Customer Collaboration - PacBio HiFiViral for SARS-CoV-2 Workflow

Low-Throughput Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2

This protocol is the result of a collaboration between LabCorp and Pacific Biosciences. We acknowledge the contributions of:

1. LabCorp
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2. Pacific Biosciences
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This procedure provides a workflow for multiplexing up to 48 SARS-CoV-2 samples and sequencing on the Sequel® II and Sequel IIe Systems. We recommend using this procedure if your throughput needs are low (≤48 samples) and if this is your first attempt to multiplex amplicons using the asymmetric M13 barcoding strategy for SMRT Sequencing. Once you are ready to increase your sample throughput, you may use the High-Throughput Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2 Procedure.

First-strand cDNA is synthesized using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). The resulting cDNA is then PCR amplified, asymmetrically barcoded and subsequently pooled with other samples for SMRTbell® library construction and multiplex sequencing on the Sequel II and IIe Systems.

This procedure recommends amplification and sequencing of twenty-nine overlapping 1.2 kb amplicons that are tiled across the full 29.9 kb SARS-CoV-2 genome (Figure 1). The PCR primers were designed by Nikki E. Freed of the School of Natural and Computational Sciences, Massey University, Auckland, New Zealand.

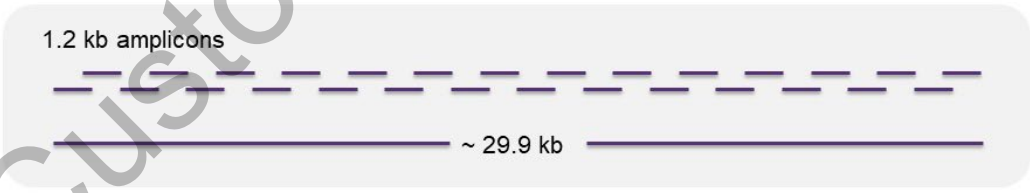

Figure 1: 29 x 1.2 kb amplicons tiled across the SARS-CoV-2 genome.

The procedure requires two rounds of PCR amplification, first using M13-tailed target specific primers to tail the PCR products with a universal M13 sequence, followed by a second-round PCR using barcoded M13 primers. A total of 32 Forward (F) and 32 Reverse (R) barcoded M13 primers are available for barcoding but you can choose a subset of the primers, e.g., 8 Forward and 6 Reverse, to enable asymmetric barcoding of 48 samples. When you are ready to increase your throughput needs, you can use the full set of 32 Forward and 32 Reverse primers which when used in different pairwise combinations allow multiplexing of up to 1024 samples.

For any questions or additional information about this procedure, please contact support@pacb.com.

The general workflow described in this procedure is summarized in Figure 2 below.

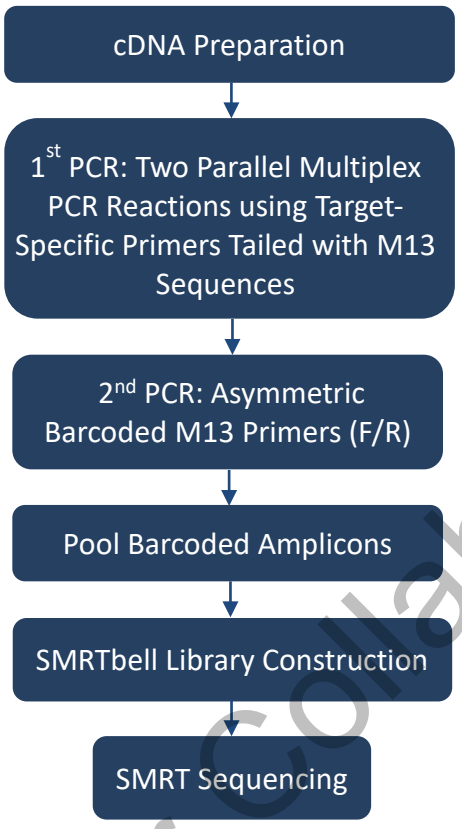

Figure 2: General workflow for full viral genome sequencing of SARS-CoV-2 on the Sequel II and Sequel IIe Systems.

