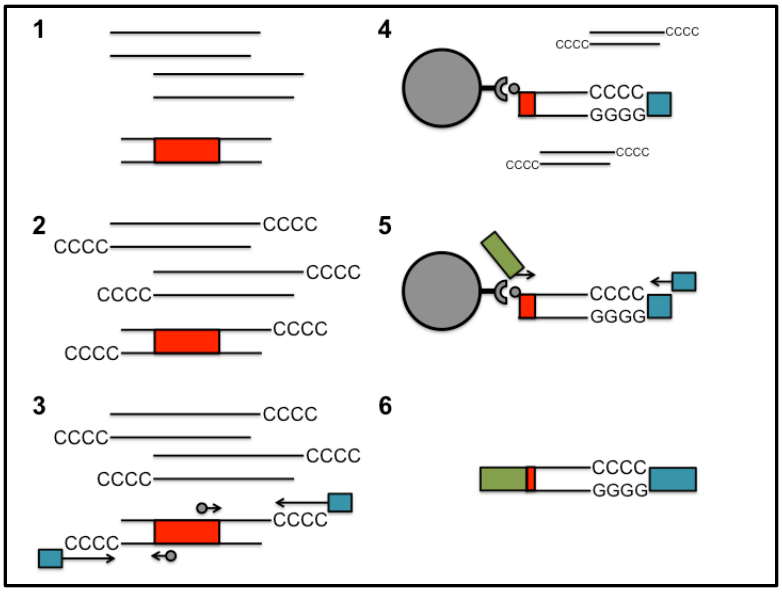
TdT tailing method for Tn-seq library construction

Overview



(1) Genomic DNA from a transposon mutant pool is extracted and sheared. Only some of the fragments contain a transposon insert (red). (2) A C-tail is added to the 3' end of each fragment using the TdT enzyme, providing a universal priming site. (3) PCR-1: A biotinylated primer (grey) targets the transposon (red). A C-tail-specific primer appends the Illumina Read 2 primer site (blue). (4) Biotinylated products from PCR-1 are purified from background genomic DNA using streptavidin-coupled DynaBeads. (5) PCR-2: A second transposon-specific primer builds the entire P5 Illumina adapter/flow cell capture site and the Read 1 primer site (green). The other primer targets the Read 2 primer site and contains the library-specific barcode and the P7 Illumina adapter/flow cell capture site (blue). (6) The final product contains both the Illumina P5 and P7 adapters and Read 1 and Read 2 primer sites for sequencing. See accompanying PDF (Tn-seq primer details) for complete details.

References

Klein *et al.*, 2012 (*BMC Genomics*) http://www.biomedcentral.com/1471-2164/13/578
Goodman *et al.*, 2011 (*Nature Protocols*) http://www.nature.com/nprot/journal/v6/n12/abs/nprot.2011.417.html
Mintz, 2004 (*Microbiology*) http://mic.sgmjournals.org/content/150/8/2677.short
Jacobs *et al.*, 2003 (*PNAS*) http://www.pnas.org/content/100/24/14339.short
Bae *et al.*, 2004 (*PNAS*) http://www.pnas.org/content/101/33/12312.short

Materials

-recombinant terminal deoxynucleotidyl transferase (rTdT) (Promega, cat. no. M1875)
-5x rTdT reaction buffer
-dCTP/ddCTP mixture
  -dCTP, 9.5 mM
  -ddCTP (VWR, cat. no. 101172-042), 0.5 mM
-Expand Long Template DNA polymerase (Roche, cat. no. 11681842001) (see note 1)
-Expand Long Template Buffer 2, 10x concentrated
-dNTPs mixture, 10 mM
-DynaBeads® M-280 Streptavidin (Life Technologies, cat. no. 11205D)
-2x binding & wash (B&W) buffer
  -2M NaCl
  -10 mM Tris-HCl
  -1 mM EDTA, pH 7.5
-low TE (LoTE) buffer
  -3 mM Tris-HCl
  -0.2 mM EDTA, pH 7.5
-AMPure XP beads (Beckman Coulter, cat. no. A63880)
-magnetic separation rack (NEB, cat. no. S1506S)
-spin columns for DNA purification
-tube rotator
-thermocycler
-molecular biology-grade H_2O
-DNase-free Eppendorf tubes

-barrier pipette tips

Primer design

Tn-1
The Tn-1 primer should be 5'-biotinylated. If possible, Tn-1 should be designed to amplify in only one direction off the transposon.

