Modified NEBNext® VarSkip Long SARS-CoV-2 Enrichment and library prep (SMRTbell prep kit 3.0 Pacific Biosciences)- adapted for wastewater samples

Forked from Modified NEBNext® VarSkip Short SARS-CoV-2 Enrichment and library prep for Oxford Nanopore Technologies- adapted for wastewater samples

Kathryn Judy[1], Padmini Ramachandran[1], Amanda Windsor[1], Tamara Walsky[1], Chris Grim[1], Maria Hoffmann[1]

[1]FDA CFSAN

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Protocol status: In development
We are still developing and optimizing this protocol

GenomeTrakr
Tech. support email: **genomeTrakr@fda.hhs.gov**

Kathryn Judy

ABSTRACT

This protocol details methods for the preparation of SARS-CoV-2 sequencing library using VSL primers from NEB, adapted for wastewater samples. This protocol produces multiplexed amplicon libraries suitable for sequencing on PacBio systems (e.g., Sequel® IIe) using the SMRTbell® prep kit 3.0 and SMRTbell® barcoded adapter plate 3.0.

GUIDELINES

Overview

Sequences and information on the NEBNext VarSkip Long primers can be found at https://github.com/nebiolabs/VarSkip. All other enzymes, buffers, beads and oligos required to convert cDNA into targeted, high quality libraries for next-generation sequencing on the PacBio platform are available.

MATERIALS

The Library Kit Includes SMRTbell® prep kit 3.0. Reagents for VSL amplification must be purchased individually. Information on NEBNext® VarSkip Long primers is available at https://github.com/nebiolabs/VarSkip**.**

Kit Components

PacBio SMRTbell® prep kit 3.0 (102-182-700) **Table of Components**

	A	B	C	D	E
	Component	Part Number	Quantity	Color	Volume
	Repair buffer	102-166-000	1	purple	220µL

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	A	B	C	D	E
	Endrepair mix	102-166-100	1	blue	110µL
	DNA repair mix	102-167-700	1	green	55µl
	SMRTbell adapter	102-167-800	1	orange	125µl
	Ligation mix	102-167-200	1	yellow	860µl
	Ligation enhancer	102-179-100	1	red	55µl
	Nuclease buffer	102-167-900	1	light purple	155µl
	Nuclease mix	102-166-200	1	light green	155µl
	Elution buffer	100-159-800	2	white	1.5ml
	SMRTbell cleanup beads	100-158-300	1	clear	10ml
	SMRTbell Low TE buffer	102-178-400	1	clear	10ml

Required Materials Not Included

- **Q5® Reaction buffer** (NEB #B9027S)
- **Q5® Hot Start High-Fidelity DNA Polymerase** (NEB #M0493L)
- **NEBNext® VarSkip Long primer mixes 1** and **2** (NEB, https://github.com/nebiolabs/VarSkip)
- **50mM $MgCl_2$** (Thermo Fisher Scientific, Inc.® V0216 or equivalent)
- **Deoxynucleotide (dNTPs) Solution** (NEB #N0447L)
- Nuclease-free water, molecular biology grade
- AMPure® XP beads (Beckman Coulter A63880) or equivalent
- 80% Ethanol (freshly prepared, molecular biology grade)
- DNA LoBind Tubes (Eppendorf® #022431021)
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.® Q32851)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables (#4150 or #4200 TapeStation System)
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)
- 1.5 ml tube magnet stand (NEB #S1506)
- **SMRTbell® barcoded adapter plate 3.0** (102-009-200)

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

BEFORE START INSTRUCTIONS

Note: We recommend setting up a no template control reaction and **all reactions are set-up in a hood**.

The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

Before you start

1

Note

To use this protocol, we recommend wastewater extraction using either of the protocols linked below. Extraction using the Promega Enviro Total Nucleic Acid Kit may be more robust to PCR inhibitors in wastewater. Other wastewater extraction methods have not been tested.