Required Materials

Item	Where Used	Vendor	Part Number
cDNA Synthesis			
SuperScript™ VILO™ cDNA Synthesis Kit	cDNA Preparation	Thermo Fisher Scientific	11754-050
Oligo(dT) (100 µM)	cDNA Preparation	IDT	Custom*
PCR Reaction			
Target-Specific F/R Primers tailed with M13 Sequences (Customer-supplied)	PCR Amplification (1st-Round)	Oligo Synthesis Company	N/A
F/R PacBio-Barcoded M13 Primers (Customer-supplied)	PCR Amplification (2nd-Round)	Oligo Synthesis Company	N/A
Q5 Hot Start High-Fidelity DNA Polymerase	PCR Amplification (1st-Round)	NEB	M0493L
dNTPS	PCR Amplification (1st-Round)	NEB	N0447L
KAPA HiFi HotStart ReadyMix	PCR Amplification (2nd-Round)	Roche	7958935001
Nuclease-Free Water	PCR Amplification	Any	Vendor-specific
SMRTbell Library Construction			
SMRTbell® Express Template Prep Kit 2.0	Library Preparation	PacBio	100-938-900
SMRTbell Enzyme Cleanup Kit	Library Preparation	PacBio	101-746-400
DynaMag-2 Magnet	Purification	Invitrogen	12321D
100% Ethanol, Molecular Biology Grade	Purification	Any	Vendor-specific
AMPure® PB Beads	Purification	PacBio	100-265-900
Sequencing			
Sequel® II Bind Kit 2.1 and Int Ctrl 1.0	Sequencing short (<3 kb) amplicon samples on the Sequel® II or Sequel IIe Systems	PacBio	101-843-000
Sequel II Sequencing Kit 2.0	Supports 4 sequencing reactions on the Sequel II or Sequel IIe Systems	PacBio	101-820-200
SMRT® Cell 8M Tray	Sequencing on the Sequel II or Sequel IIe Systems	PacBio	101-389-001
Sequel Pipette Tips v2	Sequencing on the Sequel II or Sequel IIe Systems	PacBio	100-667-601
Sequel Mixing Plates	Sequencing on the Sequel II or Sequel IIe Systems	PacBio	100-667-500
Sample Plate	Sequencing on the Sequel II or Sequel IIe Systems	BioRad	HSP9601
Tube Septa	Sequencing on the Sequel II or Sequel IIe Systems	PacBio	001-292-541
Foil Heat Seals	Sequencing on the Sequel II or Sequel IIe Systems	Thermo Fisher Scientific	AB0757

Other Equipment and Materials			
DNA LoBind Tubes, 1.5 mL	Library Preparation	Eppendorf	22431021
DNA LoBind Tubes, 2.0 mL	Library Preparation	Eppendorf	22431048
8- or 12-Multichannel Pipettor	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
Thermal Cycler, 96 well	PCR Amplification	Thermo Fisher Scientific	A48141
MicroAmp Optical 96-well Reaction Plate	PCR Amplification	Thermo Fisher Scientific	N8010560
96-welll Plate Centrifuge		Any MLS	

*See Oligo(dT) sequence in the "Reverse Transcription" section below.

Table 1. List of Required Materials and Equipment.

Before you Begin

To proceed with your experiments, you must have the following items:

Reverse Transcription

1. The recommended kit for reverse transcription is the **SuperScript VILO cDNA Synthesis Kit,** which includes an enzyme mix and a reaction mix sufficient for 50 reactions.
2. For the RT reaction, Oligo(dT) is required for synthesis. Note that the Oligo(dT) is not included in the kit and must be ordered from any oligo synthesis provider.
   The Oligo(dT) sequence is **5' TTT TTT TTT TTT GTC ATT CTC CTA AG 3'** and HPLC purification is recommended. Dilute the Oligo(dT) with nuclease-free buffer (10mM Tris-HCl pH 7.5) to a 100 µM stock concentration.

Amplification Kits

For this step, two types of high-fidelity PCR kits are required.

1. For the first-round PCR, the **Q5 Hot Start High-Fidelity DNA Polymerase** is required.
2. For the second-round PCR, **KAPA HiFi HotStart ReadyMix** is required. This step adds barcodes to both ends of each amplicon sample.

