Tn5 Library Prep Protocol

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* This protocol is adapted from previous versions, but some of the language has been modified for the sake of clarity. Read through the entire protocol before starting! *

Annealing Oligos and Loading Tn5 (duration 2 hours)

1. Thaw out **100 µM** aliquots of the three oligos you'll need to anneal:
    a. Tn5ME - R: `5'-[phos]CTGTCTCTTATACACATCT-3'`
    b. Tn5ME - A: `5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′`
    c. Tn5ME - B: `5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′`
2. Label one PCR tube "A + R" and another one "B + R".
3. You'll need 0.286 µl of annealed oligos and 2 µl of charged Tn5 for every 25 µl library you intend on making. For this annealing step, make enough oligos for 4 more libraries than you will actually make, to account for pipetting error and evaporation.
    a. You'll eventually be combining the annealed "A + R" and "B + R" oligos once you've loaded them onto Tn5, so each annealing reaction will be half of the total volume of oligos you'll use.
4. If you plan on making 20 libraries, prepare enough oligos for 24. Each of the two annealing reactions will be half of this amount (12). In the "A + R" PCR tube, add 2 µl of oligo A to 2 µl of oligo R (0.286 * 6 = 1.71). This will leave you with excess annealed oligo, which is okay. Don't worry about wasting some. Do the same in the "B + R" tube, only with the B oligo this time. Mix the contents of each tube by pipetting, and then give them a quick spin down to get the contents to the bottom.
5. Annel the oligos using the following protocol:
    a. 95ºC for 5 minutes
    b. 25ºC (-0.1ºC/second)
    c. Hold at 25 ºC for ∞
6. Spin the PCR tubes briefly to bring any condensation liquid down to the bottom of the tube. Label two more PCR tubes "Tn5 + AR" and "Tn5 + BR". Again, each of these will contain half of the total volume of material you'll use in the tagmentation.
7. Continuing with the example of preparing 20 libraries, but compensating for 24, for each library you intend on making you'll need to add 0.286 µl of annealed oligo to 2 µl of Tn5. In the "Tn5 + AR" tube, add 3.43 µl (0.286 * 12) of annealed A + R oligo to 24 µl (2 * 12) of Tn5. Do the same thing in the "Tn5 + BR" tube, only with the annealed B + R oligos this time. Mix via pipetting and then give them a quick spin down to get the contents to the bottom.
8. Incubate for 1 hour at room temperature to charge the Tn5.
9. Combine the total volumes of each loaded Tn5 tube and mix via pipetting. Do not vortex. Give the mixed aliquot a quick spin down to bring the contents to the bottom.

DNA Tagmentation (duration 45 minutes)

1. For each library, prepare the following reaction:

Component	Volume or Mass
Loaded Tn5	2 µl
5X TAPS-PEG 8000	4 µl
DNA Input	10 ng
DEPC H_2O	Fill to 20 µl
Total Volume	**20 µl**

2. Incubate each reaction in the thermocycler at 55ºC for 10 minutes.
    a. This incubation time can be modulated, and so can the volume of Tn5 added to the tagmentation reaction to optimize the tagging of a given sample. If your DNA is highly degraded, consider using less Tn5 in the tagmentation, as loading too much will fragment your DNA too much, generating primarily short reads. Always keep in mind the quality of your sample going into this reaction.
3. Kill the Tn5 reaction by quickly adding 5 µl of 0.2% SDS and mix via pipetting up and down. Let this incubate at room temperature for at least 7 minutes.
    a. It's useful to use a multichannel pipette for this step since you want to add the SDS as quickly as possible.

PCR Indexing and Amplification (duration 1.5 hours)

1. Make a master mix with the following components, per reaction:

Component	Volume (µl)
DEPC H_2O	11.75
5X KAPA HiFi Buffer	5
dNTPs	0.75
HiFi Polymerase	0.5
Total Volume	**18 µl**

2. Add separately to each reaction:

Component	Volume (µl)
10 µM i5 Index Primer	1
10 µM i7 Index Primer	1
Tn5 Product	5
Total Volume	**25**

3. Mix the components of each PCR reaction mix pipetting. Spin down all the reactions briefly in the centrifuge to bring the contents to the bottom.
4. Run the following PCR protocol:
    a. 72ºC for 5 minutes
    b. 95ºC for 3 minutes
    c. 98ºC for 20 seconds
    d. 65ºC for 15 seconds
    e. 72ºC for 30 seconds
    f. Repeat from part (c) 11X
    g. 72ºC for 5 minutes
    h. Hold at 4ºC ∞
5. Qubit some of the libraries to make sure you've obtained sufficient amplification.
    a. Remember, if 10 ng of DNA went into the tagmentation reaction that was brought to 25 µl, and only 5 µl of that was put into the PCR reaction, then your initial DNA concentration of the 25 µl PCR reaction is ~0.08 ng/µl. Good amplification after 12 cycles should yield anywhere from 20 -- 70 ng/µl.

