PacBio

PacBio HiFiViral high throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0

Procedure & checklist

April 2022

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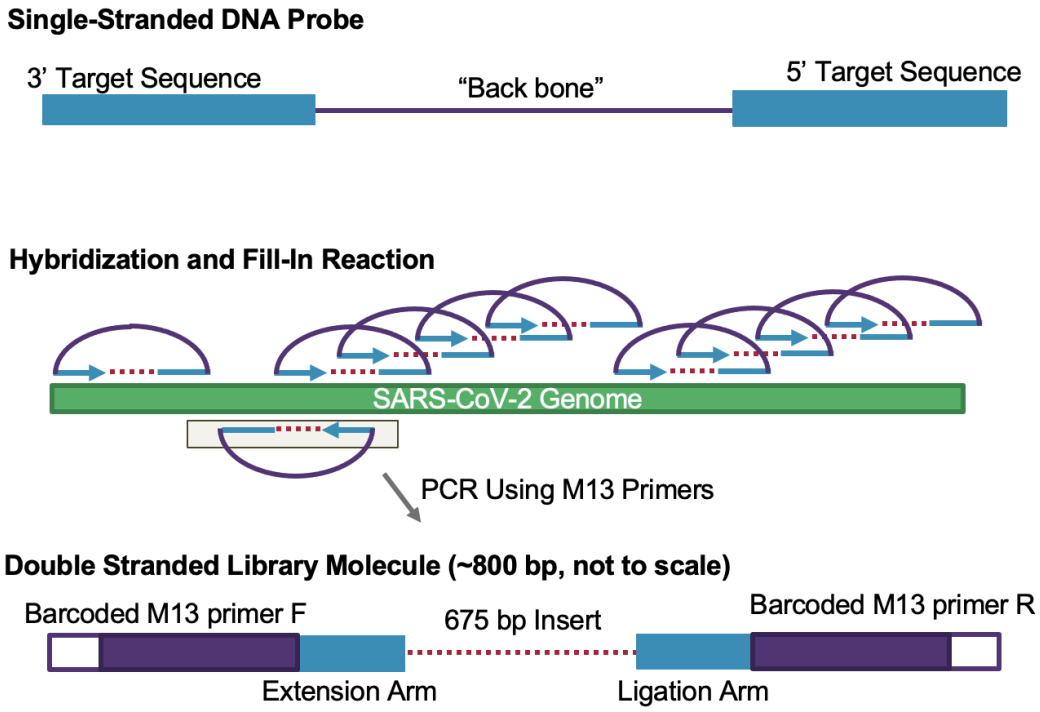
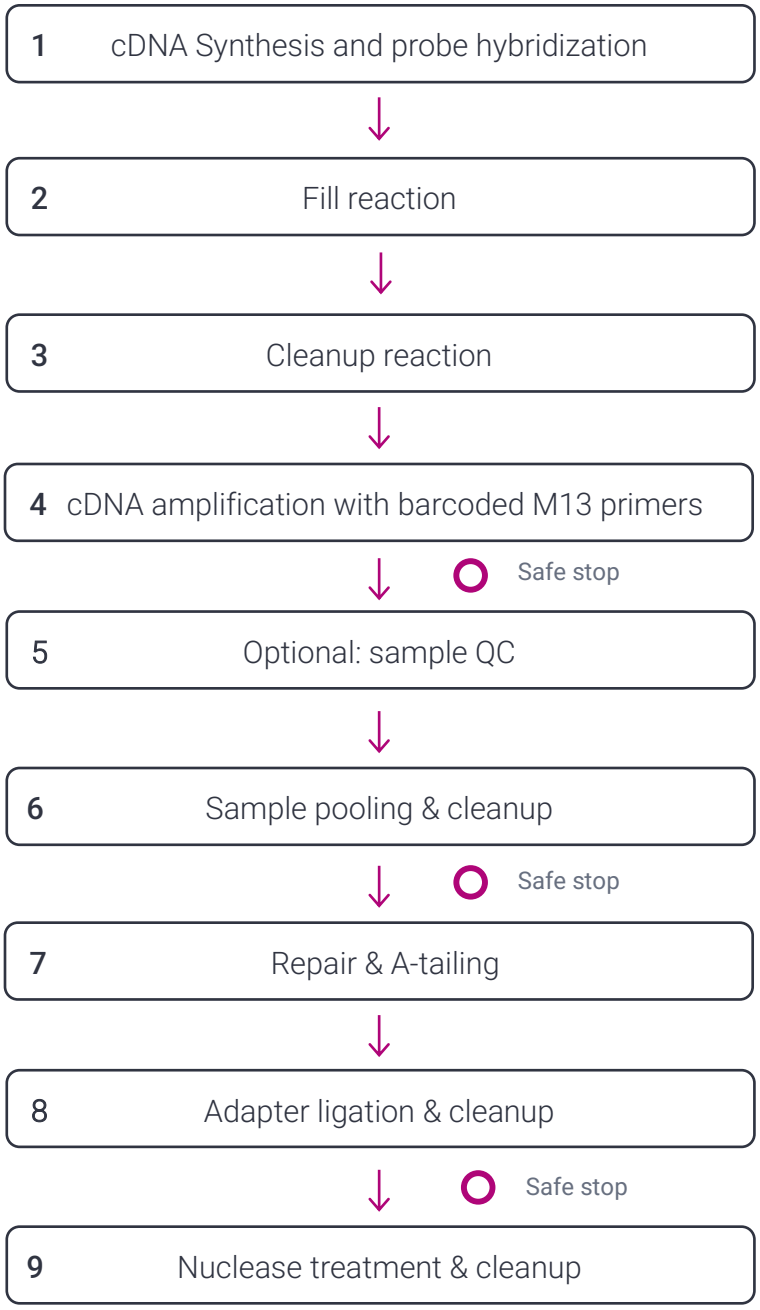
Overview

