (catalog # E7805S or E7805L)
- PCR plate and seals, or PCR strip tubes
- iTru y-yoke adapter (5 µM)
- 10 µM i7 iTru index plate(s) & 10 µM i5 iTru index plate(s)
- KAPA Pure beads (or AMPure XP)
- Magnetic rack for bead separation
- Freshly prepared 80% ethanol
- 10 mM Tris, pH 8 or 0.1X TE

PROTOCOL:

Step 1 – Fragmentation/End Prep

1.1 Retrieve the Ultra II FS reaction buffer (yellow cap) from the freezer and thaw at room temperature. Vortex to mix. If there is white precipitate, warm the buffer at 37°C briefly, then pipette up and down to break up the precipitate. Spin briefly, then keep on ice until use.

1.2 Retrieve the Ultra II FS enzyme mix from the freezer and vortex for 5-8 seconds. Spin briefly, then place on ice until use. *Vortexing the enzyme mix is critical – do not omit this step!*

1.3 Set up the following reactions in a PCR plate or strip tubes on ice. It is acceptable to make a master mix of the FS reaction buffer, enzyme mix, and any TE or Tris as appropriate.

Component	Volume per library (µL)	Master Mix (x _____reactions)
DNA	X	----
1X TE[1]	(13 - X)	
NEBNext Ultra II FS Reaction Buffer	3.5	
NEBNext Ultra II FS Enzyme Mix	1	
Total	**17.5**	

[1] Can also use 0.1X TE or 10 mM Tris

1.4 Vortex the reactions to mix, then spin briefly.

1.5 Place your reactions into a thermocycler and run the following program, ensuring that ***the heated lid is set to 75°C:***

Temperature	Time
37°C	5-10 min[1]
65°C	30 min
10°C	Hold

[1] This time can be adjusted from 5-30 minutes, pending your desired fragment size. You may need to optimize this for your specific sample type.

1.6 We recommend that you continue directly to the adapter ligation (Step 2). *If absolutely necessary, you can pause here and store your samples at -20°C. However, expect to see a loss in yield if so.*

Step 2 – Adapter Ligation

2.1 Remove your FS reactions (step 1.5) from the thermocycler, spin briefly, and place on ice.

2.2 Remove the NEBNext Ultra II Ligation Master Mix (red cap), NEBNext Ligation Enhancer, and iTru y-yoke adapter from the freezer and thaw at room temperature. Once thawed, pipette or vortex each to mix, spin briefly, and place on ice until use.

2.3 Assemble the following ligation reactions using the following steps:

a. Make a pre-mix of the Ligation Master Mix and Ligation Enhancer (make sure to calculate excess, to allow for pipetting error). Vortex to mix and spin briefly.

Component	Volume per library (µL)	Ligation Pre-Mix (x _____reactions)
FS reaction mixture (step 1.5)	17.5	------
NEBNext Ultra II Ligation Master Mix	15	
NEBNext Ligation Enhancer	0.5	
5 µM iTru y-yoke adapter	1.25	------
Total	**34.25**	

b. Add 15.5 µL of the ligation pre-mix to your samples on ice. Make sure to change tips between samples.
c. Next, add 1.25 µL of y-yoke adapter to each tube.
d. Set a P200 pipette to 25 µL, then pipette your reactions up and down 10 times to mix. Spin reactions briefly.

2.4 Incubate your reactions in a thermocycler with the following protocol, making sure ***the heated lid is off:***

Temperature	Time
20°C	15 min
10°C	Hold

2.5 Continue to on to Step 3 (Size Selection). Samples may also be safely stored at -20°C overnight if needed.

Step 3 – Size Selection of Adapter-ligated DNA

If possible, we recommend following step 3A, the double-sided bead cleanup to select for specific library sizes. Make sure to select the size most appropriate for your needs. Alternatively, you can use a 0.9X cleanup (Step 3B) instead of this double-sided cleanup if you prefer to skip size selection.