Protocol

NAME

Extraction of Total Nucleic Acid from Wastewater Using the Promega Wizard Enviro Total Nucleic Acid Kit

CREATED BY

Chris Grim

PREVIEW

Protocol

NAME

SARS-CoV-2 RNA extraction with Ceres Nanotrap and Zymo Environ Water

CREATED BY

Amanda Windsor

PREVIEW

Note

This protocol requires cDNA as input.

We recommend cDNA synthesis using the Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Catalog number:18091200), as described in the SNAP protocol with modifications (random hexamers, RT incubation of 30 min.). Before cDNA synthesis, samples must be DNase-treated (with Invitrogen™ ezDNase™ (Catalog number:11766051) or equivalent).

The presence of genomic DNA or carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful.

Absolutely no vortexing of cDNA, amplicons, or libraries at any point.

Targeted cDNA Amplification

2

Note

4.5 µl cDNA input is recommended. If using less than 4.5 µl of cDNA, add nuclease-free water to a final volume of 4.5 µl. We recommend setting up the cDNA synthesis and cDNA amplification reactions in different rooms to minimize cross-contamination of subsequent reactions.

3 Prepare master mixes fresh immediately before performing cDNA amplification.

- **Q5 Hot Start High-Fidelity Polymerase should stay on ice at all times. Do not vortex.**
- Thaw Q5 Reaction Buffer, $MgCl_2$, dNTPs, and water.
- Mix thawed tubes, spin down, and place on ice.
- Thaw VarSkip Long Primer Mix 1 and VarSkip Long Primer Mix 2.
- Mix by flicking and spin down both the tubes.
- Keep on ice.

Prepare the split pool amplification reactions as described below:

For Pool set A:
Prepare the master mix below in sufficient volume for your samples.

	A	B
	COMPONENT	VOLUME
	Q5 Reaction Buffer	2.5 µl
	50mM Magnesium Chloride	0.5 µl
	Deoxynucleotide (dNTP) Solution	0.75 µl
	Nuclease-free water	1.75 µl
	NEBNext VarSkip Long Primer Mix 1	2.25 µl
	Total Volume	7.5 µl

For Pool Set B:
Prepare the master mix below in sufficient volume for your samples.

	A	B
	COMPONENT	VOLUME
	Q5 Reaction Buffer	2.5 µl
	50mM Magnesium Chloride	0.5 µl
	Deoxynucleotide (dNTP) Solution	0.75 µl
	Nuclease-free water	1.75 µl
	NEBNext VarSkip Long Primer Mix 2	2.25 µl
	Total Volume	7.5 µl

Mix the two master mix tubes by flicking and spin down. Dispense 7.5 µl master mix from each tube into separate PCR tube strips (**A** and **B**), two PCR tubes (one for each master mix) per sample to amplify.

4 Add 4.5 µl cDNA into each pre-filled PCR tube, ensuring each sample to be amplified is added into exactly 1 tube in strip A and 1 tube in strip B.

5 While keeping the polymerase on ice, add 0.5 µL Q5 Hot Start High-Fidelity Polymerase to each tube.

6 Gently flick the tube strips to mix and spin down briefly.

7 Incubate **Pool A** reactions in a thermocycler* with the following steps:

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denature	95°C	15 seconds	38
	Annealing	59°C	1 minute	
	Extension	72°C	2 minutes	
	Hold	4°C	∞	1

* Set heated lid to 105°C.

Incubate **Pool B** reactions in a thermocycler* with the following steps:

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denature	95°C	15 seconds	38
	Annealing	61°C	45 seconds	
	Extension	72°C	2 minutes	
	Hold	4°C	∞	1

* Set heated lid to 105°C.

Note

Samples can be stored at 4 °C **if they are not used immediately.**

Cleanup of cDNA Amplicons

21m 1s

8 We highly recommend this clean up step using AMPure® XP beads, though NEBNext sample purification beads can be used as well.