This procedure captures the SARS-CoV-2 genome with tiled molecular inversion probes that create highly redundant overlapping amplicons resulting in comprehensive sequence coverage on the Sequel® II and Sequel IIe Systems (Sequel II Systems). Target capture uses an addition-only 4-step workflow with color-coded master mixes to simplify setup.



Workflow



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Required materials

Item	Where used	Vendor	Part number
RNA Preparation			
Nuclease-Free Water	RNA Preparation	Any	Vendor-specific
RNaseZap	RNA Preparation	Thermo Fisher Scientific	AM9780
Viral Enrichment with HiFiViral SARS-CoV-2 Kit (Includes 102-135-400 & 102-135-500)		PacBio	102-132-000
SARS-CoV-2 Enrichment Kit	Viral Enrichment	PacBio	102-135-400
Barcoded M13 Primer Plate	Asymmetric Sample Barcoding (Dual Indexing)	PacBio	102-135-500
SMRTbell Library Construction			
SMRTbell® prep kit 3.0	Library Preparation	PacBio	102-182-700
DynaMag-2 Magnet	Purification	Invitrogen	12321D
100% Ethanol, Molecular Biology Grade	Purification	Any	Vendor-specific
Others			
96-well PCR plates	cDNA Preparation	Bio-Rad	HSS9601
Microseal 'B' Film	cDNA Preparation	Bio-Rad	MSB1001
Film sealing roller for PCR plates	cDNA Preparation	Bio-Rad	MSR0001
DNA LoBind Tubes, 1.5 mL	Library Preparation	Eppendorf	22431021
DNA LoBind Tubes, 2.0 mL	Library Preparation	Eppendorf	22431048
8- or 12-Multichannel Pipette	High Throughput Pipetting	Any	Vendor-specific
Qubit™ 4 fluorometer	Quantification	Thermo Fisher Scientific	Q33238
Qubit™ 1x dsDNA HS Assay Kit	Quantification	Thermo Fisher Scientific	Q33230
Bioanalyzer 2100	Library QC (Optional)	Agilent	G2939A
Agilent DNA 12000 Kit	Library QC (Optional)	Agilent	5067-1508
VeritiPro Thermocycler, 96 well	PCR Amplification	Thermo Fisher Scientific	A48141
ProFlex PCR System	PCR Amplification	Thermo Fisher Scientific	4483636
PCR Tube Strips, 0.2 mL	PCR Amplification (Optional)	USA Scientific	1402-4708
96-well plate centrifuge		Any	

General best practices

RNA Input

1. Best results will be achieved if reactions contain at least 10,000 copies of RNA.
2. Best results will be achieved from nasopharyngeal extracts. Saliva and wastewater extracts are not supported.
3. Purified RNA should be resuspended in RNase-free water or TE with a pH no greater than 7.5. Contaminants including ethanol, sodium azide, sodium acetate, and guanidine salts may affect performance.
4. DNase treatment (followed by full and complete inactivation/removal) is optional and the presence of small amounts of human DNA should not affect performance.
5. If RNA is quantified, a method that is specific for RNA is recommended (e.g., Qubit RNA BR Assay Kit or qRT-PCR), rather than one that will also detect DNA.
6. To reduce inter-sample performance variability, all samples in a batch should be quantified using the same method and normalized to the same concentration.

