Adapted Tn-Seq Library Prep Protocol with Tagmentation

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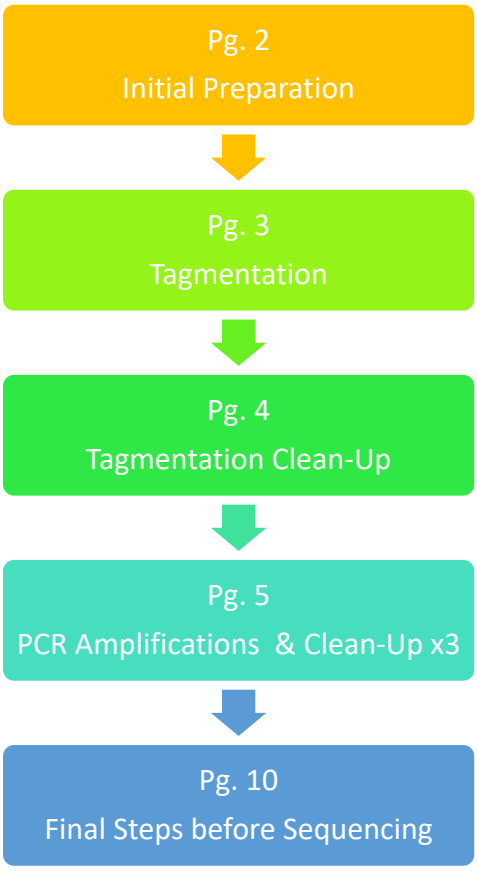
Workflow



Initial Preparations

DNA Input

Ensure that DNA input is of pure quality by assessment of the UV absorbance ratio.

260 / 280 nm = 1.8 – 2.0

260 / 230 nm = 2.0 – 2.2

Dilute samples so that a volume of 2 – 30 µl contains 100 – 500 ng of DNA.

Ideally, 30 µl will be used, so base calculations on that volume.

Materials

Illumina DNA prep, Tagmentation kit (Cat. No. 20060060)
PCR plastics
Ampure XP beads
Magnetic 96 rack fitting PCR tubes
200 proof pure EtOH
Primers
Nuclease-free H2O
Gel electrophoresis materials
2X KAPA HiFi HotStart ReadyMix
Illumina Nextera DNA CD Indexes (24 or 96 samples, review Adaptor pooling guide here)

Note: It is **strongly encouraged** that all instructions provided in the Illumina DNA Prep kit are reviewed first and followed where applicable. While much of the kit's protocols are described here, additional information, including preparation of materials and troubleshooting guides are described by Illumina.

Tagmentation

1. Let the following components warm to room temperature:
    a. Bead-Linked Transposomes (BLT)
    b. Tagmentation Buffer 1 (TB1)
2. Once thawed, vortex both of these well.
3. Set up the thermocycler for the following custom Tag program:
    a. Preheat lid at 100˚C
    b. 55˚C for 15 mins
    c. Hold at 10˚C
4. Prepare the tagmentation master mix (volumes account for residual) and multiply the number of samples used:
    a. 11 µl BLT
    b. 11 µl TB1
5. Add 20 µl of the tagmentation master mix to each 30 µl diluted sample of DNA in PCR tubes/96 well plate. Total reaction volume is 50 µl.
6. Place tubes/plate in the thermocycler and run the custom Tag program.

Tagmentation Clean-Up

1. Prepare the following buffer: Tagmentation Stop Buffer (TSB)
    a. If the TSB precipitates, heat it to 37˚C for 10 minutes then vortex and cool to room temperature.
2. Set up the following Stop Tagmentation protocol on the thermocycler:
    a. Preheat lid at 100˚C
    b. 37˚C for 15 minutes
    c. Hold at 10 ˚C
3. Add 10 µl of TSB to each tube/well from tagmentation reaction
    a. Resuspend the beads by pipetting slowly 10 times
4. Return tubes/plate to thermocycler and run the Stop Tag protocol.
5. Place the tubes on a magnetic rack and wait until the liquid clears ~2 – 5 mins
6. Aspirate and discard the supernatant.
7. Wash step:
    a. Remove the tube/plate from magnetic stand.
    b. Add 100 µl Tagmentation Wash Buffer to the beads, pipetting slowly to avoid foaming
    c. Pipet slowly to resuspend
    d. Place tubes/plate back on magnetic stand and wait until clears again
    e. Remove & discard the supernatant
8. Repeat the wash steps except do not remove supernatant at final step until ready for amplification!!
    a. Cannot let the beads dry out!

