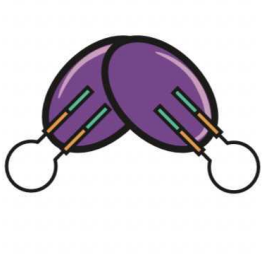
Jun 03, 2024 Version 2

SMRT-Tag - Direct transposition of native DNA for sensitive multimodal single-molecule sequencing V.2



DOI
dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v2

Scott Nanda[1,2], Ke Wu[1], Iryna Irkliyenko[1], Siva Kasinathan[3,4], vijay.ramani[1,2,5,6]

[1]Gladstone Institute for Data Science and Biotechnology, Gladstone Institutes;
[2]Department of Biochemistry and Biophysics, University of California, San Francisco;
[3]Gladstone-University of California, San Francisco Institute for Genomic Immunology, Gladstone Institutes;
[4]Division of Rheumatology, Department of Pediatrics, Stanford University; [5]Helen-Diller Cancer Center;
[6]Bakar Computational Health Sciences Institute

Ke Wu
Gladstone Institutes

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v2

External link: https://doi.org/10.1038/s41588-024-01748-0

Protocol Citation: Scott Nanda, Ke Wu, Iryna Irkliyenko, Siva Kasinathan, vijay.ramani 2024. SMRT-Tag - Direct transposition of native DNA for sensitive multimodal single-molecule sequencing. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v2**Version created by **Ke Wu**

Manuscript citation:
Nanda, A.S., Wu, K., Irkliyenko, I. *et al.* Direct transposition of native DNA for sensitive multimodal single-molecule sequencing. *Nat Genet* (2024). https://doi.org/10.1038/s41588-024-01748-0

Protocol status: Working
We use this protocol and it's working

Created: February 07, 2023

Last Modified: June 03, 2024

Protocol Integer ID: 76489

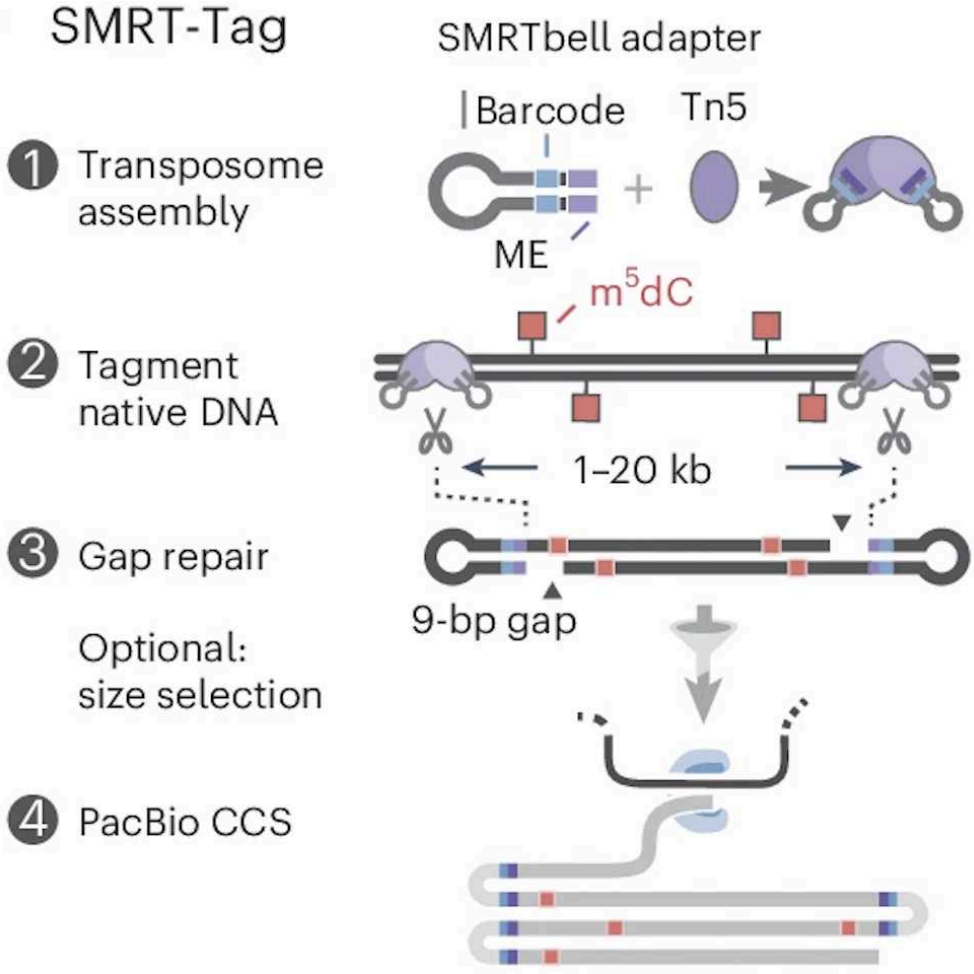
Keywords: Long-read sequencing, low-input genomics, epigenetics, chromatin biology, single-molecule sequencing, chromatin accessibility, DNA transposition, direct transposition of native dna, sequencing library, native dna, steps from purified gdna, purified gdna, quality genomic dna, using tn5 transposase, existing pacbio protocol, 40ng gdna, sequencing, pacbio protocol, dna, low as 40ng gdna, tn5 transposase, protocol for smrt, smrt, free pacbio, pacbio sequel ii platform, utility of smrt, cpg methylation, simultaneous detection of sequence variation

Abstract

Here we describe a protocol for SMRT-Tag: **S**ingle-**M**olecule **R**eal-**T**ime sequencing by **Tag**mentation - a transposase-mediatedstrategy for producing low-input, multiplexable, PCR-free PacBio sequencing libraries.

