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Hybrid BAG-Seq Protocol for DNA-RNA Co-assay

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Abstract

This protocol describes Hybrid BAG-seq, a high-throughput droplet-based split-pool barcoding technique that simultaneously captures RNA and DNA from individual nuclei. The method integrates nuclear isolation, droplet encapsulation, split-pool barcoding, and library preparation steps to generate paired genome–transcriptome profiles from thousands of single nuclei. This protocol describes the complete experimental procedures, and the resulting sequencing libraries are compatible with Illumina platforms.

Attachments



[BAG-split-pool-barco...](#)

24KB

Materials

Equipment:

- Inverted Microscope, Motic, AE31E
- Legato 100 Syringe Pumps (3), KD Scientific, 788100
- Tube Revolver, Thermo Fisher Scientific, 88881001
- Isotemp 60L incubator, Thermo Fisher Scientific, 15-103-0513
- Eppendorf ThermoMixer heat block (2), Eppendorf, 2231001127
- 3 mL Syringes, Becton Dickinson, 309657
- 10 mL Syringes, Becton Dickinson, 302995
- PE-2 Tubing, Scientific Commodities, BB31695-PE/2
- 27 Gauge x 1/2" Needles, Becton Dickinson, 305109
- 21 Gauge x 1" Needles, Becton Dickinson, 305165
- 16 Gauge x 1-1/2" Needles, Becton Dickinson, 305197
- PDMS co-flow microfluidic droplet generation device for "Drop-Seq", Nanoshift, LLC
- 150 µm PluriStrainer, PluriSelect, 43-50150-03
- 40 µm Cell Strainer, Corning, 431750
- Microseal 'B' plate seals, Bio-Rad, MSB1001
- SimpliAmp Thermal Cycler, Thermo Fisher Scientific, A24812
- FACSAria II SORP Cell Sorter, Becton Dickinson

Primers:

- TG-vt-K: /5ACryd//iSp18/TGTGTTGGGTGTGTTTGG NBBBB KKKKKKKGKKKKKKKKNN
- TG-vt-polyT: /5ACryd//iSp18/TGTGTTGGGTGTGTTTGG DDDDDNNN T(30)
- Template-switch-oligo (TSO): AAGGCTCTTGACATGAAGGATAGrGrGrG
- Adapter1-BC1-vt1-X (X = 1,2,...,96): ACGCAGAGTCCGCTC <BAG-barcode1> NNNN CA+T+G
- Adapter2-BC2-vt2-Adapter1-Y (Y = 1,2,...,96): GACCGACTCGCATTACCCTAT <BAG-barcode2> NNNN ACGCAGAGTCCGCTC/3InvdT/
- Adapter3-BC3-Adapter2-Z (Z = 1,2,...,96): AAGGCTCTTGACACGAAGGATAG <BAG-barcode3> GACCGACTCGCATTACCCT*A*T
- P5-Adapter3: AATGATACGGCGACCACCGAGATCTACAC CGCTGTCC AAGGCTCTTGACACGAAGGAT*A*G
- TG primer: TGTGTTGGGTGTGTTT*G*G
- Read1-Adapter3: CGCTGTCC AAGGCTCTTGACACGAAGGATAG
- Nextera_N70x (x = 1,2,...,12): CAAGCAGAAGACGGCATACGAGAT <index_x> GTCTCGTGGGCTCGG

Chemical Reagents:

Nuclei Preparation

- 10× PBS, Thermo Fisher Scientific, AM9624
- UltraPure BSA, Thermo Fisher Scientific, AM2618
- DAPI, Thermo Fisher Scientific, 62248
- RNase Plus RNase Inhibitor, Promega Corporation, N2615
- Surfact-Amps NP-40 (10% solution), Thermo Fisher Scientific, 28324