3A. Size Selection using a double-sided bead cleanup.

Pick the size that is most appropriate based on your sequencing plans. In most genome skimming cases, insert sizes of 275-475 bp or 350-600 bp will be best.

3A.1 Allow KAPA beads to come to room temperature (~30 minutes). While you wait, make fresh 80% ethanol.
3A.2 Briefly spin your samples, then set at room temperature.
3A.3 Bring to volume of your samples up to 50 µL by adding 15.75 µL 10 mM Tris (or 0.1X TE).
3A.4 Vortex the KAPA Pure Beads to thoroughly resuspend.
3A.5 Select the most appropriate bead ratios using the below table, based on your desired library size.

	Approx. insert size	150-250 bp	200-350 bp	275-475 bp	350-600 bp
LIBRARY PARAMETERS	Approx. final library size	270-370 bp	320-470 bp	400-600 bp	470-800 bp
BEAD VOLUME TO BE ADDED	1st bead addition	20 µL	15 µL	12.5 µL	10 µL
	2nd bead addition	10 µL	7.5 µL	5 µL	5 µL

3A.6 Add your ***first*** bead addition to your samples, based on the table above. Mix thoroughly by pipetting up/down 10 times with a P200 pipette. Alternatively, you can vortex for 3-5 seconds if your samples are sealed.
3A.7 Incubate the samples at room temperature for 5 minutes.
3A.8 Spin briefly to collect liquid to the bottom of the tubes.
3A.9 Plate the tubes/plate on a magnetic stand. Wait for beads to pellet and supernatant to clear, which should take 3-5 minutes.
3A.10 Carefully transfer all of the supernatant to a new tube/plate, making sure to not disturb the bead pellet.
a. ***Do NOT discard the supernatant! It currently contains the DNA you want!***
b. Discard the original tubes/plate that now only contain pelleted beads.

3A.11 To the supernatant in the new tubes/plate, add your ***second*** bead addition. Mix thoroughly by pipetting up/down 10 times with a P200 pipette. Alternatively, you can vortex for 3-5 seconds if your samples are sealed.
3A.12 Incubate the samples at room temperature for 5 minutes.
3A.13 Spin briefly to collect liquid to the bottom of the tubes.
3A.14 Plate the tubes/plate on a magnetic stand. Wait for beads to pellet and supernatant to clear, which should take 3-5 minutes.
3A.15 Carefully remove and discard the supernatant, making sure to not disturb the bead pellet.
3A.16 Add 200 µL of freshly prepared 80% ethanol to your samples. Do not mix. Incubate at room temperature for 30 seconds.
3A.17 Carefully remove and discard the ethanol using a P200 pipette.
3A.18 Repeat the ethanol wash (steps 3A.16-17).
3A.19 After removing the second ethanol wash, spin the tubes/plate briefly and return to the magnet. Carefully remove any residual ethanol using a P10 or P20 pipette.
3A.20 Allow beads to air dry for 2 minutes.
    a. All ethanol should be gone, but you do not want the beads to appear dry/cracked. Beads should appear relatively dark brown and glossy.
3A.21 Remove your samples from the magnet. Add 9 µL of 10 mM Tris (or 0.1X TE) to each well and use a pipette to fully resuspend the beads. Alternatively, you can vortex to resuspend if your samples are sealed.
3A.22 Incubate at room temperature for 3-5 minutes.
3A.23 Spin samples briefly, then place back on the magnet for ~3 minutes, or until the supernatant clears.
3A.24 Carefully transfer 7.5 µL of the clear supernatant to a new plate/tube, making sure to not disturb the bead pellet.
3A.25 After the cleanup, proceed to the Indexing PCR (Step 4). Alternatively, samples may be stored at -20°C.

3B. Single 0.9X bead cleanup (no size selection)
You may opt for this protocol instead of 3A when you have extremely low amounts of input DNA, as a single-sided bead selection will result in less sample loss than a double-sided selection. However, this will typically result in a smaller average library size (bp).