SMRT-Tag requires only 1-10% as much input material as existing PacBio protocols (typically at least 1–5 µg, or 150,000–750,000 human cells) and enables highly-sensitive and simultaneous detection of sequence variation and CpG methylation comparable to the current state-of-the-art.

In brief, high-quality genomic DNA is tagmented using Tn5 transposase loaded with custom hairpin adaptors, gap-repaired to generate closed and circular templates, exo-digested to remove unrepaired molecules, and then sequenced on the PacBio Sequel II platform. All steps from purified gDNA to sequencing-ready libraries can be performed in one day. We have demonstrated the utility of SMRT-Tag on input as low as 40ng gDNA (~ 7000 cell equivalents) sequenced monoplex.



Schematic workflow of the SMRT-Tag sequencing library preparation.

Guidelines

Direct transposition of native DNA for sensitive multimodal single-molecule sequencing

Arjun S Nanda[1,2*], Ke Wu[1*], Iryna Irkliyenko[1*], Brian Woo[2,6], Megan S Ostrowski[1], Andrew S Clugston[5,6], Leanne C. Sayles[5,6], Lingru Xu[6], Ansuman T Satpathy[4,7,8], Hao G. Nguyen[6], E Alejandro Sweet-Cordero[5,6], Hani Goodarzi[2,6,8,9], Sivakanthan Kasinathan[3,4*#], Vijay Ramani[1,2,6,9#]

[1]Gladstone Institute for Data Science & Biotechnology, Gladstone Institutes, San Francisco, CA 94158
[2]Department of Biochemistry & Biophysics, UCSF, San Francisco, CA 94143
[3]Division of Rheumatology, Department of Pediatrics, Stanford University, Stanford, CA 94305
[4]Gladstone-UCSF Institute for Genomic Immunology, Gladstone Institutes, San Francisco, CA 94143
[5]Department of Pediatrics, UCSF, San Francisco, CA 94143
[6]Helen-Diller Cancer Center, San Francisco, CA 94158
[7]Department of Pathology, Stanford University, Stanford, CA 94305
[8]Parker Institute for Cancer Immunotherapy, San Francisco, CA 94129
[9]Bakar Computational Health Sciences Institute, San Francisco, CA 94143
[*]These authors contributed equally to this work.
[#]correspondence to **skas@stanford.edu** or **vijay.ramani@gladstone.ucsf.edu**

Materials

- LoBind microfuge tubes (1.5 mL)
- PCR tubes (0.2 mL)
- $Tn5_{R27S,E54K,L372P}$ purified enzyme (QB3 Core)
- Barcoded SMRT-Tag Adaptor sequence (IDT, see supp. table)
- 20x NativePAGE Running Buffer (Invitrogen, cat. no. BN2001)
- 20x NativePAGE Cathode Buffer Additive (Invitrogen, cat. no. BN2002)
- GeneRuler Ultra Low Range DNA Ladder (Thermo Fisher, cat. no. SM1213)
- 4x NativePAGE Sample Buffer (Invitrogen, cat. no. BN2003)
- Coomassie Blue SafeStain (Invitrogen, cat. no. LC6060)
- NativePAGE 4-16% Bis-Tris Gel (Invitrogen, cat. no. BN1002BOX)
- 10,000x SYBR Gold (Invitrogen, cat. no. S11494)
- Agarose (Fisher Scientific, cat. no. BP160-500)
- 50x Tris/Acetic Acid/EDTA (TAE; Bio-rad, cat. no. 1610773)
- 6x DNA gel loading dye (Thermo Scientific, cat. no. R0611)
- 1kb extend ladder (New England Biolabs, cat. no. N3239S)
- High Molecular Weight genomic DNA (HMW gDNA; Coriell Institute, cat. no. HM24385, HM24149, HM24143)
- Distilled, deionized or RNAse-free H_2O (dH_2O; e.g. Invitrogen, cat. no. 10977023)
- Elution buffer (1x EB; Qiagen, cat. no. 19086)
- Dimethylformamide (DMF; Sigma-Aldrich, cat. no. 319937)
- 1 M TAPS buffer pH 8.5 (Boston BioProducts, cat. no. BB-2375)
- 1 M Magnesium Chloride (MgCl2; G-Bioscience, cat. no. R004)
- dNTP mix (100mM Thermo Fisher Scientific, cat. no. R0181)
- 10x Taq DNA Ligase Reaction Buffer (New England Biolabs, cat. no. B0208S)
- Phusion High-Fidelity DNA Polymerase (2,000U/mL New England Biolabs, cat. no. M0530L)
- Taq DNA Ligase (40,000U/mL New England Biolabs, cat. no. M0208S)
- 10x NEBuffer 2 (New England Biolabs, cat. no. B7002S)
- Exonuclease III (100,000U/mL New England Biolabs, cat. no. M0206L)
- Ethanol (Decon Labs, cat. no. V1016)
- SPRI beads (e.g. Agencourt AMPure XP, Beckman Coulter, cat. no. A63880)
- AMPure PB beads (Pacific Biosciences, cat. no. 100-265-900)
- Qubit 1X dsDNA HS Assay Kit (Invitrogen, cat. no. Q33231)
- Bioanalyzer High Sensitivity DNA Reagent Kit (Agilent Technology, cat. no. 5067-4627)
- Bioanalyzer DNA 12000 Reagent Kit (Agilent Technology, cat. no. 5067-1508)