Preparation of M13-tailed Target-specific Primers for First-Round PCR

1. There are 29 pairs of M13-tailed Forward and Reverse Primers that amplify overlapping 1.2 kb regions tiled across the entire 29.9 kb genome of SARS-CoV-2. The forward and reverse primers tailed with M13 sequences may be ordered from any oligo synthesis provider.
   - Add a 5' blocker (e.g., 5AmMC6) to ensure that carryover amplicons from the first-round PCR are not ligated to the SMRTbell adapters during SMRTbell library construction.
   - Desalted primers are sufficient for PCR amplification.
2. Before use, dilute oligos with nuclease-free buffer (10mM Tris-HCl pH 7.5) to 100 µM stock concentration.
3. Prepare two pools of primers as follows, where **Primer Pool 1** contains the odd primer pairs and **Primer Pool 2** contains the even primer pairs. Into a 1.5 mL DNA LoBind tube, prepare 100 µM stocks of **Primer Pool 1** and **Primer Pool 2** by adding the recommended volumes listed in the tables below. Mix well and perform a quick-spin. To minimize multiple freeze/thaw cycles, store 50.0 µL aliquots of Primers Poo1 and 2 at -20ºC.

Primer Pool 1 (100 µM Stock; Total Volume = 250 µL)

Primer Pair	Forward Primer to Add (µL)	Reverse Primer to Add (µL)
SARSCoV_1200_1	10.0	10.0
SARSCoV_1200_3	10.0	10.0
SARSCoV_1200_5	5.0	5.0
SARSCoV_1200_7	10.0	10.0
SARSCoV_1200_9	10.0	10.0
SARSCoV_1200_11	5.0	5.0
SARSCoV_1200_13	10.0	10.0
SARSCoV_1200_15	10.0	10.0
SARSCoV_1200_17	5.0	5.0
SARSCoV_1200_19	10.0	10.0
SARSCoV_1200_21	5.0	5.0
SARSCoV_1200_23	5.0	5.0
SARSCoV_1200_25	10.0	10.0
SARSCoV_1200_27	10.0	10.0
SARSCoV_1200_29	10.0	10.0

Primer Pool 2 (100 µM Stock; Total Volume = 200 µL)

Primer Pair	Forward Primer to Add (µL)	Reverse Primer to Add (µL)
SARSCoV_1200_2	5.0	5.0
SARSCoV_1200_4	5.0	5.0
SARSCoV_1200_6	5.0	5.0
SARSCoV_1200_8	10.0	10.0
SARSCoV_1200_10	10.0	10.0
SARSCoV_1200_12	5.0	5.0
SARSCoV_1200_14	10.0	10.0
SARSCoV_1200_16	5.0	5.0
SARSCoV_1200_18	5.0	5.0
SARSCoV_1200_20	5.0	5.0
SARSCoV_1200_22	10.0	10.0
SARSCoV_1200_24	10.0	10.0
SARSCoV_1200_26	5.0	5.0
SARSCoV_1200_28	10.0	10.0

4. Next, dilute the 100 µM Pool 1 and Pool 2 stock solutions to 10 µM working solutions as shown in the reaction table below. Mix well and perform a quick spin. Store the Primer Pools in small aliquots (e.g., 100 µL) and store at -20ºC until ready to use.

Reagent	Volume	✓	Notes
Primer Pool 1 Stock (100 µM)	50.0 µL		
Nuclease Free Water	450.0 µL		
Total Volume	500.0 µL		

Reagent	Volume	✓	Notes
Primer Pool 2 Stock (100 µM)	50.0 µL		
Nuclease Free Water	450.0 µL		
Total Volume	500.0 µL		

Preparation of Barcoded M13 Primers for Second-Round PCR

1. For sample multiplexing, there are a total of 32 Forward and 32 Reverse barcoded M13 primers that are available and when used in different combinations allow multiplexing of up to 1024 samples. For a 48-plex design, we recommend using 8 of the Forward Primers and 6 of the Reverse Primers as shown in the table below.
2. Resuspend PacBio-barcoded M13 primers with nuclease-free buffer (10mM Tris-HCl pH 7.5) to a concentration of 3.0 µM and aliquot into a 96-well plate as shown in the example layout below (or use a different configuration that facilitates aliquoting the desired combinations of forward and reverse barcoded primers). Note that QC analysis of barcoded primers is highly recommended, as oligos with missing 5' bases may appear to produce the expected amounts of PCR products but will generate low (de-multiplexed) sequencing data yields if they lack the complete barcode sequence.

