PACIFIC BIOSCIENCES®

# PacBio HiFiViral Workflow Overview: Multiplexed Amplicon Library Preparation for Full-Viral Genome Sequencing of SARS-CoV-2

*Sequel II System ICS v9.0 / Sequel II Chemistry 2.0 / SMRT Link v9.0*

*Sequel IIe System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0*



PN 102-084-800 Version 2021-02-25-A (February 2021)

# Multiplexed Amplicon Library Preparation for Full-Viral Genome Sequencing of SARS-CoV-2

1. PacBio HiFiViral for SARS-CoV-2 Workflow Overview
2. Multiplexed SARS-CoV-2 Amplicon Library Preparation Using PacBio Barcoded M13 Primers
3. Multiplexed SARS-CoV-2 Amplicon Library Sequencing Workflow Recommendations
4. Multiplexed SARS-CoV-2 Amplicon Data Analysis Recommendations
5. Technical Documentation & Applications Support Resources

**Appendix:** RNA Isolation Kit Options for Full-Viral Genome Sequencing of SARS-CoV-2

# SARS-CoV-2 FULL-VIRAL GENOME SEQUENCING: HOW TO GET STARTED

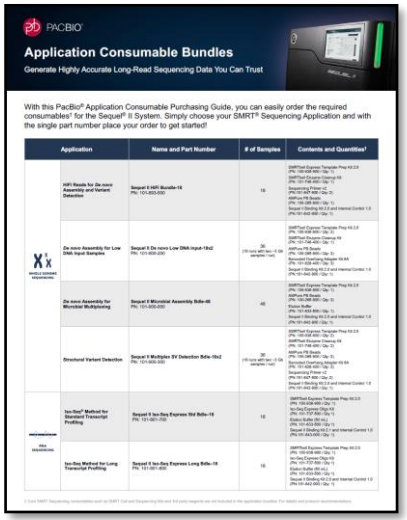
**Application-Specific Resources Website** → **Application-Specific Procedure & Checklist** → **Application Consumable Bundle Purchasing Guide** → **Library Construction, Sequencing & Analysis**


[PacBio COVID-19 Sequencing Tools and Resources Website]

Summary overview of application-specific sample preparation and data analysis workflow recommendations


[Customer Collaboration – PacBio HiFiViral for SARSCoV-2 Workflow: High-Throughput Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2] (102-075-000 / 102-082-500)

Technical documentation containing sample library construction and sequencing preparation protocol details




[PacBio Application Consumable Bundle Purchasing Guide] (PG100-082620)

Purchasing Guide enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II and IIe Systems.*

## cDNA Synthesis & Multiplexed PCR Amplicon Generation

Amplify and asymmetrically barcode SARS-CoV-2 samples for multiplexing in a single library using barcoded M13 Primers

Low-Throughput and High-Throughput HiFiViral sample prep workflows available

## Library Construction (SMRTbell Express TPK 2.0)

## HiFi Sequencing (Sequel II and IIe Systems)

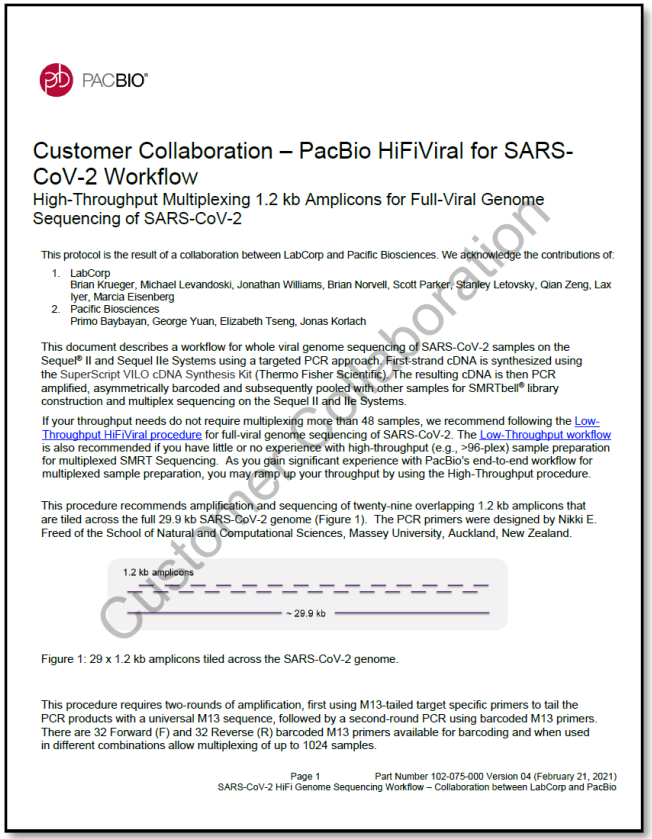
Recommend pooling up to 900 SARS-CoV-2 samples per SMRT Cell 8M

## Data Analysis

Perform assembly or variant calling with HiFi data using Coronavirus (SARS-Cov-2) sequencing analysis (CoSA) tools on GitHub

* Note: For SARS-CoV-2 amplicon sequencing, users can choose the 'Amplicons for <3kb with Barcoded Primers' consumables bundle (101-901-000) and order other required kit products separately – refer to the **HiFiViral protocol** for specific details.

# Detailed, End-to-End PacBio Protocol for SARS-CoV-2 Full-Viral Genome Sequencing



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

High-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
   Brian Krueger, Michael Levandoski, Jonathan Williams, Brian Norvell, Scott Parker, Stanley Letovsky, Qian Zeng, Lax Iyer, Marcia Eisenberg
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   Primo Baybayan, George Yuan, Elizabeth Tseng, Jonas Korlach

This document describes a workflow for whole viral genome sequencing of SARS-CoV-2 samples on the Sequel® II and Sequel IIe Systems using a targeted PCR approach. 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.

If your throughput needs do not require multiplexing more than 48 samples, we recommend following the Low-Throughput HiFiViral procedure for full-viral genome sequencing of SARS-CoV-2. The Low-Throughput workflow is also recommended if you have little or no experience with high-throughput (e.g., >96-plex) sample preparation for multiplexed SMRT Sequencing. As you gain significant experience with PacBio's end-to-end workflow for multiplexed sample preparation, you may ramp up your throughput by using the High-Throughput procedure.

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.

This procedure requires two-rounds of 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. There are 32 Forward (F) and 32 Reverse (R) barcoded M13 primers available for barcoding and when used in different combinations allow multiplexing of up to 1024 samples.





[PacBio COVID-19 Sequencing Tools and Resources]

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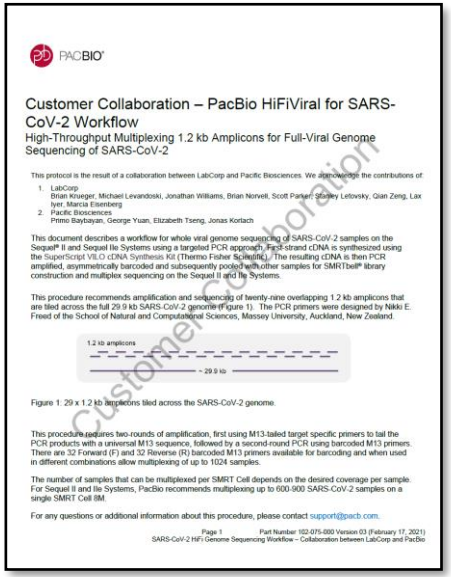
## Highly accurate long reads offer critical advantages for surveillance

- Easier Primer Balancing
- Fewer Amplicon Dropouts
- Catch All Variants
- Flexible Batch Size
- Cost Effective

# PacBio HiFiViral for SARS-CoV-2 Workflow Overview

# MULTIPLEXED SARS-CoV-2 AMPLICON LIBRARY PREPARATION PROCEDURE DESCRIPTION

- Customer Collaboration – PacBio HiFiViral for SARS-CoV-2 Workflow documents (PN 102-082-500 / 102-075-000) describe a procedure for whole viral genome sequencing of asymmetrically barcoded SARS-CoV-2 samples on the Sequel II and IIe Systems using a 2-step targeted PCR amplicon approach.
- The procedure involves amplification and sequencing of twenty-nine overlapping 1.2 kb amplicons that are tiled across the full 29.9 kb SARS-CoV-2 genome
- This library preparation workflow is amenable to automation and was developed in collaboration with Laboratory Corporation of America
- Two versions of the HiFiViral protocol are available:
  1. **Low-throughput sample preparation workflow** (PN 102-082-500) supports multiplexed library construction for up to 48 SARS-CoV-2 samples at a time
  2. **High-throughput sample preparation workflow** (PN 102-075-000) supports multiplexed library construction for up to 900 SARS-CoV-2 samples at a time
- PacBio recommends pooling **up to 900 SARS-CoV-2 samples for sequencing on a single SMRT Cell 8M.**


PACBIO

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

High-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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This document describes a workflow for whole viral genome sequencing of SARS-CoV-2 samples on the Sequel® II and Sequel IIe Systems using a targeted PCR approach. 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.

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1.2 kb amplicons

~ 29.9 kb

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

This procedure requires two-rounds of 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. There are 32 Forward (F) and 32 Reverse (R) barcoded M13 primers available for barcoding and when used in different combinations allow multiplexing of up to 1024 samples.

The number of samples that can be multiplexed per SMRT Cell depends on the desired coverage per sample. For Sequel II and IIe Systems, PacBio recommends multiplexing up to 600-900 SARS-CoV-2 samples on a single SMRT Cell 8M.

