Technical overview: Multiplexed SARS-CoV-2 library preparation for full-viral genome sequencing using SMRTbell prep kit 3.0

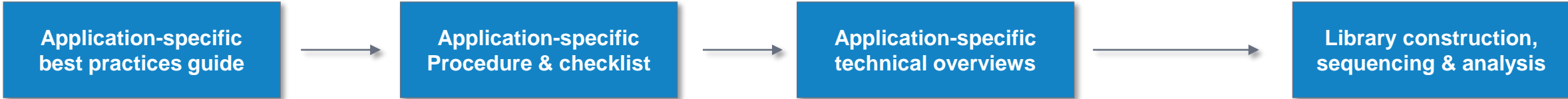Sequel II and IIe systems ICS v11.0 / SMRT Link v11.0

PN 102-399-300 Version 01 (April 2022)

Multiplexed SARS-CoV-2 library preparation for full-viral genome sequencing using SMRTbell prep 3.0

Technical overview

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
7. APPENDIX 1: RNA isolation kit options for full-viral genome sequencing of SARS-CoV-2

8. APPENDIX 2: Guidance on workflow automation for multiplexed library SARS-CoV-2 library preparation

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SARS-CoV-2 full-viral genome sequencing: How to get started

Application-specific best practices guide	→	Application-specific Procedure & checklist	→	Application-specific technical overviews	→	Library construction, sequencing & analysis

Application brief: HiFiViral SARS-CoV-2 for COVID-19 whole genome sequencing – Best practices ([102-193-692](#))

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

Procedure & checklist – PacBio HiFiViral high-throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0 ([102-396-100](#))

Technical documentation containing sample library construction and sequencing preparation protocol details

Technical overview: Multiplexed SARS-CoV-2 library preparation for full-viral genome sequencing using SMRTbell prep 3.0 ([102-399-300](#))

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 prep kit 3.0)

- Multiplex 24 – 384 SARS-CoV-2 samples per SMRT Cell 8M

Sequencing (Sequel II and IIe systems)

- ABC* with Sequel II binding kit 3.1
- 8-hr movie collection time

Data analysis (SMRT Link)

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

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**

SARS-CoV-2 enrichment kit

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End-to-end PacBio protocol for full-viral genome sequencing using the HiFiViral SARS-CoV-2 kit
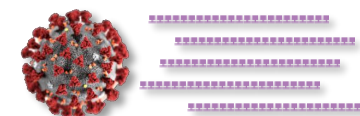
Extracted viral RNA samples

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

Procedure & checklist
April 2022

PacBio Documentation (102-396-100)

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

For targeted enrichment and barcoding Of SARS-CoV-2 PCR-positive samples*

HIFIVIRAL SARS-COV-2 ASSAY KIT

- ☑ 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

Workflow

cDNA synthesis & probe hybridization — **4 – 16 h**

Circularization (Fill-in) & cleanup

PCR with barcoded M13 primers
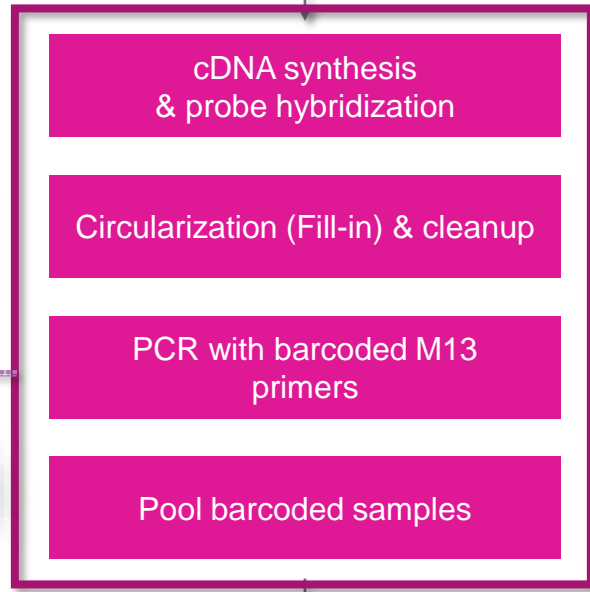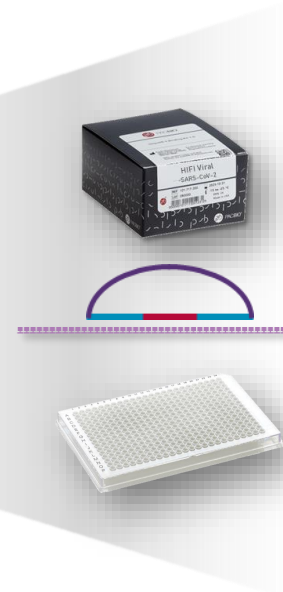
Pool barcoded samples — **4 – 5 h**