Example Plate Layout for 8 Forward and 6 Reverse PacBio-Barcoded M13 Primers.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FWD_1001	x	x	x	x	x	REV_1049	x	x	x	x	x
B	FWD_1002	x	x	x	x	x	REV_1050	x	x	x	x	x
C	FWD_1003	x	x	x	x	x	REV_1051	x	x	x	x	x
D	FWD_1004	x	x	x	x	x	REV_1052	x	x	x	x	x
E	FWD_1005	x	x	x	x	x	REV_1053	x	x	x	x	x
F	FWD_1006	x	x	x	x	x	REV_1054	x	x	x	x	x
G	FWD_1007	x	x	x	x	x	x	x	x	x	x	x
H	FWD_1008	x	x	x	x	x	x	x	x	x	x	x

Column 1 is M13 forward primers tailed with PacBio barcode 1001 to barcode 1008.

Column 7 is M13 reverse primers tailed with PacBio barcode 1049 to barcode 1054.

3. Any of the 8 Forward primers may be combined with any of the 6 Reverse primers to create asymmetrically barcoded pairs. Plan out your specific barcoding strategy to use prior to preparing PCR reaction mixes. See **Appendix 1** for an example of an Asymmetric Barcode Plate Map for 48 samples.

DNA Input Requirements for Library Construction

When planning your amplification experiments, always consider the total input DNA required for SMRTbell library construction. In this procedure, **500 – 1000** ng of **pooled** (barcoded) PCR products are required. If using more than 1000 ng of total pooled PCR products, scale the reaction volumes accordingly. Samples should be present at equimolar concentrations after pooling (see Best Practices for Equimolar Pooling section below).

If necessary, replicate PCR reactions should be set up to obtain the required total amount of DNA product needed for library construction. This approach also minimizes PCR sampling bias for samples containing heterogeneous templates.

Best Practices for Equimolar Pooling

To obtain equal representation of each amplicon in the data, we highly recommend following the guidelines below. As you gain significant experience with PacBio's end-to-end workflow for multiplexed sample preparation, you may evaluate other approaches to simplify the guidelines below. The High-Throughput Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2 procedure describes a workflow to multiplex 500 – 900 samples.

1. Ideally, amplicons should be AMPure PB bead purified prior to pooling.
2. To obtain more balanced coverage in the sequencing data, it is important to pool samples in equimolar concentrations. To do this, it is a best practice to purify samples with AMPure PB beads and perform a sizing QC step using the Agilent Bioanalyzer System, Agilent TapeStation, or Agilent Fragment Analyzer system. We recommend quantifying purified amplicon samples using the Qubit dsDNA assay.
3. Remove non-specific PCR products (contaminating bands) prior to pooling. The presence of non-specific products in the pool will impact sequencing data yield.
   a. If the contaminating bands are close in size to or are larger than the desired amplicon size selection using an automated size selection tool or other gel-based method may be necessary.
   b. If removal of contaminating bands is not possible, we recommend re-optimization of the amplification reaction using more stringent PCR conditions.
   c. Always determine the concentration of the amplicon **target band** or **peak only** and use this value to calculate the mass or volume of the amplicon sample to be used during pooling.
   d. If presence of contaminating bands is determined to be acceptable (i.e., their presence has minimum impact on the sequencing yield of the desired target), you may choose to include the amplicon in the sample pool. In such cases, however, it may be necessary to increase the relative input amounts of such amplicons (containing non-specific products) during pooling in order to achieve adequate sequencing data yields for each amplicon in the sample pool.
4. For highly multiplexed experiment designs, purifying and quantifying each individual PCR product may be difficult or impractical. A QC method that may work well is to load samples on an agarose gel to view the PCR products prior to pooling. This QC method may work well if PCR conditions are fully optimized to generate clean specific PCR products consistently.
   a. PCR products that show the same band intensity on a gel may be pooled by volume or mass. To do this, include control fragments of known concentrations when loading samples to perform agarose gel electrophoresis. The pooled samples must meet the minimum DNA input requirements for SMRTbell library construction (see DNA Input Requirements for Library Construction section).
   b. For samples that show weak signals on a gel, increase the volume or mass used during pooling.

Before you begin your experiments, review the diagram shown in Figure 3 which provides an overview of the workflow starting from up to 48 cDNA synthesis reactions to pooling for SMRTbell library construction.