Supplementary Table - SMRT-Tag Adaptor sequence:

	A	B
	Barcode Name	**Sequence**
	SMRT-A_bc-none	/5Phos/CTG TCT CTT ATA CAC ATC TAT CTC TCT CTT TTC CTC CTC CTC CGT TGT TGT TGT TGA GAG AGA TAG ATG TGT ATA AGA GAC AG
	SMRT-A_bc001	/5Phos/CTG TCT CTT ATA CAC ATC TTT CTT CCG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CGG AAG AAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc003	/5Phos/CTG TCT CTT ATA CAC ATC TTT CCA CAC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GTG TGG AAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc006	/5Phos/CTG TCT CTT ATA CAC ATC TTT GTC GCA ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT TGC GAC AAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc010	/5Phos/CTG TCT CTT ATA CAC ATC TTT AGC TGC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GCA GCT AAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc011	/5Phos/CTG TCT CTT ATA CAC ATC TTC CTA AGG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CCT TAG GAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc012	/5Phos/CTG TCT CTT ATA CAC ATC TTC CGT TGT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT ACA ACG GAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc013	/5Phos/CTG TCT CTT ATA CAC ATC TTC GAA TCG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CGA TTC GAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc014	/5Phos/CTG TCT CTT ATA CAC ATC TTC ACT GTG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CAC AGT GAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc015	/5Phos/CTG TCT CTT ATA CAC ATC TTG CAG GAT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT ATC CTG CAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc016	/5Phos/CTG TCT CTT ATA CAC ATC TTA TGG CGT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT ACG CCA TAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc017	/5Phos/CTG TCT CTT ATA CAC ATC TTA CCG ACT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT AGT CGG TAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc018	/5Phos/CTG TCT CTT ATA CAC ATC TTA CAA GCC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GGC TTG TAA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc019	/5Phos/CTG TCT CTT ATA CAC ATC TCT GAC CAA ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT TTG GTC AGA GAT GTG TAT AAG AGA CAG

	A	B
	SMRT-A_bc020	/5Phos/CTG TCT CTT ATA CAC ATC TCC TCT CTA ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT TAG AGA GGA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc021	/5Phos/CTG TCT CTT ATA CAC ATC TCC TGT AAC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GTT ACA GGA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc022	/5Phos/CTG TCT CTT ATA CAC ATC TCC GCA TAA ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT TTA TGC GGA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc023	/5Phos/CTG TCT CTT ATA CAC ATC TCA AGT GGA ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT TCC ACT TGA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc024	/5Phos/CTG TCT CTT ATA CAC ATC TGT GCA TTC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GAA TGC ACA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc025	/5Phos/CTG TCT CTT ATA CAC ATC TGG CTT CAT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT ATG AAG CCA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc026	/5Phos/CTG TCT CTT ATA CAC ATC TGG AAC TAC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GTA GTT CCA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc027	/5Phos/CTG TCT CTT ATA CAC ATC TGA CGT TAG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CTA ACG TCA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc028	/5Phos/CTG TCT CTT ATA CAC ATC TGA GTG TCT ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT AGA CAC TCA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc029	/5Phos/CTG TCT CTT ATA CAC ATC TGA AGA AGG ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT CCT TCT TCA GAT GTG TAT AAG AGA CAG
	SMRT-A_bc030	/5Phos/CTG TCT CTT ATA CAC ATC TAA CAC CTC ATC TCT CTC TTT TCC TCC TCC TCC GTT GTT GTT GTT GAG AGA GAT GAG GTG TTA GAT GTG TAT AAG AGA CAG

Troubleshooting

Safety warnings

DMF is toxic and extra care should be taken. Use full PPE including a mask, lab coat, and gloves while handling any amount of either chemical.

Annealing SMRT-Tag adaptors

1 Reconstitute HPLC-purified SMRT-Tag adaptors (IDT) to 100 µM in nuclease-free water.

2 Dilute adaptors to 20 µM in 1x annealing buffer (10mM Tris-HCl pH 7.5 and 100mM NaCl) in PCR tubes.

3 Place the tubes in a thermocycler at 95°C with a heated lid at 105ºC for 5 min, then remove them from the heat source and slowly cool to room temperature for at least 30 min.

[NOTE] Annealed adaptors can be stored at -20°C for up to 6 months.

Assembling SMRT-Tn5 transposomes (Tn5 loaded with SMRT-Tag adaptors)

4 Thaw Tn5 enzyme stock (3.9mg/mL) suspended in Storage Buffer (50mM Tris-HCl pH 7.5, 800mM NaCl, 0.2mM EDTA, 2mM DTT, 10% glycerol) on ice.

5 Dilute to ~1mg/mL (or 18.9 µM monomer) in Tn5 dilution buffer (50mM Tris-HCl pH 7.5, 200mM NaCl, 0.1mM EDTA, 2mM DTT, and 50% glycerol) and slowly mix 10-15 times with a wide-bore pipette. The mixture is extremely viscous.

6 Let sit on a rotational mixer at 4ºC for at least 3 hr until the mixture becomes fully homogenized.

7 Mix 1.02x volume of 1mg/mL Tn5 with 1x volume of 20 µM annealed SMRT-Tag adaptors using a wide-bore pipette.

8 Incubate the mixture at 23ºC for 55 min with continuous shaking at 350 rpm. After 55 min, place on ice. Loaded SMRT-Tn5 (~9.4µM monomer) can be supplemented with glycerol up to a final concentration of 50% and stored at −20° for up to 6 months.

