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Technical Overview: Multiplexed Library Preparation for Full-Viral Genome Sequencing Using HiFiViral SARS-CoV-2 Kit

Sequel II and IIe Systems ICS v10.1 / SMRT Link v10.2

PN 102-205-300 Version 01 (November 2021)

Multiplexed Library Preparation for Full-Viral Genome Sequencing Using HiFiViral SARS-CoV-2 Kit

1. HiFiViral SARS-CoV-2 Kit Workflow Overview
2. Multiplexed Library Preparation Using Molecular Inversion Probe-Based Enrichment with the HiFiViral SARS-CoV-2 Kit
3. Multiplexed SARS-CoV-2 Library Sequencing Workflow Recommendations
4. Multiplexed SARS-CoV-2 Data Analysis Recommendations
5. Multiplexed SARS-CoV-2 Library Example Performance Data
6. Technical Documentation & Applications Support Resources

APPENDIX 1: RNA Isolation Kit Options for Full-Viral Genome Sequencing of SARS-CoV-2

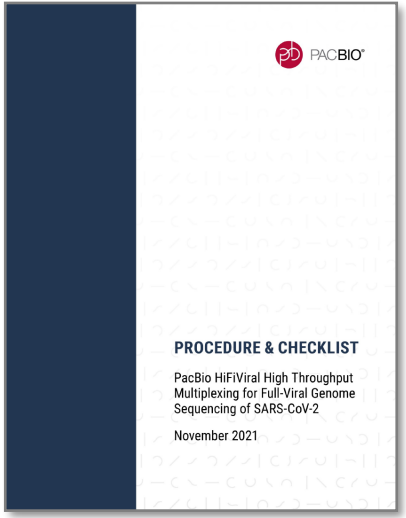
APPENDIX 2: Guidance on Workflow Automation For Multiplexed Library SARS-CoV-2 Library Preparation

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

Application-Specific Resources Website → **Application-Specific Procedure & Checklist** → **Application-Specific Technical Overviews** → **Library Construction, Sequencing & Analysis**

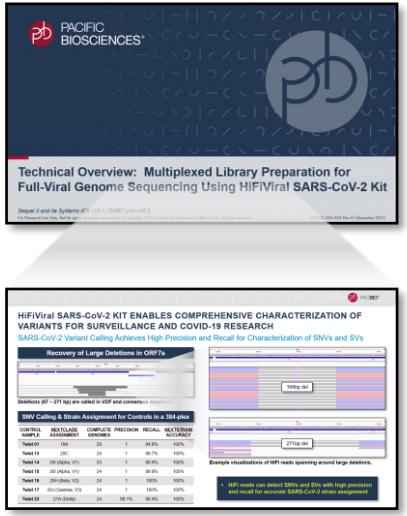
[PacBio COVID-19 Sequencing Tools and Resources Website]

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



Procedure & Checklist: HiFiViral SARS-CoV-2 Workflow (102-188-800)*

Technical documentation containing sample library construction and sequencing preparation protocol details



Technical Overview: Multiplexed Library Preparation for Full-Viral Genome Sequencing Using HiFiViral SARS-CoV-2 Kit (102-205-300)

Technical Overview presentations describe sample preparation details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

SARS-CoV-2 Viral Enrichment

Perform molecular inversion probe (MIP)-based enrichment procedure using HiFiViral SARS-CoV-2 Kit (102-132-000)

Library Construction (SMRTbell Express TPK 2.0)

HiFi Sequencing (Sequel II and IIe Systems)

Recommend pooling up to 384 SARS-CoV-2 samples per SMRT Cell 8M

Data Analysis

Perform variant calling with HiFi data using SMRT Link HiFiViral SARS-CoV-2 analysis application

** For Research Use Only. Not for use in diagnostic procedures.*

HiFiViral SARS-COV-2 KIT USES MOLECULAR INVERSION PROBES FOR EFFICIENT ENRICHMENT OF VIRAL RNA SEQUENCES FOR ANALYSIS

Robust performance

Easier workflow

Capture all variants

Flexible batch size

Cost-effective

Better Performance with Molecular Inversion Probes (MIPs)

- Differentiated enrichment technology
- Robust genome coverage across a range of Ct-values
- Probe design resilient to novel variants
- Capture mutations of all types
- Detect multiple strains in one sample

Easier Workflow and Faster Turnaround Times

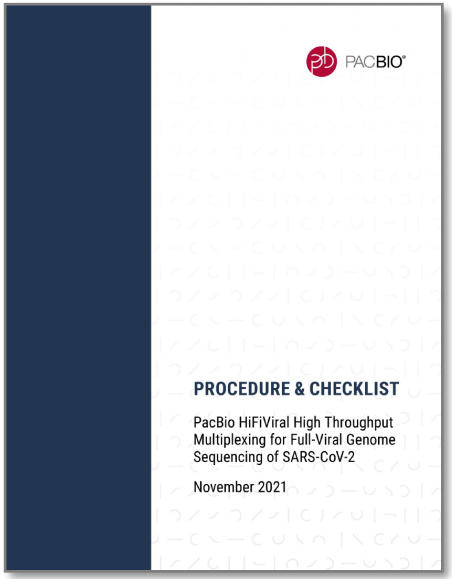
- Easier workflow compared to targeted PCR amplicons
- All ready-to-use reagents in one kit
- Color change indicator confirms correct reagent was added
- Addition-only workflow can be automated
- Automated sequencing and analysis runs overnight

Flexible Scaling

- 384 reactions per kit
- Scalable batching: 24 – 384 samples per run

Quickly and efficiently scale genomic surveillance by sequencing with **an accurate and robust kit solution to capture all variants**

END-TO-END PACBIO PROTOCOL FOR FULL-VIRAL GENOME SEQUENCING USING HiFiViral SARS-CoV-2 KIT



PacBio Documentation (102-188-800)

HiFiViral SARS-CoV-2 Kit (102-132-000)

For Targeted Enrichment and Barcoding of SARS-CoV-2 PCR-Positive Samples*

- ☑ Robust Performance
- ☑ Easier Workflow
- ☑ Capture All Variants
- ☑ Flexible Batch Size
- ☑ Cost Effect

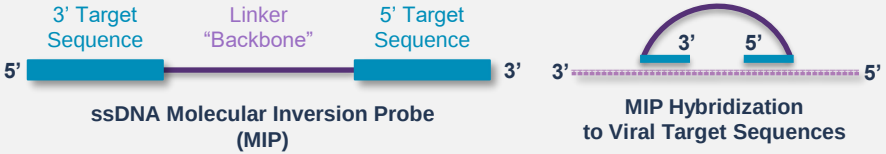
- Full workflow can be completed from **sample to answer in as short as ~28 – 42 h** (1 – 2.5 h hands-on time)
- Multiplex 24 – 384 samples per SMRT Cell 8M and load up to 8 SMRT Cells per Sequel IIe System to **run up to 3,072 samples per week**

* *HiFiViral SARS-CoV-2 Kit demonstrated use cases include RNA-extracted samples such as nasopharyngeal or saliva swabs from human SARS-CoV-2 PCR+ cohort samples.*

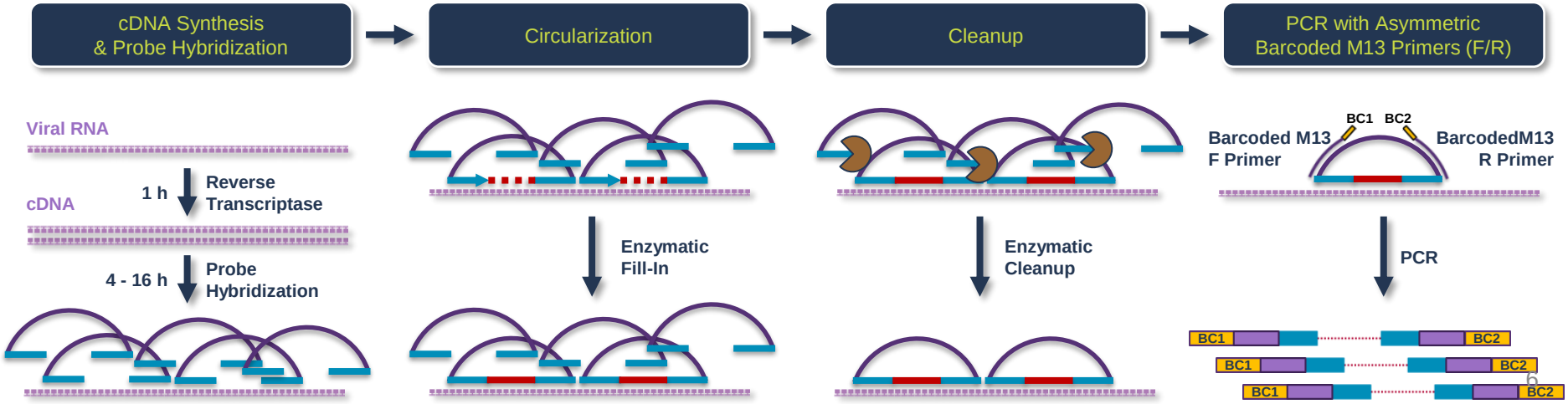
** *For multi-SMRT Cell runs, sequencing + data analysis time is ~14 – 16 h for the first cell. For subsequent SMRT Cells, sequencing + data analysis time is ~9 – 10 h per cell*

HiFiViral SARS-CoV-2 KIT USES *MOLECULAR INVERSION PROBE* TECHNOLOGY FOR EFFICIENT VIRAL GENOME ENRICHMENT

Overview of MIP-Based Viral Enrichment Enzymatic Reaction Steps

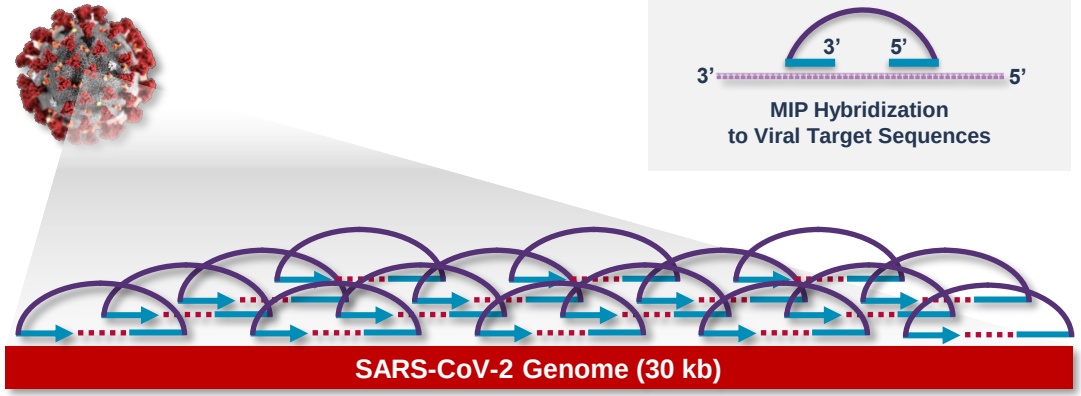


- Single pool of 969 ssDNA MIPs comprised of two probe arms connected by a common linker (30 bp)
- MIPs tile SARS-CoV-2 genome at 22-fold target coverage
- Capture of 675-bp target sequences is performed by circularization of MIPs *via* "fill-In" enzymatic reaction
- PCR amplification using universal (M13) primers adds unique (dual index) asymmetric barcodes to each sample to enable multiplexed analyses



HiFiViral SARS-CoV-2 KIT USES *MOLECULAR INVERSION PROBE* TECHNOLOGY FOR EFFICIENT VIRAL GENOME ENRICHMENT (CONT.)

Dense MIP-Based Tiling of Target Sequences Enables Robust Coverage



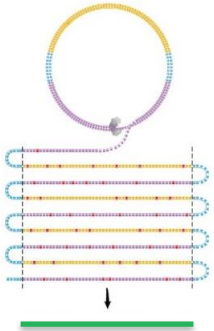
Asymmetrically Barcoded Double-Stranded Library Molecules (~800 bp)*

Advantages of MIPs

- **Higher Specificity**
  - Each MIP molecule contains two probe arms
- **Easier Workflow**
  - Unlike traditional PCR-based targeting with overlapping primers, overlapping MIPs can be used in a single reaction leading to fewer plates and fewer touch points
- **More Robust Probe Design**
  - ~1000 ssDNA probes tile target SARS-CoV-2 genome at 22-fold coverage
  - More tolerant to viral RNA sample degradation and a wider range of input RNA quantities
  - Resilient to mutation-induced probe dropouts with new viral genomic variants

** Not to scale.*

HIFIVIRAL SARS-COV-2 SEQUENCING REQUIRES FEWER READS FOR COMPLETE VIRAL GENOME COVERAGE



HiFi Read

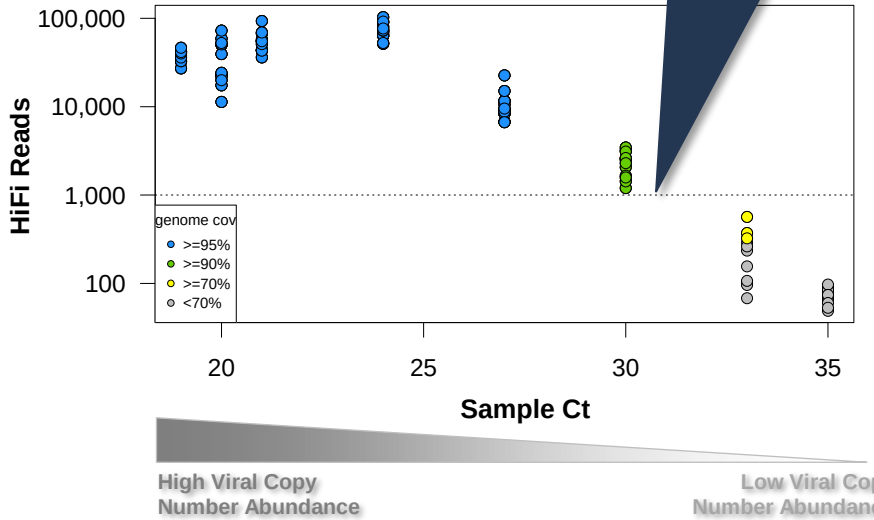
PacBio HiFi reads achieve >99.9% accuracy

TECHNOLOGY	# OF READS FOR COMPLETE COVERAGE	MINIMUM READ DEPTH
PacBio HiFi	1,000	4-fold
Oxford Nanopore	10,000	20-fold
Illumina	1,000,000	10-fold

HiFi reads are more accurate
→ Fewer reads simplifies analysis

96-Plex of Twist Control Samples

Samples with Ct ≤ 30 achieved complete genome coverage* with 1000 HiFi reads



** Complete = ≥90% genome coverage*

HiFiViral SARS-CoV-2 Kit Workflow Overview

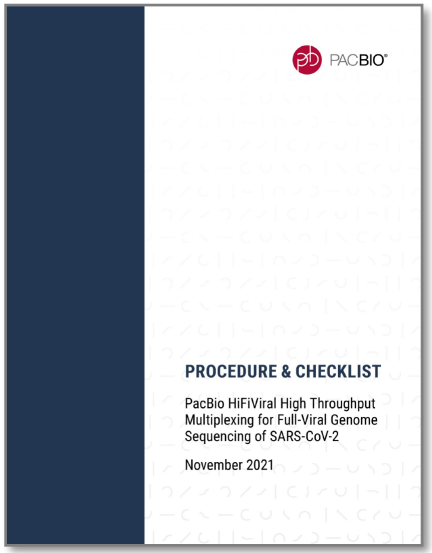
HIFIVIRAL SARS-COV-2 KIT LIBRARY PREPARATION PROCEDURE DESCRIPTION

- ***Procedure & Checklist – HiFiViral for SARS-CoV-2 Workflow*** (102-188-800) describes a viral enrichment and library preparation procedure for whole viral genome sequencing of multiplexed SARS-CoV-2 samples on the Sequel II and IIe Systems using HiFiViral SARS-CoV-2 Kit (102-132-000) and SMRTbell Template Prep Kit 2.0 (100-938-900)

HiFiViral SARS-CoV-2 Kit (102-132-000)

SMRTbell Express TPK 2.0 (100-938-900)

- This procedure utilizes **molecular inversion probe** (MIP)-based chemistry to enrich the SARS-CoV-2 genome with tiled probes that create highly-redundant overlapping amplicons, which are barcoded and pooled for construction into a single SMRTbell library for sequencing
- Viral enrichment uses an **addition-only 4-step workflow with color-coded master mixes** to simplify setup
- End-to-end workflow from cDNA synthesis through to SMRTbell library construction, sequencing & analysis can be completed in as short as 28 – 42 hours depending on desired hybridization time



PacBio Documentation (102-188-800)

RESEARCH FOCUS

MICROBIOLOGY AND INFECTIOUS DISEASE

PacBio COVID-19 Sequencing Tools and Resources

HiFiViral SARS-CoV-2 KIT PRODUCT DESCRIPTION

HiFiViral SARS-CoV-2 Kit (102-132-000)

- Assay kit designed for targeted enrichment and barcoding of up to 384 human SARS-CoV-2-positive samples for full-length viral genomic sequencing on PacBio Sequel II or IIe Systems
- Kit contains two components: 1) SARS-CoV-2 Enrichment Kit; and 2) Barcoded M13 Primer Plate

HiFiViral SARS-CoV-2 Kit (102-132-000)

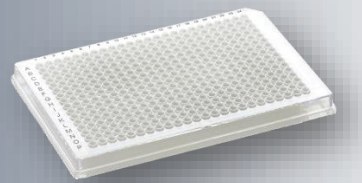
SARS-CoV-2 Enrichment Kit

- The SARS-CoV-2 Enrichment Kit contains all reagents for enrichment using Molecular Inversion Probes (MIPs) of extracted RNA virus from cohort samples infected with the SARS-CoV-2 virus. This kit is to be used in conjunction with the Barcoded M13 Primer Plate.
- The results of the kit are enriched DNA fragments of ~800 bp in length that can be used to prepare a SMRTbell library for sequencing.
- Reagent quantities support preparation of 384 samples with flexible scaling down to batches of 24 samples.