Aqueous Phase 2



- Acrylamide/bis-acrylamide, 40% solution, Sigma-Aldrich, A9926
- Acrylamide solution, 40%, Sigma-Aldrich, A4058
- 0.5 M EDTA, Thermo Fisher Scientific, AM9260G
- 20% Sarkosyl, Sigma-Aldrich, L7414
- 0.1 M DTT, Thermo Fisher Scientific, 707265ML
- Ammonium persulfate, Sigma-Aldrich, 09913-100G
- 1M Tris-HCl pH 7.5, Thermo Fischer Scientific 15567027

Oil Phase

- HFE-7500, Oakwood Chemical, 051243
- 008-Fluorosurfactant, Ran Biotechnologies, 008-FluoroSurfactant-5G
- TEMED, Bio-Rad, 1610801

Post-Droplet Generation

- Mineral Oil, Sigma-Aldrich, 69794-500ML
- 20× SSC, Thermo Fisher Scientific, 15557044
- Perfluorooctanol (PFO), Sigma-Aldrich, 370533
- 10 mM dNTP, Sigma-Aldrich, 11814362001
- DNA Polymerase I, Large (Klenow), New England BioLabs, M0210M
- Fragment Recombinant RNase Inhibitor, Takara Bio USA, Inc., 2313A
- Exonuclease I, New England BioLabs, M0293L
- NlaIII, New England BioLabs, R0125L
- Klenow Fragment (3-5' exo minus), New England BioLabs, M0212L
- Quick Ligase, New England BioLabs, M2200
- KAPA HiFi HotStart ReadyMix, Roche, KK2602
- Agencourt AMPure XP beads, Beckman Coulter, A63880
- Nextera XT DNA Library Prep Kit, Illumina, FC-131-1024
- 1M Tris-HCl pH 8.0, Thermo Fischer Scientific, 15568025

Chemical Solutions to Prepare:

- NST buffer (Recipe refers to Navin, Nicholas, et al. "Tumour evolution inferred by single-cell sequencing." Nature 472.7341 (2011): 90-94.)
- PBS-BSA-DAPI buffer (1× PBS, 0.05% BSA, 0.5 µg/mL DAPI, 0.8 u/µL RNasein Plus RNase Inhibitor)
- 40% Surfactant in HFE-7500
- 10% Ammonium persulfate (make fresh solution for every experiment)
- 6× SSC buffer
- TE-SDS buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% SDS)
- STOP-25 buffer (10 mM Tris pH 8.0, 25 mM EDTA, 0.1% Tween-20, 0.1 M KCl)
- STOP-10 buffer (10 mM Tris pH 8.0, 10 mM EDTA, 0.1% Tween-20, 0.1 M KCl)
- STOP-1 buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.1% Tween-20, 0.1 M KCl)
- HBW buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.1% Tween-20)



Equipment Preparation

- 1 Prepare heat blocks and incubator:
 - Set one heat block to 50°C
 - Set another heat block to 85°C
 - Set Isotemp 60L incubator to 37°C

Nuclei Preparation

2 Thaw and Wash Nuclei

Prepare an ice bucket. Perform the following nuclei preparation steps on ice:

- 2.1 Take a tube containing frozen pulverized tissue out of -80°C freezer and keep the tube on ice.

Add 1 mL ice-cold NST buffer into the sample tube. Pipette up and down several times. (NST buffer recipe: <https://doi.org/10.1038/s41467-017-00244-w>)

- 2.2 Transfer nuclei solution to a 15 mL conical tube prefilled with 5 mL NST buffer on ice. Pipette to mix.

Place the conical tube on ice for 15 minutes with regular shaking by hand (every 3 minutes).

- 2.3 Centrifuge nuclei at 300 rcf and 4°C for 5 minutes. Discard supernatant.

Resuspend nuclei pellet using 2 mL ice-cold PBS-BSA-DAPI buffer.

- 2.4 Pass nuclei solution through 40 µm cell strainer.

3 Sort Nuclei

Perform single-nucleus sorting based on DAPI-H vs. DAPI-A to remove debris and clumps. Sort nuclei into a 1.5 mL Eppendorf tube prefilled with 100 µL PBS-BSA buffer. This constitutes **Aqueous Phase 1** (also called the **Cell Phase**), with PBS-BSA buffer used to adjust the nuclei concentration.

