

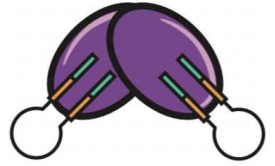
Aug 10, 2022

Version 1

🌐 SMRT-Tag - Sensitive multimodal profiling of native DNA by transposase-mediated single-molecule sequencing V.1

DOI

dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v1



Scott Nanda¹, Ke Wu¹, Siva Kasinathan², vijay.ramani¹

¹Gladstone Institute for Data Science & Biotechnology;

²Division of Allergy, Immunology, and Rheumatology, Department of Pediatrics, Stanford University



Coco Wu

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v1>

Protocol Citation: Scott Nanda, Ke Wu, Siva Kasinathan, vijay.ramani 2022. SMRT-Tag - Sensitive multimodal profiling of native DNA by transposase-mediated single-molecule sequencing. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.e6nvwk3b9vmk/v1>



License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: July 26, 2022

Last Modified: August 10, 2022

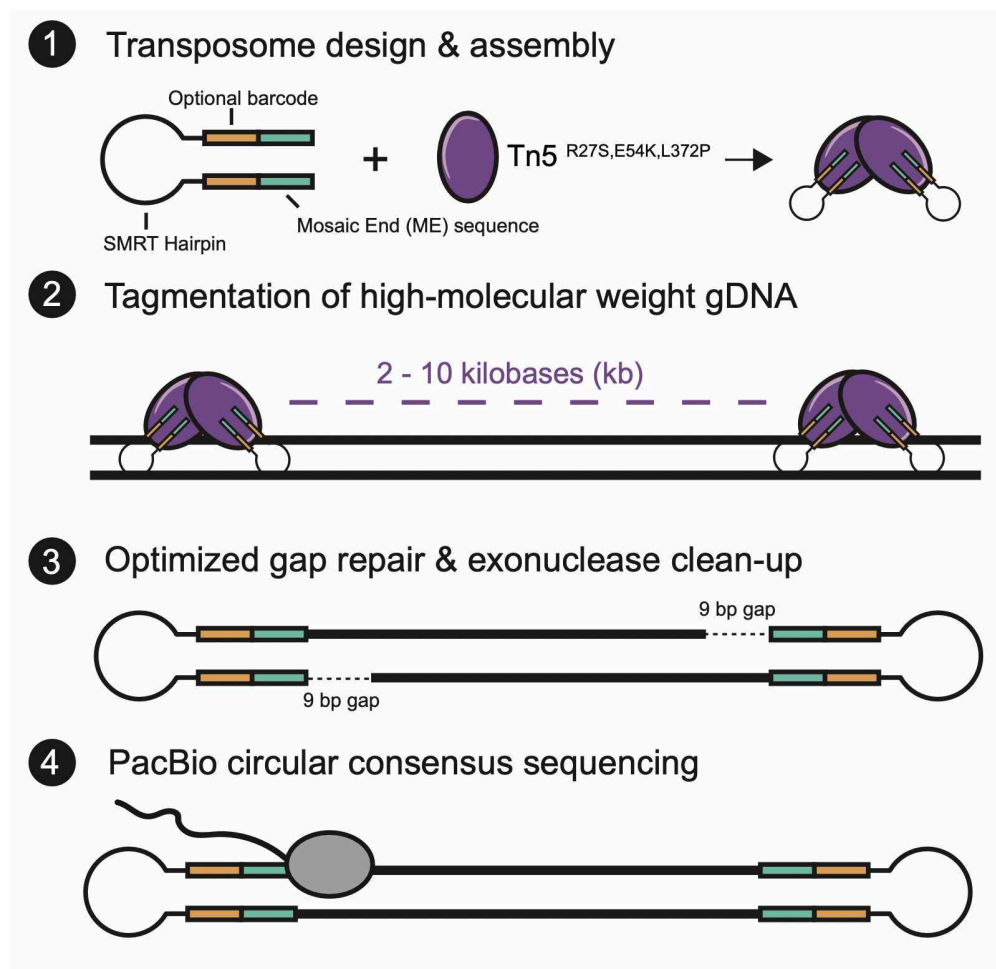
Protocol Integer ID: 67586

Keywords: Long-read sequencing, low-input genomics, chromatin, epigenetics, sensitive multimodal profiling of native dna, simultaneous detection of single nucleotide variant, sequencing library, native dna, single nucleotide variant, steps from purified gdna, quality genomic dna, purified gdna, sequencing, dna, protocol for smrt, smrt, utility of smrt, sensitive multimodal profiling, pacbio sequel ii platform, free pacbio, cpb methylation

Abstract

Here we describe a protocol for SMRT-Tag: **S**ingle-**M**olecule **R**eal-**T**ime sequencing by **T**agmentation - a transposase-mediated strategy for producing low-input, multiplexable, PCR-free PacBio sequencing libraries. SMRT-Tag requires only 1-5% as much input material as existing protocols (15,000–50,000 human cell equivalents) and enables highly-sensitive and simultaneous detection of single nucleotide variants, small insertions / deletions, and CpG methylation comparable to the current state-of-the-art.

In brief, high-quality genomic DNA are tagmented using hairpin-loaded Tn5-adaptors, gap-repaired, exo-digested, 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 80ng in HG002 - HG004.