SARS-CoV-2 Enrichment Kit

*Barcoded M13 Primer Plate**

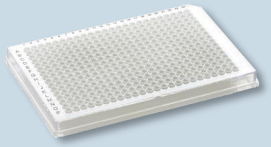
- 1 premixed primer plate containing 384 barcoded M13 primer pairs for asymmetric (dual index) barcoding of multiplexed SMRTbell libraries
- Single-use per well with pierceable foil (can reseal between sample batches)



Barcoded M13 Primer Plate

** Barcoded M13 Primer Plate (102-135-500) part may also be ordered separately for use with other multiplexed SMRT Sequencing applications.*

HiFiViral SARS-CoV-2 KIT COMPONENTS

KIT PRODUCT OR COMPONENT	KIT SUBCOMPONENT	PART NUMBER	QUANTITY	NO. OF REACTIONS SUPPORTED
HiFiViral SARS-CoV-2 Kit (NEW) 		**102-132-000**		**384**
SARS-CoV-2 Enrichment Kit 	1 Probe Mix		1 Tube	
	2 Fill-In Mix		1 Tube	
	3 Cleanup Mix		1 Tube	
	4 Reverse Transcriptase Mix		1 Tube	
	5 PCR Mix		3 Tubes	
Barcoded M13 Primer Plate* 	Premixed Primer Plate		1 Plate	

** Barcoded M13 Primer Plate (102-135-500) part may also be ordered separately for use with other multiplexed SMRT Sequencing applications.*

HiFiViral SARS-CoV-2 KIT WORKFLOW OVERVIEW

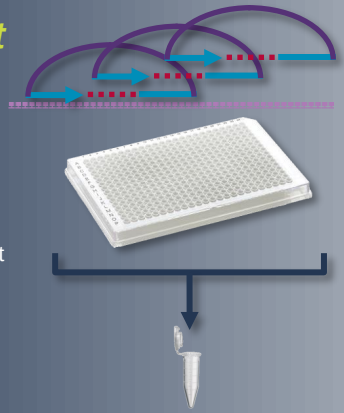
Sample RNA Extraction

- Isolate viral RNA from human SARS-CoV-2 PCR+ samples using third-party protocols

Sample RNA Extraction

Viral Genome Enrichment with HiFiViral SARS-CoV-2 Kit

- Addition-only workflow features a visible color change with each reagent addition step to signal success
- All reactions performed on one sample plate
- MIP-based viral enrichment workflow times:
  - Overnight cDNA Synthesis (1 h) + Hybridization (16 h) with option to reduce hybridization time to 4 h for faster turnaround time. (A longer hybridization time boosts HiFi Data Yield for high-Ct samples
  - MIP Circularization, Cleanup, PCR and Pooling steps can be completed in ~4 – 5 h
- Amplify and asymmetrically barcode up to 384 SARS-CoV-2 samples (per SMRT Cell 8M) for multiplexing in a single library using PacBio-Barcoded M13 Primers



cDNA Synthesis & Probe Hybridization

Circularization & Cleanup

PCR with Asymmetric Barcoded M13 Primers (F/R)

Pool Barcoded Samples

4 – 16 h

4 – 5 h

SMRTbell Library Construction & Sequencing Prep

- Library Prep: SMRTbell Express TPK 2.0; SMRTbell Enzyme Cleanup Kit 2.0
- Sequencing Prep: Sequencing Primer v5; Binding Kit 2.1; ProNex Bead Cleanup

SMRTbell Library Construction & Sequencing Preparation

6 h

Sequencing & Data Analysis

- Use 8-h movie collection time per SMRT Cell 8M
- Load up to 8 SMRT Cells per run to analyze up to 3,072 samples in 1 week
- Use SMRT Link HiFiViral SARS-CoV-2 analysis application for data analysis

SMRT Sequencing & Analysis

14 – 16 h

Multiplexed Library Preparation Using Molecular Inversion Probe-Based Enrichment with the HiFiViral SARS-CoV-2 Kit

PROCEDURE & CHECKLIST – PACBIO HIFIVIRAL HIGH-THROUGHPUT MULTIPLEXING FOR FULL-VIRAL GENOME SEQUENCING OF SARS-COV-2

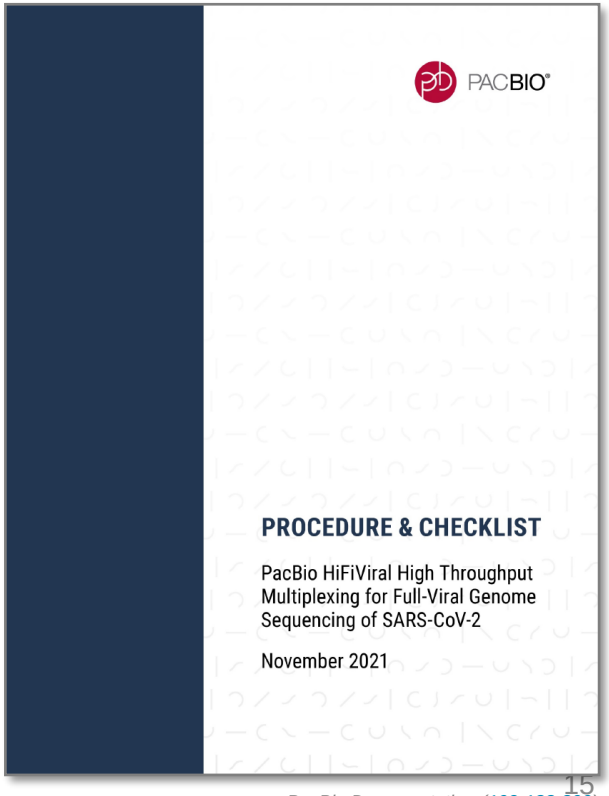
Procedure & Checklist 102-188-800 describes a viral enrichment and library preparation procedure for whole viral genome sequencing of multiplexed SARS-CoV-2 samples on the Sequel II and IIe Systems using HiFiViral SARS-CoV-2 Kit (102-132-000) and SMRTbell Template Prep Kit 2.0 (100-938-900)

HiFiViral SARS-CoV-2 Kit (102-132-000)

SMRTbell Express TPK 2.0 (100-938-900)

Protocol Contents

1. RNA input requirements and best practices recommendations for preparing master mixes, handling RNA samples, and sealing reaction plates
2. Instructions for performing enrichment of SARS-CoV-2 viral cDNA products using HiFiViral SARS-CoV-2 Kit (102-132-000)
3. Instructions for pooling amplified SARS-CoV-2 cDNA products and constructing SMRTbell libraries using SMRTbell Express Template Prep Kit 2.0 (100-938-900)



PacBio Documentation (102-188-800)

REQUIRED MATERIALS & EQUIPMENT

ITEM	WHERE USED	VENDOR	PART NUMBER
RNA Preparation			
Nuclease-Free Water	RNA Preparation	Any	Vendor-specific
RNaseZap	RNA Preparation	Thermo Fisher Scientific	AM9780
SARS-CoV-2 RNA Viral Enrichment			
HiFiViral SARS-CoV-2 Kit (Includes items below) SARS-CoV-2 Enrichment Kit Barcoded M13 Primer Plate	cDNA Synthesis & Probe Hybridization Reaction Fill Reaction Cleanup Reaction PCR & Barcoding Reaction Library Pooling	PacBio	102-132-000
SMRTbell Library Preparation			
SMRTbell Express Template Prep Kit 2.0	Library Construction	PacBio	100-938-900
SMRTbell Enzyme Cleanup Kit 2.0	Library Construction	PacBio	101-932-600
DynaMag-2 Magnet	Library Purification	Thermo Fisher Scientific	12321D
Absolute Ethanol, Molecular Biology or ACS Grade	Library Purification	Any	Vendor-specific
ProNex Beads	Library Purification	Promega	NG2001-10mL / NG2002-125mL / NG2003-500mL
DNA LoBind Tubes	Library Construction	Eppendorf	22431021 (1.5 mL) / 22431048 (2.0 mL)

REQUIRED MATERIALS & EQUIPMENT (CONT.)

ITEM	WHERE USED	VENDOR	PART NUMBER
Sequencing Preparation			
Sequel II Binding Kit 2.1 and Int Ctrl 1.0	Sequencing on the Sequel II and IIe Systems	PacBio	101-843-000
Sequel II Sequencing Kit 2.0	Sequencing on the Sequel II and IIe Systems	PacBio	101-820-200
SMRT Cell 8M Tray	Sequencing on the Sequel II and IIe Systems	PacBio	101-389-001
Sequel SMRT Oil	Sequencing on the Sequel II and IIe Systems	PacBio	100-621-300
Sequel Pipette Tips v2	Sequencing on the Sequel II and IIe Systems	PacBio	100-667-601
Sequel Mixing Plates	Sequencing on the Sequel II and IIe Systems	PacBio	100-667-500
Sample Plate	Sequencing on the Sequel II and IIe Systems	PacBio	HSP9601
Tube Septa	Sequencing on the Sequel II and IIe Systems	PacBio	001-292-541
Sequel Sample Plate Foil	Sequencing on the Sequel II and IIe Systems	PacBio	100-667-400
DNA / Library QC Evaluation			
Qubit 4 Fluorometer	DNA Quantitation	Thermo Fisher Scientific	Q33238
Qubit 1x dsDNA HS Assay Kit	DNA Quantitation	Thermo Fisher Scientific	Q33230
Bioanalyzer 2100	DNA Sizing	Agilent	G2939A
Agilent DNA 12000 Kit	DNA Sizing	Agilent	5067-1508

REQUIRED MATERIALS & EQUIPMENT (CONT.)

ITEM	WHERE USED	VENDOR	PART NUMBER
General			
96-well PCR plates	SARS-CoV-2 RNA Viral Enrichment	Bio-Rad	HSP9601
Microseal 'B' Film	SARS-CoV-2 RNA Viral Enrichment	Bio-Rad	MSB1001
Film sealing roller for PCR plates	SARS-CoV-2 RNA Viral Enrichment	Bio-Rad	MSR0001
Thermal Cycler With Heated Lid (Examples below) VeritiPro Thermal Cycler, 96 well ProFlex PCR System	SARS-CoV-2 RNA Viral Enrichment	Thermo Fisher Scientific	A48141 (VentiPro) / 4483636 (ProFlex)
PCR Tube Strips, 0.2 mL	SARS-CoV-2 RNA Viral Enrichment	USA Scientific	1402-4708
96-Well Plate Centrifuge	SARS-CoV-2 RNA Viral Enrichment	Any Vendor	Vendor-specific
8- or 12-Multichannel Pipette	SARS-CoV-2 RNA Viral Enrichment	Any Vendor	Vendor-specific

HIFIVIRAL SARS-COV-2 KIT BARCODED M13 PRIMER PLATE

Asymmetric barcode plate map for Barcoded M13 Primer Plate*

- Ready-to-use premixed primer plate containing 384 barcoded M13 primer pairs for asymmetric (dual index) barcoding of multiplexed SMRTbell libraries
  - Plate includes 40 different oligos (16 M13 Forward Primers + 24 M13 Reverse Primers)
- Single-use per well with pierceable foil (can reseal between sample batches)
  - Fill volume in each well = 12 µl (at 10 µM primer concentration)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1002 1050	1002 1051	1002 1052	1002 1053	1002 1054	1002 1055	1002 1056	1002 1057	1002 1058	1002 1059	1002 1060	1002 1061	1002 1062	1002 1063	1002 1064	1002 1065	1002 1066	1002 1067	1002 1068	1002 1069	1002 1070	1002 1071	1002 1072	1002 1073
B	1003 1050	1003 1051	1003 1052	1003 1053	1003 1054	1003 1055	1003 1056	1003 1057	1003 1058	1003 1059	1003 1060	1003 1061	1003 1062	1003 1063	1003 1064	1003 1065	1003 1066	1003 1067	1003 1068	1003 1069	1003 1070	1003 1071	1003 1072	1003 1073
C	1004 1050	1004 1051	1004 1052	1004 1053	1004 1054	1004 1055	1004 1056	1004 1057	1004 1058	1004 1059	1004 1060	1004 1061	1004 1062	1004 1063	1004 1064	1004 1065	1004 1066	1004 1067	1004 1068	1004 1069	1004 1070	1004 1071	1004 1072	1004 1073
D	1005 1050	1005 1051	1005 1052	1005 1053	1005 1054	1005 1055	1005 1056	1005 1057	1005 1058	1005 1059	1005 1060	1005 1061	1005 1062	1005 1063	1005 1064	1005 1065	1005 1066	1005 1067	1005 1068	1005 1069	1005 1070	1005 1071	1005 1072	1005 1073
E	1006 1050	1006 1051	1006 1052	1006 1053	1006 1054	1006 1055	1006 1056	1006 1057	1006 1058	1006 1059	1006 1060	1006 1061	1006 1062	1006 1063	1006 1064	1006 1065	1006 1066	1006 1067	1006 1068	1006 1069	1006 1070	1006 1071	1006 1072	1006 1073
F	1007 1050	1007 1051	1007 1052	1007 1053	1007 1054	1007 1055	1007 1056	1007 1057	1007 1058	1007 1059	1007 1060	1007 1061	1007 1062	1007 1063	1007 1064	1007 1065	1007 1066	1007 1067	1007 1068	1007 1069	1007 1070	1007 1071	1007 1072	1007 1073
G	1008 1050	1008 1051	1008 1052	1008 1053	1008 1054	1008 1055	1008 1056	1008 1057	1008 1058	1008 1059	1008 1060	1008 1061	1008 1062	1008 1063	1008 1064	1008 1065	1008 1066	1008 1067	1008 1068	1008 1069	1008 1070	1008 1071	1008 1072	1008 1073
H	1009 1050	1009 1051	1009 1052	1009 1053	1009 1054	1009 1055	1009 1056	1009 1057	1009 1058	1009 1059	1009 1060	1009 1061	1009 1062	1009 1063	1009 1064	1009 1065	1009 1066	1009 1067	1009 1068	1009 1069	1009 1070	1009 1071	1009 1072	1009 1073
I	1010 1050	1010 1051	1010 1052	1010 1053	1010 1054	1010 1055	1010 1056	1010 1057	1010 1058	1010 1059	1010 1060	1010 1061	1010 1062	1010 1063	1010 1064	1010 1065	1010 1066	1010 1067	1010 1068	1010 1069	1010 1070	1010 1071	1010 1072	1010 1073
J	1011 1050	1011 1051	1011 1052	1011 1053	1011 1054	1011 1055	1011 1056	1011 1057	1011 1058	1011 1059	1011 1060	1011 1061	1011 1062	1011 1063	1011 1064	1011 1065	1011 1066	1011 1067	1011 1068	1011 1069	1011 1070	1011 1071	1011 1072	1011 1073
K	1012 1050	1012 1051	1012 1052	1012 1053	1012 1054	1012 1055	1012 1056	1012 1057	1012 1058	1012 1059	1012 1060	1012 1061	1012 1062	1012 1063	1012 1064	1012 1065	1012 1066	1012 1067	1012 1068	1012 1069	1012 1070	1012 1071	1012 1072	1012 1073
L	1013 1050	1013 1051	1013 1052	1013 1053	1013 1054	1013 1055	1013 1056	1013 1057	1013 1058	1013 1059	1013 1060	1013 1061	1013 1062	1013 1063	1013 1064	1013 1065	1013 1066	1013 1067	1013 1068	1013 1069	1013 1070	1013 1071	1013 1072	1013 1073
M	1014 1050	1014 1051	1014 1052	1014 1053	1014 1054	1014 1055	1014 1056	1014 1057	1014 1058	1014 1059	1014 1060	1014 1061	1014 1062	1014 1063	1014 1064	1014 1065	1014 1066	1014 1067	1014 1068	1014 1069	1014 1070	1014 1071	1014 1072	1014 1073
N	1015 1050	1015 1051	1015 1052	1015 1053	1015 1054	1015 1055	1015 1056	1015 1057	1015 1058	1015 1059	1015 1060	1015 1061	1015 1062	1015 1063	1015 1064	1015 1065	1015 1066	1015 1067	1015 1068	1015 1069	1015 1070	1015 1071	1015 1072	1015 1073
O	1016 1050	1016 1051	1016 1052	1016 1053	1016 1054	1016 1055	1016 1056	1016 1057	1016 1058	1016 1059	1016 1060	1016 1061	1016 1062	1016 1063	1016 1064	1016 1065	1016 1066	1016 1067	1016 1068	1016 1069	1016 1070	1016 1071	1016 1072	1016 1073
P	1017 1050	1017 1051	1017 1052	1017 1053	1017 1054	1017 1055	1017 1056	1017 1057	1017 1058	1017 1059	1017 1060	1017 1061	1017 1062	1017 1063	1017 1064	1017 1065	1017 1066	1017 1067	1017 1068	1017 1069	1017 1070	1017 1071	1017 1072	1017 1073

FORWARD
Reverse

** Barcoded M13 Primer Plate (102-135-500) part may also be ordered separately for use with other multiplexed SMRT Sequencing applications.*

HiFiViral SARS-CoV-2 KIT WORKFLOW DETAILS

1 Viral Genome Enrichment with HiFiViral SARS-CoV-2 Kit (102-132-000)

i. cDNA Synthesis & Hybridization With Molecular Inversion Probes (MIPs)

- For input into cDNA synthesis & probe hybridization, aim to start with ≥10,000 copies of RNA
- For SARS-CoV-2 viral enrichment using ssDNA MIPs, a 16-h hybridization time (55°C) is recommended for high-Ct samples (Ct >25). Optionally, can reduce hybridization time to as short as 4 h for low-Ct samples

ii. Circularization of MIPs

- Circularize MIPs bound to target SARS-CoV-2 sequences *via* fill-in reaction between ssDNA probe arms

iii. Enzymatic Cleanup

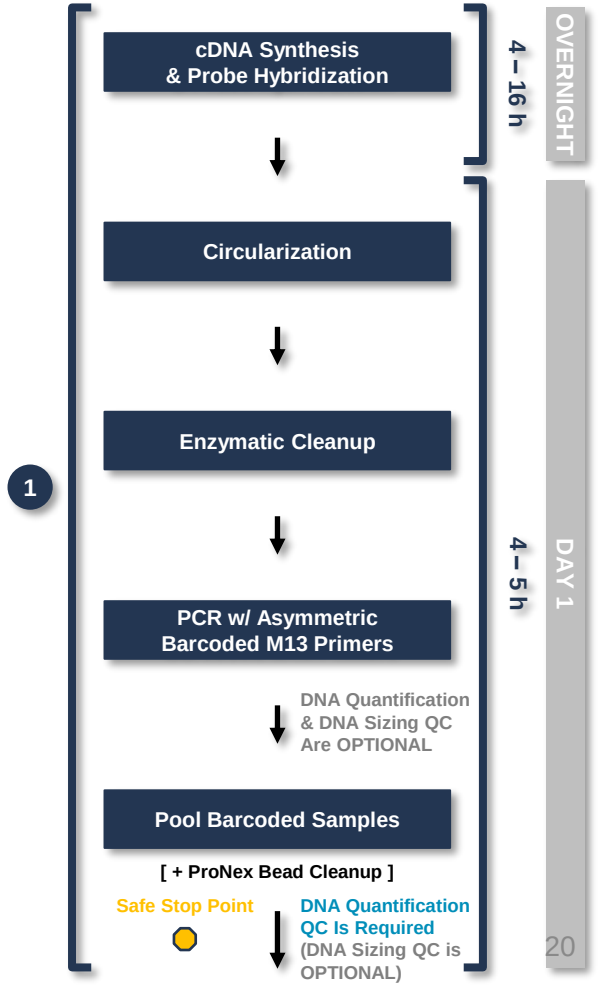
- Remove unbound MIPs via enzymatic cleanup

iv. PCR Amplification with Asymmetric Barcoded M13 Primers

- Amplify captured SARS-CoV-2 target sequences via 1-step PCR with asymmetric barcoded M13 primers (included with HiFiViral SARS-CoV-2 Kit) to produce barcoded ~800 bp dsDNA amplicon products

v. Pool Barcoded Samples

- Perform equal-volume pooling of barcoded SARS-CoV-2 amplicon products from up to 384 cohort samples (per SMRT Cell 8M) and purify the resulting mixture using ProNex Beads.
- After ProNex Bead cleanup, the pooled sample is used for construction into a single, multiplexed SMRTbell library



HiFiViral SARS-CoV-2 KIT WORKFLOW DETAILS (CONT.)

