Customer Collaboration - PacBio® Compatible CDC Protocols for SARS-CoV-2 Sequencing

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These protocols were developed, tuned and validated by the Viral Discovery laboratory at CDC/NCIRD, where they were used to generate the first 16 SARS-CoV-2 genome sequences from the United States.

These approaches were originally developed for Sanger sequencing, but products from the Singleplex PCR method have also been pooled to sequence multiple patient samples at once on the PacBio Sequel System at the CDC. For clarity, we have copied only the sections relevant for PacBio sequencing, then added additional information on PacBio barcoding options for multiplexing (*See CDC SARS-CoV-2 Primers with PacBio Barcoding Options*).

If you have your own barcoding system you prefer to use or have amplicons that are already barcoded, those will work equally well on PacBio systems. Complete information on all CDC protocols for SARS-CoV-2 sequencing can be found here.

Please note that none of the protocols below have been tested at PacBio, and we make no claims as to their reproducibility. If you have questions about the protocols, please reach out to support@pacb.com and we will be happy to assist you.

Singleplex PCR protocol with ~900 bp amplicons

Multiplex PCR protocol with ~300 bp amplicons

Appendix A – AMPure PB Bead Clean Up

PacBio barcoding options and protocols

Singleplex PCR – 38 Amplicons, ~900 bp Protocol Notes

With this protocol, the shortest amplicon is 280 bp, the median is 900 bp, and the maximum is 1030 bp. To complete this protocol, 185 µL of extracted template is needed. For samples between Ct 27 and 35, two rounds of nested RT-PCR are recommended; for samples up to Ct 27, one round of RT-PCR is recommended. The resulting PCR products can be pooled for sequencing on the PacBio Sequel or Sequel II Systems, depending on the number of samples. See *CDC SARS-CoV-2 Primers with PacBio Barcoding Options* for primer lists.

Required Materials

Item	Vendor	Catalog number
Superscript III one-step RT-PCR with Platinum Taq High Fidelity DNA polymerase	Thermo (Invitrogen)	12574035
Protector RNase inhibitor	Sigma Aldrich (Roche)	33354020
Superscript III one-step RT-PCR with Platinum Taq High Fidelity DNA polymerase	Thermo (Invitrogen)	12574035
Nuclease Free water		
50 µM Primers		

Procedure

First round of RT-PCR

Prepare the first-round master mix as below. Please note, the protocol is generic as all 38 primer pairs require the same master mix (see companion excel sheet, 'Singleplex Primers'). For each SARS-CoV-2 sample to be sequenced, 38 individual PCR reactions are required.

Component	Volume(µL)
Water	1.75
2x Buffer (2.4mM MgSO4)	12.5
5mM MgSO4	4.5
50uM Primer For	0.25
50uM Primer Rev	0.25
RNase Inhib. 40U/µL	0.25
SSIII / Platinum Taq high fidelity	0.5
Pre-mix	20
Template (RNA)	5
Total	25

1. Add 5µL of RNA template to each of the 38 PCR reactions. Spin tubes/plates down and proceed to PCR.
2. Perform first round PCR with the cycling parameters as below. 60°C 1min, decrease 0.5°/sec, 94°C, 2 min; 40 cycles of 94°C 15 seconds, 55°C 15 seconds, 72°C 60 seconds; 72°C 7 minutes, 12°C

Second round of semi-nested or nested PCR

1. After first round RT-PCR is complete, prepare the master mix for 2nd round of semi-nested or nested PCR as below. Please note, the protocol is generic as all 38 second round primer pairs require the same master mix. Primer information is located in the companion excel spreadsheet. For the 2nd round of semi-nested- or nested PCR, there are 38 individual PCR reactions for each sample to be sequenced.