An overview of SMRT-Tag schematic design.

Guidelines

Sensitive multimodal profiling of native DNA by transposase-mediated single-molecule sequencing

Arjun S Nanda^{1,2*}, Ke Wu^{1*}, Sivakanthan Kasinathan^{3,4*#}, Megan SOstrowski¹, Andrew S Clugston^{5,6}, Ansuman T Satpathy^{4,7,8}, E Alejandro Sweet-Cordero^{5,6}, Hani Goodarzi^{2,6,8,9}, Vijay Ramani^{1,2,6,9#}

¹Gladstone Institute for Data Science & Biotechnology, Gladstone Institutes, San Francisco, CA 94158

²Department of Biochemistry & Biophysics, UCSF, San Francisco, CA 94143

³Division of Allergy, Immunology, and Rheumatology, Department of Pediatrics, Stanford University, Stanford, CA 94305

⁴Gladstone-UCSF Institute for Genomic Immunology, Gladstone Institutes, San Francisco, CA 94143

⁵Department of Pediatrics, UCSF, San Francisco, CA 94143

⁶Helen-Diller Cancer Center, San Francisco, CA 94158

⁷Department of Pathology, Stanford University, Stanford, CA 94305

⁸Parker Institute for Cancer Immunotherapy, San Francisco, CA 94129

⁹Bakar 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 H₂O (dH₂O; e.g. Invitrogen, cat. no. 10977023)
- Elution buffer (1x EB; Qiagen, cat. no. 19086)
- Dimethylformamide (DMF; Sigma-Aldrich, cat. no. 319937)
- 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



A	B
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 μ M in RNase-free water.
- 2 Dilute adaptors to 20 μ M in 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 105°C 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) on ice.
- 5 Dilute to 1mg/mL (or 18.9 μ M monomer) in Tn5 dilution buffer (50mM Tris pH 7.5, 0.065mM EDTA, 2mM DTT, 60% 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 4°C 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 μ M annealed SMRT-Tag adaptors using a wide-bore pipette.
- 8 Incubate the mixture at 23°C for 55 min with continuous shaking at 350 rpm. After 55 min, place on ice. Loaded SMRT-Tn5 transposome (~9.4 μ M 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