2 SMRTbell Library Construction with SMRTbell Express Template Prep Kit 2.0 (100-938-900)

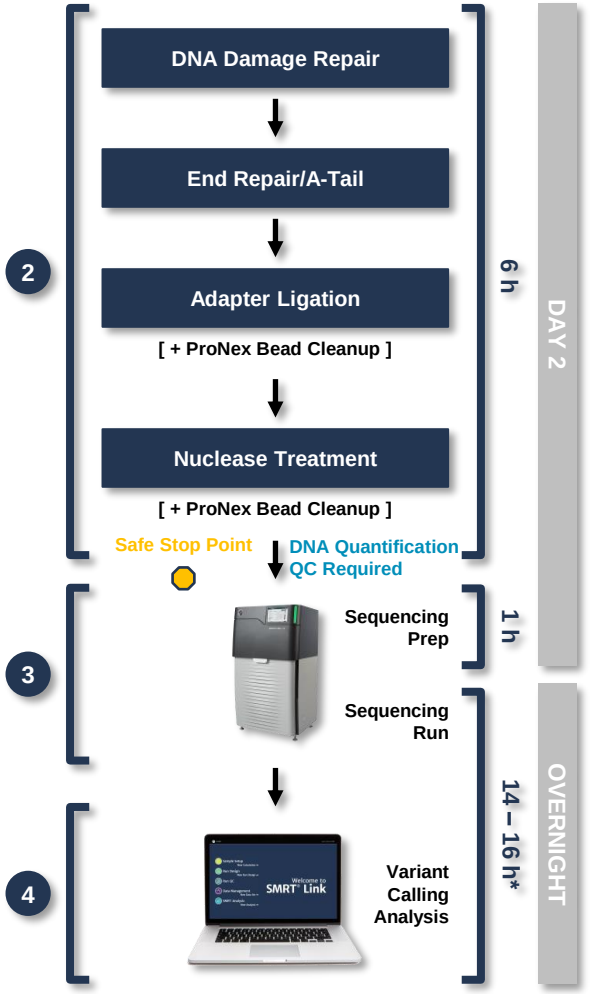
- The amount of total pooled (barcoded) DNA required for SMRTbell library construction is 500 ng – 1000 ng.
- Library construction can be completed in ~5 h
- Typical library construction yield is ≥40%

3 SMRT Sequencing

- Anneal Sequencing Primer v5 (15 min), bind Sequel Polymerase 2.2 (15 min), and perform complex cleanup with ProNex Beads (0.5 h)
- Use 8-h movie collection time per SMRT Cell 8M for sequencing HiFiViral SARS-CoV-2 Kit samples on the Sequel II or IIe System
- Load up to 8 SMRT Cells per run to analyze up to 3,072 samples in 1 week

4 Data Analysis

- Use SMRT Link HiFiViral SARS-CoV-2 analysis application to perform variant calling



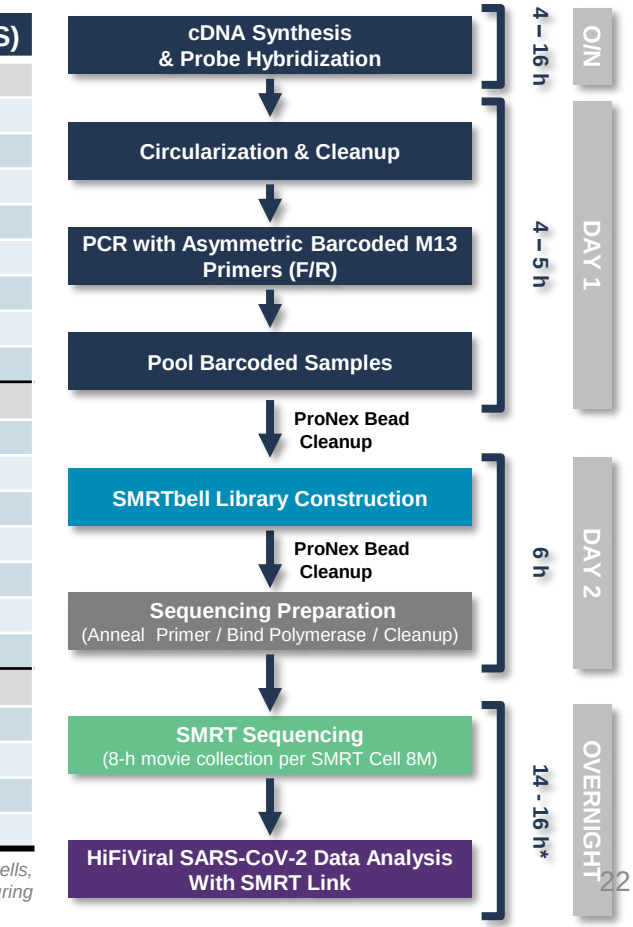
* *For multi-SMRT Cell runs, sequencing + data analysis time is ~14 – 16 h for the first cell. For subsequent SMRT Cells, sequencing + data analysis time is reduced to ~9 – 10 h per cell due to parallelization of sequencing and analysis functions during the instrument run.*

HiFiViral SARS-CoV-2 KIT SAMPLE PREP WORKFLOW TIMING SUMMARY

Efficient Workflow (~1 – 2.5 Hours Hands-On Time) Enables Sample to Answer in ~28 – 42 Hours

WORKFLOW STEP	HANDS-ON (MIN)	WALK-AWAY (HRS)
SARS-CoV-2 RNA Enrichment (~22 h)		
cDNA Synthesis	5 – 15	1.0
Probe Hybridization with MIPs	5 – 15	4.0 – 16.0
Circularization (Fill-in Reaction)	5 – 15	1.0
Enzymatic Cleanup Reaction	5 – 15	1.2
PCR with Barcoded M13 Primers	10 – 30	1.5
Pooling (DNA sizing QC is optional)	5 – 10	—
1.3X ProNex Bead Cleanup + Qubit Assay	5 – 10	0.3
Total	**~40 – 110**	**~9.0 – 21.0**
SMRTbell Library Construction (~5 h)		
DNA Damage Repair	2 – 4	0.5
End Repair / A-Tailing	2 – 4	1.0
Adapter Ligation	2 – 4	1.2
1.3X ProNex Bead Cleanup	2 – 4	0.3
Nuclease Treatment	2 – 4	0.5
1.3X ProNex Bead Cleanup + Qubit Assay	5 – 10	0.3
Total	**~15 – 30**	**~3.8**
Sequencing Preparation (~1.5 h)		
Anneal Sequencing Primer	2.5 – 5	0.25
Bind Polymerase	2.5 – 5	0.25
1.2X ProNex Bead Complex Cleanup	5 – 10	0.5
Total	**~10 – 20**	**~1.0**

** For multi-SMRT Cell runs, sequencing + data analysis time is ~14 – 16 h for the first cell. For subsequent SMRT Cells, sequencing + data analysis time is reduced to ~9 – 10 h per cell due to parallelization of sequencing and analysis functions during the instrument run.*



RNA INPUT REQUIREMENTS FOR VIRAL ENRICHMENT USING HiFiViral SARS-CoV-2 KIT

- Best results will be achieved if reactions contain **at least 10,000 copies of RNA**.
  - Samples with higher copy numbers of RNA virus will generally produce superior results.
  - See at table at right for example viral copy number values converted from a Ct scale*
- Purified RNA should be resuspended in RNase-free water or TE with a pH no greater than 7.5.
- Contaminants including ethanol, sodium azide, sodium acetate, and guanidine salts may affect performance.
- DNase treatment is optional but the presence of small amounts of human DNA should not affect performance.
- If RNA is quantified, a method that is specific for RNA is recommended (e.g., Qubit RNA BR Assay Kit or qRT-PCR), rather than one that will also detect DNA.
- To reduce inter-sample performance variability, all samples in a batch should be quantified using the same method and normalized to the same concentration.

Example viral copy number values shown in Table below are converted from a Ct scale after **Han *et al*. 2021.**

Sample Ct	Viral Copy Number*
19	6 Million
20	3 Million
21	1 Million
24	100,000
27	10,000
30	1,000
33	100
35	3

*** NOTE:** A Ct value itself **cannot** be directly interpreted as viral load without a standard curve using reference materials. [See *Han M.S., et al. (2021). RT-PCR for SARS-CoV-2: quantitative versus qualitative. The Lancet Infectious Disease 21(2) p165*]

GENERAL BEST PRACTICES RECOMMENDATIONS FOR VIRAL ENRICHMENT USING HiFiViral SARS-CoV-2 KIT

Best Practices

Master Mixes

1. Prepare master mixes in a PCR workstation.
2. The PCR workstation should be UV-irradiated after each setup. If unsure, UV-irradiate the workstation before setting up a master mix.
   NOTE: do not turn on the UV light when reagents are in the workstation.
3. Master mixes are prepared in 0.5mL, 1.5 mL or 2 mL
   down.
4. If using multichannel pipette to transfer master mixes, pre-aliquot appropriate volume with overage into PCR strip tubes.

Samples

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

Reaction Plates

1. Always seal plates with Microseal 'B' Film (clear adhesive). Foil seals are not recommended for any step in this protocol. However, they can be used for plates that will be placed in the freezer for storage.
2. Using a roller for Microseal 'B' Film, apply firm pressure and seal over the tops of all wells. Ensure all wells, especially those along the edges of the plate, are visibly sealed.
3. Inspect the corners of the plate to confirm that the seal is in contact with the plate. If not, apply firm pressure and roll until the film is in contact with the plate.
4. When removing plate seals, a heated plate sealer
   loosen the adhesive.
5. Centrifuge in an Eppendorf 5810 fitted with a swinging bucket plate rotor at maximum rpm for approximately 30 sec.
6. After centrifugation, inspect the bottom of the plate to ensure the expected volume is present in every well.

Preparing Master Mixes

- Prepare master mixes in a PCR workstation and have them ready before the end of the prior incubation steps
- Use multichannel or electronic pipettes to facilitate transfer of master mixes to sample wells

Handling RNA Samples

- Use special care when handling small volumes of reagents
- Be careful when removing plate seals to avoid cross contamination

Preparing Reaction Plates

- Always perform a visual check of liquid volumes before and after each incubation step
- Verify that the liquid solution color at each reach step is correct
- Proper plate sealing is **critical**, especially for the overnight probe hybridization step

HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES

1. cDNA Synthesis and Probe Hybridization

cDNA Synthesis and Probe Hybridization

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

1. Prepare labware and reagents.
   a. Label one or more 96-well PCR plates. Alternatively, for a small number of reactions, PCR tube strips may be used.
   b. Retrieve extracted RNA samples from storage.
2. Pipette 6 µL of sample RNA into each well of the reaction plate. Be sure to follow RNA input recommendations. Use nuclease-free water to adjust sample RNA volume, if needed.
3. Prepare RT-Hybridization Master Mix on ice.
   a. Allow RT Mix and Probe Mix to fully thaw. Briefly vortex and spin
   b. Prepare master mix with 12.5% overage as indicated in the table below. Preparing fewer than 24 reactions at a time is not recommended.
   c. RT Mix is viscous, pipette slowly.

Reagent	1X reaction	96 reactions (with overage)	✓	Notes
RT Mix	1.6 µL	172.8 µL		
Probe Mix	0.4 µL	43.2 µL		

4. Transfer **2 µL** of RT-Hybridization Master Mix into each sample-containing well in the reaction plate. RT-Hybridization Master Mix is viscous, pipette
5. Seal the plate tightly with a film. Poor sealing could result in significant
6. Spin down the 96-well plate.
7. Vortex a few times with short pulses and spin down.
8. Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **pale blue** color.
9. Place the reaction plate in the thermal cycler and run the following program (set the heated lid at 105ºC).

Step	Temperature	Time
1	25°C	10 minutes
2	50°C	50 minutes
3	95°C	
4	55°C	24 hours * (16hrs for Probe Hybridization and 1hr for Fill-in Reaction
5	55°C	Hold

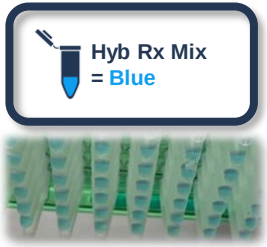
10. Make a note of the thermal cycler start time. A hybridization time of 16 hours (the 55°C step) is recommended for high Ct samples (Ct >25). A 4hr hybridization could be considered if most of samples have low Ct value (Ct <25). Start preparing for the fill reaction just prior to the end of hybridization (approximately 17 hours from the start of the cycling program).

Preparing Master Mixes

- Slowly pipette small reaction volumes and viscous reagents (Master Mix volumes shown in the table only include 12.5% overage)

Preparing Reaction Plates

- Seal reaction plates tightly with Microseal 'B' Film to minimize evaporation, especially along the plate edges and corners
- Verify that the liquid solution color for each Hybridization reaction is **blue** and homogeneous



Starting and Monitoring Hyb Reactions

- Do not use questionable or problematic thermal cycler equipment for this viral enrichment workflow
- A 16-hour hybridization time is recommended – Make note of the reaction start time (incubating slightly longer than 16 hours should not have a negative impact)
- Keep the thermal cycler program running after probe hybridization is completed to maintain proper temperature control of the heating block

HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES (CONT.)

2. Circularization (Fill Reaction)

Fill Reaction

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

1. Remove the sample plate from the thermal cycler. **Keep the program running**.
2. Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
3. At room temperature, transfer **2 µL** of Fill-in Mix to each sample well.

   It is important to perform the transfer as fast as possible to minimize non-specific binding; aim to finish within minutes.
4. Reseal the plate tightly with a new film, vortex a few times with short pulses, and spin down the plate.
5. Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **pale green** color.
6. reaction plate in the thermal cycler and continue the program for another 60 minutes.

   time the reaction plate was returned to the thermal cycler; correct timing is important to result quality.

Preparing Fill Reaction Plates

- Add reagents at room temperature, DO NOT cool on ice
- Fill Reaction steps are time sensitive – Work quickly with a multichannel pipettor to complete all liquid transfer steps within 5 minutes for best capture results
- Verify that the liquid solution color for each Fill Reaction is **green** and homogeneous

3. Cleanup Reaction

Cleanup Reaction

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

1. Remove the sample plate from the thermal cycler.
2. Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
3. At room temperature, transfer **2 µL** of Cleanup Mix to each sample well.

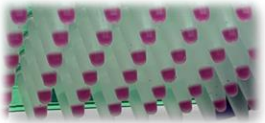
   It is important to perform the transfer to minimize non-specific binding; aim to finish within 10 minutes.
4. Reseal the plate tightly with a new film, vortex a few times with short pulses, and spin down the plate.
5. Perform a quick visual check of the liquid level and take note of any well with low volume. The reaction should now be a homogenous **red** color.
6. Place the reaction plate in the thermal cycler and run the following program (set the heated lid at 105°C).

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

7. The approximately 65 minutes to run; proceed immediately to the cDNA amplification st

Preparing Cleanup Reaction Plates

- Add reagents at room temperature, DO NOT cool on ice
- Cleanup Reaction steps are time sensitive – Work quickly with a multichannel pipettor to complete all liquid transfer steps within 5 minutes for best capture results
- Verify that the liquid solution color for each Cleanup Reaction is **red** and homogeneous



HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES (CONT.)

4. PCR Amplification With Barcoded M13 Primers

cDNA Amplification

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

1. Remove the sample plate from the thermal cycler.
2. Spin down the plate, perform a quick visual check of the liquid level to make sure there are no droplets on the top seal or side walls, and remove the seal carefully to avoid cross contamination.
3. Prepare the PCR reaction as follow.

Reagent	Stock Conc.	1X reaction	✓	
Cleanup reaction		9.6		
PCR Mix		12 µL		
Asymmetric Barcoded M13 Primer Mix	10 µM	2.4 µL		
Total Volume		24 µL		

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

4. Using a multichannel pipette, transfer 12 µL of PCR Mix to the sample plate.
5. Transfer 2.4 µL of premixed asymmetric barcoded M13 primers from the primer plate to the corresponding sample wells.
6. The total reaction volume in each well is approximately 24.0 µL.
7. Reseal the plate tightly with a new film, vortex a few times with short pulses, and spin down the plate.
8. Perform a quick visual check of liquid level and take note of any well with low volume. The reaction should now be a homogenous **magenta** color.
9. Place the PCR reactions in a thermal cycler and run the following program (set the heated lid at 105°C).

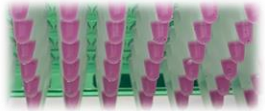
Step	Temperature	Time
1	95°C	3 minutes
2	98°C	15
3	55°C	5 seconds
4	72°C	1 minute 30 seconds
5	Repeat steps 2 to 4 (26 times)	
6	4°C	Hold

10. After amplification, briefly spin down the plate.
11. Immediately proceed to the "Sample Pooling for Library Construction" section if not performing the optional Library Quantitation/QC step. Alternatively, the reaction plate can be stored at -20°C until further processing.

Preparing PCR Reaction Plates

- Expected sample volume after cleanup step is ~9.6 µL.
- PCR amplification step is not time-sensitive
- Verify that the liquid solution color for each PCR Reaction is **magenta** and homogeneous

PCR Rx Mix = Magenta



Starting and Monitoring PCR Reactions

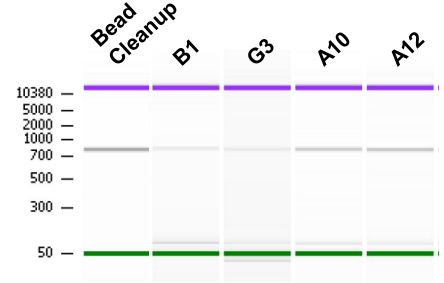
- PCR thermal cycler program at this step takes ~1.5 hours to complete (27 cycles)
- Expect some degree of cumulative evaporation loss to occur from completing previous steps in the workflow – If any sample in a well has significantly less than 9.6 µL, add nuclease-free water to bring up the sample volume and document this action
- After completing the PCR step, amplified cDNA samples can be stored at -20ºC until further processing

HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES (CONT.)