This step replaces the input DNA quality control and cleanup step from the amplicon library preparation using SMRTbell® prep kit 3.0. It may be possible to omit this cleanup in favor of the PacBio initial cleanup, but this has not been tested.

Note

If using AMPure® XP Beads, allow the beads to warm to Room temperature for at least 30 minutes before use. These bead volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

9 For each sample, combine pool A and pool B PCR products (amplicons), measuring the pooled volume.

10 Vortex AMPure® XP beads for 00:00:30 to resuspend. 30s

11 Add 0.6 X resuspended AMPure® XP beads to the combined PCR product. Mix well by flicking the tube and a very short 2-3 seconds quick centrifugation. Be sure to stop the centrifugation before the beads start to settle out.

12 Incubate samples at Room temperature for 00:05:00. 5m

13 Quickly spin samples to collect the liquid from the sides of the tube before placing on the magnetic stand for 00:05:00 to separate the beads from the supernatant. 5m

14 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note

Caution: do not discard the beads.

15 Add 200 µL freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 30s

16 Repeat previous step once for a total of two washes: 31s
Add 200 µL freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at Room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube for 00:00:01, place back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.

17 Air dry the beads for up to 00:03:00 while the tube is on the magnetic stand with the lid open. 3m

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking. When the beads turn lighter brown and start to crack, they are too dry.

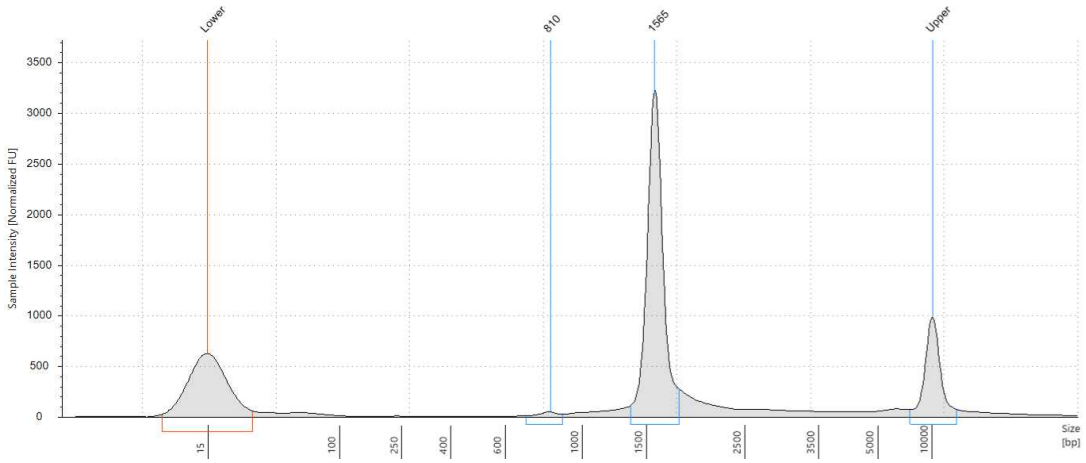
18 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 18 µL 0.1x TE buffer.

19 Mix well by flicking the tube followed by a very short centrifugation. Incubate for 00:05:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 5m

20 Place the tube on the magnetic stand. After 00:02:00 (or when the solution is clear), transfer 17 µL to clean PCR tubes. 2m

21 Assess the concentration of the DNA targets. We recommend using a Qubit fluorometer for concentration assessment. Use 1 µl of sample for the Qubit fluorometer. Amplicons should also be run on Femto or Bioanalyzer® or Tape Station using High Sensitivity (HS) 5000 tape to confirm ~1500-1600 bp size of amplicons.

Note



A high positive wastewater sample.

Note

Samples can be stored at 4 °C if they are not used immediately.

DNA Repair and A-tailing

22

Note

If amplicons were cleaned following steps 8-21 (Cleanup of cDNA Amplicons), each sample must be made up to 46 µL in SMRTbell Low TE buffer. If amplicons were cleaned using the SMRTbell prep kit 3.0 input DNA quality control & cleanup protocol, this step (22) can be omitted.