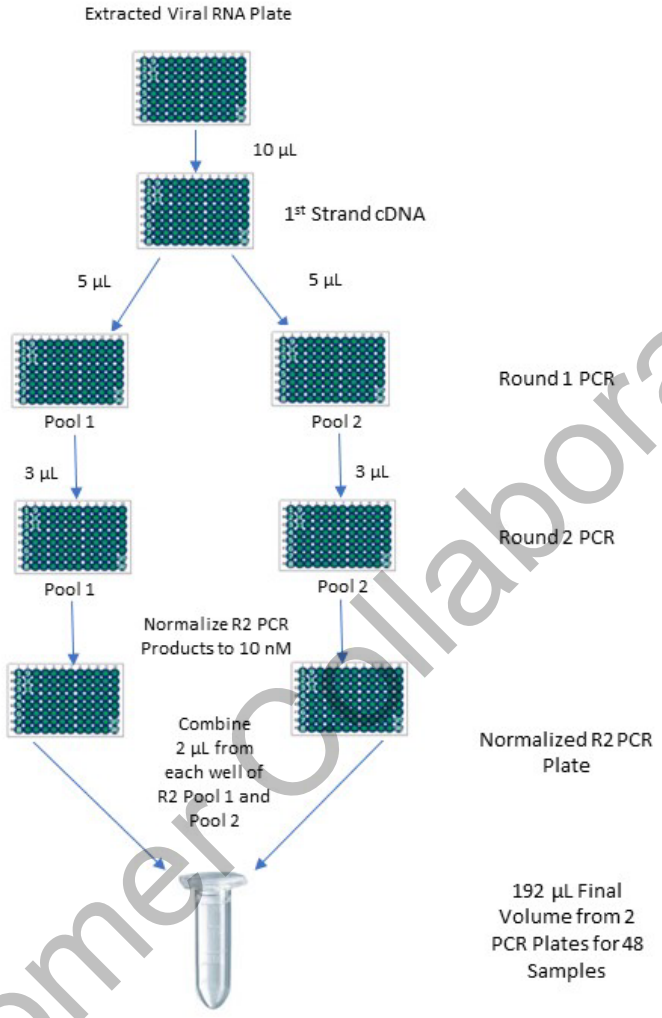

Figure 3: Illustration of sample preparation workflow for whole viral genome analysis of multiplexed SARS-COV-2 amplicon samples. To obtain more balanced coverage in the sequencing data, it is recommended to pool samples in equimolar concentrations by first normalizing the Round 2 PCR products.

First-strand cDNA Synthesis

The procedure below was optimized for generating first-strand cDNA using the SuperScript VILO cDNA Synthesis Kit. Work on ice at all times.

1. Thaw the 5X VILO Reaction Mix to room temperature. If precipitates have formed, incubate at 37°C for 5 minutes.
2. Prepare the following first-strand synthesis reaction Master Mix. Combine the following components into a 1.5 mL DNA LoBind tube on ice.

Reagent	Stock Conc.	1 Rxn	N Rxn*	48 Rxn*	✓	Notes
5X VILO Reaction Mix	5X	6.0 µL	N X 6.0 X 1.2	345.6 µL		
Nuclease Free Water		1.9 µL	N X 1.9 X 1.2	109.4 µL		
Oligo(dT)	100 µM	0.1 µL	N X 0.1 X 1.2	5.8 µL		
10X SuperScript Enzyme Mix	10 X	2.0 µL	N X 2.0 X 1.2	115.2 µL		
Total Volume		**10.0 µL**		**576.0 µL**		

* Note that indicated volumes include a 20% overage

3. Invert tube to mix 2 times. Perform a quick spin.
4. Add 10 µL of RNA from the source plate and 10 µL of Master Mix to each well of a new 96-well plate.
5. Seal the plate. Ensure that any liquid on the side is in the bottom of each well by performing a quick spin.
6. Place the 96-well plate in a thermocycler and run the following program:

Step	Temperature	Time
1	23ºC	10 minutes
2	50ºC	60 minutes
3	80ºC	10 minutes
4	4ºC	Hold

7. When the program is complete, briefly spin down the plate and place on ice.
8. Immediately proceed to the “First Round PCR” section of this procedure.

cDNA Amplification

Sections A and B below describe the preparation of the first- and second-round PCR reactions needed for producing barcoded amplicon samples.

For the first-round PCR, each sample requires that two multiplex PCR reactions using Primer Pool 1 and Pool 2 be performed in parallel.

For the second-round PCR, PCR products from the first-round PCR reactions are re-amplified using barcoded M13 primers to generate asymmetric barcoded amplicons.

