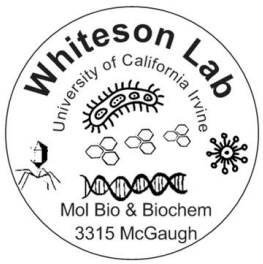
Oct 21, 2020

Low Volume Methodology for Nextera DNA Flex Library Prep Kit (96 Samples)



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DOI
https://dx.doi.org/10.17504/protocols.io.be6rjhd6

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.be6rjhd6

Protocol Citation: Eric Adams, Stephen Wandro, Julio Avelar-Barragan, Andrew Oliver, Katrine Whiteson 2020. Low Volume Methodology for Nextera DNA Flex Library Prep Kit (96 Samples). **protocols.io** https://dx.doi.org/10.17504/protocols.io.be6rjhd6

Manuscript citation:
Adams, E. et al. Comparing Fecal, Saliva, and Chicha Microbiomes Between Mothers and Children in an Indigenous Ecuadorian Cohort. bioRxiv 2020.10.02.323097 (2020) doi:10.1101/2020.10.02.323097.

Protocol status: Working
We use this protocol and it works for us in our lab.

Created: April 15, 2020

Last Modified: October 21, 2020

Protocol Integer ID: 35761

Keywords: nextera dna flex library prep kit, nextera dna flex library prep, illumina dna prep, sequencing library, dna, kit

Disclaimer

Abstract

This protocol presents a low volume methodology for the Nextera DNA Flex Library 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 × 96-wells). While other low-cost methods have been deveolped for this purpose, this protocol is a very straightforward method utilizing reduced reagent and sample volumes.

Update: The "Nextera DNA Flex Library Prep" kit has been renamed to "Illumina DNA Prep" with the appropriate indexes having been similarly renamed. While all reagents and steps remain identical, an updated version of this protocol may be published to account for any differences between the protocols.

Guidelines

Primers used with the Kapa HiFi ReadyMix:
KAPA-PCR-F: AATGATACGGCGACCACCG*A
KAPA-PCR-R: CAAGCAGAAGACGGCATACG*A

Order these from IDT at 1umole scale with HPLC purification.
The * is a phosphothioate bond that prevents polymerases with 3′-5′ proof-reading activity from chewing back the oligos

Materials

MATERIALS

- Proteinase K
- Nuclease-free Water
- Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #**KK2612
- Fresh 80% Ethanol
- Magnetic Stand-96 **Thermofisher Catalog #**AM10027
- Sterile reagent reservoirs
- Nextera DNA Flex Library Prep **Illumina, Inc. Catalog #**20018705
- Adhesive PCR Plate Foils **Thermo Fisher Catalog #**AB0626
- 0.2mL PCR Tube Strips, 8-Tube Strip; 120/Pk. **Thermo Fisher Catalog #**E0030124286
- 96-well PCR plates
- IDT for Illumina Nextera DNA Unique Dual Indexes **Illumina, Inc. Catalog #**20027213

Before start

1. Enter and save the **TAGflex** tagmentation protocol on the thermal cyclers:
   a. Enable the heated lid (to 100°C if the option is available)
   b. 55°C for 15m followed by a hold at 10°C
2. Enter and save the **TAGstop** tagmentation stop protocol on the thermal cyclers:
   a. Enable the heated lid (to 100°C if the option is available)
   b. 37°C for 15m followed by a hold at 10°C
3. Enter and save the **flexPCR** protocol on the thermal cyclers:
   a. 72°C for 3m
   b. 98°C for 3m
   c. 12 cycles of:
      i. 98°C for 45s
      ii. 62°C for 30s
      iii. 72°C for 2m
   d. 72°C for 1m
   e. 4°C hold
4. Enter and save the **protK** protocol on the thermal cyclers:
   a. 37°C for 30m
   b. 68°C for 10m
   c. 4°C hold