Tn-2
The Tn-2 primer should hybridize downstream of Tn-1 but *not* at the very edge of the transposon. Instead, Tn-2 should be nested at least 10-15 bases into the transposon. This is for bioinformatics purposes. Primers can be non-specific, and these 10-15 bases serve as a way to bioinformatically distinguish sequencing reads that are derived from your transposon from those that are present because of offsite priming. The Tn-2 primer is very long and should be PAGE purified (an option that should be available when the primer is ordered). The five random bases (N's) are meant to help with generating cluster diversity on the Illumina flow cell. Please e-mail us if you have any questions!

Barcode (BC) primers
These primers are identical except for a 6 bp 'barcode' sequence. These barcodes give each library a unique ID and allow them to be sequenced on the same Illumina flow cell. Labs that plan to do many Tn-seq experiments should have several barcode primers. Barcode sequences can be found here: https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors. Since these are reverse primers, please note to use the *reverse complement* of what is listed on the website (see table below for examples).

The melting temperatures (T_m) in the tables listed below were calculated using the default settings in OligoAnalyzer 3.1 (https://www.idtdna.com/calc/analyzer) and correspond to the underlined regions.

Transposons

Transposon	Plasmid	Organism	Reference
Tn10	pVT1542	*Aggregatibacter actinomycetemcomitans*	Mintz, 2004 (*Microbiology*)
T8 (Tn5 derviative)	pIT2	*Pseudomonas aeruginosa*	Jacobs *et al.*, 2003 (*PNAS*)
mariner	pMR361-K*	*Aggregatibacter actinomycetemcomitans*	Bae *et al.*, 2004 (*PNAS*)
*This is a mariner delivery plasmid constructed by Dr. Matthew Ramsey.			

PCR-1 primers

Primer	Description	Sequence (5'->3')	T_m
Forward			
Tn-1	biotinylated; targets the Tn	biotin-(insert your sequence)	
Tn10-1	Tn-1 primer for Tn10	biotin-TTTACACTGATGAATGTTCCGTTGCGCTGC	63ºC
T8-1	Tn-1 primer for T8	biotin-GGGTTTTCCCAGTCACGACGTTG	61ºC
mariner-1	Tn-1 primer for mariner	biotin-ACTCACTATAGGAGGGCGGGAATCATTTGAAGGTTGGTAC	
Reverse			
olj376	targets the C-tail; adds the Read 2 primer site	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG	72ºC

PCR-2 primers

Primer	Description	Sequence (5'->3')	T_m
Forward			
Tn-2	targets the Tn; adds the P5 adapter and Read 1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN-(insert your sequence)	

	primer site		
Tn10-2	Tn-2 primer for Tn10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN AGATGTGTATCCACCTTAACTTAATGATTTTTACC	57ºC
T8-2	Tn-2 primer for T8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN CGTCCAGGACGCTACTTGTG	58ºC
mariner-2	Tn-2 primer for mariner	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNN GTGTCAGACCGGGGACTTATCAG	59ºC
Reverse			
BC--	barcode primers; add the library-specific barcode and the P7 adapter	CAAGCAGAAGACGGCATACGAGATxxxxxxGTGACTGGAGTTCAGACGTGTG	58ºC
BC39	primer with the TruSeq 39 barcode	CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGTG	58ºC
BC40	primer with the TruSeq 40 barcode	CAAGCAGAAGACGGCATACGAGATTCTGAGGTGACTGGAGTTCAGACGTGTG	58ºC
BC42	primer with the TruSeq 42 barcode	CAAGCAGAAGACGGCATACGAGATCGATTAGTGACTGGAGTTCAGACGTGTG	58ºC

Protocol

1. Shear genomic DNA to a 100-700 bp size range (ideally 200-400 bp). We use a QSonica 800R with the settings given below, but for some *in vivo* samples, we have found that it helps to further size-select the DNA using AMPure beads (see note 2 for AMPure bead size selection protocol). This ensures that all of the DNA that goes through the Tn-seq protocol is exactly within the target size range, especially small fragments which may be preferentially amplified (PCR bias) or large fragments which may inhibit Illumina bridge amplification. Sheared DNA should be run out on a gel to determine its size range (see note 3 for example gels).