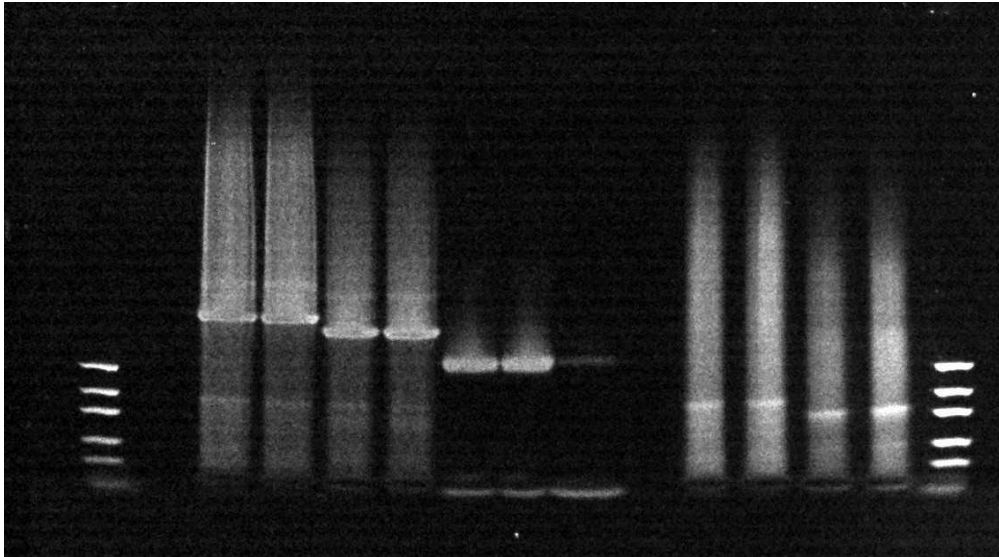
- 9 Add 1-2 μ L of each SMRT-Tn5 transposome with 2.5 μ L of 4x Native Gel Loading Buffer and dilute up to 10 μ L with dH₂O.



- 10 Load each SMRT-Tn5 transposome per well 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 4°C, 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 transposomes on a NativePAGE 4-16% Bis-Tris Gel.



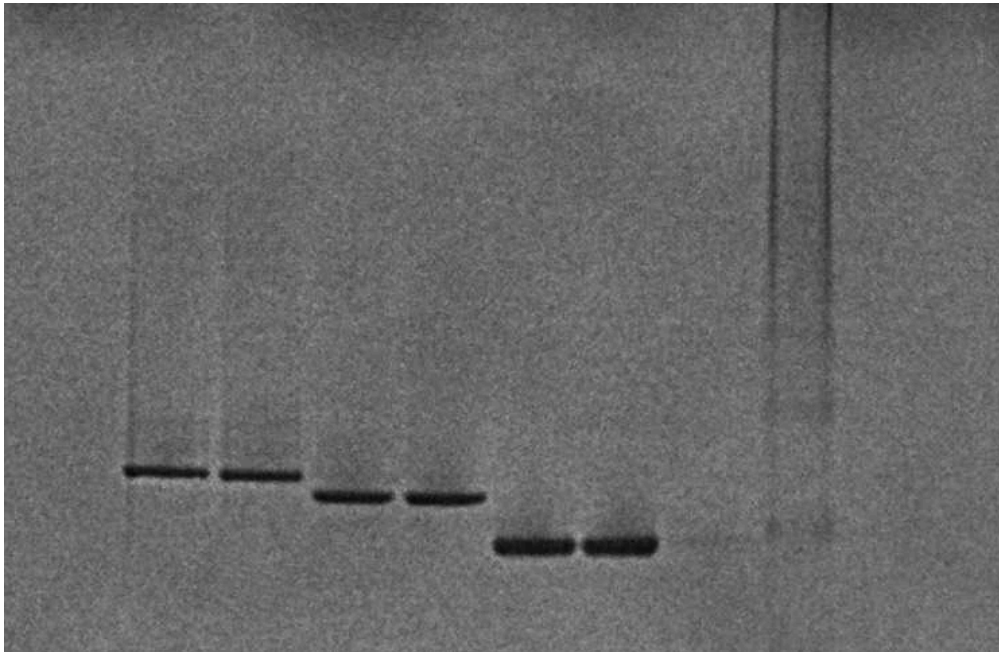
Left to Right: GeneRuler ULR ladder, 2 replicates of Tn5 loaded with barcoded SMRT-Tag adaptors, 2 replicates of Tn5 loaded with unbarcoded SMRT-Tag adaptors, 2 replicates of Tn5 loaded with Nextera N5/N7 adaptors (shorter than SMRT-Tag adaptors), Tn5 monomer without adaptors, 2 replicates of annealed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