A. First-Round PCR

For each sample to be processed, prepare two separate multiplex PCR reactions using the 10 µM Primer Pool 1 and Pool 2 working solutions prepared in the "Preparation of M13-tailed Target-specific Primers for First-Round PCR" section.

1. Allow the Q5 Reaction Buffer to thaw to room temperature. If precipitates have formed, incubate at 37°C for 5 minutes.
2. Label a clean 2.0 mL DNA LoBind tube **"Pool 1 Master Mix"** and add the following reagents. Slowly aspirate and dispense several times to mix contents. Quick-spin the tubes to bring contents to the bottom.

Reagent	Stock Conc.	1X Rxn	N Rxn*	48 Rxn*	✓	Notes
Q5 Reaction Buffer	5X	5.0 µL	N X 5.0 X 1.2	288.0 µL		
dNTPs	10 mM	1.0 µL	N X 1.0 X 1.2	57.6 µL		
Q5 Hot Start High-Fidelity DNA Polymerase	2000 units/mL	0.5 µL	N X 0.5 X 1.2	28.8 µL		
Primer Pool 1 Working Solution	10 µM	1.5 µL	N X 1.5 X 1.2	86.4 µL		
Nuclease Free Water		12.0 µL	N X 12.0 X 1.2	691.2 µL		
Total Volume		**20.0 µL**		**1152.0 µL**		

* Note that indicated volumes include a 20% overage

3. Label a new clean 2.0 mL DNA LoBind tube **"Pool 2 Master Mix"** and add the following reagents. Slowly aspirate and dispense several times to mix contents. Quick-spin the tubes to bring contents to the bottom.

Reagent	Stock Conc.	1X Rxn	N Rxn*	48 Rxn*	✓	Notes
Q5 Reaction Buffer	5X	5.0 µL	N X 5.0 X 1.2	288.0 µL		
dNTPs	10 mM	1.0 µL	N X 1.0 X 1.2	57.6 µL		
Q5 Hot Start High-Fidelity DNA Polymerase	2000 units/mL	0.5 µL	N X 0.5 X 1.2	28.8 µL		
Primer Pool 2 Working Solution	10 µM	1.5 µL	N X 1.5 X 1.2	86.4 µL		
Nuclease Free Water		12.0 µL	N X 12.0 X 1.2	691.2 µL		
Total Volume		**20.0 µL**		**1152.0 µL**		

* Note that indicated volumes include a 20% overage

4. Label two 96-well PCR plates. Label one plate as **"R1 Pool 1**" and the other plate as **"R1 Pool 2".**
5. Using a multichannel pipette, transfer 20 µL of "**Pool 1 Master Mix"** to the appropriate wells of the 96-well plate labeled **"R1 Pool 1"** and 20 µL of "**Pool 2 Master Mix"** to the appropriate wells of the 96-well plate labeled **"R1 Pool 2".**

6. Using a multichannel pipette, transfer **5.0 µL** of first-strand cDNA to both the Pool 1 and Pool 2 plates. The total reaction volume in each well is 25.0 µL.
7. Slowly aspirate and dispense several times to mix contents.
8. Quick-spin the 96-well plates.
9. Place the PCR reactions in a thermocycler and run the following program (set the heated lid at 105°C).

Step	Temperature	Time
1	98ºC	30 seconds
2	98ºC	15 seconds
3	65ºC	5 minutes
4	Repeat steps 2 to 3 (34 times)	
5	4ºC	Hold

10. After amplification, briefly spin down plate.
11. Immediately proceed to the "Second-Round PCR" section.

B. Second-Round PCR

For each of the Pool 1 and Pool 2 plates prepared above, PCR products from the first-round PCR reactions are re-amplified using barcoded M13 primers to generate asymmetric barcoded amplicons.

1. Prepare a 96-well plate containing the desired asymmetric pairs of barcoded M13 primers.
   a. Into a clean 96-well plate, add aliquots of the appropriate forward and reverse M13 barcoded primer solutions into the corresponding wells. See **Appendix 1** for an example of an asymmetric barcode plate map for 48 samples.
   b. In each plate well, prepare 12 µL of a M13 forward and reverse barcoded primer mixture as follows:
      - Add 6 µL of the desired 3 µM M13 forward primer solution
      - Add 6 µL of the desired 3 µM M13 reverse primer solution

      Note: If desired, additional quantities of the M13 forward and reverse barcoded primer mixture can be prepared and stored at -20°C for future use.
   c. Slowly aspirate and dispense several times to mix contents. Quick-spin the 96-well plate.
2. Thaw the KAPA HiFi HotStart ReadyMix for 10 minutes.
3. In a 2.0 mL DNA LoBind tube, prepare the Round 2 PCR Master Mix as shown in the table below.