Master mixes

1. Prepare master mixes in a PCR workstation.
2. The PCR workstation should be UV-irradiated after each setup. If unsure, UV-irradiate the workstation before setting up a master mix.

Note: do not turn on the UV light when reagents are in the workstation

3. Master mixes are prepared in 0.5mL, 1.5 mL or 2 mL microfuge tubes. Briefly vortex to mix and spin down.
4. If using multichannel pipette to transfer master mixes, pre-aliquot appropriate volume with overage into PCR strip tubes.

Samples

1. RNA samples should be stored at -80ºC until use and thawed on ice.
2. Heavily degraded RNA or RNA samples with many freeze-thaw cycles should be avoided.
3. All work surfaces and gloves should be sanitized with RNaseZap (or the equivalent) prior to setup.
4. For most consistent performance, all samples included in a batch, including control samples, should be from the same sample type and extracted by the same RNA extraction procedure.
5. A no-RNA control is recommended but not required.
6. Upon thawing frozen samples, briefly vortex and spin down prior to use.

Reaction plates

1. Always seal plates with Microseal 'B' Film (clear adhesive). Foil seals are not recommended for any step in this protocol. However, they can be used for plates that will be placed in the freezer for storage.
2. Using a roller for Microseal 'B' Film, apply firm pressure and seal over the tops of all wells. Ensure all wells, especially those along the edges of the plate, are visibly sealed.

3. Inspect the corners of the plate to confirm that the seal is in contact with the plate. If not, apply firm pressure and roll until the film is in contact with the plate.
4. When removing plate seals, a heated plate sealer can be used if desired to briefly warm the seal and loosen the adhesive.
5. Centrifuge in an Eppendorf 5810 fitted with a swinging bucket plate rotor at maximum rpm for approximately 30 sec.
6. After centrifugation, inspect the bottom of the plate to ensure the expected volume is present in every well.

Reagent handling

1. Room temperature is defined as any temperature in the range of 18-23°C for this protocol.
2. Thaw the repair buffer, nuclease buffer, and elution buffer at room temperature.
3. Mix reagent buffers and SMRTbell adapter with a brief vortex prior to use. Enzyme mixes do not require vortexing.
4. Quick spin all reagents in microcentrifuge to collect liquid at bottom prior to use.
5. Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Repair & A-tailing	Blue	End repair mix
	Green	DNA repair mix
Adapter ligation	Orange	SMRTbell adapter
	Yellow	Ligation mix
	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix

6. Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30-60 minutes prior to use.
7. Pipette mix all bead binding and elution steps until beads are distributed evenly in solution.
8. Pipette mix all SMRTbell prep reactions by pipetting up and down 10 times.
9. Samples can be stored at 4°C at all safe stopping points listed in the protocol.
10. Puncture the top of the seal on the barcoded M13 primer plate with a clean, empty pipette tip before pipetting the primer mix.

Thermocycler programs

Program thermocycler(s) prior to beginning the protocol for the first time.

Set the lid temperature to 105°C for all cDNA synthesis, probe hybridization, fill, cleanup, and amplification steps.

cDNA synthesis, probe hybridization, fill, cleanup, and amplification thermocycler programs

1. Hybridization and fill program

Step	Time	Temperature
1	10 min	25°C
2	50 min	50°C
3	1 minute	95°C
4	24 hours*	55°C
5	Hold	55°C

*16hrs for probe hybridization and 1hr for fill reaction plus some margin

2. Cleanup program

Step	Time	Temperature
1	60 min	45°C
2	3 min	95°C
3	Hold	4°C

3. cDNA amplification with barcoded M13 primers program

Step	Time	Temperature
1	3 min	95°C
2	15 sec	98°C
3	15 sec	55°C
4	1 minute, 30 sec	72°C
5	Repeat steps 2 to 4 for 26 cycles	
6	Hold	4°C

SMRTbell prep kit 3.0 thermocycler programs

Repair and A-tailing, adapter ligation, and nuclease treatment thermocycler steps can be combined into a single program and paused in between prep treatments if preferred.