QSonica 800R settings for shearing DNA to 200-400 bp	
amplitude	60%
pulse rate	10 seconds on
	10 seconds off
total sonication on time	10 minutes
water bath temperature	4ºC
sample volume	≥250 µl

2. Set up the TdT tailing reaction:

Reagent	**Volume (µl)**
sheared DNA (2.5 µg)	-
9.5 mM dCTP / 0.5 mM ddCTP	2.5
5x rTdT reaction buffer	10
rTdT	1.25
H_2O	to 50 µl

3. Incubate the reaction at 37ºC for 1 hour.

4. Purify either with AMPure beads (see step 12 for protocol) or with spin columns (follow the manufacturer's protocol; if given the option, add isopropanol). Elute in 25 µl H_2O.

5. Set up PCR-1:

Reagent	**Volume (µl)**
tailed template (250-500ng)	-
10x Buffer 2	5
dNTPs (10 mM)	2.5
Tn-1 primer (30 µM)	1
olj376 primer (30 µM)	3
DNA polymerase	0.75
H_2O	up to 50 µl

6. Run the following thermocycler program:

Temperature	**Time**	
95ºC	5 minutes	
94ºC	30 second	10x
60ºC*	30 seconds	
68ºC	2 minutes	
68ºC	10 minutes	
16ºC	hold	

*The annealing temperature may be different depending on the design of your Tn-1 primer. The temperature shown here has been successful in making libraries with our Tn10-1, T8-1, and mariner-1 primers.

7. Purify (either with AMPure beads or columns). Elute in 50 µl H_2O.

8. Bind purified product to Dynabeads:
-resuspend beads, add the following volume to a 1.5 ml Eppendorf tube:
32 µl x the total number of reactions
-on magnetic stand, wash beads 3x with 1 ml 1x B&W buffer
-remove final wash, resuspend beads in the following volume of 2x B&W buffer:
52 µl x the total number of reactions
-add 50 µl purified product to 50 µl beads, rotate at room temperature for 30 minutes
-wash 1x with 100 µl 1x B&W buffer, 2x with 100 µl LoTE, remove final wash

9. Set up PCR-2 by resuspending the beads in the following reaction mixture:

Reagent	**Volume (µl)**
PCR-1 product	DynaBeads
10x Buffer 2	5
dNTPs (10 mM)	2.5
Tn-2 primer (30 µM)	1
BC-- primer (30 µM)	1
DNA polymerase*	0.75
H_2O	39.75

*To avoid shearing the polymerase, add it to the PCR-2 mixture after the DynaBeads are fully resuspended.

10. Run the following themocycler program:

Temperature	**Time**	
95ºC	5 minutes	
94ºC	30 second	15x
58ºC*	30 seconds	
68ºC	2 minutes#	
68ºC	10 minutes	
16ºC	hold	

*The annealing temperature may be different depending on the design of your Tn-2 primer. The temperature shown here has been successful in making libraries with our Tn10-2, T8-2, and mariner-2 primers.
#We recommend gently flicking the tube at the 1-minute mark of each 68ºC extension segment to keep the DynaBeads in solution.

11. On a magnetic stand, collect the supernatant from the beads and transfer it to a new tube. Purify the supernatant with 40 µl AMPure beads (see protocol below). Elute in a small volume (10-15 µl) H_2O. Do not use spin columns here since they will not remove adapter contamination.

1. Adjust sample volume to 50 µl with H_2O
2. Add a 0.8X ratio of AMPure beads (40 µl)
3. Mix well by thoroughly pipetting and/or gently vortexing
4. Incubate at room temperature for 15 minutes
5. Place tube on magnetic stand, let suspension clear
6. Remove supernatant, add 200 µl 80% ethanol, wait 30 seconds
7. Remove ethanol, add 200 µl 80% ethanol, wait 30 seconds
8. Carefully remove all ethanol, let dry on magnetic stand for 10 minutes
   *Do not over-dry the sample as this can lead to lower DNA recovery
9. Take tube off magnetic stand, resuspend in elution volume of H_2O
10. Incubate at room temperature for 5 minutes
11. Place tube back on magnetic stand, let suspension clear
12. Transfer supernatant to new tube

12. At this point, we submit our samples to the core facility at UT-Austin for quality control, including a Bioanalyzer run (see note 4 for example Bioanalyzer results) and qPCR.