Oil Phase & Aqueous 2 Phase Preparation

- 4 **Prepare Oil Phase** in a 5 mL tube, vortex to mix:
 - 300 µL 40% 008-FluoroSurfactant in HFE-7500 (from Ran Biotechnologies)

- 10 μ L TEMED
- 2.1 mL HFE-7500

5 Prepare Aqueous Phase 2 in a 1.5 mL Eppendorf tube, pipet to mix:

- 180 μ L Acrylamide/bis-acrylamide, 40%
- 130 μ L Acrylamide solution, 40%
- 130 μ L H₂O
- 80 μ L 1 M Tris pH 7.5
- 50 μ L 0.5 M EDTA pH 8.0
- 40 μ L 500 μ M TG-vt-polyT primer
- 80 μ L 500 μ M TG-vt-K primer
- 20 μ L 20% Sarkosyl
- 100 μ L RNasin Plus RNase Inhibitor
- 30 μ L 0.1 M DTT (1.5 mM)
- 100 μ L 10% NP-40
- 60 μ L 10% APS (add last)

Microfluidics Setup & Oil Droplet Collection

6 Set up microfluidic device

Turn on microscope. Place a two-aqueous-phase/co-encapsulation droplet-generation microfluidic device (such as a Drop-Seq device) on the microscope stage.

7 Set up microfluidics outlet tube:

Insert a piece of PE/2 tubing into the outlet and place the other end into a waste container (such as a 15 mL conical tube).

8 Load Cell Phase, Aqueous Phase 2, and Oil Phase into syringe pump

8.1 Cell Phase: Use a 3 mL syringe and a 21G x 1" needle to aspirate the solution into syringe.

8.2 Aqueous Phase 2: Use a 3 mL syringe and a 21G x 1" needle to aspirate the solution into syringe.

8.3 Oil Phase: Use a **10 mL** syringe with a 16G x 1-1/2" needle to aspirate oil phase into syringe.

8.4 For each syringe, change the needle to a 27G x 1/2" needle and insert it into a piece of PE/2 tubing.

8.5 Place syringes in syringe pumps and insert tubing into the corresponding inlets of the microfluidics device.

9 Initiate droplet creation

9.1 Set flow rates:

- Cell Phase: 750 $\mu\text{L/hr}$
- Aqueous Phase 2: 750 $\mu\text{L/hr}$
- Oil Phase: 3,000 $\mu\text{L/hr}$

(flow rates are adjustable depending on the desired droplet size and the specific microfluidic device used)

9.2 Turn on the Cell Phase pump.

Once Cell Phase is flowing stably, turn on the Aqueous Phase 2 pump.

Once the interphase between Cell Phase and Aqueous Phase 2 has stabilized, turn on the Oil Phase pump and wait for the flow to stabilize and droplets to form.

9.3 Check droplet size:

Place the output tubing over a glass slide under the microscope to assess the size uniformity of oil droplets until they are the correct size.

10 Collect droplets

Collect ~400 μL of droplets in 1.5 mL Eppendorf tube prefilled with 300 μL mineral oil.

11 Incubate BAGs at:

1. 37°C for 2.5 hours
2. 85°C heat block for 5 minutes
3. 50°C for 10 minutes
4. Room temperature for 15 minutes

12 Set Isotemp 60L incubator to 42°C.



BAG Collection

13 Perform BAG collection

- 13.1 Centrifuge BAGs at 600 rcf for 1 minute. Remove the top oil layer using pipette. Remove the bottom oil layer using syringe and 21G ½ needle, leaving the middle layer.

Add 600 µL 6× SSC buffer and 150 µL perfluorooctanol (PFO) to BAGs. Invert the tube several times by hand to separate the BAGs from the oil phase.

- 13.2 Centrifuge again and discard the top and bottom layers, leaving the middle BAG layer again.

Add 800 µL 6× SSC buffer and shake the tube multiple times.