3B.1 Allow KAPA beads to come to room temperature (~30 minutes). While you wait, make fresh 80% ethanol.
3B.2 Briefly spin your samples, then set at room temperature.
3B.3 Bring to volume of your samples up to 50 µL by adding 15.75 µL 10 mM Tris (or 0.1X TE).
3B.4 Vortex the KAPA Pure Beads to thoroughly resuspend.
3B.5 Add 45 µL beads (0.9X) to each sample. Mix thoroughly by pipetting up/down 10 times with a P200 pipette. Alternatively, you can vortex for 3-5 seconds if your samples are sealed.
3B.6 Incubate the samples at room temperature for 5 minutes.
3B.7 Spin briefly to collect liquid to the bottom of the tubes.
3B.8 Plate the tubes/plate on a magnetic stand. Wait for beads to pellet and supernatant to clear, which should take 3-5 minutes.
3B.9 Carefully remove and discard the supernatant, making sure to not disturb the bead pellet.
3B.10 Add 200 µL of freshly prepared 80% ethanol to your samples. Do not mix. Incubate at room temperature for 30 seconds.
3B.11 Carefully remove and discard the ethanol using a P200 pipette.
3B.12 Repeat the ethanol wash (steps 3B.10-11).

3B.13 After removing the second ethanol wash, spin the tubes/plate briefly and return to the magnet. Carefully remove any residual ethanol using a P10 or P20 pipette.

3B.14 Allow beads to air dry for ~2 minutes.

a. All ethanol should be gone, but you do not want the beads to appear dry/cracked. Beads should appear relatively dark brown and glossy.

3B.15 Remove your samples from the magnet. Add 9 µL of 10 mM Tris (or 0.1X TE) to each well and use a pipette to fully resuspend the beads. Alternatively, you can vortex to resuspend if your samples are sealed.

3B.16 Incubate at room temperature for 3-5 minutes.

3B.17 Spin samples briefly, then place back on the magnet for ~3 minutes, or until supernatant clears.

3B.18 Carefully transfer 7.5 µL of the clear supernatant to a new plate/tube, making sure to not disturb the bead pellet.

3B.19 Proceed to the Indexing PCR (Step 4). Alternatively, samples may be stored at -20°C.

Step 4 – Indexing PCR

4.1 Retrieve your index plates from the freezer and thaw at room temperature. Once thawed, vortex and spin briefly.

a. If you are getting new aliquots from LAB's freezer, make sure to pay for them on the iPad near the door.

4.2 Retrieve the NEBNext Ultra II Q5 Master Mix (blue cap) from the freezer and thaw if frozen. Vortex and spin briefly, then place on ice.

4.3 Set up the following PCR reactions by adding master mix and index primers to your samples on ice. Each sample should receive a unique i7 and i5. Make sure to record what sample gets which indices.

Component	Volume per library (µL)
cleaned, ligated DNA fragments (from step 3A.35 or 3B.19)	7.5
NEBNext Ultra II Q5 Master Mix	12.5
10 µM iTru i7 index primer	2.5
10 µM iTru i5 index primer	2.5
Total	**25**

4.4 Set a P200 pipette to 20 µL, then pipette your reactions up/down 10 times to mix thoroughly. *Avoid making bubbles, as this may reduce PCR success!* Spin briefly.

4.5 Place your reactions into a thermocycler with a ***105°C heated lid***, and use the following cycling protocol:

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	3-13[1]
Annealing & Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	10°C	Hold	1

[1] Select the most appropriate number of cycles based on amount of input DNA you used (see table on right). We often recommend ~8 cycles as a starting point. Please note that 3 cycles is the absolute minimum required.