(Optional) Validation of SMRT-Tn5 loading

9 Add 1-2 µL of each loaded SMRT-Tn5 transposome with 2.5µL of 4x Native Gel Loading Buffer and dilute up to 10 µL with dH2O.

10 Load each prepared sample on a NativePAGE 4-16% Bis-Tris Gel along with GeneRuler Ultra Low Range DNA Ladder, a negative DNA control (Tn5 without SMRT-Tag adaptors), a negative protein control (annealed SMRT-Tag oligos without Tn5), a positive control (Tn5 loaded with any confirmed adaptors i.e Nextera N5/N7 oligos) if available.

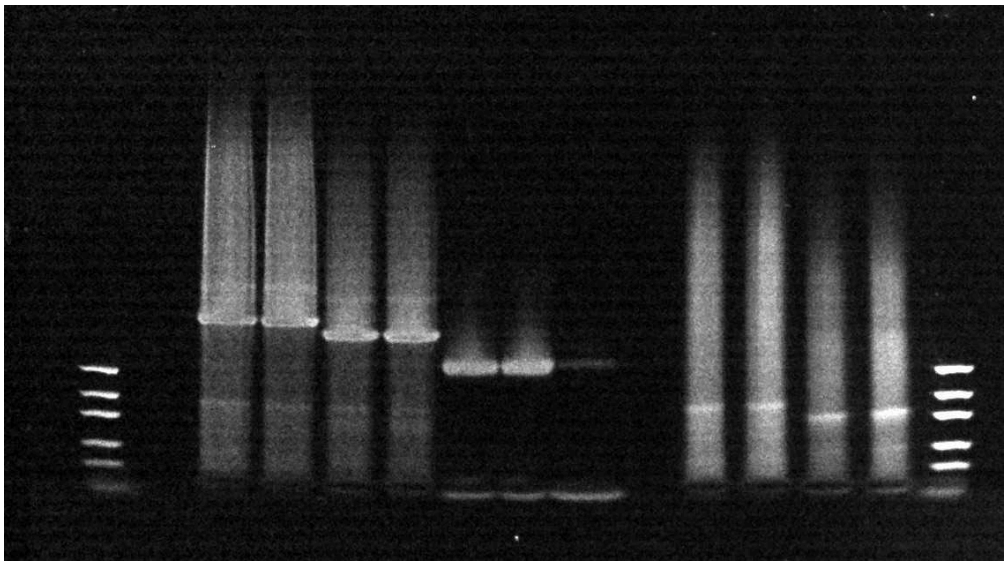
11 Add 200mL of cold cathode buffer (1x NativePAGE running buffer and 0.1x NativePAGE cathode additive) to the inner chamber. Add 600mL of cold anode buffer (1x NativePAGE running buffer) to the outer chamber.

12 Run the gel at 150V for 1 hr at 4ºC, followed by 180V for 15 min.

13 Stain the gel with 1x SYBR Gold Solution for ~30 min on a shaker. Visualize on a LiCor or any imaging system to confirm the proper loading of SMRT-Tn5 transposomes.

Note

Reference fig.1: loaded SMRT-Tn5 on a NativePAGE 4-16% Bis-Tris Gel.



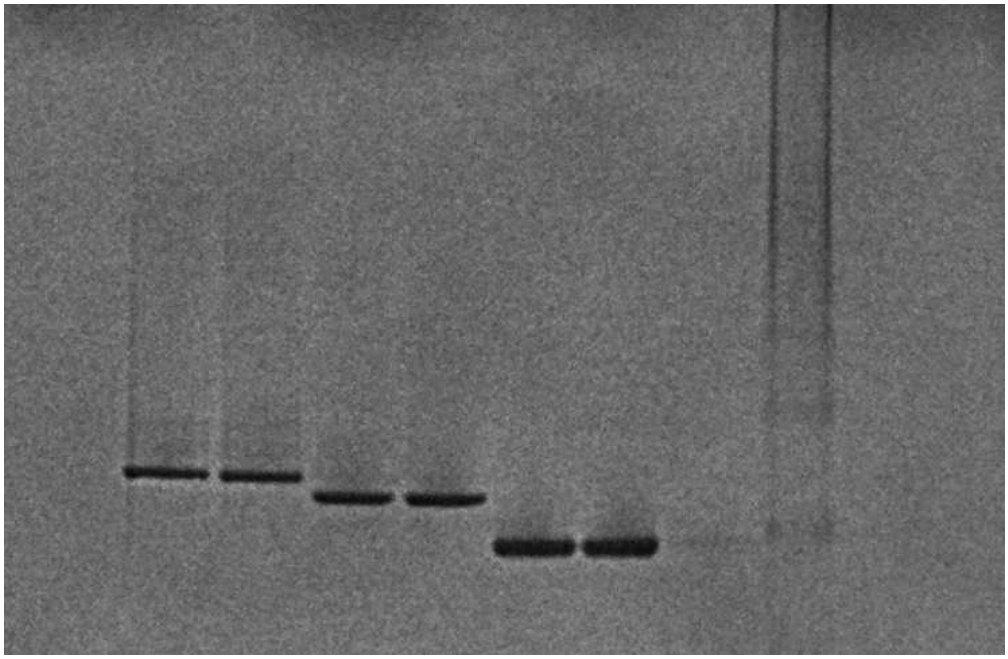
Left to Right: GeneRuler ULR ladder; 2 replicates of Tn5 loaded with barcoded SMRT-Tag adaptors; 2 replicates of Tn5 loaded with unbarcoded SMRT-Tag adaptors (shorter than barcoded SMRT-Tag adaptors); 2 replicates of Tn5 loaded with Nextera N5/N7 adaptors (shorter than SMRT-Tag adaptors); Tn5 monomer without adaptors; 2 replicates of annealed, barcoded SMRT-Tag oligos without Tn5; 2 replicates of annealed, unbarcoded SMRT-Tag oligos without Tn5; GeneRuler ULR ladder.