Calculate the volume of each sample needed to bring forward at least 150 ng DNA per sample. We recommend bringing forward approximately the same mass of DNA for each sample. DNA mass <150 ng may be usable but masses < 125 ng have not been tested.

Aliquot the volume of each sample calculated into fresh PCR tubes and make up each sample to 46 µL using SMRTbell Low TE buffer. Excess amplicons should be returned to 4 °C

23 Make the repair and A-tailing master mix by combining the reagents below in the order and amounts listed in the table. Adjust component volumes for your number of samples plus 20% overage.

- Thaw Repair Buffer at room temperature, then vortex and spin down briefly.
- Thaw End Repair and DNA Repair mixes on ice, spin down briefly, and return to ice. Do not vortex.

	A	B
	Component	Volume per Sample
	Repair buffer	8 µl
	End Repair mix	4 µl
	DNA Repair mix	2 µl
	Total volume	14 µl

Mix the master mix components by pipetting or gentle flicking and quickly centrifuge.

24 Add 14 µL master mix to each sample for a total reaction volume of 60 µl per sample.

25 Gently mix samples by flicking and quickly spin to collect liquid.

26 Incubate samples in a thermocycler* with the following settings:

	A	B	C
	TEMP	TIME	CYCLES
	37°C	30 minutes	1
	65°C	5 minutes	1
	4°C	∞	1

* Set heated lid to 75°C

Adapter Ligation and Cleanup

24m

27

Note

This cleanup requires SMRTbell® cleanup beads at room temperature. Allow beads to come to room temperature for 30 minutes before use. Removing the beads from refrigeration before performing the adapter ligation PCR (step 31) should allow the beads sufficient time to warm.

Add 4 µL SMRTbell barcoded adapter 3.0 to each sample tube from the previous step, using a different barcode for each sample. Add only one barcode to each sample.

28 Make the adapter ligation master mix by combining the reagents below in the amounts and volumes listed in the table. Adjust component volumes to your number of samples plus 20% overage.

- Thaw Ligation mix and Ligation enhancer on ice. Do not vortex.

	A	B
	Component	Volume per Sample

	A	B
	Ligation mix	30 µl
	Ligation enhancer	1 µl
	Total volume	31 µl

Mix components by pipetting or gently flicking the tube, then centrifuge briefly.

29 Add 31 µL master mix to each sample for a total volume of 95 µl per sample.

30 Gently mix samples by flicking and quickly spin to collect liquid.

31 Incubate samples in a thermocycler* with the below settings:

	A	B	C
	TEMP	TIME	CYCLES
	20°C	30 minutes	1
	4°C	∞	1

* Set heated lid to 75°C.

32
- Thaw elution buffer at room temperature.
- Room temperature SMRTbell cleanup beads should also be stored at room temperature until the protocol is completed or paused overnight at a safe stopping point.

Add 124 µL SMRTbell cleanup beads (1.3X) to each adapter-ligated sample and mix by gently flicking followed by a short spin to collect the liquid. Stop the centrifugation before the beads begin to settle.

33 Leave samples at room temperature for 00:10:00 to bind DNA to the beads. 10m

34 Place tube strip in an appropriate magnetic separation rack until the beads have separated, usually within 00:03:00 3m

35 Carefully pipette off the supernatant without disturbing the beads, discarding the supernatant. **Do not discard the beads, which contain your DNA target**.

36 Slowly dispense 200 µL freshly prepared 80% ethanol to each sample tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.** 30s

37 Repeat the previous step once for a total of two washes:
Slowly dispense 200 µL freshly prepared 80% ethanol to each sample tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.** 30s

38 To remove residual ethanol, quickly spin samples and return the tubes to the magnetic rack, allowing beads to separate fully. Pipette off residual ethanol with a P20 pipette and discard. **Do not discard the beads.**

39 Remove samples from the magnetic rack and immediately add 40 µL elution buffer. Resuspend beads by flicking, then quickly spin to collect liquid.