4. PCR Amplification With Barcoded M13 Primers (Cont.)

Library Quantification/QC (Optional)

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

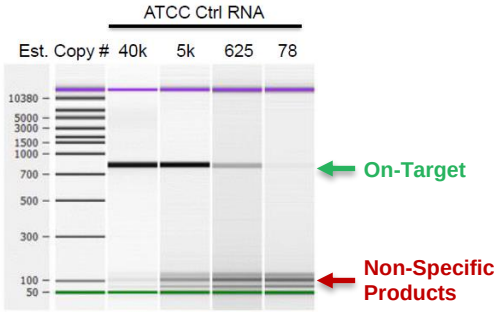


Example post-PCR DNA sizing analysis results for extracted viral RNA samples.

- Spot-checking PCR amplification products prior to pooling is highly recommended when performing the HiFiViral workflow for the first time
- 1.3X ProNex Bead purification can help remove non-specific amplification products

Post-PCR DNA Quantification and DNA Sizing QC (Optional)

Performing **post-PCR DNA sizing quantification and sizing QC steps is recommended** and can be useful for verifying sample integrity prior to SMRTbell library construction as well as downstream troubleshooting



Example post-PCR DNA sizing analysis results for ATCC Control RNA samples.

- Going from high to low copy number, the on-target band diminishes, and the amount of non-specific amplification products increases
- 1.3X ProNex Bead purification can help remove non-specific amplification products

PCR Product Yield vs. Input Control RNA Copy Number

Example post-PCR yield results for ATCC Control RNA samples.

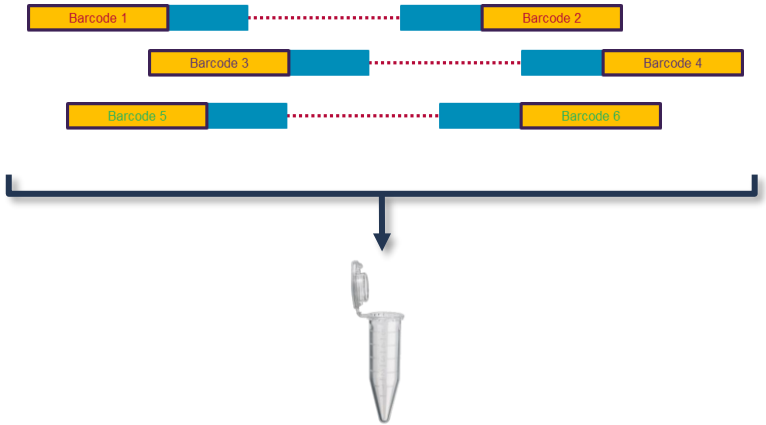
- Higher-copy number samples are generally correlated with higher PCR yields (*via* Qubit dsDNA HS assay quantification)

HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES (CONT.)

5. Sample Pooling for SMRTbell Library Construction

Sample Pooling for Library Construction

1. Remove the reaction plate from the thermal cycler.
2. Spin down the reaction plate and perform a quick visual check of the liquid level. Take note of any well with low volume, which indicates excessive evaporation during amplification.
3. Remove the seal carefully to avoid cross contamination.
4. Transfer a minimum of 5 µL per reaction into a clean 1.5 mL or 2.0 mL Lo-bind tube.
   a. The total pool should be at least 100 µL. For example, if running 8 reactions, pool 12.5 µL per reaction.
   b. If pooling 384 reactions, vortex to mix and transfer no more than 800uL to a new 2.0mL Lo-bind tube for purification. Save the rest of the sample pool at -20ºC.



Preparing Samples for Pooling

- Transfer a minimum of 5 µL per reaction into a clean 1.5 mL or 2.0 mL Lo-bind tube.
- The total pool volume should be at least 100 µL
  - If running 8 samples, pool 12.5 µL from each PCR reaction
  - For 96 samples, Total Pool Volume = 480 µL
  - For 384 samples, Total Pool Volume = 1920 µL
- Note: If pooling 384 reactions, the total volume is too large for a 1.5 mL tube
  - Transfer no more than 800 µL to a new 2.0 mL Lo-bind tube for purification. (Save the rest of the sample pool at -20ºC.)

HiFiViral SARS-CoV-2 KIT PROCEDURAL NOTES (CONT.)

5. Sample Pooling for SMRTbell Library Construction (Cont.)

Purification of Pooled Library

STEP	✓	Purification with ProNex Beads	Notes
1		Add 1.3X volume of resuspended, room-temperature ProNex beads to the pooled library. Pipette mix 10 times. Perform a quick-spin to collect all liquid from the sides of the tube.	
2		Incubate the sample on the bench top for 5 minutes at room temperature.	
3		Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipette to remove the supernatant.	
4		Wash 2 times with 1400 µL (or enough to fully cover the beads) of freshly prepa… 80% ethanol. After removal of the second 1400 µL ethanol wash, spin t… return to the magnetic stand, and remove residual ethanol wit…	
5		Remove the tube from the magnetic stand. **Imme**… …LB and pipette the mix to resuspend. Perform a quick-spin to collect all liquid from the sides of the tube. Place at room temperature for 5 minutes to elute the DNA from the beads.	
6		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA sample to a new tube.	
7		Use 1 µL of sample to quantify with a Qubit dsDNA HS kit.	

Library Quantification/QC (Optional)

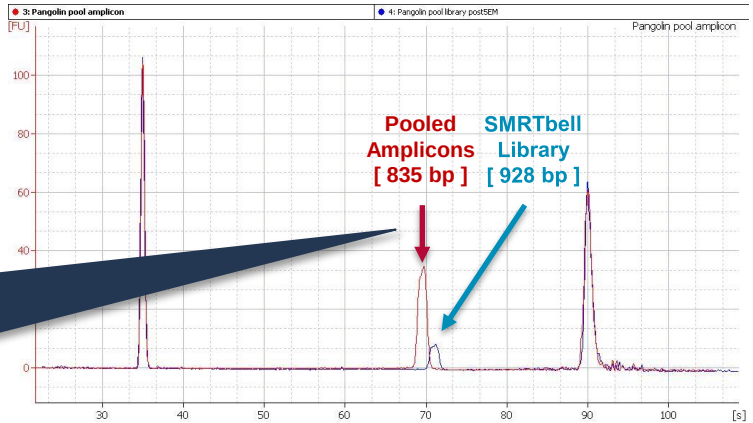
1. Pooled sample QC can be performed on the Agilent 2100 Bioanalyzer. Use a DNA12000 chip and follow the manufacturer's setup instruction.
2. A target peak of ≥700 bp should be detected. Non-specific amplicons (~170-200 bp) should be removed completely.

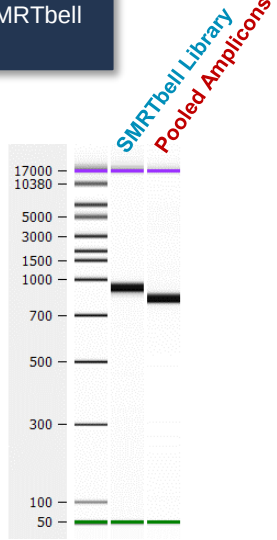
Purifying Pooled Samples

- Add 1.3X volume of resuspended, room-temperature ProNex beads to the pooled library.
  - Bead incubation: 5 mins, Room Temperature
  - Elution incubation: 5 mins, Room Temperature
- The total amount of purified pooled (barcoded) DNA required for SMRTbell library construction is 500-1000 ng.

DNA Sizing QC

- DNA sizing QC can optionally be performed on the pooled sample using an Agilent 2100 Bioanalyzer
  - A target peak of ≥700 bp should be detected
  - Non-specific amplicons (~170-200 bp) should be removed completely.





SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 AND SMRTBELL ENZYME CLEANUP KIT 2.0 REAGENT HANDLING RECOMMENDATIONS

- Several reagents in the kit are sensitive to temperature and vortexing
- PacBio highly recommends:
  - Never leaving reagents at room temperature
  - Working on ice at all times when preparing master mixes for SMRTbell library construction
  - Finger tapping followed by a quick-spin prior to use

SMRTbell Express TPK 2.0 (100-938-900)

LIST OF TEMPERATURE-SENSITIVE REAGENTS INCLUDED IN SMRTBELL EXPRESS TPK 2.0 AND SMRTBELL ENZYME CLEANUP KIT 2.0.

PACBIO KIT	REAGENT	WHERE USED
SMRTbell Express Template Prep Kit 2.0 (PN 100-938-900)	DNA Prep Additive	Remove Single-Strand Overhangs
	DNA Prep Enzyme	Remove Single-Strand Overhangs
	DNA Damage Repair Mix v2	DNA Damage Repair
	End Prep Mix	End-Repair/A-tailing
	Overhang Adapter v3	Ligation
	Ligation Mix	Ligation
	Ligation Additive	Ligation
	Ligation Enhancer	Ligation
SMRTbell Enzyme Cleanup Kit 2.0 (PN 101-932-600)	SMRTbell Enzyme Clean Up Mix	Nuclease Treatment
	SMRTbell Enzyme Clean Up Buffer	Nuclease Treatment

Multiplexed SARS-CoV-2 Library Sequencing Workflow Recommendations

SAMPLE SETUP AND RUN DESIGN RECOMMENDATIONS FOR HiFiViral SARS-Cov-2 LIBRARY SAMPLES (SEQUEL II/IIE SYSTEMS)

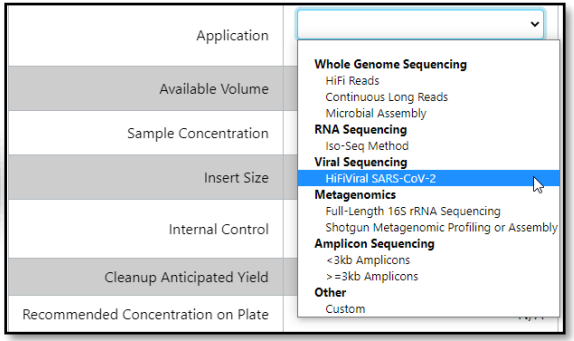
Follow **SMRT Link Sample Setup** instructions using the recommendations provided in ***Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems*** (101-769-100) for preparing HiFiViral samples for sequencing

Application	Library Prep Kit*	Sequencing Primer (Annealing Time)	Binding Kit (Binding Time)	Complex Cleanup	Loading Concentration Range (pM)
HiFiViral SARS-CoV-2 (1 kb)	Express TPK 2.0	v4 (15 min)	Binding Kit 2.1 (15 min)	1.2X ProNex Beads	100 - 300

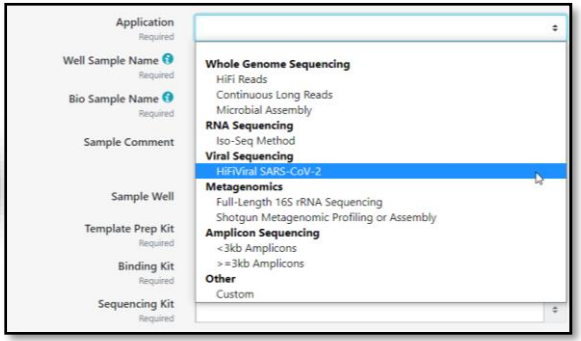
Application	Pre-Extension Time (hours)	Adaptive Loading Target (P1 + P2)	Movie Collection Time (hours)
HiFiViral SARS-CoV-2 (1 kb)	0	N/A	8

→ For **SMRT Link v10.2**: Select **'Viral Sequencing / HiFiViral SARS-CoV02'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Sample Setup



Run Design

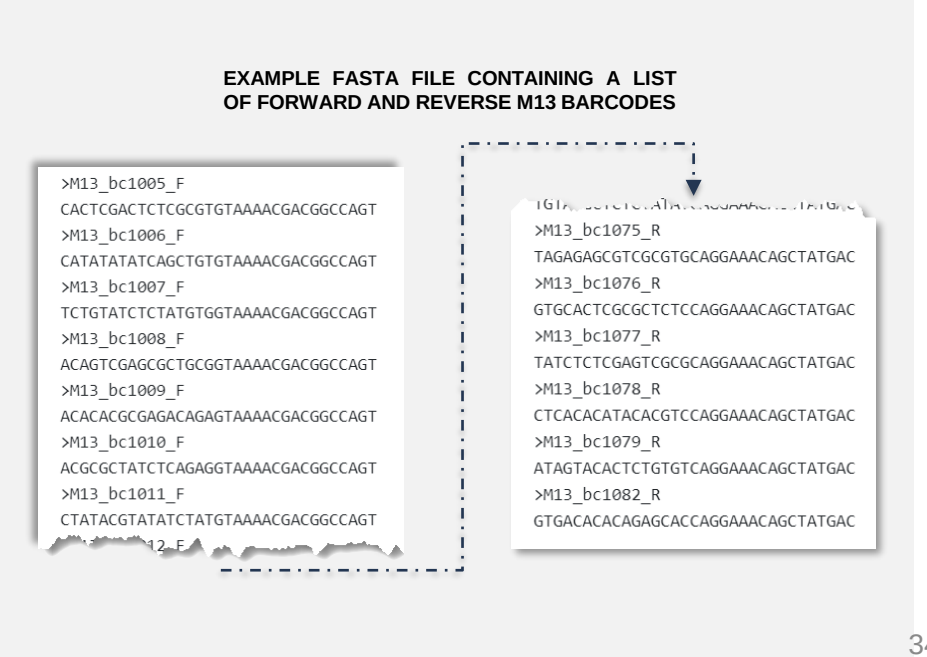


IMPORTING THE BARCODE FASTA FILE INTO SMRT LINK FOR AUTOMATED DEMULTIPLEXING OF HiFiViral SARS-Cov-2 LIBRARY SAMPLES

Note: SMRT Link v10.2 software installations by default come **pre-bundled** with FASTA files containing a list of PacBio barcodes recommended for use with specific multiplexed SMRT sequencing applications

If your SMRT Link installation does ***not*** already include an appropriate barcode FASTA file, the following steps describe how to import such a file for use in automated demultiplexing (refer to "Importing Data" section in the SMRT Link User Guide):

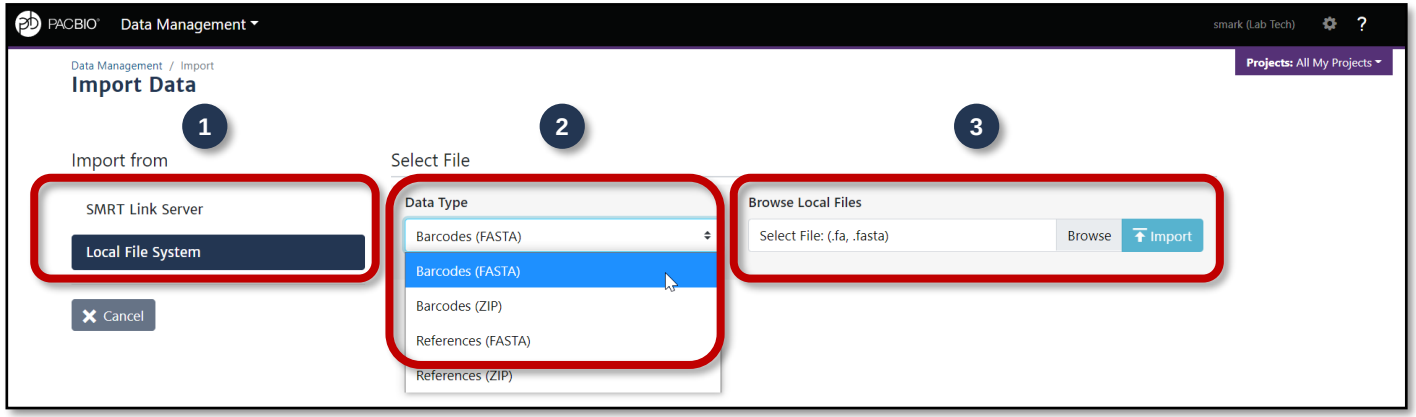
1. Download the FASTA file containing the relevant barcode sequences from PacBio's Multiplexing website or contact PacBio Technical Support to obtain a copy of the appropriate Barcode FASTA file. For example:
   - **`HiFiViral_SARS-CoV-2_M13barcodes`** FASTA file contains a list of 32 Forward and 32 Reverse M13 barcodes for use with the Barcoded M13 Primer Plate included in HiFiViral SARS-CoV-2 Kit (102-132-000)



IMPORTING THE BARCODE FASTA FILE INTO SMRT LINK FOR AUTOMATED DEMULTIPLEXING OF HiFiViral SARS-Cov-2 LIBRARY SAMPLES (CONT.)

2. Import the desired FASTA file into SMRT Link.
   i. On the SMRT Link Home Page, select **Data Management**.
   ii. Click **Import Data** and follow the steps below:

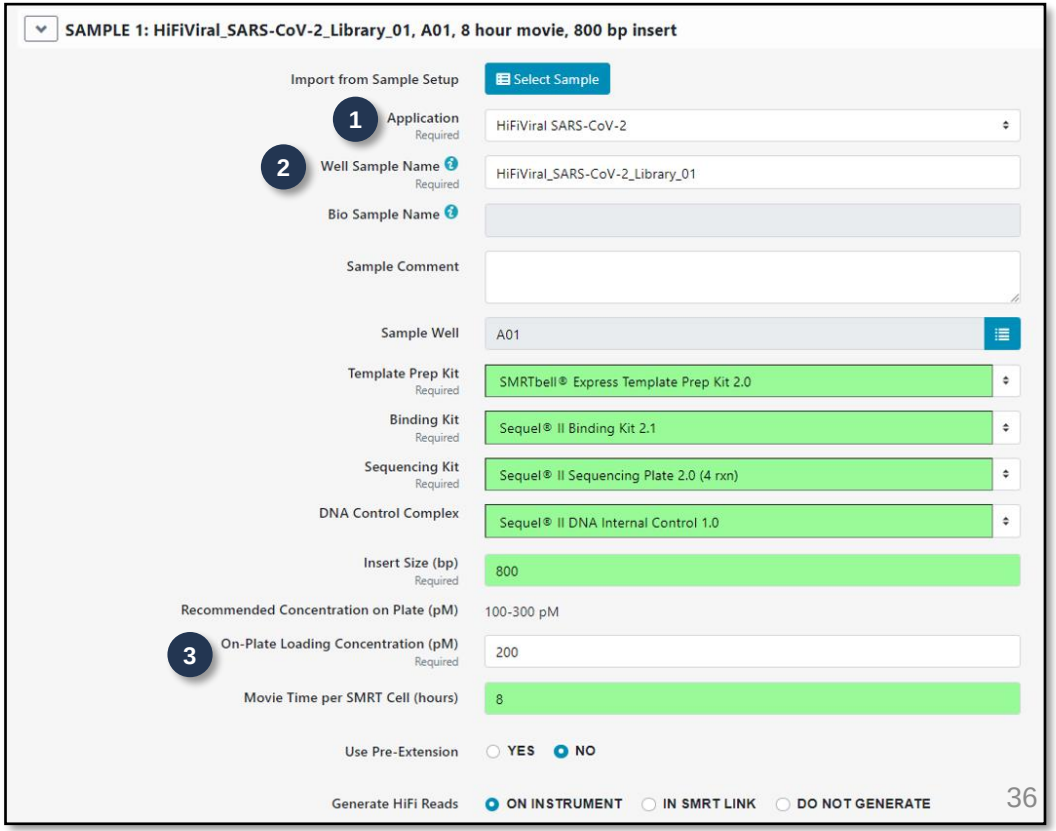
1. Specify whether to import data from the **SMRT Link Server**, or from a **Local File System**. (**Note:** Only references and barcodes are available if you select **Local File System**.)
2. Select the data type to import: **Barcodes** – FASTA (.fa or .fasta), XML (.barcodeset.xml), or ZIP files containing barcodes.
3. Navigate to the appropriate file and click **Import**. The selected barcode filed is imported and becomes available for viewing in the SMRT Link Data Management module home screen.