Initial amount of input DNA	Recommended[1] # of PCR cycles
100+ ng	5-6
50 ng	7-8
10 ng	8-10
5 ng	8-10
1 ng	8-11
0.5 ng	8-12
0.1 ng	10-13

[1] These are just an initial starting point if you're looking for guidance. We find that 8-10 cycles often works for most people.

Step 5 – PCR Cleanup

5.1 Allow KAPA beads to come to room temperature (~30 minutes). While you wait, make fresh 80% ethanol.
5.2 Briefly spin your samples, then set at room temperature.
5.3 Vortex the KAPA Pure Beads to thoroughly resuspend.
5.4 Add 20 µL beads (0.8X) to each sample. Mix thoroughly by pipetting up/down 10 times with a P200 pipette. Alternatively, you can vortex for 3-5 seconds if your samples are sealed.
5.5 Incubate the samples at room temperature for 5 minutes.
5.6 Spin briefly to collect liquid to the bottom of the tubes.
5.7 Plate the tubes/plate on a magnetic stand. Wait for beads to pellet and supernatant to clear, which should take 3-5 minutes.
5.8 Carefully remove and discard the supernatant, making sure to not disturb the bead pellet.
5.9 Add 200 µL of freshly prepared 80% ethanol to your samples. Do not mix. Incubate at room temperature for 30 seconds.
5.10 Carefully remove and discard the ethanol using a P200 pipette.
5.11 Repeat the ethanol wash (steps 10-11).
5.12 After removing the second ethanol wash, spin the tubes/plate briefly and return to the magnet. Carefully remove any residual ethanol using a P10 or P20 pipette.
5.13 Allow beads to airdry for ~2 minutes.
    a. All ethanol should be gone, but you do not want the beads to appear dry/cracked. Beads should appear relatively dark brown and glossy.
5.14 Remove your samples from the magnet. Add 21.5 µL of 10 mM Tris (or 0.1X TE) to each well and use a pipette to fully resuspend the beads. Alternatively, you can vortex to resuspend if your samples are sealed.
    a. Note – you can adjust this volume higher or lower if preferred.
5.15 Incubate at room temperature for 3-5 minutes.
5.16 Spin samples briefly, then place back on the magnet for ~3 minutes, or until the supernatant clears.
5.17 Carefully transfer 20 µL of the clear supernatant to a new plate/tube, making sure to not disturb the bead pellet.
5.18 Your libraries are now complete and ready for QC! They should be stored at 20°C when not in use.

Your libraries are now ready for QC (quality control)! The concentration of each individual library should be measured with Qubit or Quant-iT. Library size and quality can be assessed using the TapeStation or Bionanalyzer.

Libraries should be pooled in equimolar ratios for sequencing.

ADDITIONAL INFORMATION:

- When you need to convert from ng/ul to nM, please use the following equation. Average size is determined using the TapeStation or Bioanalyzer, and concentration in ng/µL measured using Qubit or Quant-iT.

$$\frac{(\text{concentration in ng}/\mu\text{L})}{(660\ \text{g/mol}) \times (\text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

- If libraries show an adapter-dimer peak around 130 bp on the TapeStation or Bioanalyzer, you should perform an additional 0.8X bead cleanup.

- iTru y-yoke adapter and index sequences can be found in Glenn *et al* (2019).

REFERENCES:

1. New England Biolabs. 2023. NEBNext Ultra II FS DNA Library Prep Kit for Illumina: NEB #E7805S/L, #E6177S/L (Version 4.0_7/23).

2. Glenn TC, Nilsen RA, Kieran TJ, Sanders JG, Bayona-Vásquez NJ, Finger JW, Pierson TW, Bentley KE, Hoffberg SL, Louha S, Garcia-De Leon FJ, del Rio Portilla MA, Reed KD, Anderson JL, Meece JK, Aggrey SE, Rekaya R, Alabady M, Belanger M, Winker K, Faircloth BC. 2019. Adapterama I: universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). PeerJ 7:e7755 http://doi.org/10.7717/peerj.7755.