Set the lid temperature to **75°C** for all SMRTbell prep kit 3.0 programs. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

1. Repair and A-tailing program

Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

2. Adapter ligation program

Step	Time	Temperature
1	30 min	20°C
2	Hold	4°C

3. Nuclease treatment program

Step	Time	Temperature
1	15 min	37°C
2	Hold	4°C

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Procedure & checklist

1. cDNA synthesis and probe hybridization

Before setting up the reaction, the workstation should be sanitized with RNaseZap and UV-irradiated without the presence of the reagents. All samples and reagents should be kept on ice while setting up the reaction.

✔	Step	Instructions
	1.1	Prepare labware and reagents. A. Label one or more 96-well PCR plates. Alternatively, for a small number of reactions, PCR tube strips may be used. B. Retrieve extracted RNA samples from storage.
	1.2	Add **6 µL** of sample RNA into each well of the reaction plate. Be sure to follow RNA input recommendations. Use nuclease-free water to adjust sample RNA volume, if needed. Keep RNA samples on ice.
	1.3	Prepare RT-Hybridization Master Mix on ice. A. Allow RT Mix and Probe Mix to fully thaw. Briefly vortex and spin down. B. Prepare master mix with 12.5% overage as indicated in the table below. Preparing fewer than 24 reactions is not recommended. C. RT Mix is viscous, pipette slowly
	1.4	Add 2 µL of RT-Hybridization Master Mix to each sample-containing well (6 µL) in the reaction plate. RT-Hybridization Master Mix is viscous, pipette slowly.
	1.5	Seal the plate tightly with the microseal 'B' film. **Poor seal will result in significant sample loss due to evaporation.**
	1.6	Spin down the 96-well plate(s) to collect liquid.
	1.7	Vortex a few times with short pulses and spin down again to collect liquid.
	1.8	Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **pale blue** color
	1.9	Place the reaction plate in the thermocycler and run the **hybridization & fill program** (set the heated lid at 105ºC). Ensure that the thermocycler lid has a tight fit with the plate to reduce evaporation.
	1.10	Make a note of the thermocycler start time. A hybridization time of 16 hours (the 55°C step) is recommended for high Ct samples (Ct >25). A 4hr hybridization could be considered if most of samples have low Ct value (Ct <25). Start preparing for the fill reaction just prior to the end of hybridization (approximately 17 hours from the start of the cycling program).

RT-Hybridization Master Mix

Component	1X reaction	96 reactions (+12.5%)
RT mix	1.6 µL	172.8 µL
Probe mix	0.4 µL	43.2 µL

2. Fill reaction

Before the end of the probe hybridization reaction, allow the Fill-in mix to fully thaw. Briefly vortex and spin down. Do not remove the reaction plate from the thermal cycler until the reagent is ready and the hybridization time is over. Correct timing is important to maximize result quality.

✔	Step	Instructions
	2.1	Remove the sample plate from the thermocycler. **Keep the hybridization and fill program running**.
	2.2	Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
	2.3	**At room temperature**, add **2 µL** of Fill-in Mix to each sample well. It is important to **finish within 10 minutes** to minimize non-specific hybridization.
	2.4	Reseal the plate tightly with a new microseal 'B' film, vortex a few times with short pulses, and spin down the plate to collect liquid.
	2.5	Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **pale green** color.
	2.6	Place the reaction plate in the thermocycler and continue the program for another 60 minutes.
	2.7	Record the time the reaction plate was returned to the thermocycler; correct timing is important to maximize result quality.

3. Cleanup reaction

Before the end of the fill reaction, allow the cleanup mix to fully thaw. Briefly vortex and spin down. Do not remove the reaction plate from the thermal cycler until the reagent is ready. Correct timing is important to maximize result quality.