SMRT LINK RUN DESIGN SETUP PROCEDURE FOR HiFiViral SARS-Cov-2 LIBRARY SAMPLES

A. Specifying Sample Information and Movie Collection Parameters

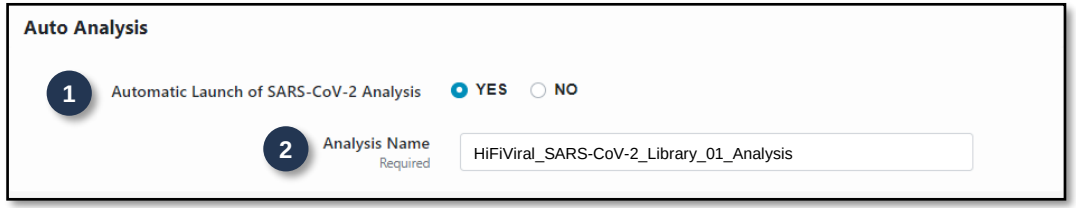
1. Under Application Type, select '**Viral Sequencing / HiFiViral SARS-CoV-2**'
   - Verify all default values in auto-filled sample information fields match the recommended values shown in ***Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems*** (101-769-100) for preparing HiFiViral samples for sequencing
2. Enter a Well Sample Name for your library sample
3. We recommend using a starting on-plate concentration (OPLC) = 200 pM and adjusting higher or lower if needed to achieve optimal *P1* loading



SMRT LINK RUN DESIGN SETUP PROCEDURE FOR HiFiViral SARS-Cov-2 LIBRARY SAMPLES (CONT.)

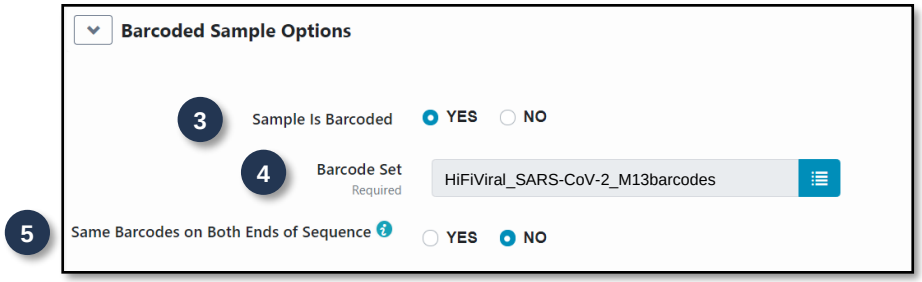
B. Enabling Automated SARS-CoV-2 Data Analysis in SMRT Link and Specifying Sample Barcoding Information

1. **To enable automated SARS-CoV-2 data analysis in SMRT Link:**



1. Select YES for '**Automatic Launch of SARS-CoV-2 Analysis**'
2. Enter an **Analysis Name**

2. **Under Barcoded Sample Options, the following options are automatically specified if *HiFiViral SARS-CoV-2* is selected for Application Type:**



3. Sample is Barcoded: **Yes**
4. Barcode Set: **`HiFiViral_SARS-CoV-2_M13barcodes`**
5. Same Barcodes on Both Ends of Sequence: **No**

SMRT LINK RUN DESIGN SETUP PROCEDURE FOR HiFiViral SARS-Cov-2 LIBRARY SAMPLES (CONT.)

3. Specify Barcode assignments and Bio Sample Names as follows:

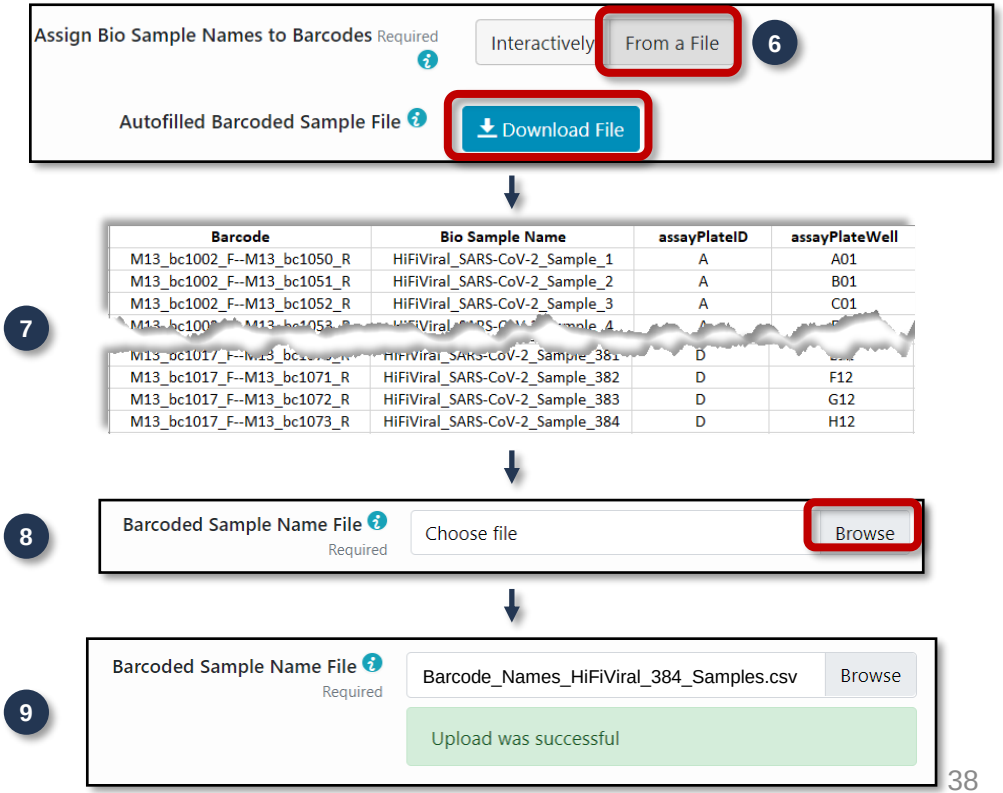
6. Under **Assign Bio Sample Names to Barcodes**: Click **From a File**, then click **Download File**.

7. Edit the file and enter the **biological sample name**, **Plate ID** and **Plate Well** associated with each unique forward + reverse barcode pair listed in the first column; then save the file.
   - Delete entire rows of barcodes not used
   - Allowed characters*: Alphanumeric; dot; underscore; hyphen. Other characters will be automatically removed.

8. Browse for the Barcoded Sample File you just edited and click on Open.

9. You will see 'Upload was successful' appear on the line below, assuming the file is formatted correctly.

Refer to "**Working with Barcoded Data**" section in the **SMRT Link User Guide** for further details on how to specify barcode setup and sample name information in a Run Design



* ***DO NOT includes spaces*** *– Sample Names must be unique and will be truncated after any spaces.*

Multiplexed SARS-CoV-2 Data Analysis Recommendations

USE SMRT LINK TO EASILY ANALYZE MULTIPLEXED HIFI DATA FROM SARS-CoV-2 SURVEILLANCE SAMPLES

Analyze HiFiViral SARS-CoV-2 HiFi Data Using SMRT Link* by Creating an **Auto Analysis** in Run Design or by Performing a **Manual Analysis** in SMRT Analysis

Creating an Auto Analysis in Run Design

- ***HiFiViral SARS-CoV-2 Analysis Application*** can be run using the **Auto Analysis** feature available in SMRT Link Run Design
- This optional Run Design feature allows users to **automatically** complete all necessary analysis steps immediately after sequencing on the Sequel II and IIe Systems **without manual intervention**
- HiFiViral Auto Analysis workflow **automatically** launches CCS Analysis, Demultiplex Barcodes, and HiFiViral SARS-CoV-2 Analysis.

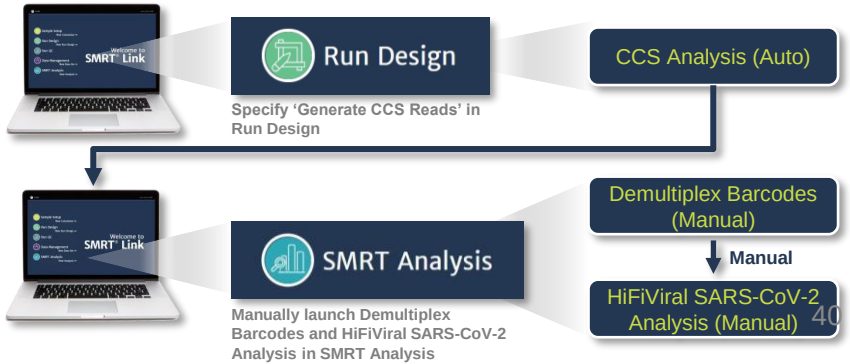
HiFiViral SARS-CoV-2 Auto Analysis Workflow

** Analysis is supported for samples isolated from individual humans and has not been designed or validated for use with other sample types (e.g., wastewater samples).*

Performing a Manual Analysis in SMRT Analysis

- ***HiFiViral SARS-CoV-2* Analysis Application** can also be run by performing a **manual analysis** in SMRT Link SMRT Analysis
- This process requires users to **manually** prepare input data for the HiFiViral SARS-CoV-2 Analysis Application
- HiFiViral manual analysis workflow requires **manually** specifying CCS Analysis ('Generate HiFi Reads') in Run Design, and then **manually** launching Demultiplex Barcodes and HiFiViral SARS-CoV-2 Analysis applications in SMRT Analysis

HiFiViral SARS-CoV-2 Manual Analysis Workflow

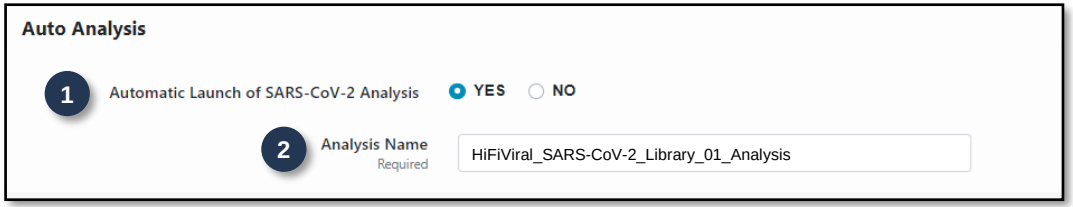


HiFiViral SARS-CoV-2 ANALYSIS SETUP – AUTO ANALYSIS

How to Use SMRT Link Run Design to Create an Auto Analysis

A. Specify Auto Analysis in Run Design

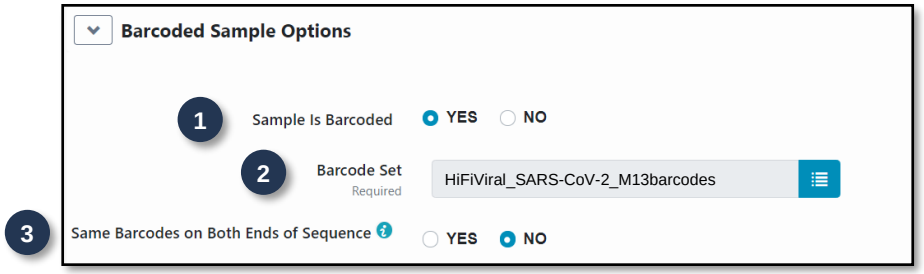
1. Under **Auto Analysis**, select YES for '**Automatic Launch of SARS-CoV-2 Analysis**'
2. Enter an **Analysis Name**



B. Specify Barcoded Sample Options

Under **Barcoded Sample Options**, the following options are automatically specified if *HiFiViral SARS-CoV-2* is selected for Application Type in Run Design:

1. Sample is Barcoded: **Yes**
2. Barcode Set: **`HiFiViral_SARS-CoV-2_M13barcodes`**
3. Same Barcodes on Both Ends of Sequence: **No**



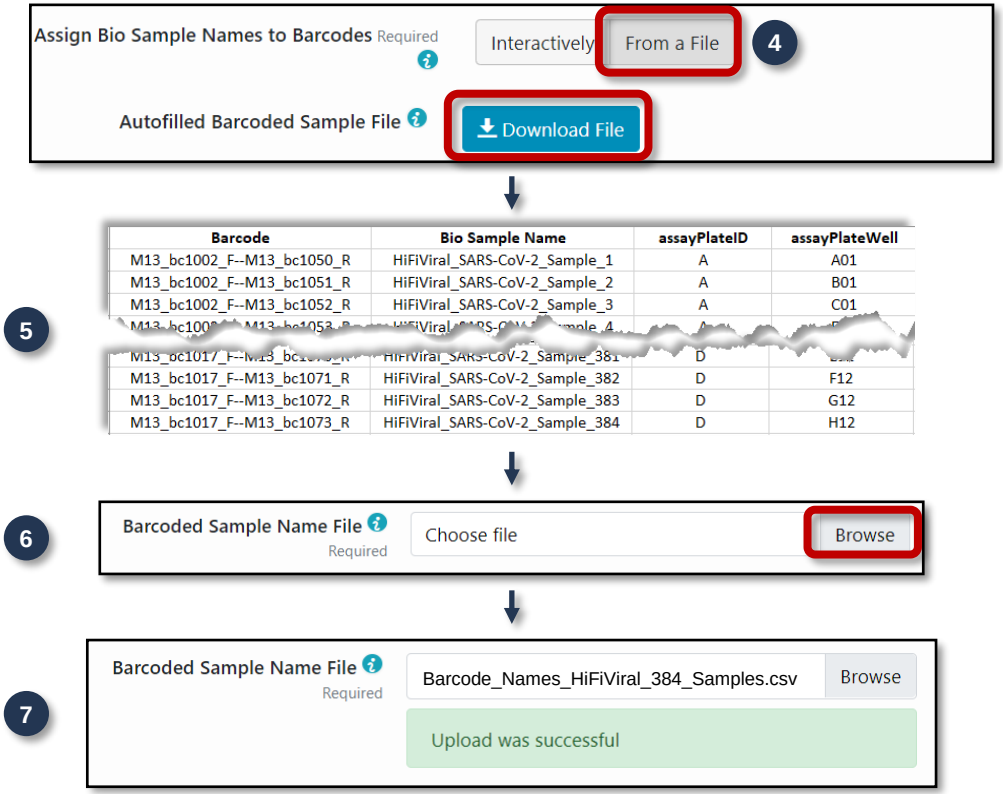
How to Use SMRT Link Run Design to Create an Auto Analysis (Cont.)

4. Under **Assign Bio Sample Names to Barcodes**: Click **From a File**, then click **Download File**.

5. Edit the file and enter the **biological sample name**, **Plate ID** and **Plate Well** associated with each unique forward + reverse barcode pair listed in the first column; then save the file.
   - Delete entire rows of barcodes not used
   - Allowed characters*: Alphanumeric; dot; underscore; hyphen. Other characters will be automatically removed.

6. Browse for the Barcoded Sample File you just edited and click on Open.

7. You will see 'Upload was successful' appear on the line below, assuming the file is formatted correctly.



Refer to "**Working with Barcoded Data**" section in the **SMRT Link User Guide** for further details on how to specify barcode setup and sample name information in a Run Design

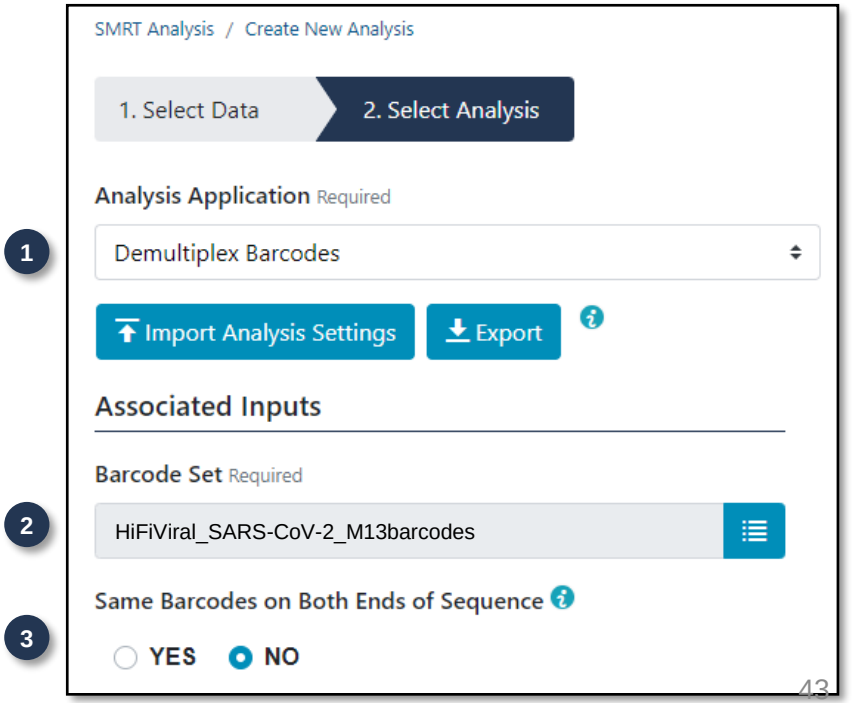
* ***DO NOT include spaces*** *– Sample Names must be unique and will be truncated after any spaces.*

HiFiViral SARS-CoV-2 ANALYSIS SETUP – MANUAL ANALYSIS

How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis

A. Prepare Input Data for the HiFiViral SARS-CoV-2 Analysis Application by Running Demultiplex Barcodes

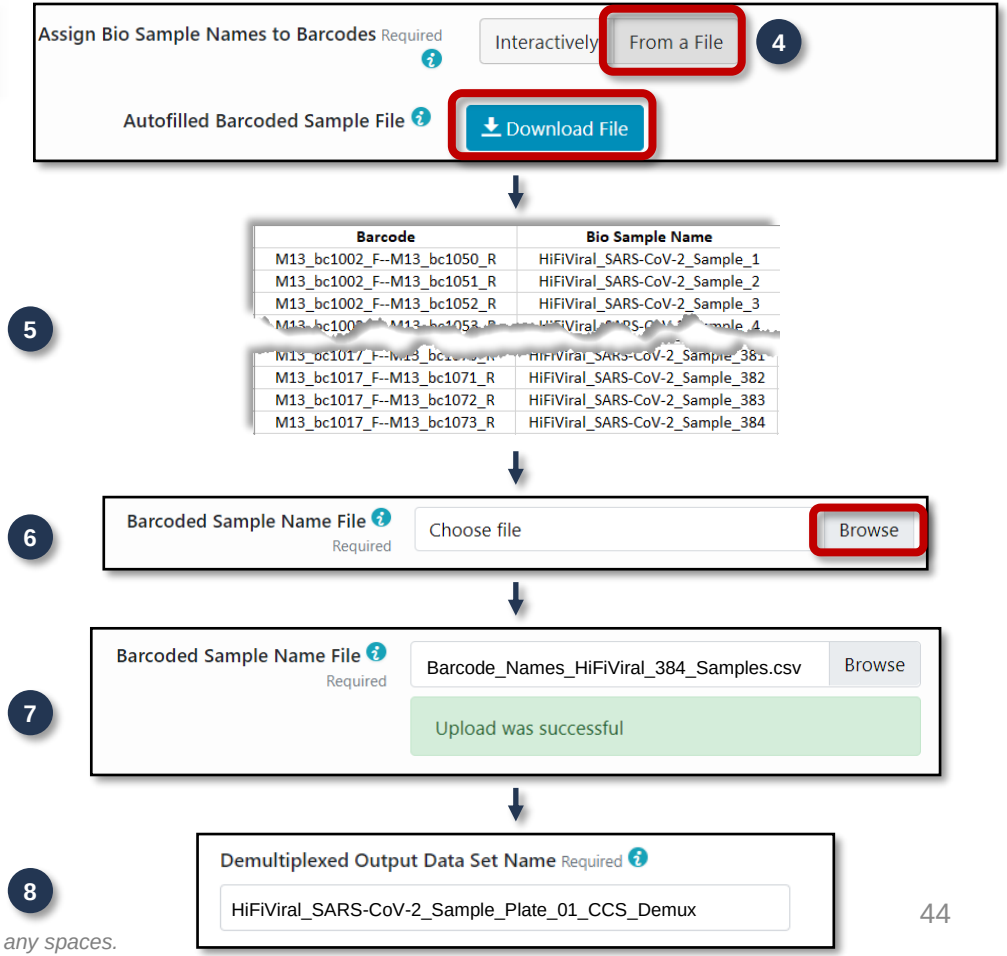
1. In SMRT Analysis, select the SMRT Link **Demultiplex Barcodes** application, where the input to that application are HiFi Reads. (If HiFi Reads have not already been generated on the instrument, run CCS Analysis first.)
2. Barcode Set: Select **`HiFiViral_SARS-CoV-2_M13barcodes`**
3. Barcodes on Both Ends of Sequence: Select **No**



How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis (Cont.)