Library Purification:

1. The library purification can be done in two ways. You can use SPRI beads to clean up the libraries, using a high bead to sample ratio to remove only the smallest DNA (primers), or you can use the ZYMO Select-a-Size Spin columns to remove fragments smaller than ~300 bp. In this protocol, I will outline the procedure for both purification techniques, however, only one of them is needed. Choose wisely.

Library Purification via Bead Cleanup (duration 1 hour)

1. Before starting, make sure that the SPRI beads are at room temperature. Take them out of the fridge at least 30 minutes before you begin the cleanup. Cold beads dramatically reduce their ability to bind the DNA.

    a. This waiting time is factored into the total time of this purification. The purification itself is super quick so don't worry.
2. While you're waiting for the beads to warm up, make a solution of 70% EtOH. Assume you'll need about 100 µl per sample you're purifying.
3. Use a bead to sample ratio of 1.8:1, so if you have 25 µl of sample pipet 45 µl of beads into the tube containing the sample. Mix by pipetting up and down 10-20 times to insure solution is thoroughly mixed. For optimal binding, allow sample to incubate at room temperature for at least 5 minutes.
4. Place the sample on the magnetic separation rack for 5 minutes, or for as long as it takes for the solution to be cleared of beads and the beads look completely collected on the magnet.
5. While keeping the tube on the magnet and trying to not move it around too much, carefully aspirate and discard the cleared solution. Avoid disturbing the bead pellet at all costs.
6. Dispense 50 µl of 70% EtOH (or enough to fill the tube above the pellet) on the side of the tube opposite of the pellet so as to not disturb the beads. Incubate for 30 seconds, aspirate, and discard all of the EtOH. DON'T DISTURB THE BEAD PELLET!
    a. Seriously, don't disturb the pellet. All of your precious library is bound to the beads so if you disturb them, you'll lose some of your sample.
7. Repeat step 6 for a total of 2 EtOH washes.
8. Let the pellet air-dry for ~5 minutes to remove the residual EtOH. Let it dry until it no longer appears shiny, but before it begins to crack.
    a. If the beads are under dried, the residual EtOH may affect downstream applications. If the beads are over dried, it may be difficult to elute the DNA from the beads completely, and you might also shear your DNA.
9. Remove the samples from the magnet and resuspend the pellet (yes, disturb the pellet now) in 20 µl of water by pipetting the elution volume up and down **lightly** 30+ times to ensure there are no clumps of beads. Be careful not to splash the sample up onto the side of the tube. Incubate the sample at room temperature for 3 minutes, then place the tube back on the magnetic rack for 2-3 minutes, or until the solution is clear.
    a. Believe it or not, the water will elute the DNA off of the beads very effectively!
10. Once the beads are collected on the magnet, carefully transfer the entire elution solution (and none of the beads) to a new tube.
11. Qubit to quantify the concentration of your purified DNA library. Run the samples on the tapestation using a HS tape if you intend on getting these libraries sequenced. Store the remaining 18 µl sample at -20ºC.

Library Purification via Size Selection Columns (duration 30 minutes)

1. To select for sequences above 300 bp, use the *ZYMO Select-A-Size DNA Clean and Concentrator* Kit and use the appropriate option for selection of ≥300 bp fragments.
2. Add 500 µl of Binding Buffer to a new 1.5 mL tube. Since we're removing all DNA smaller than 300 bp, do not add any ethanol to the Binding Buffer.
3. Add enough Elution Buffer to the DNA sample to bring it up to 100 µl. If you have 25 µl of PCR product, add 75 µl of Elution Buffer and mix by pipetting.

4. Add the entire DNA + Elution Buffer sample to the tube containing Binding Buffer and pipette up and down at least 10 times to mix.
5. Pipette all of the contents of the tube into a yellow IC-S column placed in a collection tube. Spin at 12,000 xg for 30 seconds. Discard the flow through.
6. Add 700 µl of Wash Buffer to the column and spin for 30 seconds. Discard the flow through.
7. Add 200 µl of Wash Buffer to the column and spin for 1 minute. Discard the flow through.
8. Place the column in a new 1.5 mL centrifuge tube. Add 20 µl of Elution Buffer directly to the center of the column and let it sit at room temperature for at least 5 minutes. Spin at 12,000 xg for 30 seconds.
    a. **Optional:** Pipette the entire elution volume and place it back onto the center on the column placed in the same tube Add 2 more µl's of Elution Buffer. Incubate for another 3 minutes at room temperature. Spin at 12,000 xg for 30 seconds. Qubit to quantify.
9. Qubit to quantify the concentration of your purified DNA library. Run the samples on the tapestation using a HS tape if you intend on getting these libraries sequenced. Store the remaining ~20 µl sample at -20ºC.