✔	Step	Instructions
	3.1	Remove the sample plate from the thermocycler.
	3.2	Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
	3.3	At room temperature, add **2 µL** of **Cleanup Mix** to each sample well. It is important to **finish within 10 minutes** to minimize non-specific hybridization.
	3.4	Reseal the plate tightly with a new microseal 'B' film, vortex a few times with short pulses, and spin down the plate.
	3.5	Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **red** color.
	3.6	Place the reaction plate in the thermocycler and run the **cleanup program** (set the heated lid at 105°C).
	3.7	The program will take approximately 65 minutes to run; proceed immediately to the cDNA amplification step when finished.

4. cDNA amplification

Before the end of the cleanup reaction, allow the PCR Mix and Barcoded M13 Primer Plate to fully thaw. Spin down the Barcoded M13 primer plate before opening. Briefly vortex the PCR Mix and spin down. The reaction plate and reagents should be kept on ice while setting up the reaction.

✔	Step	Instructions
	4.1	Remove the sample plate from the thermocycler.
	4.2	Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
	4.3	Using a multichannel pipette, add **12 µL** of **PCR Mix** to each sample on the plate.
	4.4	Add **2.4 µL** of primer from the barcoded M13 primer plate to the corresponding sample wells. Puncture the top of the seal on the barcoded M13 primer plate with a clean, empty pipette tip before pipetting the primer mix.
	4.6	The total reaction volume in each well is approximately **24.0 µL.** See table below:

cDNA amplification	
Component	**Volume**
Cleanup reaction mix	9.6 µL*
PCR Mix	12 µL
Barcoded M13 Primer Pair	2.4 µL
Total Volume	**24 µL**

* The expected volume after the cleanup reaction is approximately 9.6 µL, considering some degree of evaporation during the prior steps

	4.7	Reseal the plate tightly with a new microseal 'B' film, vortex a few times with short pulses, and spin down the plate.
	4.8	Perform a quick visual check of liquid level and take note of any well with low volume. The reaction should now be a homogenous **magenta** color.
	4.9	Place the PCR reactions in a thermocycler and run the **cDNA amplification** program (set the heated lid at 105°C).
	4.10	After amplification, briefly spin down the plate.
	4.11	Immediately proceed to the "Sample pooling & cleanup" section if not performing the optional Library Quantitation/QC step. Alternatively, the reaction plate can be stored at -20°C until further processing.

SAFE STOPPING POINT

5. Library quantification/QC (optional)

✔	Step	Instructions
	5.1	Remove the reaction plate from the thermocycler.
	5.2	Spin down the reaction plate and perform a quick visual check of the liquid level. Take note of any well with low volume, which indicates excessive evaporation during amplification.
	5.3	Remove the seal carefully to avoid cross contamination.
	5.4	Use **1 µL** of sample to quantify with a Qubit fluorometer using the 1x dsDNA HS assay kit.
	5.5	Individual sample QC can be performed on the Agilent 2100 Bioanalyzer. Use a DNA12000 chip and follow the manufacturer's setup instruction.
	5.6	A target peak of **≥700 bp** should be detected. A small peak of ~170-200 bp representing non-specific amplicons may or may not be present. The ~170-200 bp amplicons will be removed when the sample pool is purified.

6. Sample pooling for library construction

✔	Step	Instructions
		Sample pooling
	6.1	Remove the reaction plate from the thermocycler.
	6.2	Spin down the reaction plate and perform a quick visual check of the liquid level. Take note of any well with low volume, which indicates excessive evaporation during amplification.
	6.3	Remove the seal carefully to avoid cross contamination.
	6.4	Transfer a minimum of **5 µL** per reaction into a clean 1.5 or 2.0 mL DNA Lo-bind tube. If pooling **384 reactions**, vortex to mix and transfer no more than **800 µL** to a new 2.0mL Lo-bind tube for purification. Save the rest of the sample pool at -20ºC.
		Cleanup with 1.3X SMRTbell cleanup beads
	6.5	Add **1.3X** volume over volume (v/v) of resuspended, room-temperature SMRTbell cleanup beads to the pooled library in the 1.5 or 2.0 mL LoBind tube.
	6.6	Pipette mix beads until evenly distributed.
	6.7	Quick spin the tube in a microcentrifuge to collect liquid.
	6.8	Leave at room temperature for **10 minutes** to allow DNA to bind beads.
	6.9	Place tube in a magnetic separation rack until beads separate fully from the solution.
	6.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.11	Slowly dispense **1400 µL**, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.