40 Leave samples at room temperature for 00:05:00 to elute DNA. 5m

41 Place samples on the magnetic rack until beads separate fully from the solution, usually less than 00:05:00 5m

42 Slowly pipette 40 µL of clear supernatant without disturbing the beads and transfer to a new PCR tube strip. Discard the old sample tubes with beads. **Do not discard the supernatant.**

Note

Cleaned adapter-ligated samples can be safely stored at 4°C overnight.

Nuclease Treatment and Cleanup

43

Note

This cleanup requires SMRTbell® cleanup beads at room temperature. Ensure beads have warmed at room temperature for 30 minutes before use.

Make the nuclease treatment master mix by adding the reagents listed below in the following order and amounts. Adjust the volumes as needed for your number of samples plus 20% overage.

- Thaw Nuclease buffer at room temperature, then vortex and spin down briefly.
- Thaw Nuclease mix on ice, spin down briefly, and return to ice. Do not vortex.

	A	B
	Component	Volume per Sample
	Nuclease buffer	5 µl
	Nuclease mix	5 µl
	Total volume	10 µl

Mix components by pipetting or gently flicking the tube, then quickly centrifuge to mix.

44 Add 10 µL master mix to each sample for a total volume of 50 µl per sample.

45 Gently flick the tubes to mix and briefly spin down.

46 Incubate samples in a thermocycler* with the below settings:

	A	B	C
	TEMP	TIME	CYCLES
	37°C	15 minutes	1
	4°C	∞	1

* Set heated lid to 75°C.

47
- Thaw elution buffer at room temperature.
- Room temperature SMRTbell cleanup beads should also be stored at room temperature until the protocol is completed or paused overnight at a safe stopping point.

Add 65 µL SMRTbell cleanup beads (1.3X) to each nuclease-treated sample and mix by gently flicking followed by a short spin to collect the liquid. Stop the centrifugation before the beads begin to settle.

48 Leave samples at room temperature for 00:10:00 to bind DNA to the beads.

49 Place tube strip in an appropriate magnetic separation rack until the beads have separated, usually within 00:03:00

50 Carefully pipette off the supernatant without disturbing the beads, discarding the supernatant. **Do not discard the beads, which contain your DNA target**.

51 Slowly dispense 200 µL freshly prepared 80% ethanol to each sample tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.**

52 Repeat the previous step once for a total of two washes:

Slowly dispense 200 µL freshly prepared 80% ethanol to each sample tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.**

53 To remove residual ethanol, quickly spin samples and return the tubes to the magnetic rack, allowing beads to separate fully. Pipette off residual ethanol with a P20 pipette and discard. **Do not discard the beads.**

54 Remove samples from the magnetic rack and immediately add 15 µL elution buffer. Resuspend beads by flicking, then quickly spin to collect liquid.

55 Leave samples at room temperature for 00:05:00 to elute DNA.

56 Place samples on the magnetic rack until beads separate fully from the solution, usually less than 00:05:00

57 Slowly pipette 15 µL of clear supernatant without disturbing the beads and transfer to a new PCR tube strip. Discard the old sample tubes with beads. **Do not discard the supernatant.**

58 Dilute 1 µL from each sample in 9 µL elution buffer or water, then measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit.

Note

Cleaned nuclease-treated samples can be safely stored at 4°C overnight.

Pooling and Concentrating Barcoded Samples

59

Note

This cleanup requires SMRTbell® cleanup beads at room temperature. Ensure beads have warmed at room temperature for 30 minutes before use. We recommend to multiplex a minimum of 6 samples (lower# have not been tested).

Based on the Qubit values determined in step 58, combine an equal mass of each sample together in a single pool within a 1.5mL DNA LoBind tube. The total mass of the pooled samples should be at least 100 ng.