PCR Amplifications & Clean-Up

There will be three amplification steps using custom primers to enrich for the transposon junction. Clean-up will be using AmPure XP beads.

First Amplification
Tn-junction enrichment of tagmented DNA

Primers:

Tn-Specific Random Hybrid	ACCTGGGCACGCGACGACGCTCTTCCGATCTGG
Shortened Adaptor 1	TCGTCGGCAGCGTCA

1. Thaw the Illumina enhanced PCR mix (EPM), invert to mix and centrifuge. Thaw primers:

   Tn-specific Random Hybrid and Shortened Adaptor 1

2. Set up the following program as Tn-Amplification 1 on the thermocycler
   a. Preheat lid to 100˚C
   b. Start
      i. 68˚C 3:00 (min:sec)
      ii. 98˚C 3:00
   c. First 5 cycles
      i. 98˚C 0:45
      ii. 60˚C 0:45
      iii. 68˚C 2:00
   d. 15 more cycles
      i. 98˚C 0:15
      ii. 62˚C 0:45
      iii. 68˚C 0:30
         1. On the last cycle for the final extension step, set it to be 10 minutes
   e. Hold at 4˚C
3. Prepare the Illumina Enhanced PCR (EPM) Master mix and multiple each volume by the number of samples being used. (volumes account for residual)
   a. 22 µl EPM
   b. 22 µl nuclease-free water
   c. 5 µl Tn-specific hybrid primer
   d. 5 µl Shortened Adaptor 1 primer
4. Vortex then centrifuge the master mix to bring down droplets at 280 g x 10 seconds.

5. With bead-linked tagmented samples ready, remove & discard the supernatant while on the magnetic stand.
6. Remove from magnet stand
7. Immediately add 50 µl of PCR master mix to each sample tube/well
8. Pipet to mix until fully resuspended then centrifuge for 3 seconds.
9. Place in thermocycler and run the Tn-Amplification 1 program

Clean Up 1st Round Amplicons

1. Bring Ampure XP beads to room temperature. Thaw Resuspension Buffer (RSB) to room temp.
2. Make 80% EtOH from pure ethanol stock
3. Centrifuge the bead-linked transposome reaction after amplification at 280 G x 1 min.
4. Place tubes/plate on magnetic stand and wait until clears ~3 mins
5. Transfer 45 µl of the supernatant to a microcentrifuge tube or 96 well 0.8 ml deepwell storage (midi) plate
6. ***Before bead cleanup – assess reaction success by running the remaining 5 µl an agarose gel – add loading dye at appropriate ratio. Aim to see DNA of numerous sizes, most > 350 bp***
7. Vortex & invert the Ampure stock solution before transferring 45 µl of these beads to each tube/well
    a. *This accounts for a 1:1 ratio*
8. Pipet each tube/well ten times or on shaker for 1 min.
    a. Solution is viscous, pipet slowly
9. Incubate at room temp for 5 mins
10. Place tubes/plate on magnetic stand and allow to clear
11. Remove & discard the supernatant
12. Wash the beads twice:
    a. Add 200 µl of fresh 80% EtOH to the beads while they are on the magnet stand
        i. Do NOT mix or disturb beads!!
    b. Incubate for 30 sec

c. Remove & discard the supernatant
d. Repeat for second wash
e. After second wash, all beads to air dry for 5-15 minutes

13. Remove tubes from magnetic stand. Add 32 µl of resuspension buffer and pipet to resuspend.
14. Incubate at room temp for 2 mins
15. Place back on magnet stand and allow to clear
16. Transfer 20 µl to a new PCR tube/plate for each sample.
    a. Store remaining 10-12 µl of cleaned up library sample at -20˚C

It is safe to store purified samples for 1 month at -20˚C

Second Amplification
Reattach full adaptor 2 sequence to Tn-site 3' end

PCR Mix (Note: this mix differs from the first amplification):
The 2X ReadyMix contains KAPA HiFi HotStart DNA Polymerase (0.5 U per 25 µL reaction) in a proprietary reaction buffer containing dNTPs (0.3 mM of each dNTP at 1X), MgCl2 (2.5 mM at 1X) and stabilizers.