Preparation

1 Equilibrate the following reagents at Room temperature for 00:30:00

Bead-Linked Transposomes **(BLT)**
Tagmentation Buffer **(TB1)**
Tagment Stop Buffer **(TSB)**
Tagment Wash Buffer **(TWB)**
Sample Purification Beads **(SPB)**

2 Thaw the **Resuspension Buffer (RSB)** and bring to Room temperature then vortex to mix

3 Prepare a **proteinase K** solution at [M] 300 µg/mL

> Note
>
> For a 1mL solution: add 15µL of 20mg/mL proteinase K to 985µL of nuclease-free water

4 Thaw **KAPA HiFi HotStart ReadyMix (2X)** On ice

5 Thaw the **i5 and i7 indexes** at Room temperature

> Note
>
> Index tubes: vortex and spin down briefly
> Index plates: spin down at 1500 rpm to ensure no droplets remain on the plate cover
>
> Index stocks should be diluted to [M] 10 micromolar (µM) working stocks

6 Prepare a **Tagmentation Master Mix (TMM)**

TMM contains 1µL BLT and 1µL TB1 per reaction

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

6.1 Make a 110X mix: 110 µL TB1 + 110 µL BLT

Vortex to mix

7 Prepare **PCR Master Mix (PCR MM)** On ice

PMM contains 2.75µL nuclease-free water, 0.5µL KAPA-PCR-F primer, 0.5µL KAPA-PCR-R primer, and 6.25µL KAPA HiFi HotStart ReadyMix (2X) per reaction

Note

See Guidelines section for KAPA primer design
KAPA primer stocks should be diluted to [M] 10 micromolar (µM)

7.1 Make a 110X mix: 302.5 µL nuclease-free water + 55 µL KAPA-PCR-F + 55 µL KAPA-PCR-R + 687.5 µL KAPA HiFi HotStart ReadyMix (2X)

Vortex to mix and briefly spin down

8 Prepare a diluted **SPB Master Mix (SPB MM)**

54 µL SPB + 48 µL nuclease-free water

96-Well Tagmentation

9 Transfer at least 100 ng genomic DNA (up to 500ng) into each well of a 96-well PCR plate in **1-5µL**

10 Adjust the volume of each well to 5 µL total volume using nuclease-free water

11 Load 27 µL TMM into each tube of an 8-strip of PCR tubes

Vortex TMM thoroughly before use and ensure the beads remain evenly suspended while being aliquoted into the 96-well plate

12 Add 2 µL TMM to each well using an 8-channel pipette

Thoroughly mix by gentle pipetting

Note

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

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

Note

It is especially important to be able to remove the plate seal as easily as possible to prevent cross contamination

Proceed **immediately** to the next steps upon completion of the TAGflex cycle

Post Tagmentation Cleanup

14 Load 15 µL TSB per tube in an 8-strip of PCR tubes

15 Gently remove the plate seal 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

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

17 Remove the seal and place the plate on a 96-well magnetic plate stand for 00:03:00 or until solution is clear

18 Carefully remove the supernatant using an 8-channel pipette without disturbing the beads

19 Load 6 mL TWB into a sterile trough to pipette from

20 Remove the plate from the magnetic stand and add 20 µL TWB to each well using an 8-channel pipette

Mix by gently pipetting (slowly, to minimize foaming) until beads are fully resuspended

21 Place the plate on the magnetic stand for 00:03:00 or until solution is clear

22 Carefully remove the supernatant using an 8-channel pipette without disturbing the beads

23 Repeat steps 20-22 one more time for a total of 2 washes

24 Remove the plate from the magnetic stand and add 20 µL TWB to each well

Gently pipette until beads are fully resuspended

25 Seal the plate and place on the magnetic stand to incubate until needed in the next section

> Note
>
> Keeping the pellet in TWB helps prevent over-drying of the beads

Amplify Tagmented DNA

26 Load 135 µL PCR MM per tube into an 8-strip of PCR tubes (store On ice until needed)

27 Carefully remove the TWB supernatant from the samples while on the magnetic stand

> Note
>
> Any remaining foam on the well walls should not adversely affect the library