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

Page 1 Part Number 102-075-000 Version 03 (February 17, 2021)
SARS-CoV-2 HiFi Genome Sequencing Workflow – Collaboration between LabCorp and PacBio

Customer Collaboration

*PacBio COVID-19 Sequencing Tools and Resources*

RESEARCH FOCUS

## MICROBIOLOGY AND INFECTIOUS DISEASE

### PacBio COVID-19 Sequencing Tools and Resources

# PACBIO HIFIVIRAL FOR SARS-CoV-2 WORKFLOW OVERVIEW

**cDNA Synthesis & Multiplexed PCR Amplicon Generation (~8 hours)**

Amplify and asymmetrically barcode SARS-CoV-2 samples for multiplexing in a single library using PacBio-Barcoded M13 Primers

Low-Throughput (≤48-plex) and High-Throughput (up to 900-plex) sample preparation workflows available

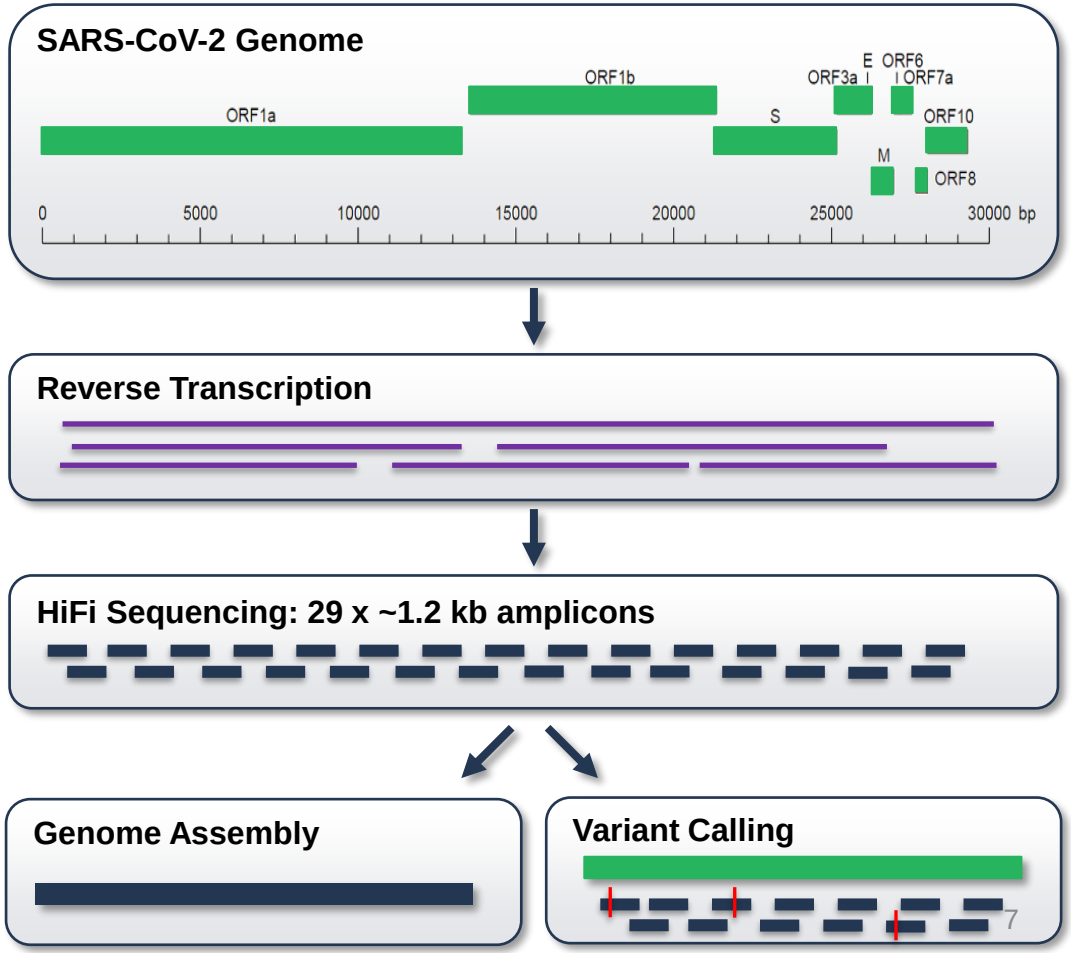
**Library Construction (SMRTbell Express TPK 2.0) (~6 hours)**

**HiFi Sequencing (Sequel II and IIe Systems) (15-hour collection time)**

Recommend pooling up to 900 SARS-CoV-2 samples per SMRT Cell 8M

**Data Analysis**

Perform assembly or variant calling with HiFi data using Coronavirus (SARS-Cov-2) sequencing analysis (CoSA) tools on GitHub



# HIFI SEQUENCING HAS HIGHER SUCCESS IN DELIVERING COMPLETE VIRAL GENOMES

## *Easier Primer Balancing:*

1.2 kb amplicons optimizes for even genome coverage by simplifying the PCR reaction while preserving performance with degraded samples

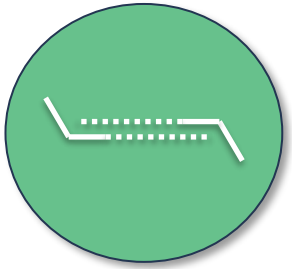
## *Fewer Amplicon Dropouts:*

With fewer amplicons, primers anneal to less of the viral genome, lowering the chance that viral mutations will cause dropouts due to primer mismatches

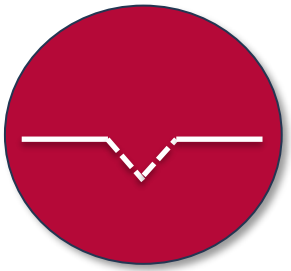
## *Catch All Variants:*

Longer, 99.9+% accurate HiFi reads detect both SNPs and indels up to 300 bp, allow phasing of variants

**Easier Primer Balancing**



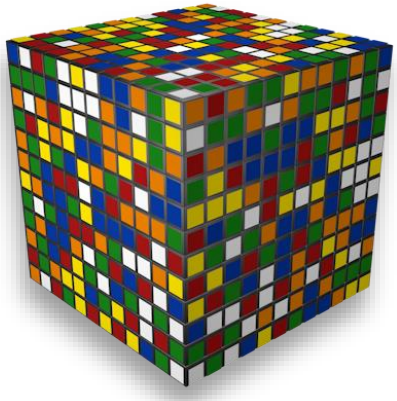
**Fewer Amplicon Dropouts**

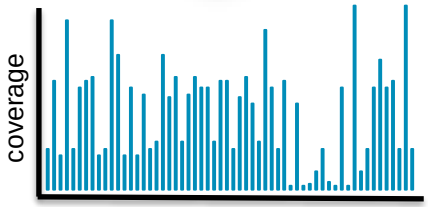


**Catch All variants**

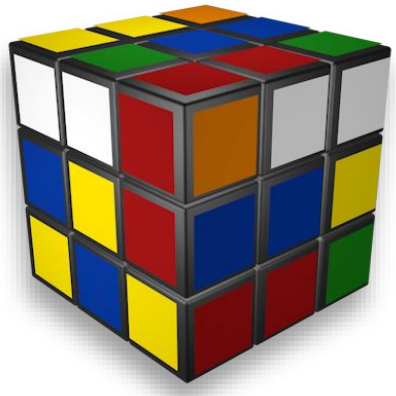
# USING FEWER REQUIRED PRIMER SETS SIMPLIFIES PCR BALANCING

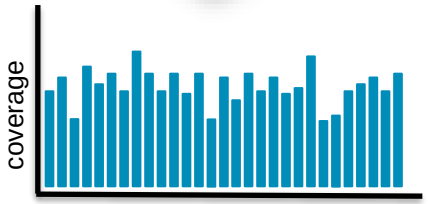
Unbalanced PCR reactions are a driver of uneven coverage, poor scale up, and missing regions






Balancing 96 or more primer sets



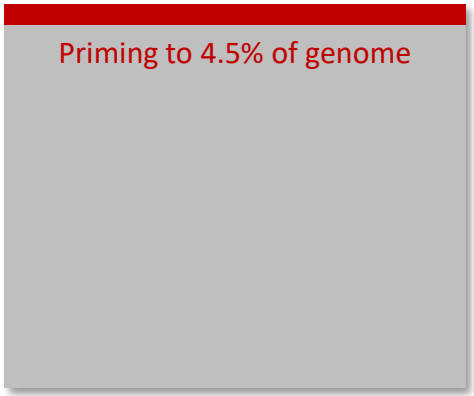



Balancing 29 primer sets for PacBio Sequencing

# USING FEWER REQUIRED PRIMER SETS LEADS TO FEWER DROPOUTS

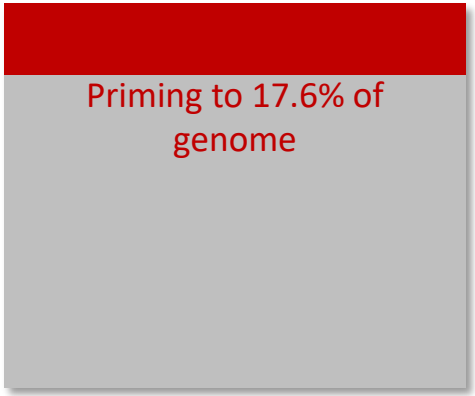
Less of the genome is covered by the primer sequences, making the assay more robust to coverage dropouts due to primer mismatches with new viral strains