4. Under **Assign Bio Sample Names to Barcodes**: Click **From a File**, then click **Download File**.
5. Edit the file and enter the **biological sample name** associated with each unique forward + reverse barcode pair listed in the first column; then save the file.
   - Delete entire rows of barcodes not used
   - Allowed characters*: Alphanumeric; dot; underscore; hyphen. Other characters will be automatically removed.
6. Browse for the Barcoded Sample File you just edited and click on Open.
7. You will see 'Upload was successful' appear on the line below, assuming the file is formatted correctly.
8. Enter a Name for the Demultiplexed Output Data Set.

Refer to "**Working with Barcoded Data**" section in the **SMRT Link User Guide** for further details on how to specify barcode setup and sample name information in a Run Design

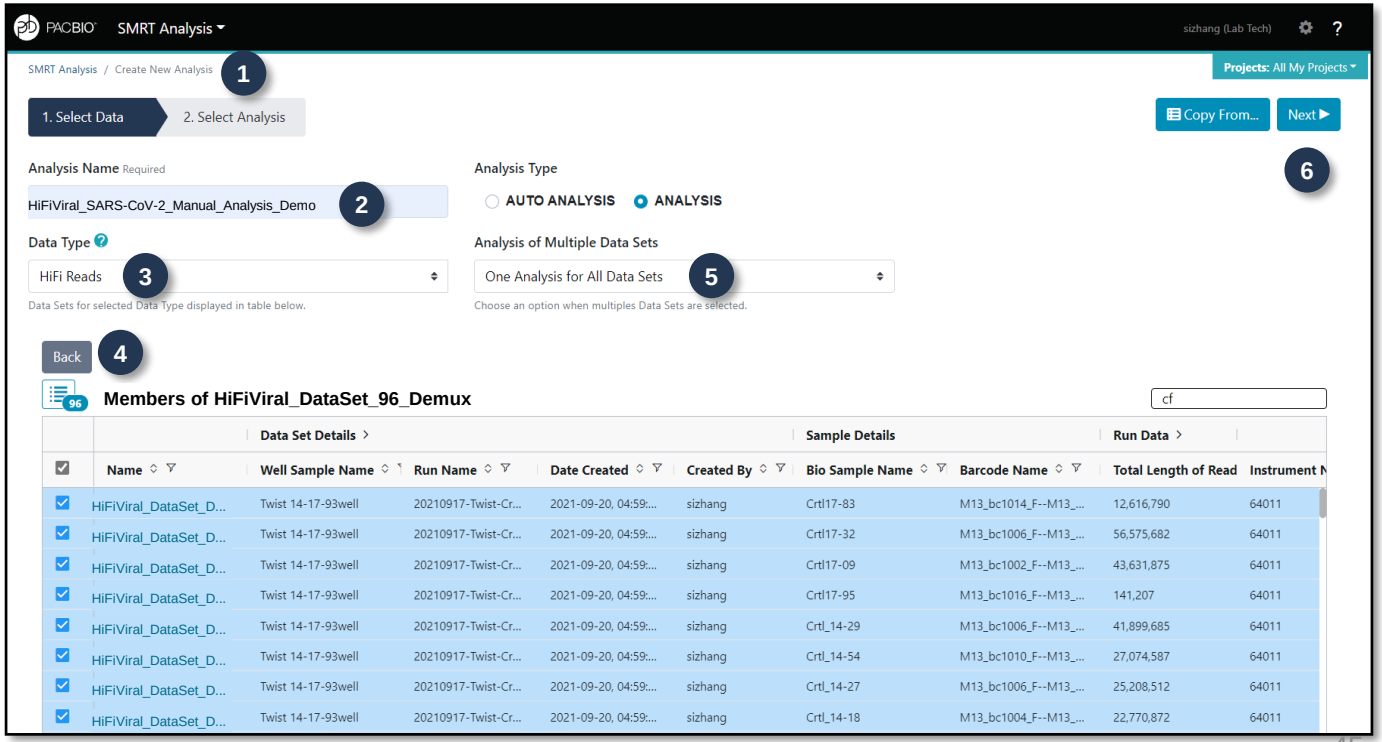


* ***DO NOT include spaces*** *– Sample Names must be unique and will be truncated after any spaces.*

How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis (Cont.)

B. Set Up and Launch HiFiViral Analysis Application

1. After running the Demultiplex Barcodes application, create a new analysis using **SMRT Analysis > Create New Analysis**.
2. Name the analysis
3. Select **Data Types > HiFi Reads**.
4. Select all the demultiplex samples contained in the Data Set and choose **Analysis of Multiple Data Sets > One Analysis for All Data Sets**.
5. Under Analysis of Multiple Data Sets, specify '**One Analysis for All Data Sets**'
5. Click **Next**.



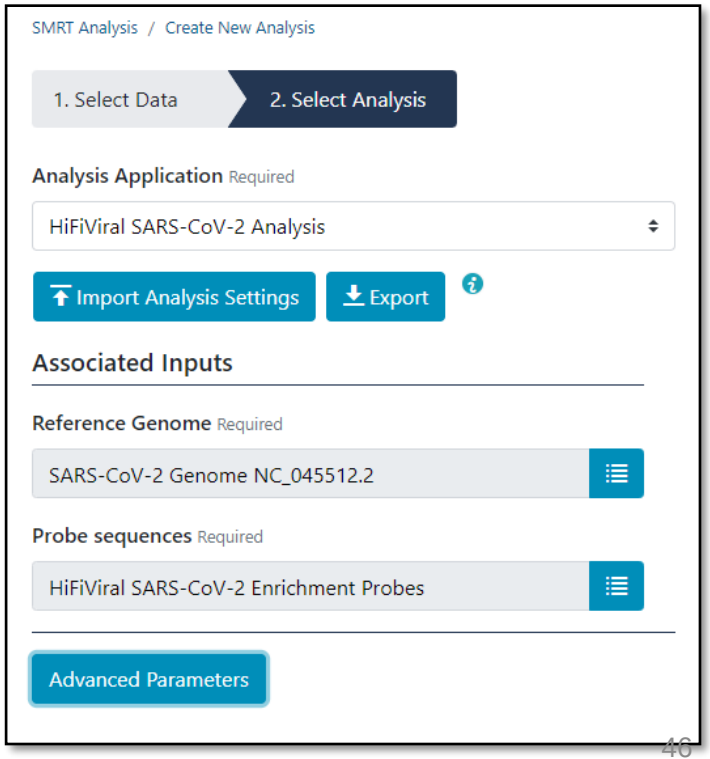
How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis (Cont.)

B. Set Up and Launch HiFiViral Analysis Application (Cont.)

6. Select HiFiViral SARS-CoV-2 Analysis from the **Analysis Application** list.

7. Under **Associated Inputs**, SARS-CoV-2 Genome NC_045512.2 (the Wuhan reference genome) and Probe Sequences v1 are automatically loaded; advanced users may select a different reference or probe set if desired.

8. To generate the optional **Plate QC** graphical summary, click **Advanced Parameters** and load a CSV file using the provided template (assayPlateQC_template_4by96.csv) as a guide.



How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis (Cont.)

B. Set Up and Launch HiFiViral Analysis Application (Cont.)

9 Under **Advanced Parameters**, download the provided CSV template (`assayPlateQC_template_4by96.csv`) as a guide and edit the file.

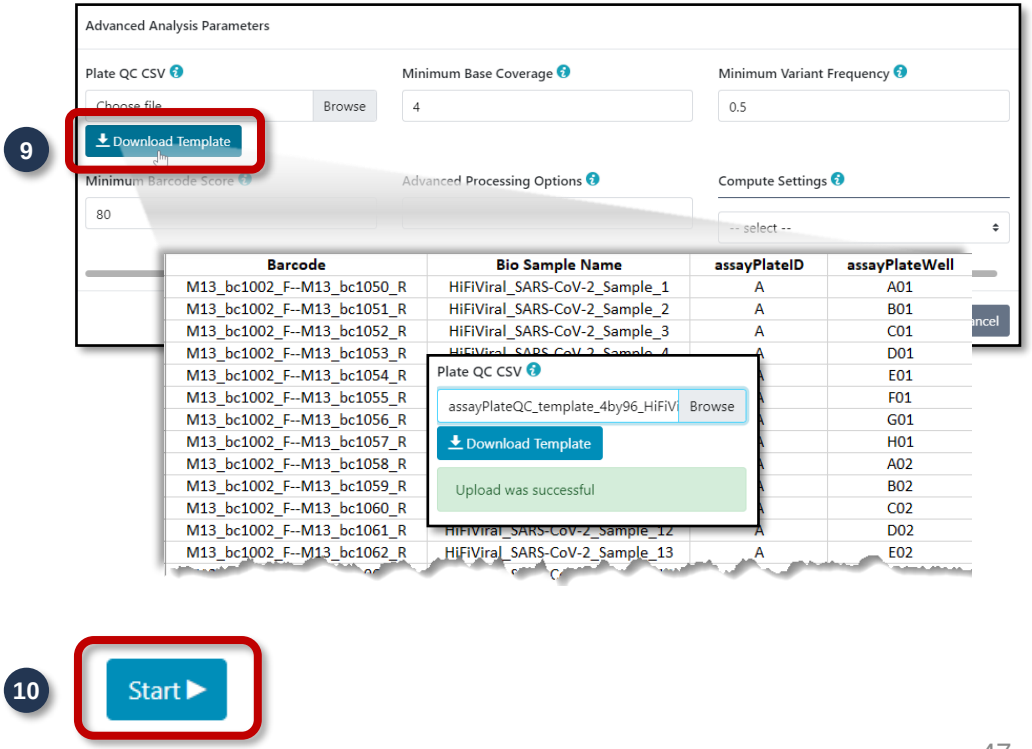
Enter the **biological sample name**, **Plate ID** and **Plate Well** associated with each unique forward + reverse barcode pair listed in the first column; then save the file.

- Delete entire rows of barcodes not used
- Allowed characters*: Alphanumeric; dot; underscore; hyphen. Other characters will be automatically removed.

Browse for the Plate QC File you just edited and click on Open.

You will see 'Upload was successful' appear on the line below, assuming the file is formatted correctly.

10 Click **Start** to start the analysis.



* ***DO NOT include spaces*** *– Sample Names must be unique and will be truncated after any spaces.*

How to Use SMRT Link SMRT Analysis to Perform a Manual Analysis (Cont.)

Comparison of CSV Templates for Demultiplex Barcodes Analysis and HiFiViral SARS-CoV-2 Assay Plate QC Analysis

Demultiplex Barcodes

Barcode	Bio Sample Name
M13_bc1002_F--M13_bc1050_R	HiFiViral_SARS-CoV-2_Sample_1
M13_bc1002_F--M13_bc1051_R	HiFiViral_SARS-CoV-2_Sample_2
M13_bc1002_F--M13_bc1052_R	HiFiViral_SARS-CoV-2_Sample_3
M13_bc1002_F--M13_bc1053_R	HiFiViral_SARS-CoV-2_Sample_4
M13_bc1017_F--M13_bc1070_R	HiFiViral_SARS-CoV-2_Sample_381
M13_bc1017_F--M13_bc1071_R	HiFiViral_SARS-CoV-2_Sample_382
M13_bc1017_F--M13_bc1072_R	HiFiViral_SARS-CoV-2_Sample_383
M13_bc1017_F--M13_bc1073_R	HiFiViral_SARS-CoV-2_Sample_384

CSV Template contains <u>two</u> columns

HiFiViral SARS-CoV-2 Assay Plate QC

Barcode	Bio Sample Name	assayPlateID	assayPlateWell
M13_bc1002_F--M13_bc1050_R	HiFiViral_SARS-CoV-2_Sample_1	A	A01
M13_bc1002_F--M13_bc1051_R	HiFiViral_SARS-CoV-2_Sample_2	A	B01
M13_bc1002_F--M13_bc1052_R	HiFiViral_SARS-CoV-2_Sample_3	A	C01
M13_bc1002_F--M13_bc1053_R	HiFiViral_SARS-CoV-2_Sample_4	A	D01
M13_bc1017_F--M13_bc1070_R	HiFiViral_SARS-CoV-2_Sample_381	D	E12
M13_bc1017_F--M13_bc1071_R	HiFiViral_SARS-CoV-2_Sample_382	D	F12
M13_bc1017_F--M13_bc1072_R	HiFiViral_SARS-CoV-2_Sample_383	D	G12
M13_bc1017_F--M13_bc1073_R	HiFiViral_SARS-CoV-2_Sample_384	D	H12

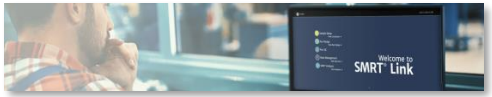
CSV Template contains <u>four</u> columns

When editing CSV templates for Demultiplex Barcodes analysis and HiFiViral SARS-CoV-2 Assay Plate QC analysis:

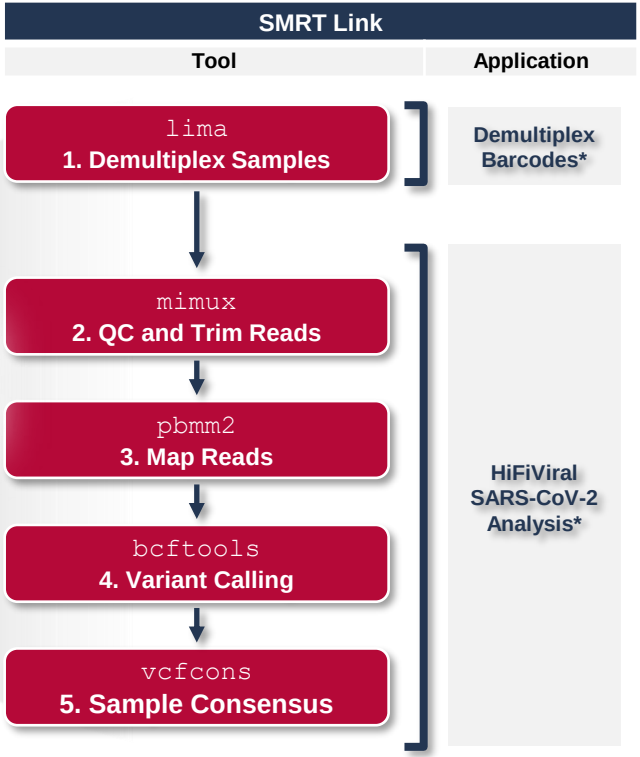
- Delete entire rows of barcodes not used
- Allowed characters*: Alphanumeric; dot; underscore; hyphen. Other characters will be automatically removed.
  - → **DO NOT** include spaces – Sample Names must be unique and will be truncated after any spaces.

HiFiViral SARS-CoV-2 ANALYSIS WORKFLOW

SMRT Link HiFiViral SARS-CoV-2 Auto Analysis Workflow Algorithm Descriptions*



1. **Demultiplex barcodes** using the `lima` tool, where the input to that application are HiFi Reads HiFi (≥Q20 CCS) Reads (BAM format).
2. **Process the reads to trim the probe arm sequences** using the `mimux` tool.
3. **Align the reads** to the reference genome using `pbmm2`.
4. **Call and filter variants** using `bcftools`, generating the raw variant calls in VCF file format. Filtering in this step removes low-quality calls (less than Q20), and normalizes indels.
5. **Filter low-frequency variants** using `vcfcons` and generate a consensus sequence by injecting variants into the reference genome. At each position, a variant is called only if both the base coverage exceeds the minimum base coverage threshold and the fraction of reads that support this variant is above the minimum variant frequency threshold.