[NOTE] Not all samples loaded on the reference gel are required. The negative DNA control (Tn5 monomer without adaptors) should not be detected. The positive control (Tn5 loaded with Nextera N5/N7 adaptors) shows a lower DNA band than Tn5 loaded with SMRT-Tag adaptors due to the shorter oligo sequence.

14 Stain the gel with 1x Coomassie Blue for 1 hr at RT on a shaker, destain for at least 20 min with dH2O, and image using visible light to confirm the presence of protein band.

Note

Reference fig. 2: loaded SMRT-Tn5 transposomes on a NativePAGE after Coomassie stain.



Same gel as above, visualized with Coomassie stain. Left to Right: 2 replicates of Tn5 loaded with barcoded SMRT-Tag adaptors; 2 replicates of Tn5 loaded with unbarcoded SMRT-Tag adaptors; 2 replicates of Tn5 loaded with Nextera N5/N7 adaptors (shorter than SMRT-Tag adaptors); Tn5 monomer without adaptors (very faint).

[NOTE] Not all samples loaded on the reference gel are required. All six loaded Tn5 transposomes can be visualized. The negative protein controls (annealed SMRT-Tag oligos without Tn5) are NOT visualized by the Coomassie stain.

(Optional) Validation of SMRT-Tn5 enzymatic activity

15 For each tagmentation reaction, add 2 µL of 5x TAPS buffer (50mM TAPS-NaOH pH 8.5, 25mM $MgCl_2$), 1 µL of 100% DMF, 160ng of genomic DNA (gDNA) as a template, 1 µL of serially diluted SMRT-Tn5 (i.e 1x, 4x, 16x, 64x), and dilute up to 10 µL with dH2O.

16 Incubate reactions (10 µL) at a range of temperatures (i.e. 37°C, 55°C) for a range of incubation periods (i.e 30 min, 60 min). To validate activity, we recommend 37ºC and 55ºC for 30 min.

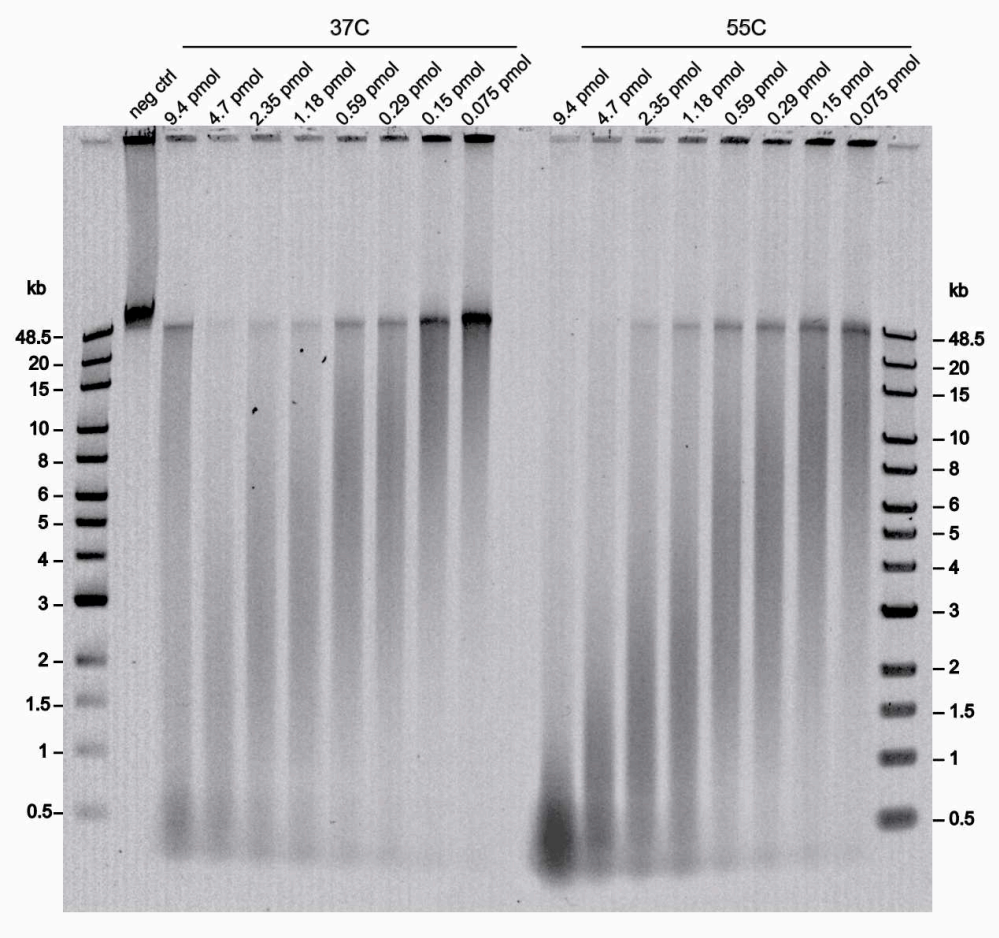
17 Terminate the reactions by adding 2.5 µL of 0.2% SDS and incubate for 5 min at RT.