**29 amplicons**



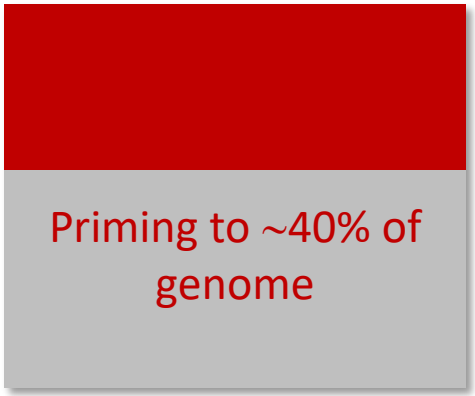

**PacBio solution**

**98 amplicons**



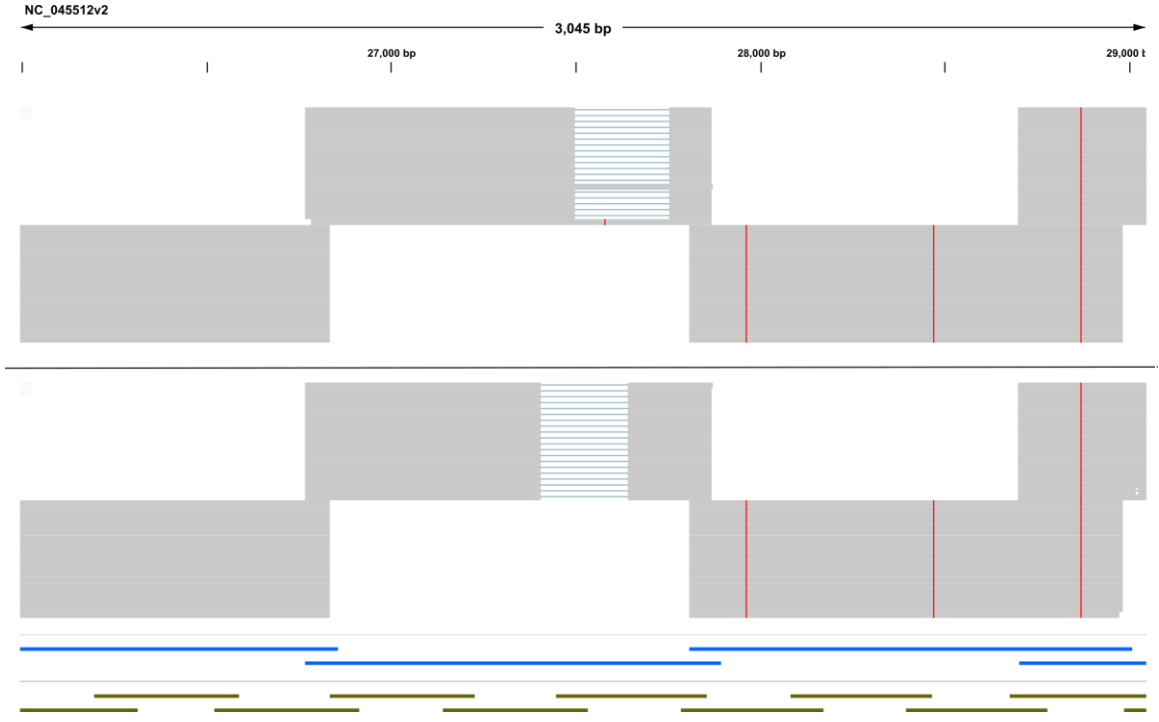

**Illumina / ONT ARTIC solution**

**237 amplicons**



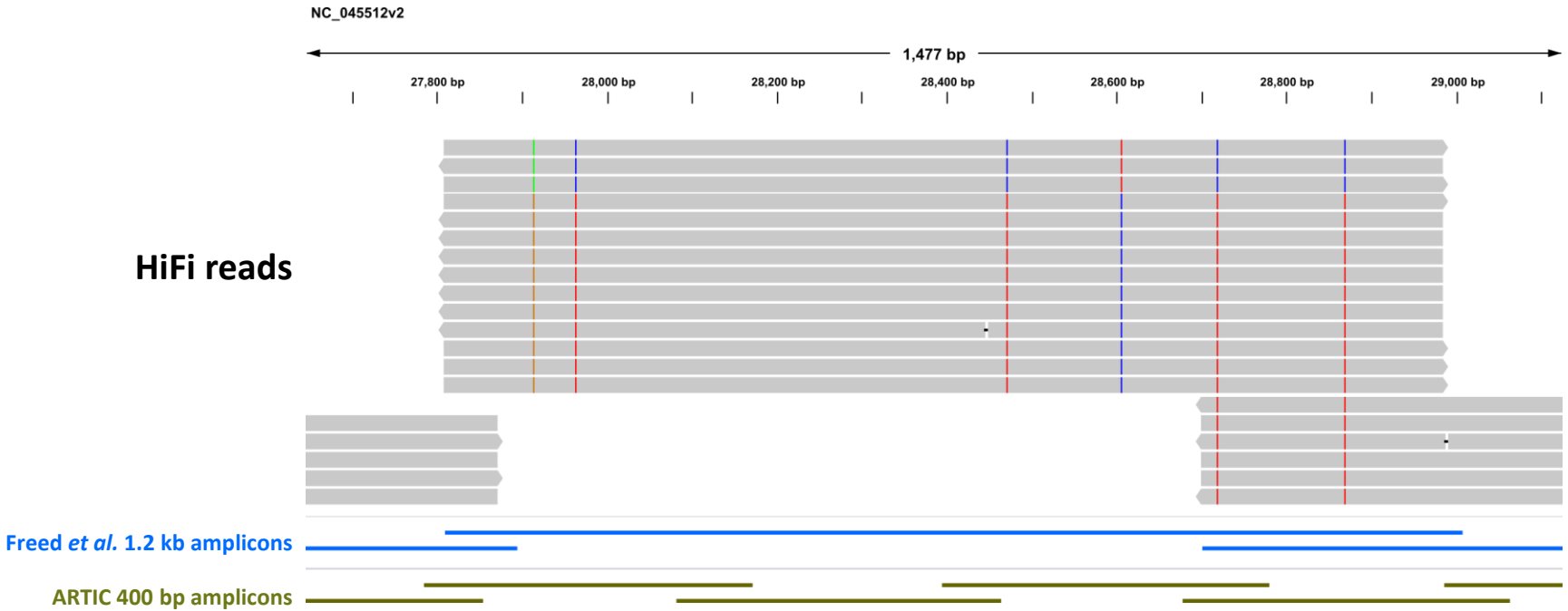

**Ion Torrent solution**

# HIFI SARS-CoV-2 SEQUENCING ENABLES DETECTION OF LARGER VARIANTS




**In collaboration with Labcorp**

# HIFI SARS-CoV-2 SEQUENCING ENABLES PHASING OF VARIANTS IN A SAMPLE CONTAINING MULTIPLE SUBTYPES




**In collaboration with Labcorp**

# PACBIO'S HiFiViral SEQUENCING WORKFLOW PROVIDES THE RIGHT SCALE AT THE RIGHT COST PER SAMPLE

## *Flexible Batch Size:*

The assay can be scaled to sequence from 48-900 samples per SMRT Cell on the Sequel IIe System

## *Cost Effective:*

Per sample costs are well within average reimbursement rates for successfully recovered, complete sequences

**Flexible Batch Size**

**Cost Effective**

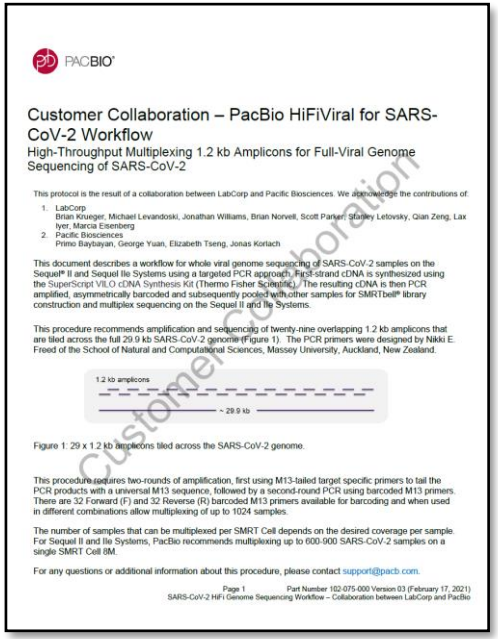
# Multiplexed SARS-CoV-2 Amplicon Library Preparation Using PacBio-Barcoded M13 Primers

# PACBIO HiFiViral FOR SARS-CoV-2 WORKFLOW PROTOCOL DOCUMENT

- Customer Collaboration – PacBio HiFiViral for SARS-CoV-2 Workflow protocol describes a procedure for amplification and sequencing of twenty-nine overlapping 1.2 kb amplicons* that are tiled across the full 29.9 kb SARS-CoV-2 genome
  - **Low-Throughput procedure** (PN 102-082-500) supports multiplexed library construction for sequencing up to 48 samples at a time
  - **High-Throughput procedure** (PN 102-075-000) supports multiplexed library construction for sequencing up to 900 samples at a time
- Following first-strand cDNA synthesis, the resulting cDNA is then amplified using a 2-step PCR method, asymmetrically barcoded and subsequently pooled with other samples for SMRTbell library construction and multiplexed sequencing on the Sequel II or IIe System.

## Protocol Document Contents

1. Link to HiFiViral for SARS-CoV-2 Oligo Ordering Sheet for ordering M13-tailed target-specific primers and barcoded M13 primers
2. Instructions for performing first-strand SARS-CoV-2 cDNA synthesis and amplification of cDNA products prior to SMRTbell library construction
3. Instructions for pooling amplified SARS-CoV-2 cDNA products and constructing SMRTbell libraries using SMRTbell Express Template Prep Kit 2.0
4. Sample setup guidance for preparing multiplexed SARS-CoV-2 amplicon SMRTbell libraries for sequencing on the Sequel II and IIe Systems


PACBIO

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

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This document describes a workflow for whole viral genome sequencing of SARS-CoV-2 samples on the Sequel® II and Sequel IIe Systems using a targeted PCR approach. 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.

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1.2 kb amplicons

~ 29.9 kb

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

This procedure requires two-rounds of 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. There are 32 Forward (F) and 32 Reverse (R) barcoded M13 primers available for barcoding and when used in different combinations allow multiplexing of up to 1024 samples.

