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Next generation shotgun library preparation for Illumina sequencing - low volume

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ABSTRACT

Adapted from: https://www.protocols.io/view/low-volume-methodology-for-nextera-dna-flex-librar-be6rjhd6 and Julio Avelar-Barragan protocol from Katrine Whiteson's lab

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Objective: To prepare a DNA sequencing library for Illumina sequencing.
This protocol can be done over two days, with a stopping point after PCR amplification.
This protocol presents a low volume methodology for the Illumina DNA Prep Kit (96 Samples). This method increases the number of sequencing libraries which can be generated using each kit from 384 samples (4 x 96-wells) to 864 samples (9 x 96-wells).

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MATERIALS TEXT

●Previously known as Nextera DNA Flex Library Prep is now called Illumina DNA Prep (Cat. No. 20025519, 20025520, 20018704, and 20018705), which includes:
○Sample purification beads (SPB) (15052363)
○Tagmentation stop buffer (TSB) (20015077)
○Tagmentation wash buffer (TWB) (20015079)
○Resuspension buffer (RSB) (15026770)
○Enhanced PCR mix (20015172)
○Bead-linked transposome (BLT) (20015173)
○Tagmentation buffer (TB1) (20015171)

●Primers used w/the Kapa HiFi Ready Mix:
○KAPA-PCR-F: AATGATACGGCGACCACCG*A
○KAPA-PCR-R: CAAGCAGAAGACGGCATACG*A
■Order these from IDT. The * is a phosphothioate bond that prevents polymerases w/ 3'-5' proof-reading activity from chewing back the oligos.
■They need to be diluted to 10 uM for protocol.
●Proteinase K (300 ug/mL):
○For a 1mL solution of 300 ug/mL, add 15uL of 20mg/mL proteinase K to 985uL of nuclease-free water.
○This is only required if you will use the stopping point. Otherwise you can just move forward without adding proteinase

●2X Kapa HiFi Ready Mix (Cat number Kk2602)
●I5 & i7 index barcodes
○Order from IDT. Will need to be diluted to 1 uM for this protocol.
●Ice / ice bucket (3 or so)
●Thermal cycler
●Tips
○1-10uL filter tips (x# boxes)
○200 uL filter tips (x# boxes)
●Pipette
○8-channel pipette
●No rim, low-profile (0.1mL) PCR plates (depends on the PCR machine used)
●8-strip PCR tubes or plates
●PCR seals
●2X 96-well plate magnets (from V&P Scientific)
●Eppendorf tubes
●Sterile reservoirs

PREPARATION

1 Take your DNA extractions from **-20 °C** and thaw on ice - spin down on bench spinner.

2 ✱

Optional: Measure DNA concentration of your DNA extractions with Qubit (see Qubit protocol).

3 ✱

Optional: Based on the DNA concentrations adjust the samples to ~equal ng for library prep.

The input volume is **5 µl** of DNA. The input range in ng can be **35 ng** to **50 ng**

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The input amount is not as critical hence step 3 is optional and if too much DNA is added it will be washed away in the downstream steps. As low as 2.5 ng input DNA for some samples created a successful library.
If low biomass samples are handled initial quantification is advisable to maximize success.

4 Take Bead linked transposons (**BLT**) and Tagmentation buffer (**TB1**) out of the fridge to bring them to room temperature for ~ **00:30:00** . 30m

5 Prepare 300 ug/mL proteinase K

6 Thaw KAPA HiFi 2X Ready Mix on ice, invert to mix and spin on a bench spinner

7 Thaw and dilute Kapa F/R primers to **10 Micromolar (µM)** , on ice.
You will need **55 µl** of each per 96-samples.

8 Thaw the i5 and i7 indexes on ice.
Index stocks should be diluted to **1 Micromolar (µM)** working stocks
You will need **1.25 µl** of each per reaction

Index tubes: spin down briefly (do not vortex as they are small DNA fragments)
Index plates: spin down at 1500 rpm to ensure no droplets remain on the plate cover

9 Enter tagmentation protocol on thermal cycler (save as TAGflex):

9.1 15m
1. **55 °C** for **00:15:00** followed by a hold at **10 °C**
2. Enable the heated lid (to 100°C if the option is available)

10 Enter tagmentation stop protocol on the thermal cyclers (save as **TAGstop**)

10.1 15m
1. **37 °C** for **00:15:00** followed by a hold at **10 °C**
2. Enable the heated lid (to 100°C if the option is available)

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11 Enter PCR protocol on thermal cycler (save as **flexPCR**)

10m 15s

11.1
a. **72 °C** for **00:03:00**
b. **98 °C** for **00:03:00**
c. 12 cycles of:
i. **98 °C** for **00:00:45**
ii. **62 °C** for **00:00:30**
iii. **72 °C** for **00:02:00**
d. **72 °C** for **00:01:00**
e. **4 °C** hold

12 Enter and save the **protK** protocol on the thermal cyclers (save as **protK**) for running the proteinase K reaction.