- 13.3 Centrifuge, discard the top layer and any leftover PFO from the bottom layer.

Reverse Transcription

14 Pre-Wash BAGs

Create pre-wash solution in 1.5 mL separate Eppendorf tube, vortex to mix:

- 850 µL 5x RT Buffer (from Thermo Fisher Maxima H- Reverse Transcriptase kit)
- 25 µL 0.1 M DTT
- 25 µL Takara 2313 Recombinant RNase Inhibitor

Transfer ~350 µL BAGs from the BAG layer to pre-wash solution, pipette to mix.

Centrifuge (600 rcf, 1 min), discard supernatant.

15 Perform Reverse Transcription and DNA Extension Reaction

- 15.1 Prepare reverse transcription solution in 1.5 mL Eppendorf tube on ice, vortex to mix:

- 100 µL 5x RT buffer
- 570 µL H₂O
- 100 µL 10 mM dNTP
- 25 µL Takara 2313 Recombinant RNase Inhibitor
- 20 µL 0.1 M DTT

- 50 µL Maxima H- Reverse Transcriptase (add last)

Immediately add the above mix into BAGs. Mix by pipetting up and down 10 times.

Incubate at room temperature with rotation for 15 minutes.

(for all subsequent BAG rotations, use 10 rpm on Thermo Scientific Tube Revolver Rotator)

- 15.2 Add 25 µL 100 µM Template-switch-oligo (TSO).

Incubate at room temperature with rotation for another 15 minutes, then incubate at 42°C with rotation for 1 hour.

- 15.3 Add 10 µL DNA Polymerase I, Large (Klenow) Fragment (M0210M).

Incubate at 42°C with rotation for another 30 minutes.

16 **Stop Reaction**

- 16.1 Prefill a 15 mL conical tube with 5 mL STOP-25 buffer.

Transfer all the BAG solution from the Eppendorf reaction tube to the 15 mL conical tube. Mix by pipetting 10 times.

Incubate at room temperature for 5 minutes.

Spin down at 300 rcf for 5 minutes. Remove supernatant.

- 16.2 Wash BAGs twice using 1 mL STOP-10 buffer.

("Wash" = add buffer to the BAGs, pipette to mix, centrifuge at 600 rcf for 1 minute, and remove the supernatant.)

-----SAFE STOPPING POINT: STORE BAGS IN STOP-10 AT 4°C-----

Exonuclease and RNase H Treatment

17 **Pre-Wash BAGs**

Set Isotemp 60L Incubator to 37°C.

- 17.1 Discard any supernatant from BAGs. Wash once with 1 mL STOP-1 buffer.

17.2 Create Exo I pre-wash buffer, vortex to mix:

- 100 μL 10 $\times$ Exo I buffer
- 900 μL H_2O

Wash BAGs in Exo I pre-wash buffer. Remove supernatant.

18 Perform Exonuclease and RNase H Reaction

Prepare exonuclease treatment solution, vortex to mix:

- 835 μL H_2O
- 100 μL 10 $\times$ Exo I buffer
- 50 μL Exo I enzyme
- 15 μL RNase H

Transfer 1 mL Exonuclease Treatment Solution to the tube of BAGs; pipette to mix.

Incubate at 37°C with rotation for 1.5 hours.

19 Stop Reaction

19.1 Centrifuge BAGs, discard supernatant.

19.2 Wash BAGs twice with 1 mL STOP-25 solution.

19.3 Wash BAGs once with 1 mL STOP-10 solution. Continue to NlaIII cutting or resuspend in 1 mL STOP-10 and store at 4°C.

-----SAFE STOPPING POINT: STORE BAGS IN STOP-10 BUFFER AT 4°C-----

NlaIII Cutting

20 Pre-wash BAGs

20.1 Remove any supernatant from BAGs. Wash once with STOP-1 solution.

20.2 Prepare NlaIII pre-wash solution, vortex to mix:

- 100 μ L 10x CutSmart buffer
- 900 μ L H₂O

Wash BAGs with NlaIII pre-wash solution.