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

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SARS-CoV-2 HiFi Genome Sequencing Workflow – Collaboration between LabCorp and PacBio

Customer Collaboration

PacBio COVID-19 Sequencing Tools and Resources

* 1.2 kb amplicon strategy for SARS-CoV-2 sequencing was adapted from Freed, N.E. et al. (2020) Biology Methods & Protocols 5(1):1 – 7

# REQUIRED MATERIALS & EQUIPMENT

| ITEM | WHERE USED | VENDOR | PART NUMBER |
|---|---|---|---|
| **cDNA Synthesis** | | | |
| **SuperScript VILO cDNA Synthesis Kit** | cDNA Preparation | Thermo Fisher Scientific | 11754250 |
| **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 |

* Oligo(dT) sequence is 5’ TTT TTT TTT TTT GTC ATT CTC CTA AG 3’ and HPLC purification is recommended

# REQUIRED MATERIALS & EQUIPMENT (CONT.)

| ITEM | WHERE USED | VENDOR | PART NUMBER |
|---|---|---|---|
| **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 |

# PACBIO HiFiViral FOR SARS-CoV-2 WORKFLOW DETAILS

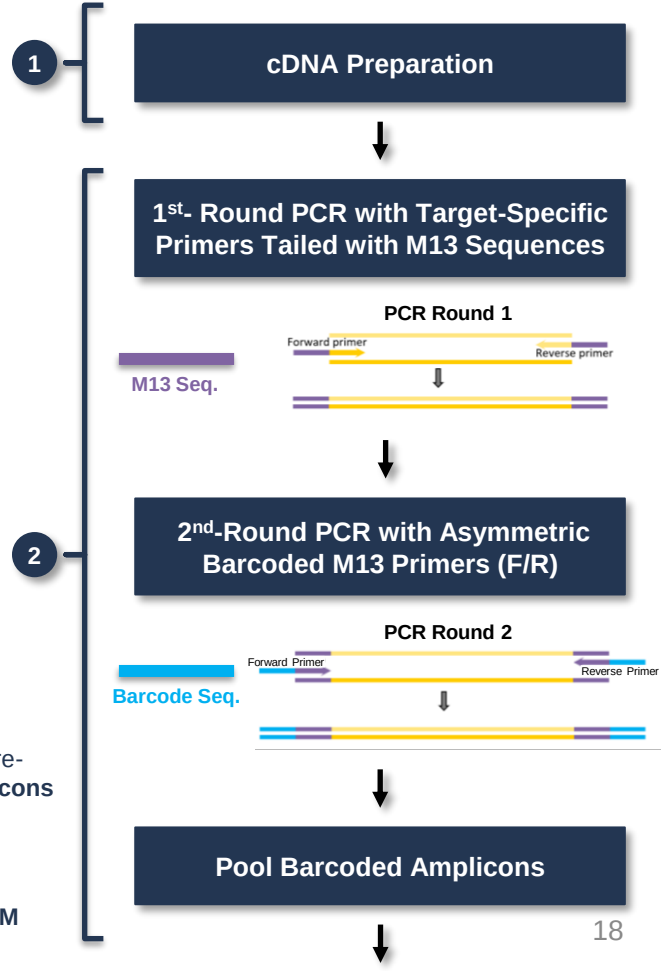
## 1. First-Strand cDNA Synthesis (~2 hrs)

- Perform 1st-strand cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit.

## 2. cDNA Amplification and Pooling (~6 hrs)

- Prepare 1st and 2nd round PCR reactions to generate overlapping barcoded 1.2 kb amplicons tiled across the full 29.9 kb SARS-CoV-2 genome


- **1st-Round PCR**
  - Perform 1st-round PCR using SARS-CoV-2 specific primers tailed with M13 sequences
  - Each sample requires that two multiplex PCR reactions using **Primer Pool 1 (15 primer pairs)** and **Primer Pool 2 (14 primer pairs)** be performed in parallel
- **2nd-Round PCR**
  - For the second-round PCR, PCR products from the 1st-round PCR reactions are re-amplified using barcoded M13 primers to generate **asymmetric barcoded amplicons**
- **Pool Barcoded Amplicons**
  - Perform pooling of PacBio-barcoded amplicon products
  - PacBio recommends pooling **up to 900 SARS-CoV-2 samples per SMRT Cell 8M**
  - Purify single, pooled amplicon sample using AMPure PB beads



# PACBIO HiFiViral FOR SARS-CoV-2 WORKFLOW DETAILS (CONT.)

### 3. SMRTbell Express TPK 2.0 Library Construction (~6 hrs)

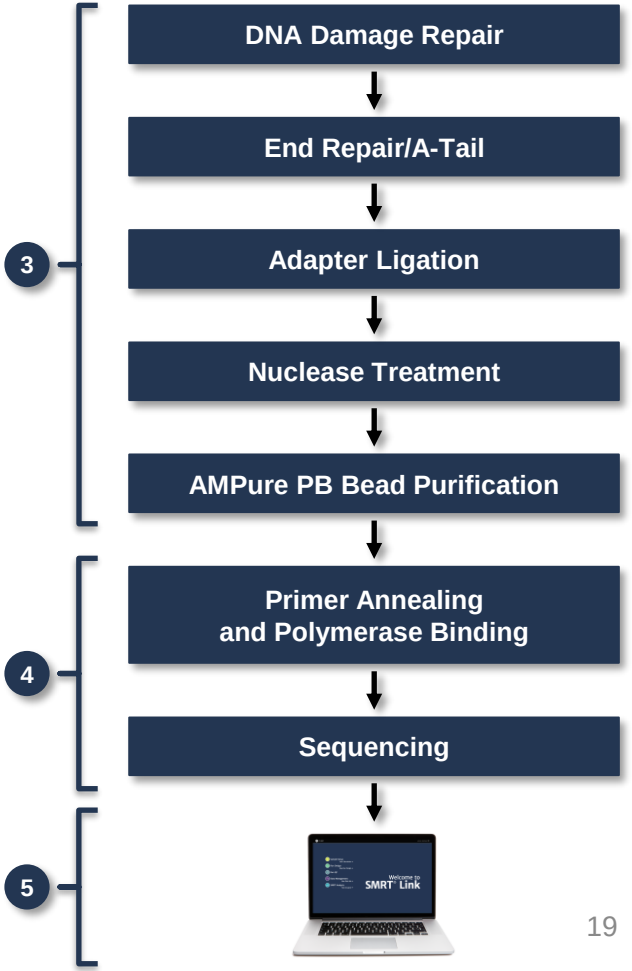
- The amount of total pooled (barcoded) amplicon DNA required for SMRTbell library construction is 500 ng – 1000 ng.
- Typical library construction yield is ≥40%

### 4. HiFi Sequencing (15-hr collection time )

- Anneal sequencing primer (1 hour), bind polymerase (1 hour), and perform complex cleanup (0.5 hours)
- Use a 15-hour movie collection time for sequencing SARS-CoV-2 samples on the Sequel II or IIe System
- Generate HiFi reads with >99.9% accuracy

### 5. Data Analysis

- Perform assembly or variant calling with HiFi data using Coronavirus (SARS-Cov-2) sequencing analysis (CoSA) tools on GitHub



# PREPARATION OF M13-TAILED SARS-CoV-2 PRIMERS FOR 1ST-ROUND PCR

- Forward and reverse SARS-CoV-2 PCR primers tailed with M13 sequences may be ordered from any oligo synthesis provider
- The HiFiViral for SARS-CoV-2 Oligo Ordering Sheet provides primer sequences (5' → 3') and recommended synthesis scale
  - Add a 5' blocker (e.g., 5AmMC6) to ensure that carryover amplicons from the 1st-Round PCR are not ligated to SMRTbell adapters during library construction. [Desalted primers are sufficient for 1st-Round PCR amplification.]
- Before use, dilute oligos with nuclease-free buffer (10mM Tris-HCl pH 7.5) to 100 µM stock concentration.
- Prepare **100 µM** stocks of **Primer Pool 1 (odd primer pairs)** and **Primer Pool 2 (even primer pairs)** by adding the appropriate volumes of F and R primers as shown in the tables below

**Primer Pool 1 (250 µL): Odd Primer Pairs**
**(15 Oligo Pairs (F/R); 30 Oligos Total)**

| 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 (200 µL): Even Primer Pairs**
**(14 Oligo Pairs (F/R); 28 Oligos Total)**

| 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 |

# PREPARATION OF PACBIO-BARCODED M13 PRIMERS FOR 2ND-ROUND PCR

- 32 forward and 32 reverse PacBio-barcoded M13 primer sequences are available for the 2nd-round PCR step to create asymmetrically barcoded SARS-CoV-2 amplicons
  - PacBio recommends multiplexing **up to 900 SARS-CoV-2 samples per SMRT Cell 8M** for HiFi sequencing
- The HiFiViral for SARS-CoV-2 Oligo Ordering Sheet provides primer sequences (5' → 3') and recommended synthesis scale
  - HPLC-purified primers are recommended for 2nd-Round PCR amplification
- 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

**EXAMPLE PLATE LAYOUT FOR RESUSPENDING 32 FORWARD AND 32 REVERSE PACBIO-BARCODED M13 PRIMER OLIGOS.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | FWD_1001 | FWD_1009 | FWD_1017 | FWD_1025 | x | x | REV_1049 | REV_1057 | REV_1065 | REV_1073 | x | x |
| B | FWD_1002 | FWD_1010 | FWD_1018 | FWD_1026 | x | x | REV_1050 | REV_1058 | REV_1066 | REV_1074 | x | x |
| C | FWD_1003 | FWD_1011 | FWD_1019 | FWD_1027 | x | x | REV_1051 | REV_1059 | REV_1067 | REV_1075 | x | x |
| D | FWD_1004 | FWD_1012 | FWD_1020 | FWD_1028 | x | x | REV_1052 | REV_1060 | REV_1068 | REV_1076 | x | x |
| E | FWD_1005 | FWD_1013 | FWD_1021 | FWD_1029 | x | x | REV_1053 | REV_1061 | REV_1069 | REV_1077 | x | x |
| F | FWD_1006 | FWD_1014 | FWD_1022 | FWD_1030 | x | x | REV_1054 | REV_1062 | REV_1070 | REV_1078 | x | x |
| G | FWD_1007 | FWD_1015 | FWD_1023 | FWD_1031 | x | x | REV_1055 | REV_1063 | REV_1071 | REV_1079 | x | x |
| H | FWD_1008 | FWD_1016 | FWD_1024 | FWD_1032 | x | x | REV_1056 | REV_1064 | REV_1072 | REV_1082 | x | x |

Columns 1-4 are M13 forward primers tailed with PacBio barcode 1001 to barcode 1032.

Columns 7-10 are M13 reverse primers tailed with PacBio barcode 1049 to barcode 1079 and barcode 1082.