6.12	Repeat the previous step.
6.13	Remove residual 80% ethanol: • Remove tube from the magnetic separation rack. • Quick spin tube in a microcentrifuge. • Place tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
6.14	Remove tube from the magnetic rack. Immediately add **47 µL** of **low TE buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
6.15	Quick spin the tube in a microcentrifuge to collect liquid.
6.16	Leave at room temperature for a minimum of **5 minutes** to elute DNA.
6.17	Place tube in a magnetic separation rack until beads separate fully from the solution.
6.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **0.2 mL PCR tube strip**. Discard old tube with beads.
6.19	**Recommended**: Evaluate sample quality (concentration and size distribution). • Take **1 µL** of eluted DNA and dilute with **9 µL** of elution buffer or water. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • (Optional): Measure DNA size distribution on the Agilent 2100 Bioanalyzer using the DNA 12000 chip. Follow all manufacturer's instructions. Target peak should be ≥700 bp with minimal non-specific peaks near 170-200 bp.
6.20	Proceed to the next step of the protocol if sample quality is acceptable.
	SAFE STOPPING POINT – Store at 4°C

7. Repair & a-tailing

✔	Step	Instructions
	7.1	Add the following components to the sample (purified pooled cDNA) in the specified order and volume listed below. The total amount of purified, pooled cDNA input should be between 100-1000 ng. If the amount exceeds 1000 ng, then dilute the sample as appropriate so that 46 µL equals 1000 ng.

✔	Tube	Component	Volume
	PREVIOUS	Contents from previous step	46 µL
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	**60 µL**

7.2	Pipette mix.
7.3	Quick spin the tube strip in a microcentrifuge to collect liquid.
7.4	Incubate in a thermocycler with the **repair & A-tailing** program.
7.5	Proceed to the next step of the protocol.

8. Adapter ligation & cleanup

✔	Step	Instructions
		Adapter ligation
	8.1	Add the following components to the sample in the specified order and volume listed below.

✔	Tube	Reagent	Volume
	PREVIOUS	Contents from previous step	60 µL
	Orange	SMRTbell adapter	4 µL
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**95 µL**

	8.2	Pipette mix.
	8.3	Quick spin the tube strip in a microcentrifuge to collect liquid.
	8.4	Incubate in a thermocycler with **the adapter ligation** program.
		Cleanup with 1.3X SMRTbell cleanup beads
	8.5	Add **124 µL** of resuspended, room-temperature SMRTbell cleanup beads to the sample.
	8.6	Pipette mix beads until evenly distributed.
	8.7	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	8.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	8.9	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	8.11	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	8.12	Repeat the previous step.
	8.13	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	8.14	Remove tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	8.15	Quick spin the tube strip in a microcentrifuge.
	8.16	Leave at **room temperature** for **5 minutes** to elute DNA.
	8.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	8.19	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C

9. Nuclease treatment & cleanup

✔	Step	Instructions
		Nuclease Treatment
	9.1	Add the following components to the sample in the specified order and volume listed below. ✓ | Tube | Reagent | Volume PREVIOUS | Contents from previous step | 40 µL Light purple | Nuclease buffer | 5 µL Light green | Nuclease mix | 5 µL **Total volume** | **50 µL**
	9.2	Pipette mix.
	9.3	Quick spin the tube strip in a microcentrifuge to collect liquid.
	9.4	Incubate in a thermocycler with the **nuclease treatment** program.
		Cleanup with 1.3X SMRTbell cleanup beads
	9.5	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads to the sample
	9.6	Pipette mix the beads until evenly distributed.
	9.7	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	9.8	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	9.9	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	9.10	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	9.11	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
	9.12	Repeat the previous step.
	9.13	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	9.14	Remove tube strip from the magnetic rack. **Immediately** add **15 µL** of **elution buffer** and resuspend the beads by pipetting 10 times or until evenly distributed.
	9.15	Quick spin the tube strip in a microcentrifuge.
	9.16	Leave at **room temperature** for **5 minutes** to elute DNA.
	9.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	9.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **tube**. Discard old tube strip with beads.
	9.19	Take a 1 µL aliquot from the sample and dilute with 9 µL of elution buffer or water. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
	9.20	Proceed to SMRT Link Sample Setup to prepare the library for sequencing or store at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.
		PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	April 2022

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