18 Mix each reaction (12.5 µL) with 2.5 µL of 6x loading dye and load on a 0.5% agarose gel. Use 5µL of NEB 1kb-Extend for the ladder. Run at 60-80V for 2-3 hr.

19 Stain the gel with 1x SYBR Gold Solution in TAE buffer for ~30 min on a shaker. Visualize on a LiCor or any imaging system to confirm the gel shift of transposase activity - DNA size distribution post tagmentation varies as a function of SMRT-Tn5 concentration.

Note

Reference fig. 3: transposase activity assay.



Two tagmentation temperatures (37C vs. 55C) and 8 serially diluted SMRT-Tn5 concentrations (9.4pmol = 1x, 0.075pmol = 128x dilution factor) are tested. A clear gel shift is observed as transposase concentration decreases from left to right across the lanes.

Quality control of gDNA

20m

20 High Molecular Weight genomic DNA (HMW gDNA) is used as standard input for the SMRT-Tag method. High quality DNA is essential for efficient sequencing.

To reduce gDNA viscosity, incubate at 37ºC for 5-10 min at low agitation speed (300 rpm). Pipette up and down 5–10 times using a P200 wide-bore pipette to ensure any clumps of DNA are dispersed.

[OPTIONAL] Storing gDNA at 4°C for days or weeks also facilitates homogenization and relaxation of HMW gDNA.

Note

For mitigating HMW gDNA viscosity, see reference: https://www.neb.com/tools-and-resources/usage-guidelines/homogenization-of-high-molecular-weight-dna-hmw-dna-samples-after-elution

21 Once homogenized, measure the concentration and purity of HMW gDNA on a microvolume spectrophotometer (e.g. Nanodrop). Qubit-based concentration values are >25% consistently lower than OD-based estimates.

Note

For QC measures of HMW gDNA, see reference: https://www.neb.com/tools-and-resources/usage-guidelines/measuring-analyzing-and-storing-high-molecular-weight-dna-hmw-dna-samples

22 Dilute QC-passing HMW gDNA to 50-100 ng/µL with 1x EB (or 10 mM Tris-HCl, pH 8.5) and measure the diluted concentration with Qubit 1x High Sensitivity dsDNA Assay.

[CRITICAL] Do not dilute gDNA in TE buffer since EDTA will decrease the Tn5 efficiency in downstream tagmentation reactions.

[OPTIONAL] Analyze 1µL of diluted HMW gDNA using Genomic DNA Screentape (Agilent) to determine a DNA Integrity Number (DIN). We recommend at minimum that samples have DIN > 9.0.

Tagmentation

2h

23 Prepare the Tagmentation Reagent Mix:

	A	B	C
	Reagent (final conc.)	**Stock conc.**	**Volume (per sample)**
	gDNA* (40 to 160ng)	-	variable
	1x TAPS Buffer	5x	2µL
	DMF (10%)	100%	1µL
	SMRT-Tn5 with varied dilution factor**	9.4µM	1µL
	dH2O	-	up to 10µL
	Total Volume		10µL

*All SMRT-Tn5 dilution factors in this protocol are normalized to 160ng of gDNA input mass. (e.g. A 1:1 (1X) dilution corresponds to using a 9.4µM SMRT-Tn5 working stock for 160ng gDNA, or a 2.35µM SMRT-Tn5 working stock for 40ng gDNA.
**A uniquely barcoded SMRT-Tn5 is added to each individual sample for the purpose of multiplexing and pooling libraries at later steps.

[CRITICAL] We recommend the following dilution factors when using barcoded SMRT-Tn5 to generate PacBio libraries of 2 different size distributions:

- Use a 1:64 (64x) dilution factor if the target library size is <3kb and using Sequel II Binding Kit 2.1.
- Use a 1:128 (1:128x) dilution factor if the target library size is >3kb and using Sequel II Binding Kit 2.0 / 2.2.

24 Incubate the tagmentation reactions at 55ºC for 30 min using a thermocycler or thermomixer and hold at 4ºC.

25 Add 2.5 µL of 0.2% SDS per sample. Pipette slowly to mix and let incubate at room temperature for 5 min.

[NOTE] Placing the tubes on ice after 5 min can help reduce bubble formation.

26 Dilute each sample (12.5 µL) to 30 µL with 1x EB and add 2x volume of resuspended, room-temperature SPRI beads to clean up the reactions. Mix well using a P200 with wide-bore tips.

27 Incubate the bead-mixed samples in a thermomixer at 23ºC for 30 min with interval mixing @ 350 rpm (1 min on, 3 min off) to keep the beads resuspended.

[NOTE] Longer binding times noticeably increase the recovery efficiency of tagmented DNA.

28 Spin down quickly. Place on the magnet and allow the solution to clear before carefully withdrawing the supernatant.

29 Wash beads twice with 80% ethanol for 30 sec. Add ethanol to the wall opposite of beads. Withdraw supernatant and after a quick spin, remove the remaining liquid with a P20 pipette. Beads take ~30 s to 1 min to dry. Do not over-dry the beads - dried beads are light-brown and appear to be fragmented and cracked.