# EXAMPLE ASYMMETRIC BARCODE PLATE MAPS FOR HIGH-THROUGHPUT MULTIPLEXING OF SARS-CoV-2 SAMPLES

- See **Appendix 1** in the high-throughput procedure (PN 102-075-000) for example asymmetric barcode plate maps for processing up to 10 x 96-well plates containing SARS-Cov-2 samples at a time

**Barcode Plate 1**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1049 | 1009-1049 | 1017-1049 | 1025-1049 | 1001-1050 | 1009-1050 | 1017-1050 | 1025-1050 | 1001-1051 | 1009-1051 | 1017-1051 | 1025-1051 |
| B | 1002-1049 | 1010-1049 | 1018-1049 | 1026-1049 | 1002-1050 | 1010-1050 | 1018-1050 | 1026-1050 | 1002-1051 | 1010-1051 | 1018-1051 | 1026-1051 |
| C | 1003-1049 | 1011-1049 | 1019-1049 | 1027-1049 | 1003-1050 | 1011-1050 | 1019-1050 | 1027-1050 | 1003-1051 | 1011-1051 | 1019-1051 | 1027-1051 |
| D | 1004-1049 | 1012-1049 | 1020-1049 | 1028-1049 | 1004-1050 | 1012-1050 | 1020-1050 | 1028-1050 | 1004-1051 | 1012-1051 | 1020-1051 | 1028-1051 |
| E | 1005-1049 | 1013-1049 | 1021-1049 | 1029-1049 | 1005-1050 | 1013-1050 | 1021-1050 | 1029-1050 | 1005-1051 | 1013-1051 | 1021-1051 | 1029-1051 |
| F | 1006-1049 | 1014-1049 | 1022-1049 | 1030-1049 | 1006-1050 | 1014-1050 | 1022-1050 | 1030-1050 | 1006-1051 | 1014-1051 | 1022-1051 | 1030-1051 |
| G | 1007-1049 | 1015-1049 | 1023-1049 | 1031-1049 | 1007-1050 | 1015-1050 | 1023-1050 | 1031-1050 | 1007-1051 | 1015-1051 | 1023-1051 | 1031-1051 |
| H | 1008-1049 | 1016-1049 | 1024-1049 | 1032-1049 | 1008-1050 | 1016-1050 | 1024-1050 | 1032-1050 | 1008-1051 | 1016-1051 | 1024-1051 | 1032-1051 |

**Barcode Plate 2**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1052 | 1009-1052 | 1017-1052 | 1025-1052 | 1001-1053 | 1009-1053 | 1017-1053 | 1025-1053 | 1001-1054 | 1009-1054 | 1017-1054 | 1025-1054 |
| B | 1002-1052 | 1010-1052 | 1018-1052 | 1026-1052 | 1002-1053 | 1010-1053 | 1018-1053 | 1026-1053 | 1002-1054 | 1010-1054 | 1018-1054 | 1026-1054 |
| C | 1003-1052 | 1011-1052 | 1019-1052 | 1027-1052 | 1003-1053 | 1011-1053 | 1019-1053 | 1027-1053 | 1003-1054 | 1011-1054 | 1019-1054 | 1027-1054 |
| D | 1004-1052 | 1012-1052 | 1020-1052 | 1028-1052 | 1004-1053 | 1012-1053 | 1020-1053 | 1028-1053 | 1004-1054 | 1012-1054 | 1020-1054 | 1028-1054 |
| E | 1005-1052 | 1013-1052 | 1021-1052 | 1029-1052 | 1005-1053 | 1013-1053 | 1021-1053 | 1029-1053 | 1005-1054 | 1013-1054 | 1021-1054 | 1029-1054 |
| F | 1006-1052 | 1014-1052 | 1022-1052 | 1030-1052 | 1006-1053 | 1014-1053 | 1022-1053 | 1030-1053 | 1006-1054 | 1014-1054 | 1022-1054 | 1030-1054 |
| G | 1007-1052 | 1015-1052 | 1023-1052 | 1031-1052 | 1007-1053 | 1015-1053 | 1023-1053 | 1031-1053 | 1007-1054 | 1015-1054 | 1023-1054 | 1031-1054 |
| H | 1008-1052 | 1016-1052 | 1024-1052 | 1032-1052 | 1008-1053 | 1016-1053 | 1024-1053 | 1032-1053 | 1008-1054 | 1016-1054 | 1024-1054 | 1032-1054 |

**Barcode Plate 3**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1055 | 1009-1055 | 1017-1055 | 1025-1055 | 1001-1056 | 1009-1056 | 1017-1056 | 1025-1056 | 1001-1057 | 1009-1057 | 1017-1057 | 1025-1057 |
| B | 1002-1055 | 1010-1055 | 1018-1055 | 1026-1055 | 1002-1056 | 1010-1056 | 1018-1056 | 1026-1056 | 1002-1057 | 1010-1057 | 1018-1057 | 1026-1057 |
| C | 1003-1055 | 1011-1055 | 1019-1055 | 1027-1055 | 1003-1056 | 1011-1056 | 1019-1056 | 1027-1056 | 1003-1057 | 1011-1057 | 1019-1057 | 1027-1057 |
| D | 1004-1055 | 1012-1055 | 1020-1055 | 1028-1055 | 1004-1056 | 1012-1056 | 1020-1056 | 1028-1056 | 1004-1057 | 1012-1057 | 1020-1057 | 1028-1057 |
| E | 1005-1055 | 1013-1055 | 1021-1055 | 1029-1055 | 1005-1056 | 1013-1056 | 1021-1056 | 1029-1056 | 1005-1057 | 1013-1057 | 1021-1057 | 1029-1057 |
| F | 1006-1055 | 1014-1055 | 1022-1055 | 1030-1055 | 1006-1056 | 1014-1056 | 1022-1056 | 1030-1056 | 1006-1057 | 1014-1057 | 1022-1057 | 1030-1057 |
| G | 1007-1055 | 1015-1055 | 1023-1055 | 1031-1055 | 1007-1056 | 1015-1056 | 1023-1056 | 1031-1056 | 1007-1057 | 1015-1057 | 1023-1057 | 1031-1057 |
| H | 1008-1055 | 1016-1055 | 1024-1055 | 1032-1055 | 1008-1056 | 1016-1056 | 1024-1056 | 1032-1056 | 1008-1057 | 1016-1057 | 1024-1057 | 1032-1057 |

**Barcode Plate 4**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1058 | 1009-1058 | 1017-1058 | 1025-1058 | 1001-1059 | 1009-1059 | 1017-1059 | 1025-1059 | 1001-1060 | 1009-1060 | 1017-1060 | 1025-1060 |
| B | 1002-1058 | 1010-1058 | 1018-1058 | 1026-1058 | 1002-1059 | 1010-1059 | 1018-1059 | 1026-1059 | 1002-1060 | 1010-1060 | 1018-1060 | 1026-1060 |
| C | 1003-1058 | 1011-1058 | 1019-1058 | 1027-1058 | 1003-1059 | 1011-1059 | 1019-1059 | 1027-1059 | 1003-1060 | 1011-1060 | 1019-1060 | 1027-1060 |
| D | 1004-1058 | 1012-1058 | 1020-1058 | 1028-1058 | 1004-1059 | 1012-1059 | 1020-1059 | 1028-1059 | 1004-1060 | 1012-1060 | 1020-1060 | 1028-1060 |
| E | 1005-1058 | 1013-1058 | 1021-1058 | 1029-1058 | 1005-1059 | 1013-1059 | 1021-1059 | 1029-1059 | 1005-1060 | 1013-1060 | 1021-1060 | 1029-1060 |
| F | 1006-1058 | 1014-1058 | 1022-1058 | 1030-1058 | 1006-1059 | 1014-1059 | 1022-1059 | 1030-1059 | 1006-1060 | 1014-1060 | 1022-1060 | 1030-1060 |
| G | 1007-1058 | 1015-1058 | 1023-1058 | 1031-1058 | 1007-1059 | 1015-1059 | 1023-1059 | 1031-1059 | 1007-1060 | 1015-1060 | 1023-1060 | 1031-1060 |
| H | 1008-1058 | 1016-1058 | 1024-1058 | 1032-1058 | 1008-1059 | 1016-1059 | 1024-1059 | 1032-1059 | 1008-1060 | 1016-1060 | 1024-1060 | 1032-1060 |

**Barcode Plate 5**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1061 | 1009-1061 | 1017-1061 | 1025-1061 | 1001-1062 | 1009-1062 | 1017-1062 | 1025-1062 | 1001-1063 | 1009-1063 | 1017-1063 | 1025-1063 |
| B | 1002-1061 | 1010-1061 | 1018-1061 | 1026-1061 | 1002-1062 | 1010-1062 | 1018-1062 | 1026-1062 | 1002-1063 | 1010-1063 | 1018-1063 | 1026-1063 |
| C | 1003-1061 | 1011-1061 | 1019-1061 | 1027-1061 | 1003-1062 | 1011-1062 | 1019-1062 | 1027-1062 | 1003-1063 | 1011-1063 | 1019-1063 | 1027-1063 |
| D | 1004-1061 | 1012-1061 | 1020-1061 | 1028-1061 | 1004-1062 | 1012-1062 | 1020-1062 | 1028-1062 | 1004-1063 | 1012-1063 | 1020-1063 | 1028-1063 |
| E | 1005-1061 | 1013-1061 | 1021-1061 | 1029-1061 | 1005-1062 | 1013-1062 | 1021-1062 | 1029-1062 | 1005-1063 | 1013-1063 | 1021-1063 | 1029-1063 |
| F | 1006-1061 | 1014-1061 | 1022-1061 | 1030-1061 | 1006-1062 | 1014-1062 | 1022-1062 | 1030-1062 | 1006-1063 | 1014-1063 | 1022-1063 | 1030-1063 |
| G | 1007-1061 | 1015-1061 | 1023-1061 | 1031-1061 | 1007-1062 | 1015-1062 | 1023-1062 | 1031-1062 | 1007-1063 | 1015-1063 | 1023-1063 | 1031-1063 |
| H | 1008-1061 | 1016-1061 | 1024-1061 | 1032-1061 | 1008-1062 | 1016-1062 | 1024-1062 | 1032-1062 | 1008-1063 | 1016-1063 | 1024-1063 | 1032-1063 |