Primers:

Adaptor 2-Random hybrid
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACCTGGGCACGCG
Full-length Adaptor 1 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

1. Thaw KAPA HiFi 2X Ready Mix, invert several times to mix and centrifuge down briefly. Thaw primers: Adaptor 2 hybrid and Shortened Adaptor 1.
2. Set up the following program as Tn-Amplification 2 on the thermocycler
    a. Preheat lid to 100˚C
    b. Start
        i. 68˚C 3:00 (min:sec)
        ii. 98˚C 3:00
    c. 5 cycles
        i. 98˚C 0:45
        ii. 62˚C 0:30
        iii. 68˚C 2:00
    d. Final cycle extension step: 68˚C for 1:00
    e. Hold at 4˚C

3. Prepare the PCR Master mix and multiple each volume by the number of samples being used. (volumes do not account for residual)
    a. 25 µl KAPA HiFi 2X ReadyMix
    b. 3 µl nuclease-free water
    c. 1 µl Adaptor 2 hybrid primer
    d. 1 µl Full-length Adaptor 1 primer
4. Vortex then centrifuge the master mix to bring down droplets at 280 g x 10 seconds.
5. Add 30 µl of the master mix to 20 µl of each library samples generated from first amplification.
6. Place in thermocycler and run the Tn-Amplification 2 program

Repeat Clean Up steps listed above

1. Add 1:1 volume (50 µl to 50 µl reaction volume) of room temperature Sample Purification Beads directly to each PCR reaction sample.
2. Incubate 15 minutes
3. Place on magnetic rack and allow to clear.
4. Leave tube(s) on magnetic rack and perform the 80% EtOH wash steps
    a. Add 200 µl of 80% EtOH to tubes without disturbing beads
    b. Incubate 30 seconds then discard supernatant
    c. Repeat for a total of two washes
    d. Allow to air dry for 5 minutes then remove from magnetic stand
5. Resuspend in either 32 µl of Resuspension buffer or EB Buffer (Tris-HCl pH 8-8.5)
6. Place tubes on magnetic stand until clear
7. Transfer 20 µl the supernatant to a new tube without any beads.
8. Store 10-12 µg of 2nd round amplicons at -20˚C

Third Amplification

Extend adaptors by adding indexes and Illumina sequencing adaptors

Primers:

Illumina Index 1 CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

Illumina Index 2 AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

1. Thaw KAPA HiFi 2X Ready Mix, invert several times to mix and centrifuge down briefly. Thaw primers: Adaptor 2 hybrid and Shortened Adaptor 1.
2. Set up the following program as Tn-Amplification 3 on the thermocycler

a. Preheat lid to 100˚C
b. Start
    i. 68˚C 3:00 (min:sec)
    ii. 98˚C 3:00
c. 5 cycles
    i. 98˚C 0:45
    ii. 62˚C 0:30
    iii. 68˚C 2:00
d. Final cycle extension step: 68˚C for 1:00
e. Hold at 4˚C

3. Prepare the PCR Master mix and multiple each volume by the number of samples being used. (volumes do not account for residual)
    a. 25 µl KAPA HiFi 2X ReadyMix
    b. 3 µl nuclease-free water
4. Vortex then centrifuge the master mix to bring down droplets at 280 g x 10 seconds.
5. Add appropriate index adaptors to each sample using the Adaptor Pooling Guide
    a. 1 µl Index with i7
    b. 1 µl Index with i5
    c. *Alternatively: 2.5 µl of each i7 and i5 index and 0 µl H2O to master mix*
6. Add 30 µl of the master mix to 20 µl of each library samples generated from second amplification.
7. Mix by pipetting then spin down.
8. Place in thermocycler and run the Tn-Amplification 3 program

<u>Repeat Clean-Up as listed above</u>

Final Steps before Sequencing

Store 10-12 µl of 3rd round amplicons at -20˚C

Combine 5 µl of each library, vortex & centrifuge. Quantify by Qubit (follow manufacturer instructions for preparation).

Submit 20 – 30 µl of final sample for fragment analysis and sequencing on appropriate platform.