Troubleshooting:
-If the Bioanalyzer shows that the library has adapter contamination, re-purify it with 0.8X AMPure beads.
-If the library is not detectable on the Bioanalyzer, you should still request for qPCR data because your sample may still contain reads. The qPCR will accurately determine the number of reads your library will yield from Illumina sequencing.
-If your sample does not contain library according to qPCR, we recommend increasing the C-tailed template in PCR-1 to 2 µg (or the remaining DNA from the C-tailing reaction). If that does not work, try scaling up the TdT reaction to ≥10 µg and adding all of it to PCR-1.
-If a library is detectable and lacks adapters, then it is ready to be sequenced!

Notes

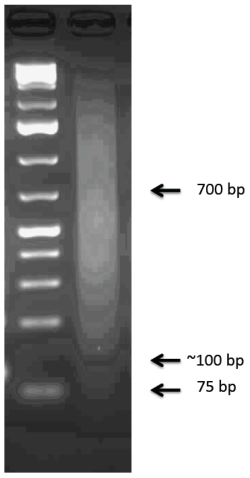
1. We have had the most success constructing Tn-seq libraries using the Expand Long Template system.

2. AMPure bead size selection protocol

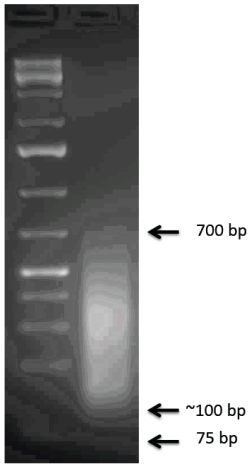
1. Remove large fragments (>700 bp)
   a. Adjust sample volume to 50 µl with H_2O
   b. Add 0.5X beads (25 µl), mix thoroughly
   c. Incubate at room temperature for 15 minutes
   d. Place sample on magnetic stand, let suspension clear
   e. Remove 72 µl supernatant, place in a new tube
      *The supernatant contains your sample, the fragments below 700 bp
2. Remove small fragments (<100 bp)
   a. To the 72 µl supernatant, add 0.7X beads (50.4 µl), mix thoroughly
   b. Incubate at room temperature for 15 minutes
   c. Place sample on magnetic stand, let suspension clear
   d. Remove supernatant and discard (optional: save supernatant at -20^{o}C in case of error)
      *Your sample is now on the beads; the supernatant contains small fragments <100 bp
3. With sample on magnetic stand, add 200 µl 80% ethanol

4. Incubate sample for 30 seconds, discard ethanol
5. Repeat steps 3 and 4 (for two total 80% ethanol washes)
6. Let sample air dry on magnetic stand at room temperature for 10 minutes
7. Remove sample from stand, resuspend in elution volume of H_2O
8. Incubate sample at room temperature for 5 minutes
9. Place sample back on magnetic stand, let suspension clear
10. Transfer clear supernatant to new tube (remove 2 µl less than elution volume added to avoid beads)

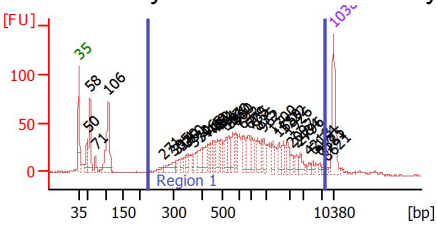
3a. Gel of a sonicated DNA sample before size selection (1 kb plus ladder):



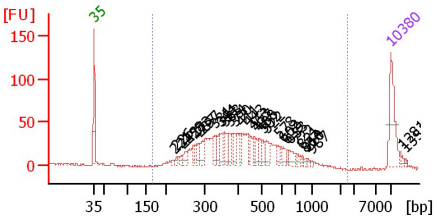
3b. Gel of a size-selected DNA sample that a Tn-seq library was made from (1 kb plus ladder):



4a. Bioanalyzer result for a library that has adapter contamination:



4b. Bioanalyzer result for a library that was sequenced:



4c. Bioanalyzer result for another library that was sequenced. Libraries are ideally in the 300-500 bp range but can still be sequenced if they have peaks much larger than that. What is key is that they do not have adapter contamination since adapters will consume a large fraction of your sequencing reads.