**Barcode Plate 6**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1064 | 1009-1064 | 1017-1064 | 1025-1064 | 1001-1065 | 1009-1065 | 1017-1065 | 1025-1065 | 1001-1066 | 1009-1066 | 1017-1066 | 1025-1066 |
| B | 1002-1064 | 1010-1064 | 1018-1064 | 1026-1064 | 1002-1065 | 1010-1065 | 1018-1065 | 1026-1065 | 1002-1066 | 1010-1066 | 1018-1066 | 1026-1066 |
| C | 1003-1064 | 1011-1064 | 1019-1064 | 1027-1064 | 1003-1065 | 1011-1065 | 1019-1065 | 1027-1065 | 1003-1066 | 1011-1066 | 1019-1066 | 1027-1066 |
| D | 1004-1064 | 1012-1064 | 1020-1064 | 1028-1064 | 1004-1065 | 1012-1065 | 1020-1065 | 1028-1065 | 1004-1066 | 1012-1066 | 1020-1066 | 1028-1066 |
| E | 1005-1064 | 1013-1064 | 1021-1064 | 1029-1064 | 1005-1065 | 1013-1065 | 1021-1065 | 1029-1065 | 1005-1066 | 1013-1066 | 1021-1066 | 1029-1066 |
| F | 1006-1064 | 1014-1064 | 1022-1064 | 1030-1064 | 1006-1065 | 1014-1065 | 1022-1065 | 1030-1065 | 1006-1066 | 1014-1066 | 1022-1066 | 1030-1066 |
| G | 1007-1064 | 1015-1064 | 1023-1064 | 1031-1064 | 1007-1065 | 1015-1065 | 1023-1065 | 1031-1065 | 1007-1066 | 1015-1066 | 1023-1066 | 1031-1066 |
| H | 1008-1064 | 1016-1064 | 1024-1064 | 1032-1064 | 1008-1065 | 1016-1065 | 1024-1065 | 1032-1065 | 1008-1066 | 1016-1066 | 1024-1066 | 1032-1066 |

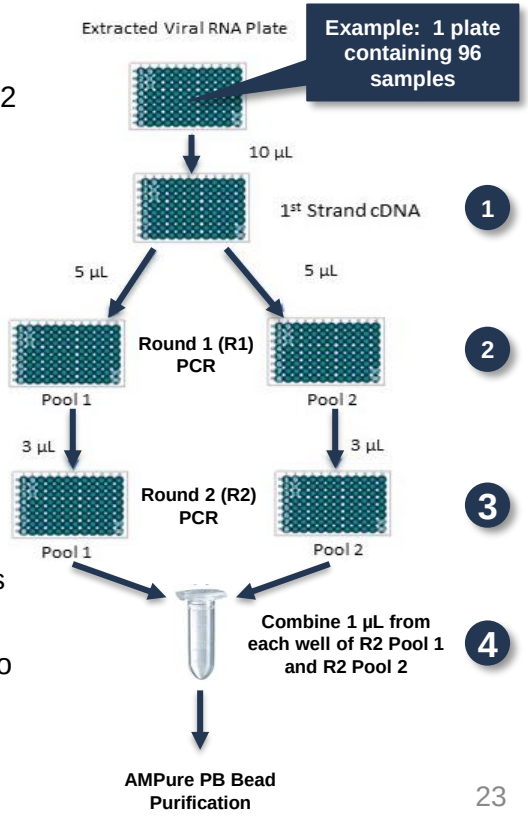
**Barcode Plate 10**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1001-1076 | 1009-1076 | 1017-1076 | 1025-1076 | 1001-1077 | 1009-1077 | 1017-1077 | 1025-1077 | 1001-1078 | 1009-1078 | 1017-1078 | 1025-1078 |
| B | 1002-1076 | 1010-1076 | 1018-1076 | 1026-1076 | 1002-1077 | 1010-1077 | 1018-1077 | 1026-1077 | 1002-1078 | 1010-1078 | 1018-1078 | 1026-1078 |
| C | 1003-1076 | 1011-1076 | 1019-1076 | 1027-1076 | 1003-1077 | 1011-1077 | 1019-1077 | 1027-1077 | 1003-1078 | 1011-1078 | 1019-1078 | 1027-1078 |
| D | 1004-1076 | 1012-1076 | 1020-1076 | 1028-1076 | 1004-1077 | 1012-1077 | 1020-1077 | 1028-1077 | 1004-1078 | 1012-1078 | 1020-1078 | 1028-1078 |
| E | 1005-1076 | 1013-1076 | 1021-1076 | 1029-1076 | 1005-1077 | 1013-1077 | 1021-1077 | 1029-1077 | 1005-1078 | 1013-1078 | 1021-1078 | 1029-1078 |
| F | 1006-1076 | 1014-1076 | 1022-1076 | 1030-1076 | 1006-1077 | 1014-1077 | 1022-1077 | 1030-1077 | 1006-1078 | 1014-1078 | 1022-1078 | 1030-1078 |
| G | 1007-1076 | 1015-1076 | 1023-1076 | 1031-1076 | 1007-1077 | 1015-1077 | 1023-1077 | 1031-1077 | 1007-1078 | 1015-1078 | 1023-1078 | 1031-1078 |
| H | 1008-1076 | 1016-1076 | 1024-1076 | 1032-1076 | 1008-1077 | 1016-1077 | 1024-1077 | 1032-1077 | 1008-1078 | 1016-1078 | 1024-1078 | 1032-1078 |

# EXAMPLE HIGH-THROUGHPUT* SAMPLE PREPARATION WORKFLOW FOR FULL-VIRAL GENOME SEQUENCING OF SARS-CoV-2 SAMPLES

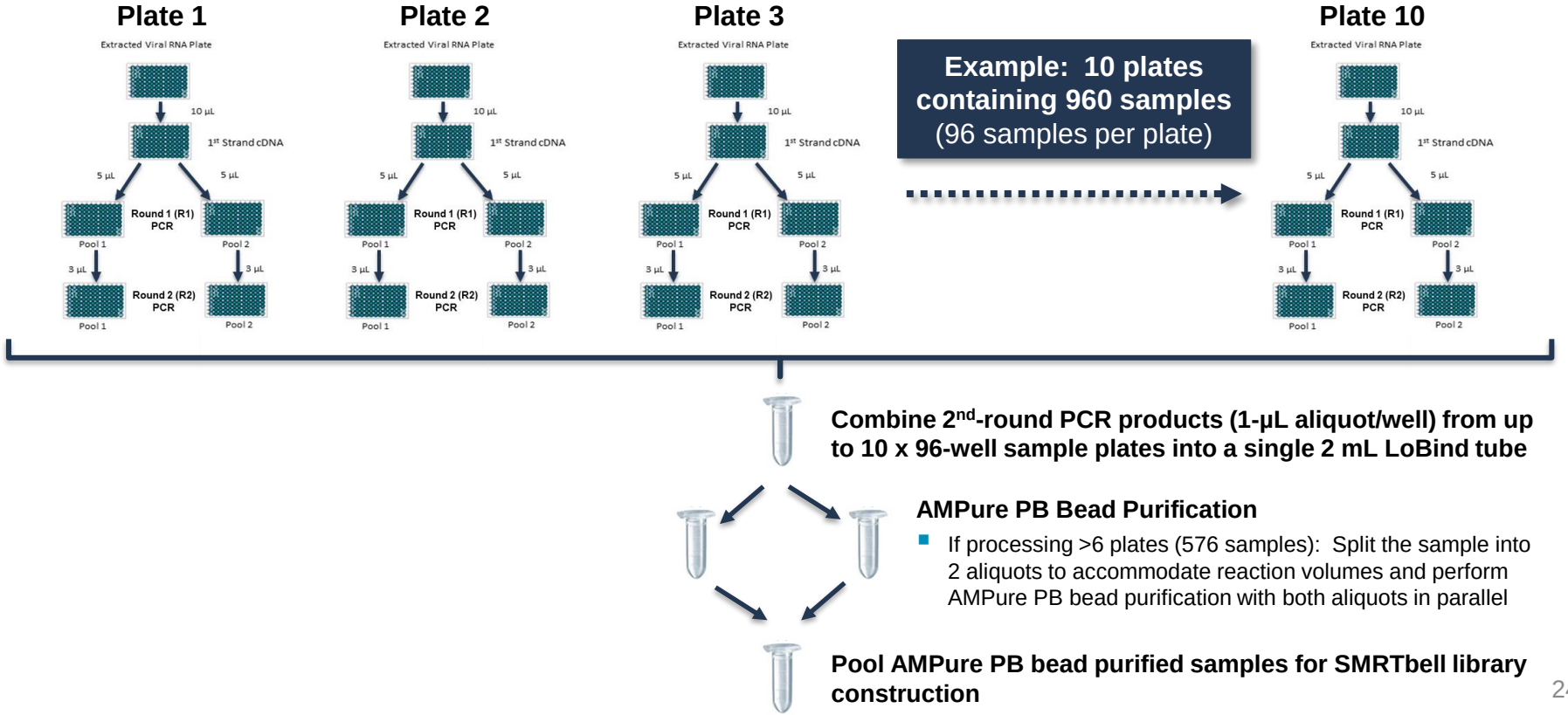
1. Perform one 1st-strand cDNA synthesis reaction per sample
2. Perform 1st-round PCR using two separate, parallel PCR reactions per sample with SARS-CoV-2 PCR primers tailed with M13 sequences
   - **Primer Pool 1** contains 15 **odd** primer pairs
   - **Primer Pool 2** contains 14 **even** primer pairs


3. Perform 2nd-round PCR using two separate, parallel PCR reactions per sample with PacBio-Barcoded M13 Primers
   - See **Appendix 1** in the high-throughput procedure for example asymmetric barcode plate maps for processing up 10 x 96-well plates containing SARS-Cov-2 samples at a time
4. Combine 2nd-round PCR products (1-µL aliquots) from each well of R2 Pool 1 and R2 Pool 2 into a single 2 mL LoBind tube and perform AMPure PB bead purification prior to proceeding with SMRTbell library construction