Centrifuge, remove supernatant.

21 Perform NlaIII reaction 1

Prepare NlaIII solution 1 on ice, vortex to mix:

- 850 μ L H₂O
- 100 μ L 10 $\times$ CutSmart buffer
- 50 μ L NlaIII enzyme

Transfer NlaIII Solution 1 to the tube of BAGs, pipette to mix.

Incubate at 37°C with rotation for 1.5 hours.

Centrifuge BAGs, discard supernatant.

22 Perform NlaIII reaction 2

Prepare NlaIII solution 2 on ice, vortex to mix:

- 880 μ L H₂O
- 100 μ L 10x CutSmart buffer
- 20 μ L NlaIII enzyme

Transfer NlaIII Solution 2 to tube of BAGs, pipette to mix.

Incubate at 37°C with rotation for 1 hour.

23 Stop reaction

23.1 Centrifuge BAGs, discard supernatant.

23.2 Wash BAGs twice with STOP-25 solution.



23.3 Wash BAGs once with STOP-10 solution.

23.4 Continue to First Split-Pool or resuspend in 1 mL STOP-10 and store at 4°C.

-----SAFE STOPPING POINT: STORE BAGS IN STOP-10 BUFFER AT 4°C-----

First Split-Pool

24 Pre-wash BAGs

Remove any supernatant from BAGs, wash twice using 1 mL HBW buffer.

25 Hybridize 1st-split-pool oligos

25.1 Prepare the following Hybridization Master Mix (HMM) in a 1.5 mL Eppendorf tube. Pipette to mix.

- 330 µL BAGs in HBW
- 550 µL 2× Quick Ligase Buffer (NEB M2200)
- 110 µL dNTP (10 mM)

Quickly add 120 µL of HMM into each of 8 tubes of a PCR strip, then *quickly* use multipipette to transfer 9 µL HMM to each well of a 96-well PCR plate.

25.2 Add 2 µL 10 µM "Adapter1-BC1-vt1-X" primers into each well. Each well should have a unique primer; avoid cross-contamination between wells. Use adhesive plate seals to seal the plate. Briefly spin down.

25.3 Incubate plate:

1. Invert plate at room temperature for 5 minutes
2. Incubate at 50°C for 5 minutes in a thermal cycler with heated (60°C) lid
3. Place plate in a cold PCR plate rack and incubate inverted at 4°C for 10 minutes
4. Rotate the plate/rack in the 4°C fridge for an additional 10 minutes

26 Extend primers

26.1 Prepare the following Extension Master Mix (EMM) on ice, vortex to mix:

- 415 µL H₂O

- 550 µL 2× Quick Ligase Buffer
- 82 µL Klenow Fragment (3'→5' exo-) (NEB M0212)
- 55 µL Quick Ligase (NEB M2200)

Transfer 135 µL Extension Master Mix to each tube of an 8-tube PCR strip on cold rack.

26.2 Transfer plate with BAGs to another cold rack. Use multipipette to add 10 µL EMM to each well of the BAG plate. Pipette up and down 10 times to mix.

26.3 Incubate plate:

1. Rotate at 4°C in the fridge for 10 minutes
2. Incubate at 10°C in a thermal cycler for 10 minutes
3. Rotate at room temperature for 20 minutes
4. Rotate at 37°C in the Isotemp incubator for 20 minutes

27 Stop reaction

27.1 Add 100 µL STOP-25 to each well of the plate. Incubate at room temperature for 5 minutes.

Centrifuge the BAG plate at 900 rcf for 2 minutes.

27.2 Without disturbing the BAG pellet, remove 85 µL solution from the top of each well.

27.3 Prefill a solution basin with 5 mL STOP-25 buffer. Transfer the remaining BAG solutions from each well of the plate to the solution basin (about 36 µL remaining volume per well).

Transfer BAG solution from the solution basin to a 15 mL conical tube.

Centrifuge at 300 rcf for 5 minutes, discard supernatant.