28 Remove the plate from the magnetic stand and proceed immediately to the next step to prevent excessive drying of the beads

29 Add 10 µL PCR MM to each sample and pipette the mix to ensure the beads are thoroughly resuspended

> Note
>
> Pipette with moderate force as the beads can be difficult to fully resuspend

30 Seal the plate and spin down briefly up to 1000 rpm

31 Add 2.5 µL of an equimolar mix of each i7 and i5 index combination to their respective wells

> Note
>
> Individual index tubes: 1.25µL i7 index (N7xx) + 1.25µL i5 index (N5xx)
>
> Pre-mixed index plate: Transfer 2.5µL of each index combination using an 8-channel pipette

32 Pipette to mix a minimum of 10 times, then seal the plate and breifly spin up to 1000 rpm

33 Place the plate in a thermal cycler and run the **flexPCR** program

34 Remove the plate from the thermal cycler and spin down for 00:01:00 at 280 x g

35 Load 35 µL of [M] 300 µg/mL proteinase K per tube into an 8-strip of PCR tubes

36 Remove the plate seal and add 2.5 µL proteinase K to each PCR reaction using an 8-channel pipette

Mix the wells by pipetting a 10uL volume at least ten times

37 Seal the plate, place in a thermal cycler, and run the **protK** program

38 If stopping, keep the plate sealed and store at 4°C for up to 3 days

Otherwise, continue with the next section

Post-PCR Library Clean Up

39 Remove the seal and place the plate on the magnetic stand for 00:05:00 or until the supernatant is clear

40 Using an 8-channel pipette, transfer 2 µL PCR supernatant from each well into an 8-strip of PCR tubes

41 From the 8-strip of PCR tubes, transfer all samples to a single 1.5mL Eppendorf tube

> Note
>
> Each tube in the strip should contain ~24µL of pooled sample, yielding ~192µL of pooled libraries

42 Vortex and briefly spin down to mix the pooled libraries evenly

43 Transfer a 45 µL aliquot of the pooled libraries to a new 1.5mL Eppendorf tube

44 Vortex and invert **SPB MM** multiple times to ensure full resuspension

45 Add 85 µL SPB MM to the pooled library aliquot

Pipette until thoroughly mixed

> Note
>
> Complete mixing is critical to proper size distribution of libraries

46 Incubate at Room temperature for 00:05:00

47 Place the Eppendorf tube on a magnetic stand for 00:05:00 or until the supernatant is clear

48 Transfer 120 µL supernatant into a new 1.5mL tube without disturbing the beads

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

50 Add 14.4 µL SPB (undiluted stock) to the new 1.5mL tube containing the supernatant

51 Pipette a 120µL volume until thoroughly mixed

52 Incubate at Room temperature for 00:05:00

53 Place the Eppendorf tube on a magnetic stand for 00:05:00 or until clear

54 Remove and discard the supernatant without disturbing the beads

55 With the tube on the magnetic stand, add 200 µL 80% ethanol without mixing and incubate for 30s

Note

Add enough 80% 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 Remove any excess liquid from the tube using a pipette without disturbing the beads

59 Air-dry beads on the magnetic stand for 00:05:00

60 Warm 35 µL RSB at 50 °C to increase final library yield

61 Remove the 1.5mL tube from the magnetic stand

62 Add 32 µL RSB (warm) to the beads

Pipette mix until thoroughly resuspended

63 Incubate on the bench at Room temperature for 00:02:00

64 Place the 1.5mL tube back on the magnetic stand for 00:02:00 or until clear

65 Transfer 30 µL supernatant into a new 1.5mL Eppendorf tube

66 Let the tube with the eluted library sit at 50 °C for 00:05:00 to ensure complete dissolution of the library

67 Quantify the library using a Qubit fluorometer

Determine fragment size distribution using a Bioanalyzer