* See *HiFiViral Workflow for High-Throughput Multiplexing of 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2* (PN 102-075-000)

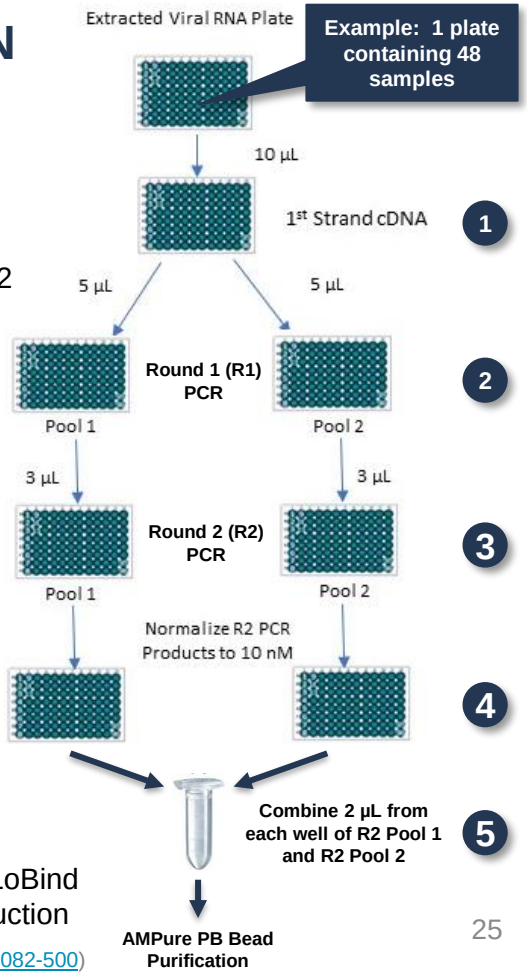
# EXAMPLE HIGH-THROUGHPUT SAMPLE PREPARATION WORKFLOW FOR FULL-VIRAL GENOME SEQUENCING OF SARS-CoV-2 SAMPLES (CONT.)



# EXAMPLE LOW-THROUGHPUT* SAMPLE PREPARATION WORKFLOW FOR FULL-VIRAL GENOME SEQUENCING OF SARS-CoV-2 SAMPLES

1. Perform one 1st-strand cDNA synthesis reaction per sample
2. Perform 1st-round PCR using two separate, parallel PCR reactions per sample with SARS-CoV-2 PCR primers tailed with M13 sequences
   - **Primer Pool 1** contains 15 **odd** primer pairs
   - **Primer Pool 2** contains 14 **even** primer pairs


3. Perform 2nd-round PCR using two separate, parallel PCR reactions per sample with PacBio-Barcoded M13 Primers
   - See **Appendix 1** in the low-throughput procedure for an example asymmetric barcode plate map for processing up to 48 SARS-Cov-2 samples in a single 96-well plate
4. Normalize R2 Pool 1 and R2 Pool 2 PCR products to 10 nM to obtain more balanced coverage.
5. Combine normalized 2nd-round PCR products (2-µL aliquots from each well) into a single 2 mL LoBind tube and perform AMPure PB bead purification prior to proceeding with SMRTbell library construction




* See *HiFiViral Workflow for Low-Throughput Multiplexing of 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2* (PN 102-082-500)

# Multiplexed SARS-CoV-2 Amplicon Library Sequencing Workflow Recommendations

# SAMPLE SETUP RECOMMENDATIONS FOR SARS-CoV-2 AMPLICON LIBRARIES – SEQUEL II AND IIe SYSTEMS

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the tables below for sequencing SARS-CoV-2 amplicon samples.
  - For **SMRT Link v10.0** (or higher): Select **'Amplicons <3 kb'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface and enter in the values shown in the tables below

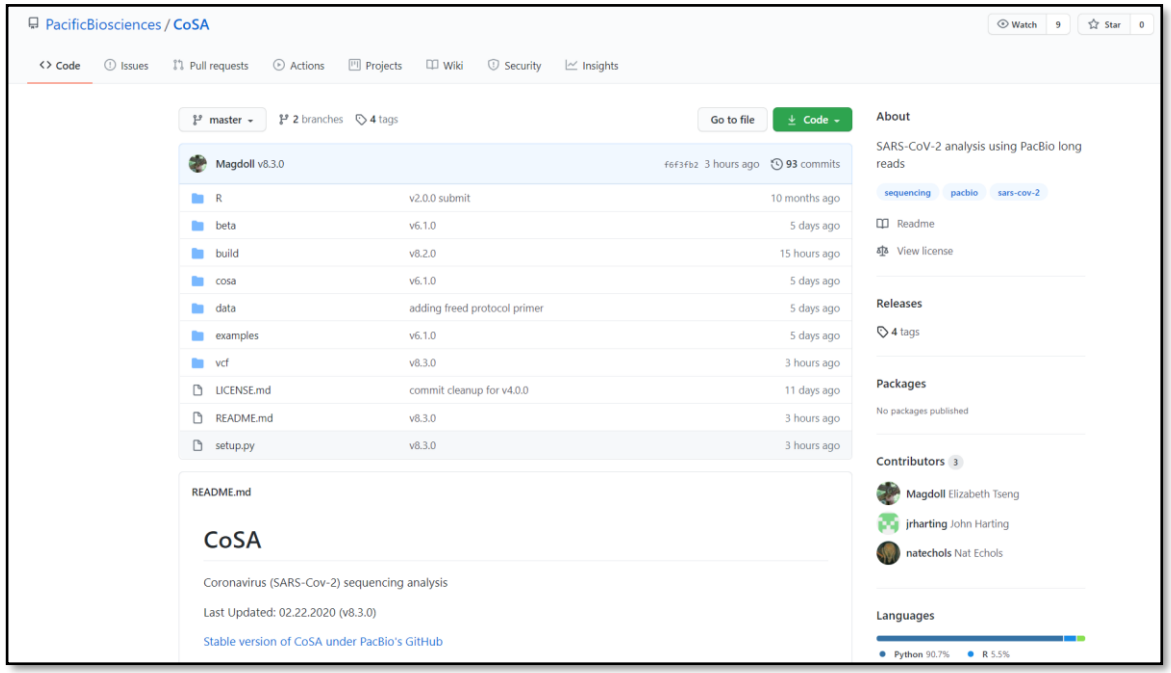
| SAMPLE SETUP CONDITIONS | SEQUEL II AND IIe SYSTEMS |
|---|---|
| **Sequencing Primer** | Sequencing Primer v4 |
| **Primer to Template Ratio** | 20:1 |
| **Polymerase to Template Ratio** | 10:1 |
| **Binding Kit** | Sequel II Binding Kit 2.1 |
| **Binding Time** | 1 hour |
| **Complex Cleanup Method** | AMPure PB Beads |
| **AMPure PB Bead Cleanup Anticipated Yield** | 35% |

| RUN DESIGN CONDITIONS | SEQUEL II AND IIe SYSTEMS |
|---|---|
| **Sequencing Kit** | Sequel II Sequencing Plate 2.0 |
| **Recommended On-Plate Loading Concentration** | 100 pM – 160 pM |
| **Movie Collection Time** | 15 hours |
| **Pre-extension Time** | 1 hour |

# Multiplexed SARS-CoV-2 Amplicon Data Analysis Recommendations

# SARS-CoV-2 SEQUENCING DATA ANALYSIS TOOLS

**Coronavirus (SARS-CoV-2) Sequencing Analysis (CoSA)** is a set of Python and R scripts available on GitHub for analyzing SARS-CoV-2 sequences from PacBio HiFi / CCS data




Join the ***COVID-19 Google Group*** for CoSA updates

*https://github.com/PacificBiosciences/CoSA*

# PREREQUISITES FOR SARS-CoV-2 VARIANT CALLING ANALYSIS USING PACBIO HIFI DATA

## Python Requirements

- Python 3.7+
- BioPython
- PyVCF
- mappy and panda (if using pbaa)

We recommend looking into **Anaconda** for easily managing Python packages

## PacBio Tool Prerequisites

- ccs
- lima
- optional - pbaa if using *pbaa* for variant calling.

These PacBio tools can be obtained either through **SMRT Link** or **pbbioconda**

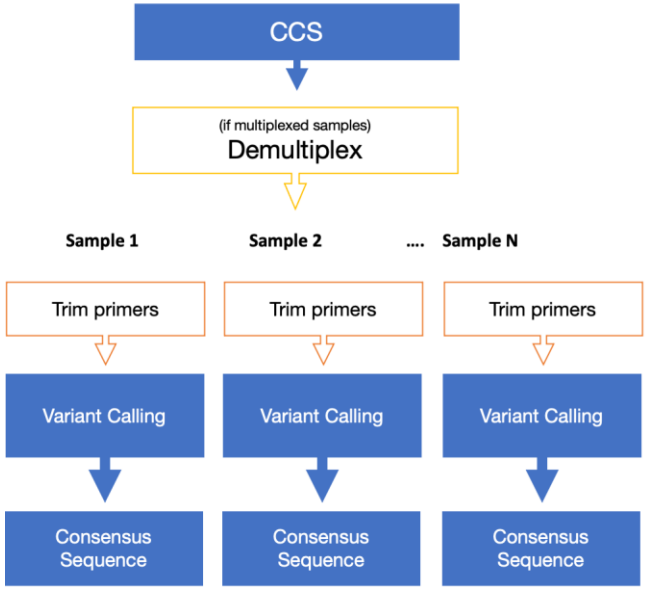
## Other Prerequisites

- CoSA
- samtools
- bamtools
- optional - bcftools, if using *bcftools* for variant calling.