27.4 Add 1 mL STOP-10, pipette up and down, and transfer BAG solution to a 1.5 mL tube.

Centrifuge, discard supernatant.

Resuspend in 1 mL STOP-10 buffer.

-----SAFE STOPPING POINT: STORE BAGS IN STOP-10 BUFFER AT 4°C-----

Second Split-Pool

28 **Pre-wash BAGs**

Discard any supernatant, wash BAGs twice with HBW buffer.

Also, preset thermal cycler to 75°C and the Isotemp incubator to 57°C.

29 **Perform Denaturation**

29.1 Prepare fresh Denaturation Solution, vortex gently to mix:

- 9.7 mL H₂O
- 150 µL 10 M NaOH
- 150 µL 30% (wt/wt) Brij-35

Add 1 mL Denaturation Solution to BAGs. Pipette to mix.

Incubate for 10 minutes at room temperature with rotation.

Centrifuge, discard supernatant.

29.2 Wash the BAGs *twice more* using the following protocol:

Add 1 mL Denaturation Solution to BAGs. Pipette to mix.

Incubate for 3 minutes at room temperature with rotation.

Centrifuge, discard supernatant.

30 **Perform Neutralization**

30.1 Prepare Neutralization Solution:

- 7.7 mL H₂O
- 1 mL 1 M Tris (pH 8.0)
- 1 mL 1 M NaCl
- 200 µL 0.5 M EDTA
- 100 µL 10% (vol/vol) Tween-20

30.2 Wash BAGs *twice* with Neutralization Buffer using the following protocol:

Add 1 mL Neutralization Solution to BAGs. Pipette to mix.

Incubate for 3 minutes at room temperature with rotation.

Centrifuge, discard supernatant.

30.3 Wash BAGs once with HBW buffer. Resuspend BAGs in HBW (400 μ L total volume)

31 Hybridize Primers

31.1 Prepare the following Isothermal Amplification Buffer (IAB) Mixture in a 2mL tube, pipette to mix:

- 1300 μ L H₂O
- 210 μ L 10 $\times$ IABuffer
- 150 μ L 10 mM dNTP
- 400 μ L BAGs in HBW

Quickly transfer 250 μ L of the IAB Mixture to each tube of an 8-tube PCR strip, then *quickly* transfer 20 μ L from each tube to each well of a 96-well plate using a multi-channel pipette.

31.2 Add 2 μ L of each of the 96 different 10 μ M "Adapter2-BC2-vt2-Adapter1-Y" (Y = 1,2,...,96) primers into separate wells. Each well should receive a unique 2nd-split primer. Avoid cross-contamination between wells.

31.3 Incubate plate:

1. Invert plate at room temperature for 2 minutes
2. Place in thermocycler at 75°C for 5 minutes.
3. Rotate in 57°C incubator for 20 minutes.

32 Add DNA Polymerase for Linear Extension

32.1 Prepare the following BST Master Mix on ice, vortex to mix.

- 372 μ L H₂O
- 48 μ L 10 $\times$ IABuffer
- 60 μ L NEB Bst2.0 DNA polymerase

Transfer 60 μ L BST Master Mix to each tube of an 8-tube PCR strip, then transfer 4 μ L to each well of the 96-well plate using a multipipette. Pipette to mix.

Incubate plate at 57°C with rotation for 40 minutes.

33 **Stop Reaction**

33.1 Add 100 µL STOP-25 to each well of the plate. Wait for at least 5 minutes.

Centrifuge plate at 900 rcf for 2 minutes at room temperature.

33.2 Without disturbing the BAG pellet, discard 85 µL solution from the top of each tube. Transfer the remaining BAG solutions from the plate to a solution basin prefilled with 5 mL STOP-25 (about 41 µL remaining volume per well).

33.3 Transfer BAG solution from the solution basin to a 15 mL conical tube.

Centrifuge at 300 rcf for 5 minutes. Discard supernatant.