Reagent	Stock Conc.	1X Rxn	N Rxn*	48 Rxn*	✓	Notes
KAPA HiFi HotStart ReadyMix	2X	12.5 µL	N X 12.5 X 1.2	720.0 µL		
Nuclease Free Water		4.5 µL	N X 4.5 X 1.2	259.2 µL		
Total Volume		**17.0 µL**		**979.2 µL**		

* Note that indicated volumes include a 20% overage

4. Slowly aspirate and dispense several times to mix contents. Quick spin the tube.
5. Label two 96-well PCR plates. Label one plate as "**R2 Pool 1**" and the other plate as "**R2 Pool 2**". Add **17 µL of "**Round 2 PCR Master Mix**" to each well in the R2 Pool 1 plate and R2 Pool 2 plate.**
6. Using a multichannel pipette, transfer **5 µL** of the appropriate M13 forward and reverse barcoded primer mixture to each well in the R2 Pool 1 plate and R2 Pool 2 plate.
7. Add first-round PCR products to each well of both 96 well plates.
   a. Using a multichannel pipette, transfer **3 µL** of 1st round PCR products from the R1 Pool 1 plate to the appropriate wells of the plate labeled "**R2 Pool 1**". The total reaction volume in each well is 25.0 µL.
   b. Using a multichannel pipette, transfer **3 µL** of 1st round PCR products from the R1 Pool 2 plate to the appropriate wells of the plate labeled "**R2 Pool 2"**. The total reaction volume in each well is 25.0 µL.

8. Slowly aspirate and dispense several times to mix contents. Quick-spin the 96-well plates.
9. Place in a thermocycler and run the following program (lid 105°C).

Step	Temperature	Time
1	98ºC	3 minutes
2	98ºC	20 seconds
3	60ºC	15 seconds
4	72ºC	1 minute
5	Repeat steps 2 to 4 (2 times)	-
6	98ºC	20 seconds
7	65ºC	15 seconds
8	72ºC	1 minute
9	Repeat steps 6 to 8 (20 times)	-
10	72ºC	5 minutes
11	4ºC	Hold

10. After amplification, briefly spin down plate.
11. Immediately proceed to the “Sample Quantitation and Sample Normalization” section.

Sample Pooling for Library Construction

To obtain equal representation of each amplicon in the data, it is important to pool the barcoded samples in equimolar concentrations**.**

1. Quantify each sample using a fluorometric-based dsDNA assay method such as Qubit (Thermo Fisher Scientific) or SpectraMax (Molecular Devices).
2. Normalize each sample to 10 nM DNA concentration.
3. Transfer **2.0 µL** of each normalized sample from R2 Pool 1 and R2 Pool 2 into a 2.0 mL DNA LoBind tube. The total volume should be 192 µL (where 96 µL of sample is transferred from R2 Pool 1 and 96 µL is transferred from R2 Pool 2) if multiplexing 48 samples.
4. Proceed to AMPure PB bead purification.

STEP	✓	Purification with AMPure PB Beads	Notes
1		If the total pooled volume is <100 µL, bring the pooled round 2 PCR products to a total volume of 100 µL with 1X Elution Buffer (EB) before proceeding with AMPure PB bead purification. If the total pooled volume is ≥100 µL, do not add additional EB buffer. Add **0.60X** of resuspended, room-temperature AMPure PB beads to the total volume of pooled round 2 PCR products. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the samples on a bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (2.0 mL for a 2.0 mL DNA LoBind tube) – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **50 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml DNA LoBind tube. – Discard the beads.	
13		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.	

SMRTbell Library Construction

The amount of total pooled (barcoded) amplicon DNA required for SMRTbell library construction is 500-1000 ng. For >1000 ng input DNA, scale reactions accordingly.

DNA Damage Repair

1. Prepare the following reaction.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Pooled and Purified PCR Product		47.0 µL		
NAD		1.0 µL		
DNA Damage Repair Mix v2		2.0 µL		
1X Elution Buffer		Up 57.0 µL		
Total Volume		57.0 µL		

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick spin in a microfuge.
4. Incubate at 37°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

End-Repair/A-tailing

1. Prepare the following reaction.

Reagent (Reaction Mix 2)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 1		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick spin in a microfuge.
4. Incubate at 20°C for 30 minutes.
5. Incubate at 65°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

Adapter Ligation

1. Prepare the following reaction, adding the components below in the order listed.

Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

1. Pipette mix 10 times. It is important to mix well.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 20°C for 60 minutes, then return the reaction to 4°C.
4. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.
5. Proceed to the next step.