*https://github.com/PacificBiosciences/CoSA*

# GENERAL WORKFLOW FOR SARS-CoV-2 VARIANT CALLING ANALYSIS

1. **Generate CCS data***
2. **De-multiplex different barcoded samples** (If dataset is multiplexed)
3. **Trim PCR amplicon primers**
4. **Perform variant calling using:**
   - bcftools; or
   - DeepVariant; or
   - pbaa
5. **Generate consensus sequences**
   - Use VCFCons.py
6. **Assign lineages**
   - Use Pangolin or Nextclade




| Tool Choices | Output Format |
|---|---|
| *lima* | BAM |
| *bcftools*<br>*DeepVariant*<br>*pbaa* | VCF |
| *VCFCons*<br>*(part of CoSA)* | FASTA |

* CCS analysis can be run using command line tools or by using the "Circular Consensus Sequencing (CCS)" application in SMRT Link. For Sequel IIe Systems, CCS analysis can also be performed on-instrument.

*https://github.com/PacificBiosciences/CoSA*

# EXAMPLE SARS-CoV-2 VARIANT CALLING ANALYSIS OUTPUT

**CLC**

```
NC_045512v2 8317    .   A    AC   200.00  .   .   GT:CLCAD2:DP    0/1:165,28:193
NC_045512v2 9867    .   T    C    200.00  .   .   GT:CLCAD2:DP    1/1:0,152:153
NC_045512v2 10450   .   C    T    200.00  .   .   GT:CLCAD2:DP    1/1:0,423:434
NC_045512v2 11287   .   GTCTGGTTTT  G   200.00  .   .   GT:CLCAD2:DP    1/1:0,424:435
```

**bcftools**

```
NC_045512v2 9867    .   T    C    228 .   DP=154;VDB=0;SGB=-0.693147;RPB=1;MQB=1;MQSB=1;BQB=1;MQ0F=0;AC=2;AN=2;DP4=0,1,56,94;MQ=60    GT:PL   1/1:255,255,0
NC_045512v2 10450   .   C    T    228 .   DP=99;VDB=0;SGB=-0.693147;RPB=0.999369;MQB=1;BQB=0.805608;MQ0F=0;AC=2;AN=2;DP4=4,0,95,0;MQ=60   GT:PL   1/1:255,108,0
NC_045512v2 11287   .   GTCTGGTTTT  G   228 .   INDEL;IDV=94;IMF=0.949495;DP=99;VDB=0;SGB=-0.693147;MQ0F=0;AC=2;AN=2;DP4=4,0,95,0;MQ=60 GT:PL   1/1:255,156,0
```

**DeepVariant**

```
NC_045512v2 8317    .   A    AC   0    RefCall .   GT:GQ:DP:AD:VAF:PL  0/0:47:200:168,30:0.15:0,46,64
NC_045512v2 9867    .   T    C    83.4    PASS    .   GT:GQ:DP:AD:VAF:PL  1/1:61:153:1,152:0.993464:83,61,0
NC_045512v2 10450   .   C    T    50.2    PASS    .   GT:GQ:DP:AD:VAF:PL  1/1:33:435:8,423:0.972414:50,33,0
NC_045512v2 11287   .   GTCTGGTTTT  G   41.2    PASS    .   GT:GQ:DP:AD:VAF:PL  1/1:27:431:7,424:0.983759:41,27,0
```

**pbaa**

```
NC_045512v2 9867    .   T    C    .   PASS    NS=1;AF=1   GT:DP:AQ:AD:VAF:TG:HP:DV:CH 1:154:63.0495:153:1:NC_045512_9351_10400:0:-1:-1
NC_045512v2 10450   .   C    T    .   PASS    NS=1;AF=1   GT:DP:AQ:AD:VAF:TG:HP:DV:CH 1:435:62.9768:424:1:NC_045512_10401_11420:0:-1:-1
NC_045512v2 11287   .   GTCTGGTTTT  G   .   PASS    NS=1;AF=1   GT:DP:AQ:AD:VAF:TG:HP:DV:CH 1:435:62.9768:424:1:NC_045512_10401_11420:0:-1:-1
```

*VCFCons.py*

**Filtered variant calls**

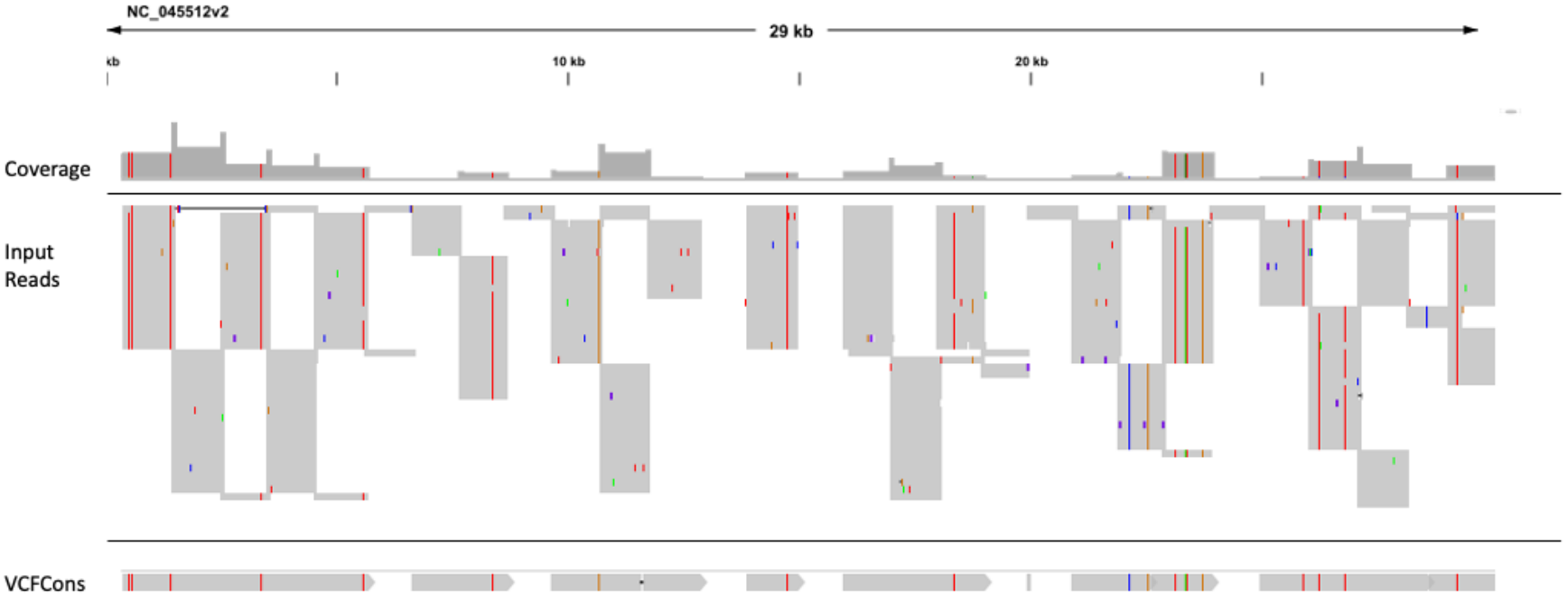
```
NC_045512v2 9867    .   T    C    .   PASS
NC_045512v2 10450   .   C    T    .   PASS
NC_045512v2 11287   .   GTCTGGTTTT  G   .   PASS
```

**Filtered consensus sequence**

```
NC_045512v2   .....T...C...GTCTGGTTTTAAGCT....TTATGG...
Sample        .....C...T...GAAGCT.............NNNNNN...
```

https://github.com/PacificBiosciences/CoSA

# EXAMPLE SARS-CoV-2 VARIANT CALLING ANALYSIS VISUALIZATION




- Example visualization of SARS-Cov-2 variant calling analysis results using Integrative Genomics Viewer (IGV)

# Technical Documentation & Applications Support Resources

# TECHNICAL RESOURCES FOR SARS-CoV-2 LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS

## Visit PacBio's ***COVID-19 Sequencing Tools and Resources Website*** for HiFiViral for SARS-CoV-2 Workflow Updates and Other Resources

### Sample Preparation Literature

- Customer Collaboration – PacBio HiFiViral for SARS-CoV-2 Workflow Protocol References
  - Low-Throughput Procedure for Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2 (PN 102-082-500)
  - High-Throughput Procedure for Multiplexing 1.2 kb Amplicons for Full-Viral Genome Sequencing of SARS-CoV-2 (PN 102-075-000)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II/IIe Systems PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Consumable Bundles Purchasing Guide (PN PG100-051320)
- PacBio HiFiViral Workflow Overview:  Multiplexed Amplicon Library Preparation for Full-Viral Genome Sequencing of SARS-CoV-2 (PN 102-084-800)

# TECHNICAL RESOURCES FOR SARS-CoV-2 LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

## Data Analysis Resources

- Coronavirus (SARS-Cov-2) Sequencing Analysis (CoSA) GitHub Page
  - CoSA is a set of Python and R scripts for analyzing SARS-CoV-2 sequences from PacBio HiFi / CCS data.
- Join the COVID19 Google Group to stay up-to-date on bioinformatics analysis recommendations and CoSA changes

## Videos & Webinars

- ASM 2020 Presentation (2020): Geographic and Temporal Mapping of the SARS-CoV-2 Pandemic in the United States [Webinar Recording]
- PacBio LabRoots Webinar (2020): Opportunities for using PacBio Long-read Sequencing for COVID-19 Research [Webinar Recording]
- PacBio Webinar (2020): Understanding SARS-CoV-2 and Host Immune Response to COVID-19 with PacBio Sequencing [Webinar Recording]

# Appendix: RNA Isolation Kit Options for Full-Viral Genome Sequencing of SARS-CoV-2

# RNA SAMPLE EXTRACTION KIT OPTIONS FOR FULL-VIRAL GENOME SEQUENCING OF SARS-CoV-2

**Note:** The products below have **not** been tested or validated by PacBio but are listed here as examples of third-party kits used by other PacBio customers for isolating SARS-CoV-2 RNA samples for multiplexed SMRTbell amplicon library preparation

| VENDOR | RNA ISOLATION KIT PRODUCT | AUTOMATION PLATFORM |
|---|---|---|
| **Thermo Fisher Scientific** | MagMAX Viral and Pathogen Nucleic Acid Isolation Kit (Link) | KingFisher Flex System |
| **Roche Molecular Systems** | MagNA Pure 96 DNA and Viral NA Small Volume Kit (Link) | Roche MagNA Pure-96 (MP6) |

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