Component	Volume(µL)
Water	5.75
2× GCBuffer I	12.5
dNTP Mixture (2.5 mM each)	4
50uM Primer For	0.25
50uM Primer Rev	0.25
TaKaRa LA Taq™ (5 units/µl)	0.25
Pre-mix	23
Template (1R product)	2
Total	25

2. Add 2 µL of the corresponding first round PCR product to the second round PCR master mix. Spin tubes/plates down and proceed to PCR.
3. Perform second round PCR with the cycling parameters as below. 94°C, 3 min; 40 cycles of 94°C 15 seconds, 55°C 15 seconds, 72 °C 60 seconds; 72 °C 7 minutes, 12 °C
4. Following the completion of second round PCR, run 3 µL of all 38 PCR reactions on 1% agarose gels or fragment analyzer to check for amplification.

If using the barcoded adaptor approach, proceed to library preparation in the PacBio protocol described below.

If using the M13 universal barcoded primer approach, proceed to the 'Second-Round PCR Using Barcoded Universal Primers' step in the PacBio protocol described below.

Multiplex PCR – 109 Amplicons, ~300-700 bp Protocol Notes

This protocol uses 10 µL of template for each sample. The pooled, multiplexed PCR products can be followed with PacBio sequencing on either the Sequel or Sequel II system, depending on the number of samples. With this protocol, the shortest amplicon is 360 bp, the median is 510 bp and the maximum length is 670 bp.
The CDC has been able to sequence full genomes reliably under Ct 30, and depending on the sample, up to Ct 33. See *CDC SARS-CoV-2 Primers with PacBio Barcoding Options* for primer lists.

This protocol was adapted from Quick J et al. *Nat Protoc*. 2017 Jun;12(6):1261-1276.

Required Materials

Item	Vendor	Catalog number
Thermo Fisher (Invitrogen)	SuperScript IV 1st strand synthesis system	18091200
NEB	NEB Next Q5 Hot Start HiFi PCR Master Mix	M0543L
PacBio	AMPure PB Kit	100-265-900
	Nuclease free water	
	Primers	

Procedure

Generate Primer Pools

1. Prepare primers as 50 µM primer stocks.
2. Add an equal volume of each 50 µM primer stock to six 1.5mL Eppendorf tubes labeled as pool 1, 2, 3, 4, 5, and 6. Primers for each pool are listed in the companion excel spreadsheet.
3. Prepare 10 µM working concentration by diluting each pool 1:5 with nuclease free water.

First-strand Synthesis

1. Mix the following components.

Component	Volume(µL)
RNA (template)	10
Random primer 25uM	2
dNTPs	1
Total	13

2. Denature the template-primer-dNTP mix at 65°C for 5 minutes.
3. Place on ice for 5 minutes.
4. Add the following components to the template-primer-dNTP mix:

Component	Volume(µL)
5x SSIV buffer	4
0.1 M DTT	1
RNAse inhibitor	1
SSIV RT (200 units/µL)	1
Total	20

5. Incubate in a thermal cycler at the following temperatures: 25°C 10 minutes, 50°C for 10 minutes, 85°C for 10 minutes, hold at 4°C.
6. Spin down. Can be stored at -20°C
7. Add 1 µL RNase H and incubate at 37°C for 20 minutes

Multiplex PCR

1. Mix the following components in 6 wells of a PCR plate or strip tube.

Component	Volume(µL)
NEBNext Q5 Hot Start HiFi PCR Master Mix	15
PCR grade water	10.2
Primer pool 1, 2, 3, 4, 5, or 6 (10uM)	1.8
Total	27

2. Add 3 µL of cDNA from above to each tube.
3. Run the following PCR program: 98°C 30 seconds, 40 cycles of 98°C 15 seconds, 65°C 5 minutes. Note: fewer cycles may be used, but 40 cycles are used to maximize detection of lower-titer samples.
4. Optional: Run a 2% agarose gel for each multiplexed PCR reaction pool 1, 2, 3, 4, 5, and 6 to check for specific bands of the correct size (0.4-0.6 kb).
5. Pool 20 µL from each of 6 tubes of multiplexed PCR reactions in a 0.3 mL tube in a PCR strip or a well in PCR plate (the total volume is 120 µL).
6. Add 1X ratio (120 µL) of AMPure PB beads to the PCR product pools.
7. Purify according to standard AMPure PB protocol (see Appendix A).
8. Elute in 80 µL water.
9. Quantitate 1 µL of cleaned PCR products using Qubit dsDNA HS kit.
10. Optional: Run a 2% agarose gel and load 3 µL of cleaned PCR products to check for specific bands of the correct size (0.4-0.6 kb).