** The SMRT Link Demultiplex Barcodes and HiFiViral SARS-CoV-2 Analysis Applications must each be launched **manually** if Auto Analysis is not specified in Run Design when setting up a sequencing run on Sequel II or IIe Systems with HiFiViral SARS-CoV-2 Kit library samples.*

HiFiViral SARS-CoV-2 ANALYSIS OUTPUTS

SMRT Link HiFiViral SARS-CoV-2 Analysis Application Outputs

- Per-sample analysis outputs include:
  - ❑ Consensus sequence (FASTA)
  - ❑ Variant calls (VCF)
  - ❑ HiFi Reads aligned to the reference (BAM)
  - ❑ Sample Summary table including: Count of variable sites, genome coverage, read coverage, and probability of multiple strains, and other metrics
  - ❑ Plot of HiFi Read coverage across the SARS-CoV-2 genome

File Downloads

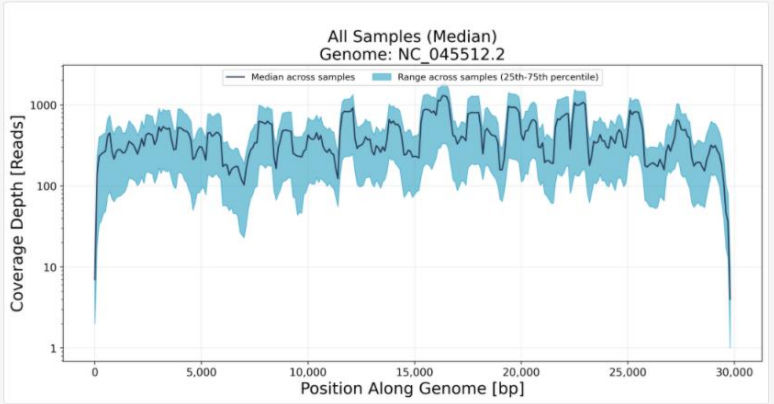
Edit Output File Name Prefix **Example:**analysis-Twist Bioscience Control 17-136917

File	Size	Type
All Samples, Probe Counts TSV	935 KB	zip
Sample Summary Table CSV	9 KB	csv
All Samples, Raw Variant Call VCF	267 KB	zip
All Samples, Consensus Sequence Aligned BAM	819 KB	zip
All Samples, HiFi Reads Mapped BAM	515 MB	zip
All Samples, Variant Call VCF	250 KB	zip
Analysis Log	737 KB	log
All Samples, Genome Coverage Plots	30 MB	zip

Sample Summary

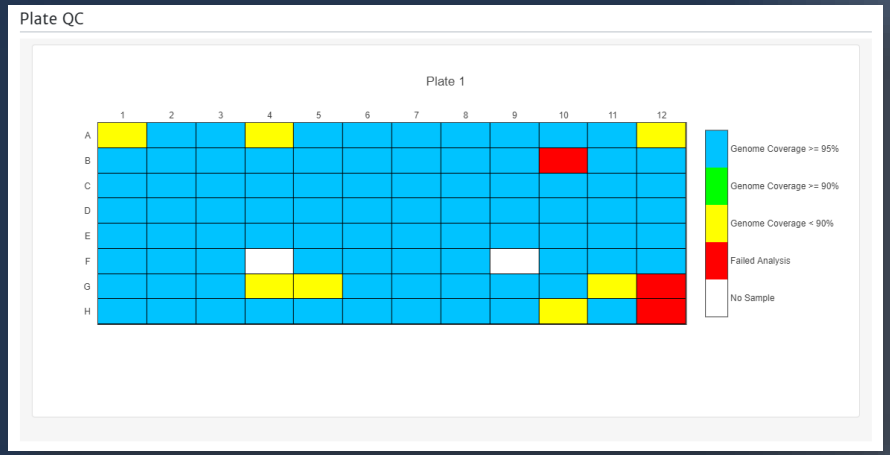
Bio Sample Name	Substitutions	Insertions	Deletions	Reads	Read Coverage	On-Target Rate	Multiple Strains (Probability)	Ns	Genome Coverage
Sample 1	36	0	3	12,964	288	99.99%	No (0.00)	156	99.47%
Sample 2	38	0	3	1,075	24	99.81%	No (0.00)	761	97.45%
Sample 3	40	0	3	2,289	51	99.91%	No (0.00)	219	99.26%

Genome Coverage



SMRT Link HiFiViral SARS-CoV-2 Analysis Application Outputs (Cont.)

- HiFiViral SARS-CoV-2 analysis application also outputs a graphical summary of performance across all samples in assay plate layout for Sample Plate QC evaluation



Lower HiFi read counts due to **evaporation-induced edge-effects** during viral enrichment

HiFi Reads

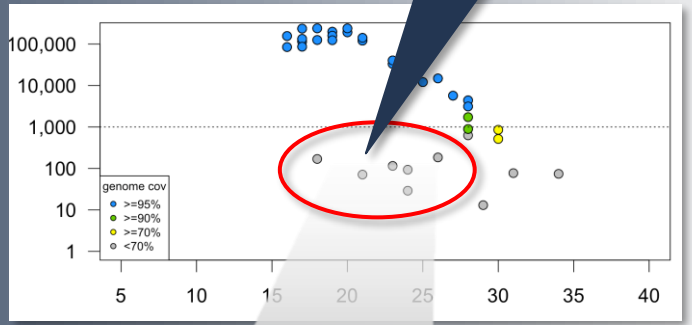
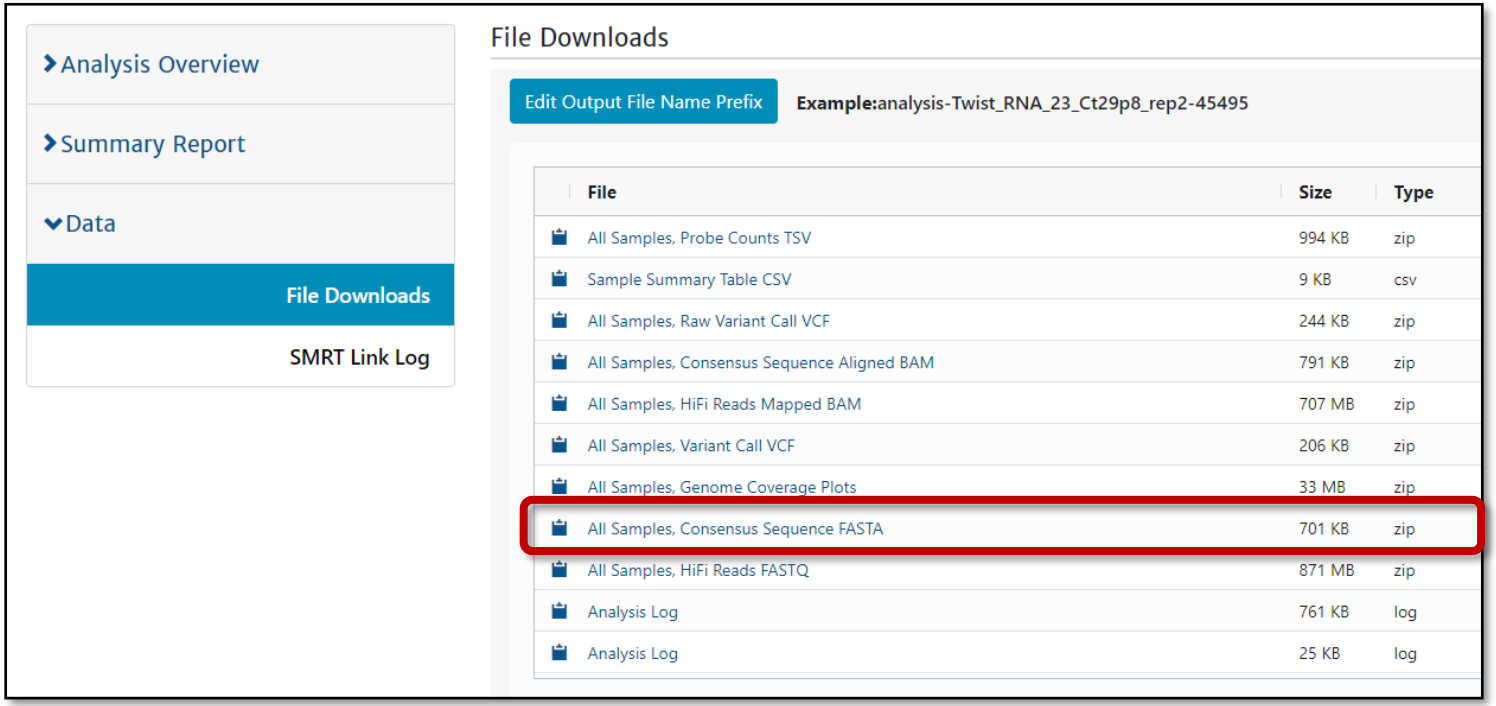


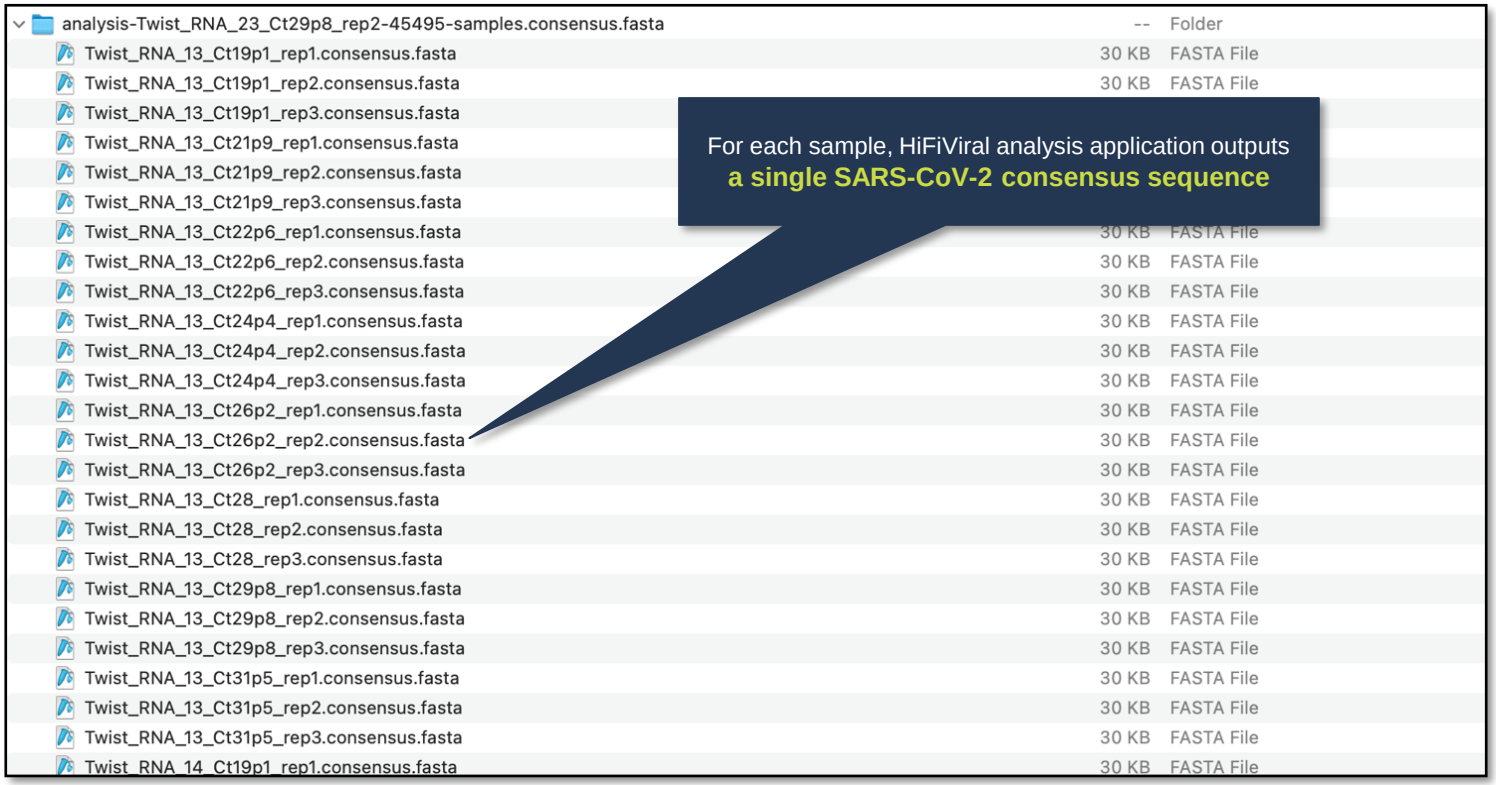
Plate QC

DOWNLOADING HiFiViral SARS-CoV-2 ANALYSIS RESULTS IN SMRT LINK V10.2

To download the HiFiViral SARS-CoV-2 analysis results, click on the File Downloads tab to download the desired output files.



DOWNLOADING HiFiViral SARS-CoV-2 ANALYSIS RESULTS IN SMRT LINK V10.2 (CONT.)

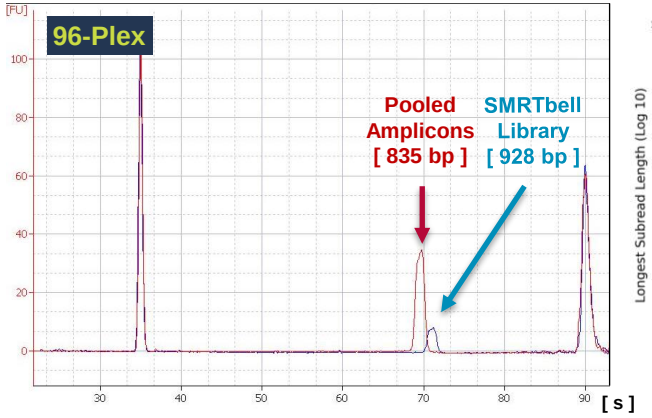


Multiplexed SARS-CoV-2 Library Example Performance Data

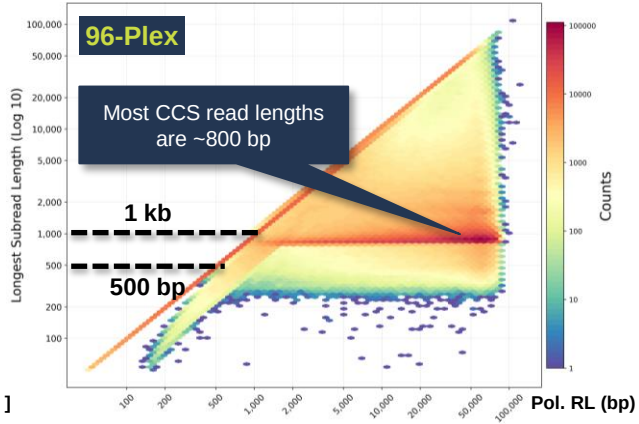
EXAMPLE SEQUENCING PERFORMANCE FOR TWIST SYNTHETIC SARS-CoV-2 RNA CONTROLS [6 X 5 KB FRAGMENTS]

SMRTbell Library QC and Primary Sequencing Metrics for 96-Plex and 384-Plex Twist Control Samples

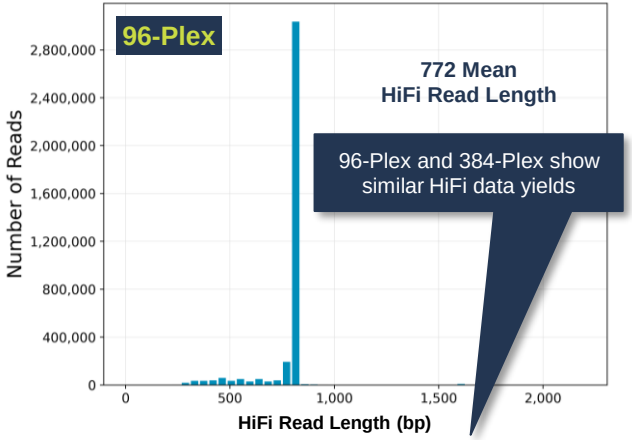
Library QC



Raw Data Report



CCS Analysis Report



	96-Plex	384-Plex
Yield of Pooled Barcoded PCR Products	2049 ng	12,400 ng
Pooled DNA Input for Library Construction	1000 ng	1000 ng
Final Yield of ProNex Bead Purified Library (%)	142 ng (14.2%)	408 ng (40.8%)

Twist 14 and 17 controls (were enriched with the HiFiViral SARS-CoV-2 Kit. Pooled barcoded PCR products were purified with 1.3X ProNex Beads and constructed into SMRTbell libraries with SMRTbell Express TPK 2.0.

	96-Plex	384-Plex
Raw Base Yield	145.6 Gb	139.4 Gb
Mean Polymerase Read Length	26.3 kb	25.1 kb
P0	18.9%	19.7%
P1	69.2%	69.4%
P2	11.9%	10.9%

200 pM on-plate concentration / 8-h movie time / No Pre-Extension Time / No Adaptive Loading

	96-Plex	384-Plex
HiFi Reads	3.6 M	3.5 M
HiFi Base Yield	2.8 Gb	2.8 Gb
Mean HiFi Read Length	772	788
Median HiFi Read Quality	QV60	QV60
HiFi Read Mean # of Passes	21	21

EXAMPLE SEQUENCING PERFORMANCE FOR TWIST SYNTHETIC SARS-CoV-2 RNA CONTROLS [6 X 5 KB FRAGMENTS] (CONT.)

HiFiViral SARS-CoV-2 Auto Analysis Outputs for 96-Plex Twist Control Samples

Summary Report

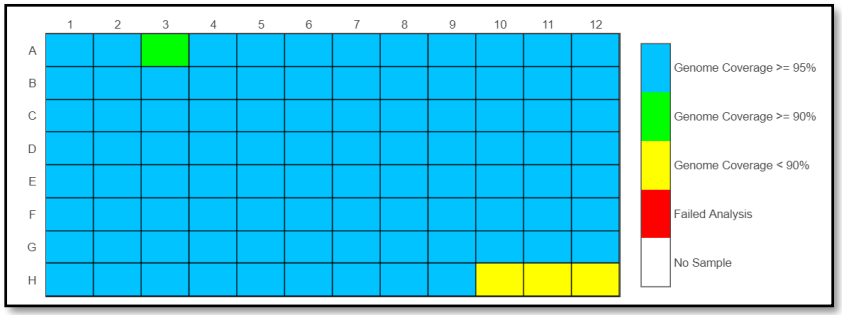
Value	Analysis Metric
96	Samples
93	Samples With Genome Coverage > 90%
92	Samples With Genome Coverage > 95%
0	Samples Failing Workflow

- 93 Positive Control samples showed ≥90% genome coverage (Blue and Green wells in Plate QC image)
- 3 Negative Control samples showed <90% genome coverage as expected (Yellow wells)

Sample Summary

Bio Sample Name	Substitutions	Insertions	Deletions	Reads	Read Coverage	On-Target Rate	Multiple Strains (Probability)	Ns	Genome Coverage
Crtl17-96	0	0	0	5	0	100.00%	No (0.00)	29,903	0.00%
Crtl17-31	32	1	1	55,235	1,197	99.99%	No (0.00)	616	97.94%
Crtl_14-27	31	0	4	35,341	762	100.00%	No (0.00)	682	97.72%
Crtl_14-03	30	0	4	9,362	177	100.00%	No (0.02)	1,556	94.79%

Plate QC



HiFiViral SARS-CoV-2 KIT DELIVERS ROBUST GENOME COVERAGE PERFORMANCE ACROSS VARIABLE INPUT QUANTITIES AND MULTIPLEX LEVELS

Example SARS-CoV-2 Genome Coverage Results Obtained for Twist Control Samples

Experimental Design

96-plex prepared with 4 Synthetic Twist RNA Controls at 8 input quantities in replicates of 3.