[NOTE] Not all samples loaded on the reference gel are required. The negative DNA control (Tn5 monomer without adaptors) is barely 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 millipore dH₂O, and image using ambient light to confirm the presence of protein band.

Note

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

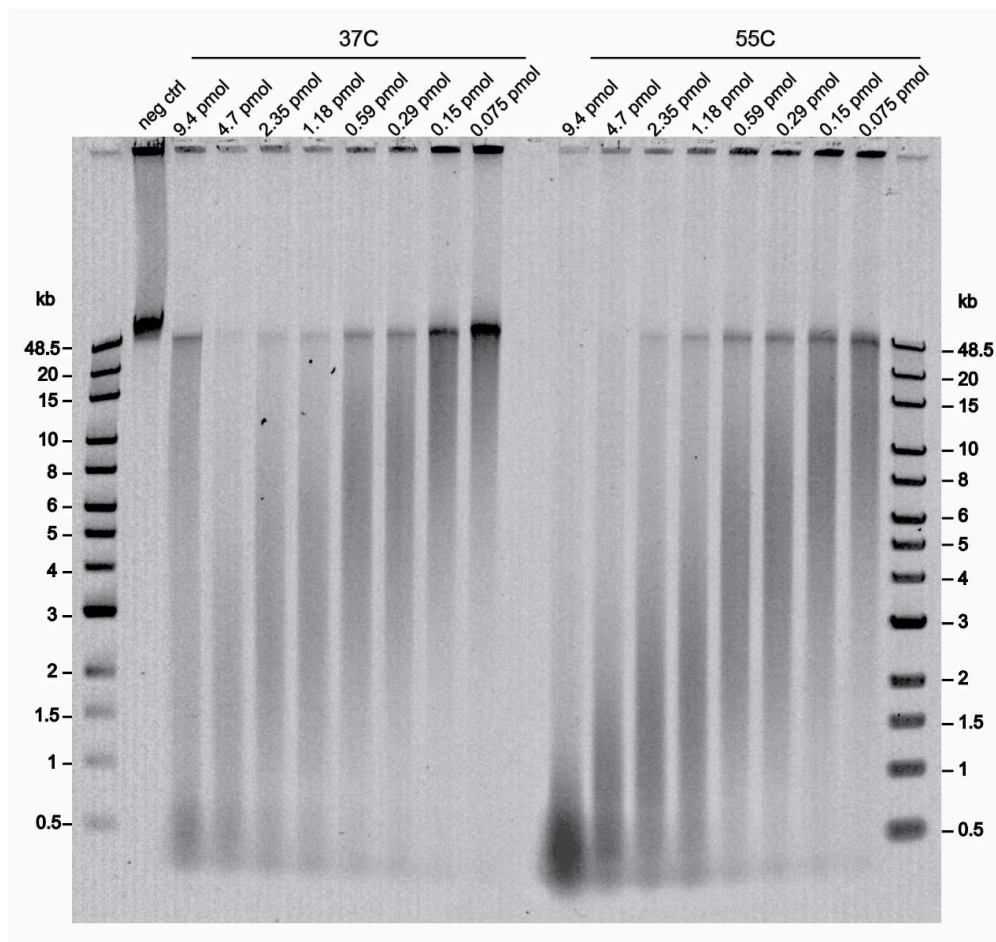
(Optional) Validation of SMRT-Tn5 enzymatic activity