If using the barcoded adaptor approach, proceed to library preparation in the PacBio protocol described below.

If using the M13 universal barcoded primer approach, proceed to the ‘Second-Round PCR Using Barcoded Universal Primers’ step in the PacBio protocol described below.

Barcoding for PacBio sequencing

Option 1: Use your own barcodes

Since PacBio produces highly accurate reads, any barcoding system you decide to implement will work well for multiplexing samples on our Sequel or Sequel II Systems. Once you have barcoded amplicons, proceed to SMRT bell library preparation, beginning at 'Sample Pooling' on page 7 in the protocol below.

Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplexing Amplicons

Option 2: Barcoded adaptor approach for low multiplexing

This approach has been used successfully at the CDC to pool up to 16 samples for sequencing on the PacBio Sequel System using our 8A / 8B barcoded overhang adaptor kits. It can also be scaled up to 96-plex by ordering additional barcoded adaptor sequences that can be ordered from your preferred oligo provider. For this approach, use the standard CDC primers (See *CDC SARS-CoV-2 Primers with PacBio Barcoding Options*) to generate amplicons.

For each sample, pool either the single-sample products the 38 individual PCR reactions of the Singleplex protocol, or the 6 multiplex PCR reactions from the Multiplex protocol, then proceed with our Barcoded Overhang Adapter protocol below.

Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons

Option 3: Barcoded Universal Primers with PacBio-supplied 96 Barcoded F/R Universal Primers Plate for higher multiplexing

To multiplex up to 96 samples, PacBio recommends using a barcoded universal primer approach. In this method, the CDC PCR primers from either the nested PCR step for the Singleplex protocol, or the multiplex PCR primers from the Multiplex protocol, are modified by adding M13 sequences. We have listed both the original CDC and modified primers in *CDC SARS-CoV-2 Primers with PacBio Barcoding Options*. In an additional PCR step, M13 universal primers are then added to each of the single-sample PCR reactions. All barcoded samples are then pooled before proceeding into a single library preparation.

Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplexing Amplicons

Option 4: Barcoded Universal Primers with self-ordered primers for very high multiplexing

This approach is like the one above but uses an asymmetric barcoding strategy to enable multiplexing of up to 1,024 samples on one SMRT Cell. We do not supply these primers in a plated format at this time, but the protocols below specify the barcoded M13 primers you can order from your preferred oligonucleotide provider.

Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing

Appendix A – AMPure® PB Bead Kit Clean-Up

This covers the basic clean-up steps of bead clean up. Make sure to check the specific protocol for the ratio of beads to use. Depending on the number of samples, the AMPure PB bead clean-up takes about 30-40 minutes.

Required Materials

Item	Vendor	Catalog number
PacBio	AMPure PB Kit	100-265-900
	10mM Tris-HCl pH 8.0	

1. Allow AMPure PB beads to warm to room temperature for at least 30 minutes before using.
2. Vortex AMPure PB beads to re-suspend.
3. Add appropriate ratio of re-suspended AMPure PB beads to the ligation reaction. Mix well by pipetting up and down at least 10 times.
4. Incubate for 5 minutes at room temperature.
5. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
6. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (do not discard beads).
7. Add 200 µL of freshly prepared 80% Ethanol to the tube/plate while in the magnetic stand.
8. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.
9. Add another 200 µL of freshly prepared 80% Ethanol to the tube/plate while in the magnetic stand.
10. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.
11. Air dry the beads for 2 minutes while the tube/plate is on the magnetic stand and with the lid(s) open.

Caution: Do not over dry the beads. This may result in lower recovery of DNA target.

12. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding appropriate volume of 10mM Tris-HCl or water.
13. Mix well by pipetting up and down or on a vortex mixer. Incubate for 2 minutes at room temperature. If necessary, quickly spin the sample to collect liquid from the sides of the tube or plate wells before placing on the magnetic stand.
14. Place the tube/plate on the magnetic stand.
15. After the solution is clear (about 5 minutes), transfer to a new tube.