TWIST CONTROL	VARIANT	PART NUMBER
14	Alpha (B.1.1.7)	103907
15	Alpha (B.1.1.7)	103909
16	Beta (B.1.351)	104043
17	Gamma (P.1)	104044

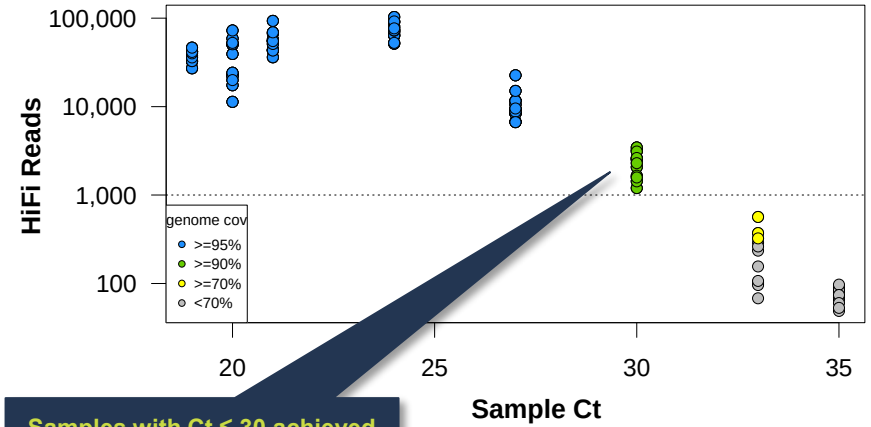
RNA Input Quantity*

SAMPLE CT	COPY NUMBER
19	6 M
20	3 M
21	1 M
24	100,000
27	10,000
30	1,000
33	100
35	3

Input Quantity Input of RNA controls ranged from 6 million copies down to 3. Copy number is converted into Ct scale after Han *et al*. 2021.*

** Han M.S., et al. (2021). RT-PCR for SARS-CoV-2: quantitative versus qualitative. The Lancet Infectious Disease 21(2) p165.*

96-Plex of Twist Control Samples



Samples with Ct ≤ 30 achieved complete genome coverage with 1000 HiFi reads**

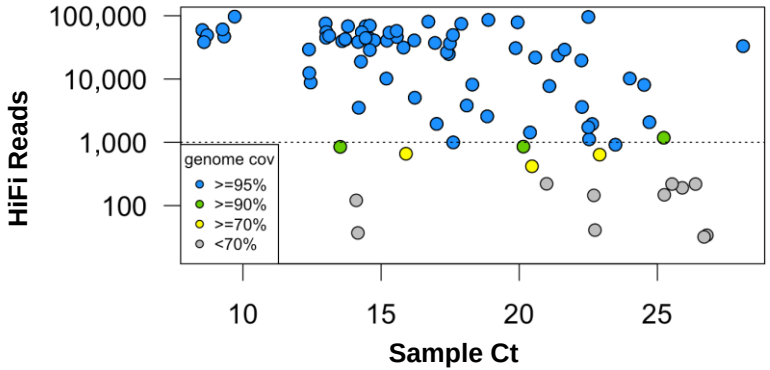
- 4-fold HiFi Read depth required to output a consensus base
- ~1,000 mapped HiFi reads reliably yields ≥90% genome coverage

***** Complete = ≥90% genome coverage***

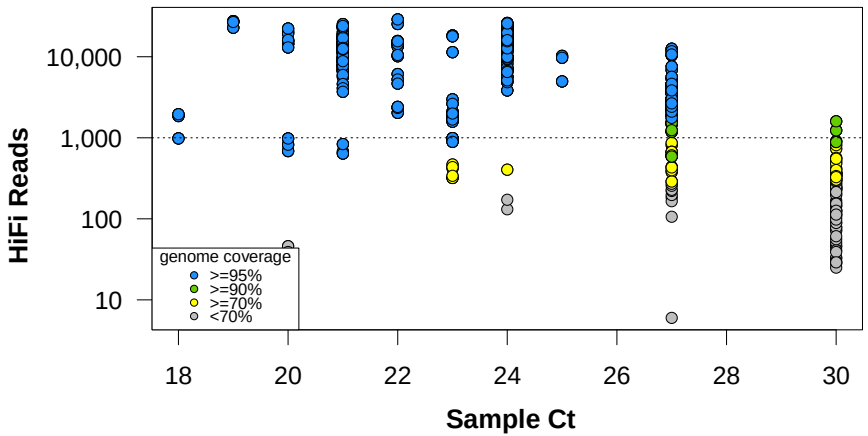
HiFiViral SARS-CoV-2 KIT DELIVERS ROBUST GENOME COVERAGE PERFORMANCE ACROSS VARIABLE INPUT QUANTITIES AND MULTIPLEX LEVELS (CONT.)

Example SARS-CoV-2 Genome Coverage Results Obtained for Surveillance Samples

96-plex of "Real" Samples for Surveillance



384-plex of Controls and Nasopharyngeal Extracts



Genome Completeness in Surveillance Samples

SAMPLE INPUT	NO. OF SAMPLES	> 90% GENOME COVERAGE
Known Ct	84	83%
Unknown Ct	9	44%
Twist Controls	2	100%
Negative Control	1	0

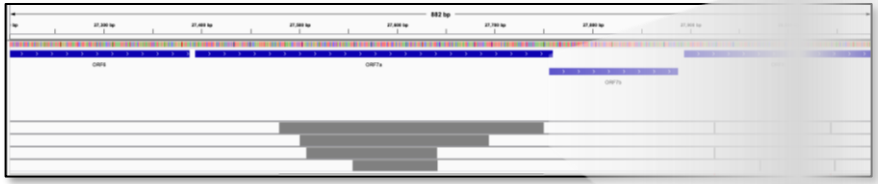
Genome Completeness in 384-plex

SAMPLE INPUT	NO. OF SAMPLES	> 90% GENOME COVERAGE
Controls (Ct<30)	216	90%
NP Extracts	144	85%

HiFiViral SARS-CoV-2 KIT ENABLES COMPREHENSIVE CHARACTERIZATION OF VARIANTS FOR SURVEILLANCE AND COVID-19 RESEARCH

SARS-CoV-2 Variant Calling Achieves High Precision and Recall for Characterization of SNVs and SVs

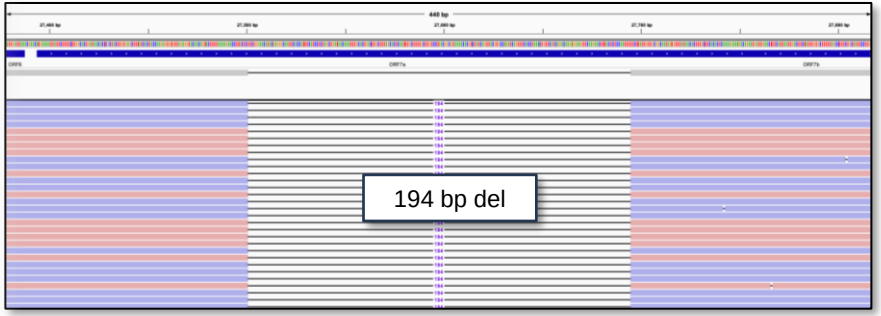
Recovery of Large Deletions in ORF7a



Deletions (87 – 271 bp) are called in VCF and consensus sequence.

SNV Calling & Strain Assignment for Controls in a 384-plex

CONTROL SAMPLE	NEXTCLADE ASSIGNMENT	COMPLETE GENOMES	PRECISION	RECALL	NEXTSTRAIN ACCURACY
Twist 01	19A	29	1	94.8%	100%
Twist 13	20C	24	1	99.7%	100%
Twist 14	20I (Alpha, V1)	25	1	99.9%	100%
Twist 15	20I (Alpha, V1)	24	1	99.9%	100%
Twist 16	20H (Beta, V2)	24	1	100%	100%
Twist 17	20J (Gamma, V3)	24	1	100%	100%
Twist 23	21A (Delta)	24	99.1%	99.4%	100%



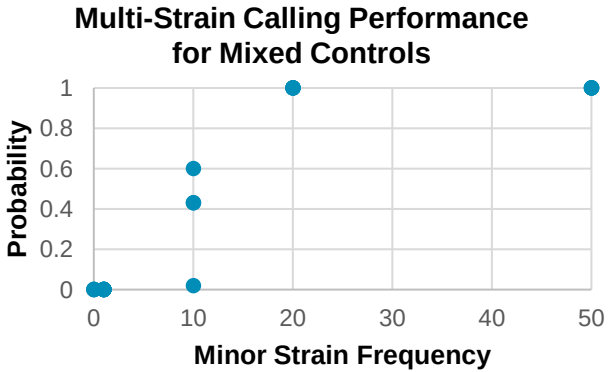
Example visualizations of HiFi reads spanning around large deletions.

- **HiFi reads can detect SNVs and SVs with high precision and recall for accurate SARS-CoV-2 strain assignment**

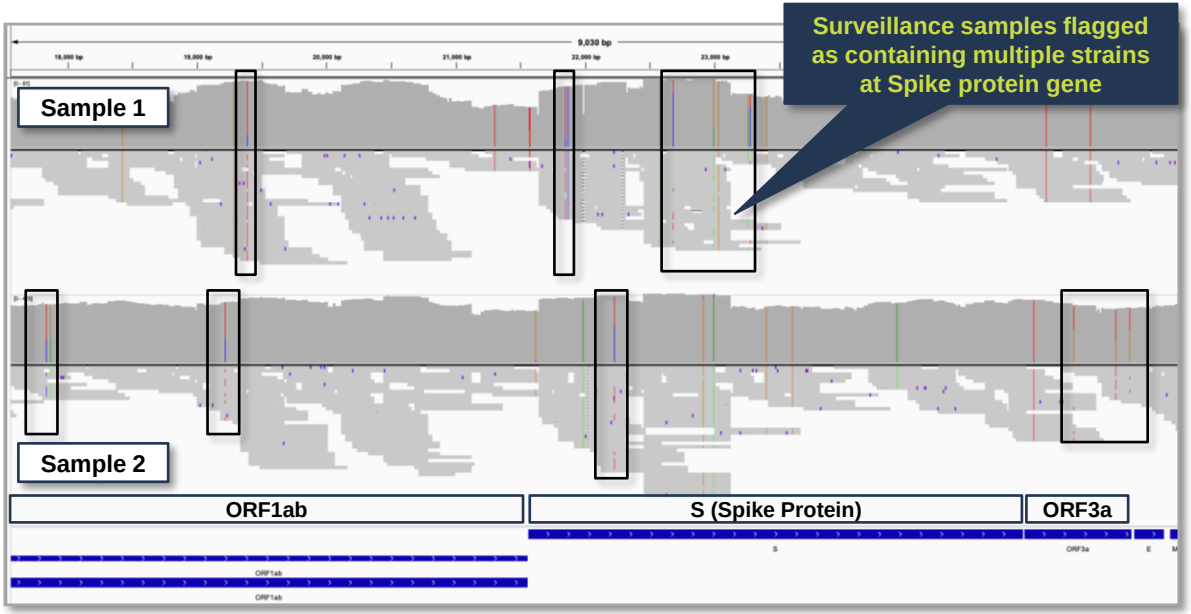
HiFiViral SARS-CoV-2 KIT ENABLES DETECTION OF MINOR VARIANTS AND MULTIPLE STRAINS* IN THE SAME SAMPLE

Mixed Control Experiment

- Titrated mixed controls
- Minor frequency: 1% to 50%
- Binomial model for multi-strain detection*
- Achieve *P* > 95% at >20% minor frequency**



Detection of Minor Variants in Surveillance Samples



Possible Sources of Multiple Strains in Sample

- Sample contamination, lab error, infection with multiple strains
- We recommend users confirm presence of multiple strains with additional experiments

* *Multi-strain detection is supported for samples with Ct < 26*
** *Power of detection increases with more variable sites.*

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 SARS-CoV-2 Workflow Updates and Other Resources

Sample Preparation Literature

- Procedure & Checklist – PacBio HiFiViral High-Throughput Multiplexing for Full-Viral Genome Sequencing of SARS-CoV-2 (102-188-800)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II/IIe Systems (101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (101-851-300)
- Application Brief: HiFiViral Full-Viral Genome Sequencing – Best practices (BP110-111121)
- Application Note: HiFiViral Full-Viral Genome Sequencing (102-194-700) [*Coming Soon*]
- Technical Overview: Multiplexed Library Preparation for Full-Viral Genome Sequencing Using HiFiViral SARS-CoV-2 Kit (102-205-300)

PacBio HiFiViral SARS-CoV-2 Kit Product Overview Video (2021) [**Link**]

FAQ

- HiFiViral SARS-CoV-2 Kit FAQ [Link]

Posters, Videos & Webinars

- PacBio HiFiViral SARS-CoV-2 Kit Product Overview Video (2021) [Link]
- SFAF Poster (2021): HiFiViral SARS-CoV-2: A kitted solution for genome surveillance that is robust across sample input quantities and new variants [Link]
- ASHG Webinar (2021): HiFiViral SARS-CoV-2 Kit: A differentiate solution for surveillance by sequencing [Link]

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

Ordering Information

CONSUMABLE PRODUCT	PART NUMBER
HiFiViral SARS-CoV-2 Kit (384 rxn)	102-132-000
SMRTbell Express Template Prep Kit 2.0 (18 rxn)	100-938-900
SMRT Cell 8M Tray	101-389-001
Sequel II Binding Kit 2.1 and Internal Control 1.0 (24 rxn)	101-843-000
Sequel II Sequencing Kit 2.0 (4 rxn)	101-820-200
SMRTbell Enzyme Cleanup Kit 2.0 (10 rxn)	101-932-600

APPENDIX 1: 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 library preparation

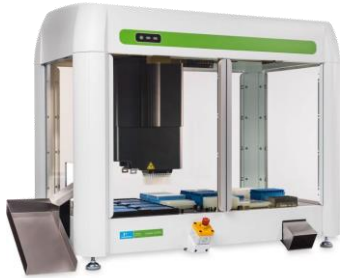
VENDOR	RNA ISOLATION KIT PRODUCT	AUTOMATION PLATFORM
Thermo Fisher Scientific	MagMAX Viral and Pathogen Nucleic Acid Isolation Kit [Link]	KingFisher Flex System
Roche Molecular Systems	MagNA Pure 96 DNA and Viral NA Small Volume Kit [Link]	Roche MagNA Pure-96 (MP6)

APPENDIX 2: Guidance on Workflow Automation For Multiplexed Library SARS-CoV-2 Library Preparation

WORKFLOW AUTOMATION OPTIONS FOR HIGH-THROUGHPUT MULTIPLEXED HiFiViral SARS-CoV-2 SAMPLE PREPARATION

Interested in automating your HiFiViral SARS-CoV-2 sample preparation workflow to achieve higher throughput? Please contact PacBio Support or your local Field Applications Scientist to discuss your needs.

Agilent Bravo Liquid Handler

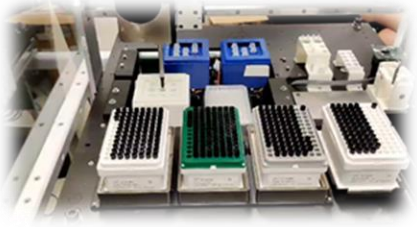


Sciclone G3 NGSx Workstation

Biomek 4000 Workstation

Hamilton Microlab VANTAGE Liquid Handler

Tecan Infinite F-Series Plate Reader



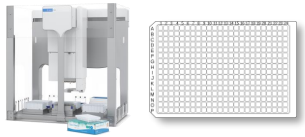
Custom Liquid Handler

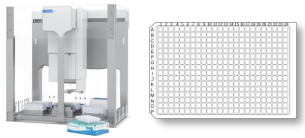
Key Considerations for Workflow Automation

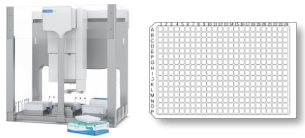
- Liquid handler capabilities, including:
  - Small volume (≥2 µL) and large volume (≥200 µL) transfers
  - Magnetic plate blocks for bead-based purification and buffer exchanges
  - Integrated heating / cooling temperature control
- Microplate reader for high-throughput DNA concentration QC

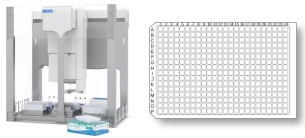
RECOMMENDED STEPS TO AUTOMATE FOR VIRAL GENOME ENRICHMENT WORKFLOW USING HiFiViral SARS-CoV-2 KIT

End-To-End Overall HiFiViral Workflow	Viral Genome Enrichment Workflow Step	On-Deck Processing Steps	Off-Deck Processing Steps*
SARS-CoV-2 Viral Genome Enrichment	cDNA Synthesis & Probe Hybridization Reaction	Dispense enzymatic reagents into 96- or 384-well plate	Thermal Cycler Incubation (O/N)
SMRTbell Library Construction	Circularization (Fill-in) Reaction	Dispense enzymatic reagents into 96- or 384-well plate	Thermal Cycler Incubation (60 min)
Sequencing Preparation	Enzymatic Cleanup Reaction	Dispense enzymatic reagents into 96- or 384-well plate	Thermal Cycler Incubation (65 min)
SMRT Sequencing & Analysis	PCR with Asymmetric Barcoded M13 Primers (F/R)	Dispense enzymatic reagents into 96- or 384-well plate	Thermal Cycler Incubation (1.5 h); DNA Quantification QC (Optional)
	Pool Barcoded Samples	Aspirate sample aliquot from each well and dispense into a single tube	DNA Sizing QC (Optional); DNA Quantification QC (Optional)









** Off-deck processing steps shown may be moved on-deck depending on liquid handler capabilities*

RECOMMENDED STEPS TO AUTOMATE FOR HiFiViral SARS-CoV-2 SMRTBELL LIBRARY CONSTRUCTION WORKFLOW

End-To-End Overall HiFiViral Workflow

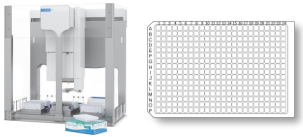
- SARS-CoV-2 Viral Genome Enrichment
- SMRTbell Library Construction
- Sequencing Preparation
- SMRT Sequencing & Analysis

SMRTbell Library Construction Workflow Step

- Prepare Master Mix Plate & DNA Damage Repair Reaction
- End Repair / A-Tailing
- Adapter Ligation
- Nuclease Treatment

On-Deck Processing Steps

Dispense enzymatic reagents into 96- or 384-well plate



Off-Deck Processing Steps*

Thermal Cycler Incubation (30 min)

Thermal Cycler Incubation (60 min)

Thermal Cycler Incubation (60 min)

ProNex Bead Purification

Thermal Cycler Incubation (30 min)

ProNex Bead Purification

DNA Sizing QC

DNA Quantification QC

** Off-deck processing steps shown may be moved on-deck depending on liquid handler capabilities*

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