30 Remove from the magnet stand, and gently resuspend the bead pellet in 12 µL 1x EB (or 10 mM Tris-HCl, pH 8.5) to elute the tagmented fragments. Once beads are resuspended, mix well.

31 Incubate the samples in a thermomixer at 37ºC for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to maximize the yield of DNA elution.

[NOTE] Incubation at 37ºC with mild agitation improves recovery. Incubations longer than 15 min have not been tested.

32 Spin samples down quickly. Place on the magnet and allow the solution to clear before carefully transferring the supernatant to a new LoBind tube.

Gap Repair

2h

33 Prepare the Repair Reaction Mix:

	A	B	C
	Reagent (final conc.)	**Stock conc.**	**Volume (per sample)**
	tagmented sample	-	12µL
	dNTP mix (0.8mM)	8mM*	2µL
	1x Taq DNA Ligase Rxn Buffer	10x	2µL
	NEB Phusion Polymerase (2U)	2U/µL	1µL

	A	B	C
	NEB Taq DNA Ligase (80U)	40U/µL	2µL
	ddH20	-	1µL
	Total Volume		20µL

*dNTP stock is 8mM total dNTPs
**Repair Mix consisting of Phusion Polymerase., Taq DNA Ligase., dNTPs in Taq DNA Ligase Rxn Buffer and ddH2O can be prepared separately and aliquoted per sample.

34 Incubate the gap-repair reactions at 37ºC for 1 hr in a thermocylcer or thermomixer and hold at 4ºC.

[NOTE] Incubation at 50ºC has been tested to improve gap repair efficiency.

35 Add 2x volume (~ 40µL) of homogeneous, room-temperature SPRI beads to clean up the reactions. Mix well using a P200 with wide-bore tips.

36 Incubate the bead-mixed samples in a thermomixer at 23ºC for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to keep the beads resuspended.

37 Spin down quickly. Place on the magnet and allow the solution to clear before carefully withdrawing the supernatant.

38 Wash beads twice with 80% ethanol for 30 sec. Add ethanol to the wall opposite of beads. Withdraw supernatant and after a quick spin, remove the remaining liquid with a P20 pipette. Beads take ~30 s to 1 min to dry. Do not over-dry the beads.

39 Remove from the magnet stand, and gently resuspend the bead pellet in 12 µL 1x EB (or 10 mM Tris-HCl, pH 8.5) to elute DNA. Once beads are resuspended, mix well.

40 Incubate the samples in a thermomixer at 37ºC for 10 min with interval mixing @350 rpm (1 min on, 3 min off) to maximize the yield of DNA elution.

41 Spin samples down quickly. Place on the magnet and allow the solution to clear before carefully transferring the supernatant to a new LoBind tube.

42 **[OPTIONAL]** QC check - take a 1µL aliquot and determine the gap-repaired sample dsDNA concentration using Qubit 1x High Sensitivity dsDNA Assay.

Exonuclease Digestion

2h

43 Prepare the ExoDigest Reaction Mix.

	A	B	C
	Reagent (final conc.)	**Stock conc.**	**Volume (per sample)**
	gap-repaired sample	-	12µL*
	NEBuffer 2 (1x)	10x	1.5µL
	Exonuclease III (100U)**	100U/µL	1µL
	ddH20	-	0.5µL
	Total Volume		15µL

*Diluted up to 12µL with 1x EB for volume <12µL.
**Normalize Exonuclease III (exo3) concentration such that 100U exo3 is used per 160ng gDNA input mass (e.g. 25U exo3 per 40ng gDNA.)
***ExoDigest Mix consisting of Exonuclease III in NEBuffer 2 and ddH2O can be prepared separately and aliquoted per sample.

44 Incubate the exonuclease digestion reactions at 37ºC for 1 hr in a thermocycler or thermomixer, then hold at 4ºC.

45 Add 2x volume (~30µL) of homogeneous, room-temperature SPRI beads to clean up the reactions. Mix well using a P200 with wide-bore tips.

46 Incubate the bead-mixed samples in a thermomixer at 23ºC for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to keep the beads resuspended.

47 Spin down quickly. Place on the magnet and allow the solution to clear before carefully withdrawing the supernatant.

48 Wash beads twice with 80% ethanol for 30 sec. Add ethanol to the wall opposite of beads. Withdraw supernatant and after a quick spin, remove the remaining liquid with a

P20 pipette. Beads take ~30 s to 1 min to dry. Do not over-dry the beads.

49 Remove from the magnet stand, and gently resuspend the bead pellet in 12 µL 1x EB (or 10 mM Tris-HCl, pH 8.5) to elute the extracted DNA. Once beads are resuspended, mix well.

50 Incubate the samples in a thermomixer at 37ºC for 10 min with interval mixing @ 350 rpm (1 min on, 3 min off) to maximize the yield of DNA elution.

51 Spin samples down quickly. Place on the magnet and allow the solution to clear before carefully transferring the supernatant to a new LoBind tube.

(Optional) Size Enrichment for HMW libraries

1h

52 **[CRITICAL]** If intending to load the target library using Sequel II Binding Kit 2.1 (i.e libraries generated using a 64x dilution factor of SMRT-Tn5 in step 23), this step can be skipped. If intending to load with Sequel II Binding Kit 2.2 (using the 128x dilution factor of SMRT-Tn5 in step 23), a size enrichment step is critical for enriching for HMW library >5kb.