- 15 For each tagmentation reaction, add 2 μL of 5x TAPS buffer, 1 μL of DMF, 160ng of gDNA as template, 1 μL of serially diluted SMRT-Tn5 transposome (i.e 1x, 4x, 16x, 64x), and dilute up to 10 μL with dH₂O.
- 16 Incubate each reaction (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).
- 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 coupled with the NEB 1kb-Extend 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.



gDNA QC

20m

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

To reduce its viscosity, incubate at 37°C 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] Store at 4°C for days or weeks also facilitates homogenization and relaxation of HMW gDNA.

Note

For homogenization of HMW DNA, 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 DNA, 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-approved 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 DNA Assay.

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

Tagmentation

2h

- 23 Prepare the Tagmentation Reagent Mix.



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

*A uniquely barcoded SMRT-Tn5 transposome 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 SMRT-Tn5 transposomes to generate PacBio libraries of 2 different size distributions:

- use 16x dilution factor (or 0.59µM) if the target library size is <3kb compatible with Sequel II Binding Kit 2.1.
- use 128x dilution factor (or 0.075µM) if the target library size is >3kb compatible with Sequel II Binding Kit 2.2.

24 Incubate the tagmentation reactions at 55°C for 30 min and hold at 4°C.

25 Add 2.5 µL of 0.2% SDS per sample. Pipette slowly to mix and sit 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 23°C for 30 min with interval mixing @ 350 rpm (1 min on, 3 min off) to keep the beads resuspended.



- 28 Spin down quickly. Place on magnet and allow to clear before carefully withdrawing 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 appear to be fragmented with cracks in light brown.
- 30 Remove from the magnet stand, and gently resuspend the bead pellet in 12 μ L 1x EB (or 10 mM Tris-HCl, pH 8.5) to elute the extracted DNA. Once beads are resuspended, mix well.
- 31 Incubate the samples in a thermomixer at 37°C for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to promote increased DNA elution.
- 32 Spin samples down quickly. Place on magnet and allow to clear before carefully transferring the supernatant to a new LoBind tube.

Gap Repair

2h

- 33 Prepare the Gap-Repair Reagent Mix.

	A	B	C
	Reagent (final conc.)	Stock conc.	Volume (per sample)
	tagmented sample	-	12 μ L
	dNTP mix (0.8mM)	8mM	2 μ L
	1x Taq DNA Ligase Rxn Buffer	10x	2 μ L
	NEB Phusion Polymerase (2U)	2U/ μ L	1 μ L
	NEB Taq DNA Ligase (80U)	40U/ μ L	2 μ L
	ddH2O	-	1 μ L
	Total Volume		20 μ L



- 34 Incubate the gap-repair reactions at 37°C for 1 hr and hold at 4°C.
- 35 Add 2x volume of resuspended, 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 23°C 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 magnet and allow to clear before carefully withdrawing 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 - dried beads appear to be fragmented with cracks in light brown.
- 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 the extracted DNA. Once beads are resuspended, mix well.
- 40 Incubate the samples in a thermomixer at 37°C for 10 min with interval mixing @350 rpm (1 min on, 3 min off) to promote increased DNA elution.
- 41 Spin samples down quickly. Place on magnet and allow to clear before carefully transferring the supernatant to a new LoBind tube.
- 42 **[OPTIONAL]** QC check - take 1µL aliquot and determine the gap-repaired concentration using Qubit 1x High Sensitivity DNA Assay.

Exonuclease Digestion

2h

- 43 Prepare the Exo-Digest Reagent Mix.

	A	B	C
	Reagent (final conc.)	Stock conc.	Volume (per sample)

	A	B	C
	gap-repaired sample	-	12µL*
	NEBuffer 2 (1x)	10x	1.5µL
	Exonuclease III (100U)	100U/µL	1µL
	ddH ₂ O	-	0.5µL
	Total Volume		15µL

*Diluted up to 12µL with 1x EB for volume <12µL.