33.4 Add 1 mL STOP-10, pipette to mix, and transfer BAG solution to a 1.5 mL Eppendorf tube.

Centrifuge at 400 rcf for 1.5 minutes. Discard supernatant.

33.5 Continue to the third split-pool or resuspend BAGs in 1 mL STOP-10 and store at 4°C.

-----SAFE STOPPING POINT: STORE BAGS IN STOP-10 BUFFER AT 4°C-----

Third Split-Pool

34 **Filter, wash, and count BAGs**

34.1 Filter BAGs through a 150 µm PluriStrainer cell strainer to remove fused BAGs.

34.2 Remove any supernatant from BAGs. Wash twice with HBW buffer.

34.3 Check the concentration and total amount of the BAGs under the microscope using a glass slide.

35 **Perform third split-pool**

35.1 Create the following TG primer/BAG mix, pipette to mix:

- 10 μL of 100 μM "TG primer"
- xx μL BAGs in HBW buffer (however much is left)
- $890 - xx$ μL H_2O

Quickly transfer 110 μL TG primer/BAG mix to each tube of an 8-tube PCR strip, then *quickly* use a multipipette to transfer 9 μL TG primer/BAG mix to each well of a 96-well plate.

35.2 Add 1 μL 10 μM "Adapter3-BC3-Adapter2-Z" ($Z = 1, 2, \dots, 96$) primers into each well. Each well should have a unique 3rd-split primer. Avoid cross-contamination between wells. Briefly spin.

35.3 On ice, add 10 μL Kapa HiFi Master Mix to each well of the 96-well plate. Pipette to mix.

35.4 Place plate in thermocycler. Run PCR:

95°C 1 min

10 cycles of:

98°C 30s

60°C 1 min

72°C 3 min

Then:

72°C 5 min

4°C infinite

36 Purify PCR Product

Allow AMPure XP beads to warm to room temperature for 30 minutes before using.

36.1 Pool 10 μL from each of the eight wells in a column of the PCR plate into a new PCR strip tube. You should now have 12 PCR tubes, each containing 80 μL of pooled PCR product.

Purify the 12 samples using 0.8 $\times$ AMPure XP beads (64 μL), and elute in 16 μL H_2O following the standard AMPure XP protocol.

36.2 Pool 14 μL of product from each of six PCR tubes into a single PCR tube. You should now have two PCR tubes, each containing 84 μL of purified product from the previous step.

Purify these two samples with another 0.8 $\times$ ratio (67 μL) of AMPure XP beads, and elute with 16 μL H_2O . Combine the products from both tubes into one (32 μL total).

- 37 Run the sample on a High Sensitivity DNA chip using the Agilent Bioanalyzer.

Tagmentation

38 Perform tagmentation

- 38.1 Prepare the following solution on ice, pipette to mix:

- 5 µL of PCR product/water (aim for 1.5 ng of DNA based on Bioanalyzer measurement)
- 10 µL Nextera TD buffer
- 5 µL Amplicon Tagment enzyme

Incubate at 55°C for 5 minutes in the thermal cycler.

- 38.2 Add 5 µL Neutralization Buffer, pipette to mix.

Incubate at room temperature for 5 minutes.

- 38.3 Add the following reagents to the tube in order on ice, pipette to mix:

- 6 µL H₂O
- 2 µL of 10 µM Nextera_N70x oligo
- 2 µL of 10 µM "P5-Adapter3" primer
- 15 µL Nextera PCR mix

(Use a different Nextera N7 primer for each sample)

- 38.4 Place on thermal cycler using the following program:

95°C 30s

10 cycles of:

95°C 10 s

55°C 30 s

72°C 30 s

Then:

72°C 5 min

4°C infinite



- 39 Purify tagmentation PCR product using 0.8× ratio (40 µL) of Ampure XP Beads, and elute using 30 µL H₂O.

Analyze Product

- 40 Run Bioanalyzer to check the final concentration.
- 41 Sequence sample using Illumina sequencer with custom Read1 sequencing primer "Read1-Adapter3".