40m

12.1
a. **37 °C** for **00:30:00**
b. **68 °C** for **00:10:00**
c. **4 °C** hold

TAGMENTATION

13 Add **5 µl** of DNA of all the samples to a 96 well plate. If less than 5 ul is used add the remaining volume as nuclease free water.

14 Seal and spin down the plate with the DNA < **1000 rpm** to ensure DNA is pelleted in the bottom of the wells

15 Make the **tagmentation master mix (TMM)**

15.1 Vortex **BLT** vigorously before use and ensure the beads are evenly resuspended

1. **TMM** contains **1 µl** **BLT** and **1 µl** **TB1** per reaction.
For 96 samples it is good to use 110 ul BLT and 110 ul TB1.

Keep the beads in the TMM mixed at all times

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16 Pre-load **27.5 µl** of **TMM** per well in an 8-strip of PCR tubes to use for easy aliquoting into the 96-well plate with DNA samples. Make sure **TMM** is well mixed *before* aliquoting and distributing it into the wells with the DNA.

17 Add **2 µl** of **TMM** to each DNA well using an 8-channel pipette and mix by pipetting gently ten times to fully mix.

If beads are adhered to the top or side of the 96-well plate, **briefly** centrifuge and fully resuspend the bead pellet by pipetting until thoroughly mixed

18 Seal the plate with a PCR plate cover, place in a thermal cycler, and run the **TAGflex** program.

1. It is especially important to be able to remove the plate seal as easily as possible to prevent cross contamination but make sure the plate is sealed well to avoid evaporation, keep it on PCR holder for stability
2. Proceed immediately to the next steps upon completion of the TAGflex cycle

POST TAGMENTATION CLEAN UP

19 While waiting for the TAGflex program, preload **15 µl** of **Tagmentation stop buffer (TSB)** into each tube of a 8 strip PCR tube for easy aliquoting.

20 1. Gently remove the plate seal from 96-well plate and add **1 µl** **TSB** into each tagmentation reaction using an 8-channel pipette
Mix by gently pipetting the **entire volume**, ensuring the beads are fully resuspend

21 Seal the plate, place in a thermal cycler, and run the **TAGstop** program

22 Remove plate seal and place the plate on a 96-well plate magnet for **00:03:00** or until solution is clear 3m

23 Load **3.5 mL** (for 96 reactions) **Tagmentation wash buffer (TWB)** into a sterile reservoir to pipette from

24 ⚠

The following 2 steps (24 and 25 1.) can be done sequentially by row while on the magnet to ensure that the beads are not drying out. This is in particular useful if you have a lot of samples.

Carefully remove the supernatant using an 8-channel pipette without disturbing the beads, change tips between rows

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25 1. Add **20 µl** **TWB** to each well using an 8-channel pipette.

More TWB might be needed to ensure that the beads will be submerged.

2. Remove the plate from the magnetic stand . Mix by gently pipetting (slowly, to minimize foaming) until beads are fully resuspended, pipette up and down at least 10 times
3. Ensure beads are submerged in TWB **at all times**

26 Place the plate on the magnetic stand for **00:03:00** or until solution is clear 3m

27 Repeat steps 24-26 for a total of two washes and finally resuspend the bead pellet in **20 µl** **TWB**. Pipet at least 10x until beads are fully resuspended. Seal the plate and keep on the magnet until step 30.
Make sure beads are submerged in **TWB** otherwise add more.

AMPLIFY TAGMENTED DNA

28 Prepare PCR master mix on ice.
Per reaction (i.e. in case of 96 samples calculate for 120) you will need: **2.75 µl** nuclease free water
0.5 µl Kapa F primer
0.5 µl Kapa R primer
6.25 µl Kapa Hifi Ready Mix

29 For 96 samples pre-load **150 µl** of PCR master mix into 8 strip PCR tubes.

30 The following steps can be done row by row to avoid excessive drying of beads and easier resuspension.

1.Remove the TWB wash buffer from the samples from step 27 while on the magnet.
2. Immediately add **10 µl** of PCR master mix to each well to avoid drying out the beads
3. After all rows received master mix, take the plate off the magnet and mix by pipetting up and down until beads are fully resuspended.

31 ⚠

Each well will receive a **different barcode combination** of i5 and i7. Both indexes will be added in equimolar amounts to each well as a total of 2.5 ul (i5= 1.25 ul + i7 =1.25 ul for each well).
It is recommended to design a plate map to keep track of the barcode combinations and to ensure that each well (sample) has a unique barcodes combination!