- 44 Incubate the exo-digest reactions at 37°C for 1 hr, then hold at 4°C.
- 45 Add 2x volume of resuspended, 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 23°C 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 magnet and allow to clear before carefully withdrawing 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 - dried beads appear to be fragmented with cracks in light brown.
- 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 37°C for 10 min with interval mixing @ 350 rpm (1 min on, 3 min off) to promote increased DNA elution.



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

(Optional) Size Enrichment for HMW libraries

1h

- 52 **[CRITICAL]** If the target library is intended to load with Sequel II Binding Kit 2.1 (using 16x dilution factor of SMRT-Tn5 in step 23), this step can be skipped. If intended to load with Sequel II Binding Kit 2.2 (using 128x dilution factor of SMRT-Tn5 in step 23), a size enrichment step is critical to 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 Pool all libraries after exo-digest and dilute up to 50 µL with 1x EB if the pooled 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.

- 54 Incubate the bead-mixed samples in a thermomixer at 23°C 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 magnet and allow to clear before carefully withdrawing supernatant.

[OPTIONAL] Save the supernatant (which contains <5kb libraries) and sequence with Sequel II Binding Kit 2.1. To recover the <5kb fraction, 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 eluting 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 - dried beads appear to be fragmented with cracks in light brown.

- 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 37°C for 15 min with interval mixing @350 rpm (1 min on, 3 min off) to promote increased DNA elution.

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

Library QC and Sequencing

- 60 Determine the concentration of pooled, enriched HMW library using Qubit 1x High Sensitivity DNA Assay.

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

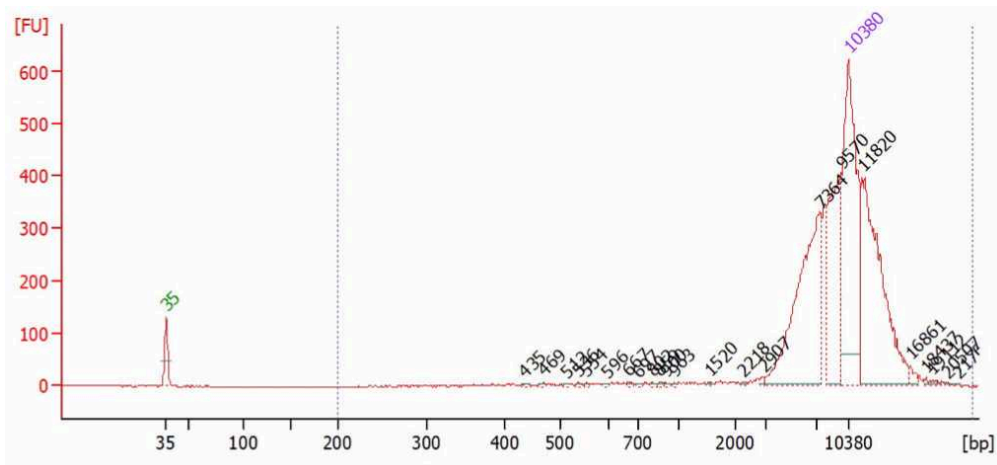
[CRITICAL] Convert the BioA trace from its default unit (fluorescent unit, FU) to "molarity per length" using the R package "bioanalyzerR". This step is critical for the evaluation of accurate library sizing required for the optimal loading on PacBio Sequel II.

Note

See more instructions on package installation and usage of "bioanalyzerR":
<https://stanford.edu/~jwfoley/bioanalyzerR.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 "bioanalyzerR", the average library size shifts down to ~7kb, which is used as the final library size for Sequel II loaded with Sequel II Binding Kit 2.2.



- 61 Sequence the multiplexed, pooled library on PacBio Sequel II 8M SMRTcells 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).