60 Add 1.3 X v/v SMRTbell cleanup beads to the sample pool and mix by gently flicking followed by a short spin to collect the liquid. Stop the centrifugation before the beads begin to settle.

61 Leave pool at room temperature for 00:10:00 to bind DNA to the beads.

62 Place the tube in an appropriate magnetic separation rack until the beads have separated, usually within 00:03:00

63 Carefully pipette off the supernatant without disturbing the beads, discarding the supernatant. **Do not discard the beads, which contain your DNA target**.

64 Slowly dispense 200 µL freshly prepared 80% ethanol into the tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.**

65 Repeat the previous step once for a total of two washes:
Slowly dispense 200 µL freshly prepared 80% ethanol into the tube. After 00:00:30, pipette off the ethanol and discard. **Do not discard the beads.**

66 To remove residual ethanol, quickly spin the tube before returning it to the magnetic rack, allowing beads to separate fully. Pipette off residual ethanol with a P20 pipette and discard. **Do not discard the beads.**

67 Remove pool from the magnetic rack and immediately add 15 µL elution buffer. Resuspend beads by flicking, then quickly spin to collect liquid.

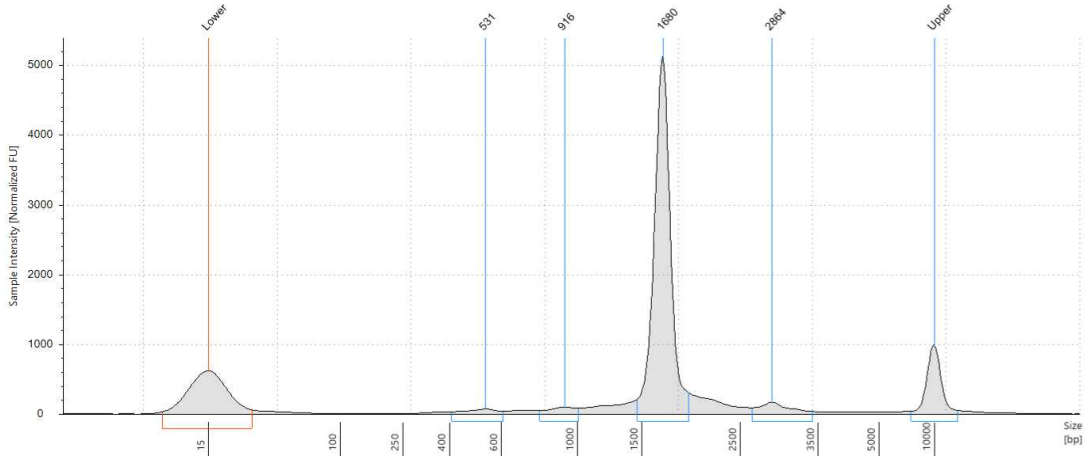
68 Leave pool at room temperature for 00:05:00 to elute DNA.

69 Place tube on the magnetic rack until beads separate fully from the solution, usually less than 00:05:00

70 Slowly pipette 15 µL of clear supernatant without disturbing the beads and transfer to a new tube. Discard the old LoBind tube with beads. **Do not discard the supernatant.**

71 Dilute 1 µL of the concentrated library in 9 µL elution buffer or water, then measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit.

We also recommend running the library on Tape Station using High Sensitivity (HS) 5000 tape to confirm ~1500-1600 bp size of the library.



Final library of a high positive sample, approx. 300 ng DNA input to the final concentration

Note

Concentrated libraries can now be stored at 4°C if sequencing within 5 days. Long-term storage of libraries should be at -20°C. Minimize freeze-thaw cycles.

End Protocol

72 Use SMRTLink Sample Setup with 150 pM on plate concentration and Binding kit 3.1 to prepare library(ies) for sequencing. Use SMRT Cell 8M tray and Sequel II sequencing kit 2.0 to sequence on the Sequel IIe instrument.