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Add **1.25 µl** of **1 Micromolar (µM)** i5 and i7 barcodes **each**.
Set a P10 to 10 ul and pipette to mix.
Then seal the plate and spin down briefly.

32 Transfer PCR plate into PCR thermocycler and run the "flexPCR" program.

33 While the PCR is running pre-load 8 strip PCR tubes with **35 µl** of **300 ug/ml** proteinase K

34 1m
Remove the PCR plate from the thermal cycler and spin down at **1000 rpm** for **00:01:00** to ensure that all the liquid is on the bottom of the wells.

35 The following 4 steps (33, 35-37) are **not** required if you directly continue to step 39.

Add **2.5 µl** of the proteinase K to each well (step 34).
Mix by pipetting 10 ul volume 10 x.
Seal the plate and spin briefly.

36 Transfer PCR plate to thermal cycler and run the "protK" program.

37 3d

If stopping at this point seal the plate with sealing foil for PCR plates and store at **4 °C** for up to **72:00:00** .

POST PCR LIBRARY CLEANUP 31m

38 31m
Prepare fresh 80% ethanol.
Thaw Resuspension buffer and bring to RT and vortex.
Equilibrate the sample purification beads (**SPB**) at RT for **00:30:00** then vortex and invert several times
If the plate was stored spin down the plate for **00:01:00** at **1000 rpm** to make sure all the liquid is on the bottom of the well.

39 5m
Place the plate on the magnet for **00:05:00** or until supernatant is clear.
Transfer supernatant into new 96 well plate, avoid the beads.

40 If equal DNA amounts were started with you can go ahead and take **2 µl** of each sample and pool into a 1.5 ml microcentrifuge tube.
Otherwise, quantify each sample using qubit or picogreen to pool equal amounts of DNA here.

41 Vortex and spin down the pool briefly.

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42 Transfer **45 µl** of the pool to another 1.5 ml microcentrifuge tube, recommended is a low binding microcentrifuge tube.
Store the remaining pool at **-20 °C** in case the size selection needs to be optimized.

43 Vortex and invert **SPB** multiple times to ensure full resuspension. To aim for an average fragment size of 300 bp follow the protocol below.
Prepare **SPB-MM** (mastemix) by mixing **56 µl** of **SPB** with **29 µl** of nuclease free water.
Mix and invert **SPB-MM** several times.

> The **SPB** amount in step 43 and step 49 are relevant for the size selection of the library. If the size of the library is not as hoped, for these steps can be repeated with another aliquot of the pool and different amounts of **SPB**.

44 ⚠

Add **85 µl** of SPB-MM to **45 µl** of the pool from step 43. Mix thoroughly by pipetting up and down several times.
This is a critical step for proper size distribution for the library.

45 Incubate at room temperature for **00:05:00** 5m

46 Place the microcentrifuge tube on a magnetic stand for **00:05:00** or until the supernatant is clear 5m

47 Transfer **125 µl** supernatant into a new 1.5mL tube without disturbing the beads

48 Vortex and invert the **SPB** (undiluted stock) until thoroughly resuspended

49 Add **12 µl** **SPB** (undiluted stock) to the new 1.5mL microcentrifuge tube containing the supernatant

50 Pipette **120 µl** volume up and down ten times to thoroughly mix

51 Incubate at room temperature for **00:05:00** . 5m

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52 Place the microcentrifuge tube on a magnetic stand for **00:05:00** . or until the supernatant is clear 5m

53 Warm up the elution buffer **RSB** at **50 °C** to increase final library yield

54 Remove and discard the supernatant from step 52 without disturbing the beads

55 With the tube on the magnetic stand, add **200 µl** **80 % (v/v)** ethanol without mixing and incubate for **00:00:30** 30s

Add enough **80 % (v/v)** ethanol so that the beads are entirely submerged

56 Pipette to remove the ethanol without disturbing the beads

57 Repeat steps 55 - 56 one more time for a total of 2 washes

58 Carefully, remove any excess liquid from the tube using a pipette without disturbing the beads, try your best to remove all liquid!

59 Air-dry beads on the magnetic stand for ~3-5 min.

60 Remove the 1.5mL tube from the magnetic stand and add **32 µl** of warm **RSB** to the beads

61 Pipette mix until thoroughly resuspended at least 15 times

62 Incubate at room temperature on bench for **00:02:00** 2m

63 Place the 1.5mL tube back on the magnetic stand for **00:02:00** or until clear 2m

64 Transfer **30 µl** supernatant into a new 1.5 ml microcentrifuge tube. Make sure no beads get transferred along with the supernatant.

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65 A subsample can now be submitted for QC: Quantification by qubit and to see the size distribution running the pool on the Bioanalyzer.
If the size distribution is not as hoped for go back to step 42 using some of the remaining pool and adjust the amounts of SPB.

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