Nuclease Treatment of SMRTbell Libraries

Use the following table to set up a reaction to remove damaged SMRTbell templates after the adapter ligation step. Enzymes A through D are found in the SMRTbell Enzyme Cleanup Kit.

1. To the remove damaged SMRTbell templates, prepare a Nuclease Treatment Master Mix:

Reagent	Tube Cap Color	Volume	✓	Notes
Ligated SMRTbell library		97.0 µL		
Enzyme A		4.0 µL		
Enzyme B		1.0 µL		
Enzyme C		1.0 µL		
Enzyme D		2.0 µL		
Total Volume		105.0 µL		

2. Mix the reaction well by pipetting up and down 10 times. It is important to mix well.
3. Incubate at 37°C for 1 hour, then return the reaction to 4°C.
4. Proceed immediately to “Purification of SMRTbell Templates.”

Purification of SMRTbell Templates

STEP	✓	First AMPure PB Bead Purification	Notes
1		Add **63 µL (0.60X)** of resuspended, room-temperature AMPure PB beads to the 105 µL. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the samples on a bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for a 1.5 mL DNA LoBind tube) – Slowly dispense the 80% ethanol against the side of the tube opposite the beads – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **100 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml DNA LoBind tube. – Discard the beads.	
13		Proceed to next step, “Second AMPure PB Bead Purification”.	

STEP	✓	Second AMPure PB Bead Purification	Notes
1		Add **60 µL (0.60X)** of resuspended, room-temperature AMPure PB beads to the 100 µL. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the samples on a bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for a 1.5 mL DNA LoBind tube) – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **20 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml DNA LoBind tube. – Discard the beads.	
13		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
14		Actual recovered DNA SMRTbell concentration (ng/µl): _____ Total recovered DNA SMRTbell amount (ng): _____	

Sequencing Preparation

Sample Setup Conditions	Sequel II and Sequel IIe Systems
Sequencing Primer	Sequencing Primer v4
Primer to Template Ratio	20:1
Polymerase to Template Ratio	10:1
Binding Kit	Sequel II Binding Kit 2.1
Binding Time	1 hr
Sample Complex Cleanup Method	AMPure PB Beads
AMPure Cleanup Anticipated Yield	35%

Sequencing Conditions	Sequel II and Sequel IIe Systems
Sequencing Kit	Sequel II Sequencing Plate 2.0
Recommended On-Plate Loading Concentration	100-160 pM
Movie Collection Time	15 hrs
Pre-extension Time	1 hr

Sequencing Data Analysis

For step-by-step instructions for analysis of SARS-CoV-2 SMRT Sequencing data, please go here.

Appendix 1 – Example Asymmetric Barcode Plate Map for Primer Preparation

Example of an Asymmetric Barcode Plate Map for processing 48 samples:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1001-1049	1001-1050	1001-1051	1001-1052	1001-1053	1001-1054						
B	1002-1049	1002-1050	1002-1051	1002-1052	1002-1053	1002-1054						
C	1003-1049	1003-1050	1003-1051	1003-1052	1003-1053	1003-1054						
D	1004-1049	1004-1050	1004-1051	1004-1052	1004-1053	1004-1054						
E	1005-1049	1005-1050	1005-1051	1005-1052	1005-1053	1005-1054						
F	1006-1049	1006-1050	1006-1051	1006-1052	1006-1053	1006-1054						
G	1007-1049	1007-1050	1007-1051	1007-1052	1007-1053	1007-1054						
H	1008-1049	1008-1050	1008-1051	1008-1052	1008-1053	1008-1054						

Revision History (Description)	Version	Date
Customer Collaboration	01	February 21, 2021
Customer Collaboration – corrected 10nM to 10mM for dNTPs in First-Round PCR on page 10.	02	March 1, 2021

The procedure described herein may not have been validated by Pacific Biosciences and is provided as-is and without any warranty. Use of this procedure is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio Systems. If this procedure is to be used in a production environment, it is the responsibility of the end user to perform the required validation.