Make a 35% v/v dilution of AMPure PB beads by mixing 1.75 mL of room-temperature, resuspended AMPure PB beads with 3.25 mL of 1X EB. The 35% dilution Ampure PB beads can be stored at 4°C for 30 days.

53 Dilute the library to 50 µL with 1x EB if the sample volume is <50 µL. Add 3.1x volume of resuspended, room-temperature 35% AMPure PB beads using a wide-bore pipette and mix gently at least 10 times.

[OPTIONAL] If multiplexing libraries, pool all libraries together before size enrichment.

54 Incubate the bead-mixed samples in a thermomixer at 23ºC for 30 min with interval mixing @350 rpm (1 min on, 3 min off) to keep the beads resuspended.

55 Spin down quickly. Place on the magnet and allow the solution to clear before carefully withdrawing the supernatant.

[NOTE] The remaining bead pellet will be small because the SPRI reagent is diluted. Adjust the pipetting technique accordingly.

[OPTIONAL] Save the supernatant (which contains <5kb library molecules) to sequence with Sequel II Binding Kit 2.1. To recover the <5kb fraction (LMW), add 0.25x regular AMPure PB, bind at RT for 10 min, wash twice with 80% EtOH, and elute in 12 µL of 1x EB following 5 min of incubation at RT.

56 Wash beads twice with 80% ethanol for 30 sec. Add ethanol to the wall opposite of beads. Withdraw supernatant and after a quick spin, remove the remaining liquid with a P20 pipette. Beads take ~30 s to 1 min to dry. Do not over-dry the beads.

57 Remove from the magnet stand, and gently resuspend the bead pellet in 12 µL 1x EB (or 10 mM Tris-HCl, pH 8.5) to elute the extracted DNA. Once beads are resuspended, mix well.

58 Incubate the samples in a thermomixer at 37ºC for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to maximize the yield of DNA elution.

59 Spin samples down quickly. Place on the magnet and allow the solution to clear before carefully transferring the supernatant to a new LoBind tube.

Library QC and Sequencing

60 Determine the concentration of SMRT-Tag libraries using a Qubit 1x High Sensitivity dsDNA Assay.

Determine the library size distribution using Agilent 2100 Bioanalyzer High Sensitivity dsDNA Assay.

If the sample is precious, library size may be roughly estimated at ~3000bp for SMRT-Tag libraries prepared with a 64x dilution factor, and ~7000bp for SMRT-Tag libraries prepared with 128x dilution factor and >5kb size selection.

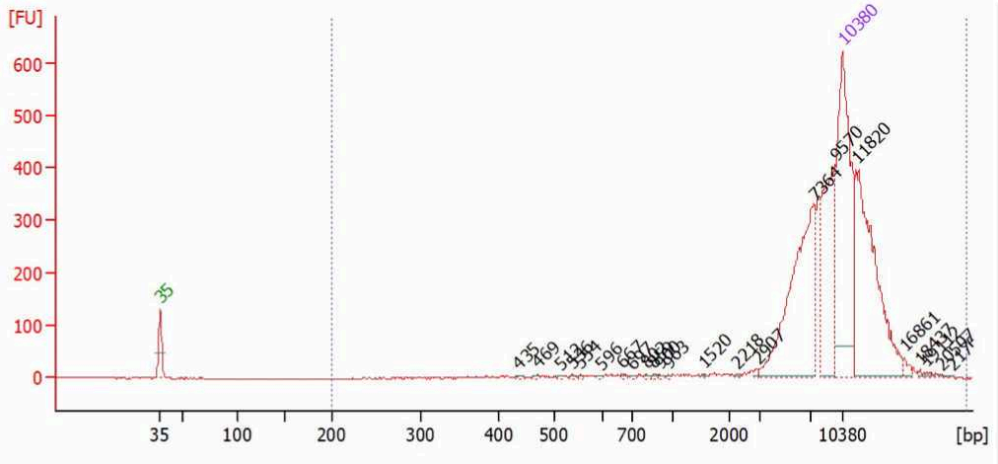
[CRITICAL] Convert the Bioanalyzer trace from its default unit (fluorescence units, FU) to "molarity per length" using the R package "**bioanalyzeR**". We have found this conversion step is critical to accurately size SMRT-Tag libraries, as is required for the optimal loading on a PacBio Sequel II.

Note

See more instructions on package installation and usage of "bioanalyzeR": https://stanford.edu/~jwfoley/bioanalyzeR.html

Note

Reference fig. 4: SMRT-Tag library



The Bioanalyzer trace shows the size distribution of a SMRT-Tag library (using 128x dilution factor of SMRT-Tn5 transposome) after pooling and HMW size enrichment with a peak of ~10kb. After unit conversions to "molarity per length" using "**bioanalyzeR**", the average library size shifts down to ~6.6kb, which is used as the final library size for Sequel II loaded with Sequel II Binding Kit 2.2.

61 Sequence SMRT-Tag libraries using a PacBio Sequel II 8M SMRTcell with either Sequel II Binding Kit 2.1 (e.g. up to 220pM loading concentration with 30h movie time, 2h pre-extension, and 4h immobilization), or Sequel II Binding Kit 2.2 (e.g. up to 120pM loading concentration with 30h movie time, 2h pre-extension, and 4h adaptive loading).

Samples may be sequenced in monoplex or multiplex formats. For sample multiplexing, we recommend pooling libraries to equalize their molar concentration (nM).

[NOTE] For precious libraries, the "before" and "after" loading cleanup Qubit measurements may be skipped, and the entire library loaded on one cell.