SMRTbell library construction & sequencing preparation — **6 h**

SMRT sequencing (Sequel II or IIe system)

HiFiViral SARS-CoV-2 data analysis in SMRT Link — **14 – 16 h****

* HiFiViral SARS-CoV-2 kit demonstrated use cases include RNA-extracted samples such as nasopharyngeal swabs from human SARS-CoV-2 PCR+ cohort samples. Note: The HiFiViral SARS-CoV-2 workflow is not recommended for analysis of wastewater samples.

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HiFiViral SARS-CoV-2 kit uses molecular inversion probe technology for efficient viral genome enrichment

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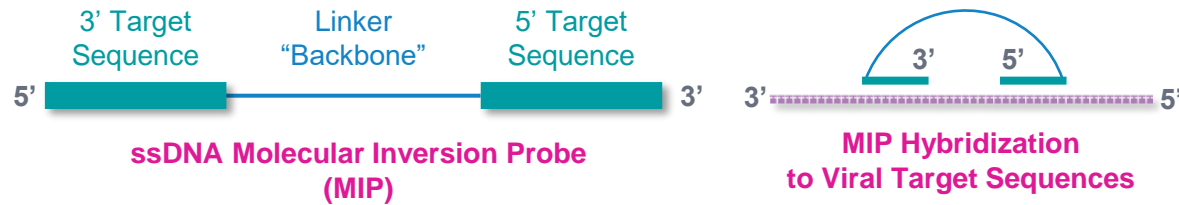
- Single pool of 969 ssDNA MIPs comprised of two probe arms connected by a common linker (30 bp)
- MIPs tile SARS-CoV-2 genome at 22-fold target coverage
- Capture of 675-bp target sequences is performed by circularization of MIPs *via* "fill-In" enzymatic reaction
- PCR amplification using universal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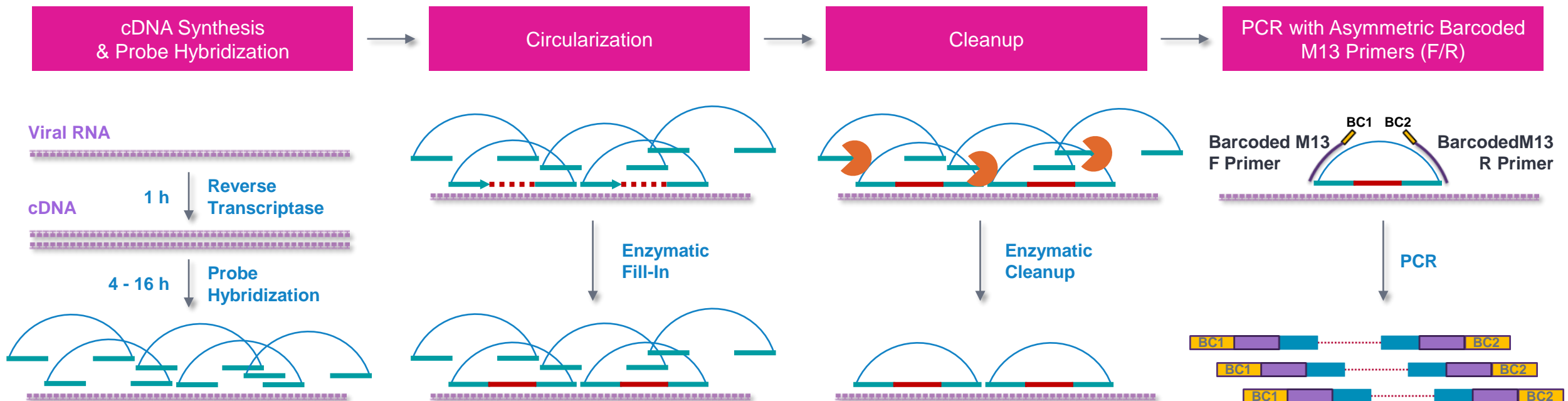
ssDNA Molecular Inversion Probe (MIP)

MIP Hybridization to Viral Target Sequences

cDNA Synthesis & Probe Hybridization → Circularization → Cleanup → PCR with Asymmetric Barcoded M13 Primers (F/R)

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

MIP Hybridization to Viral Target Sequences

3' 5'

3' 5'

SARS-CoV-2 Genome (30 kb)
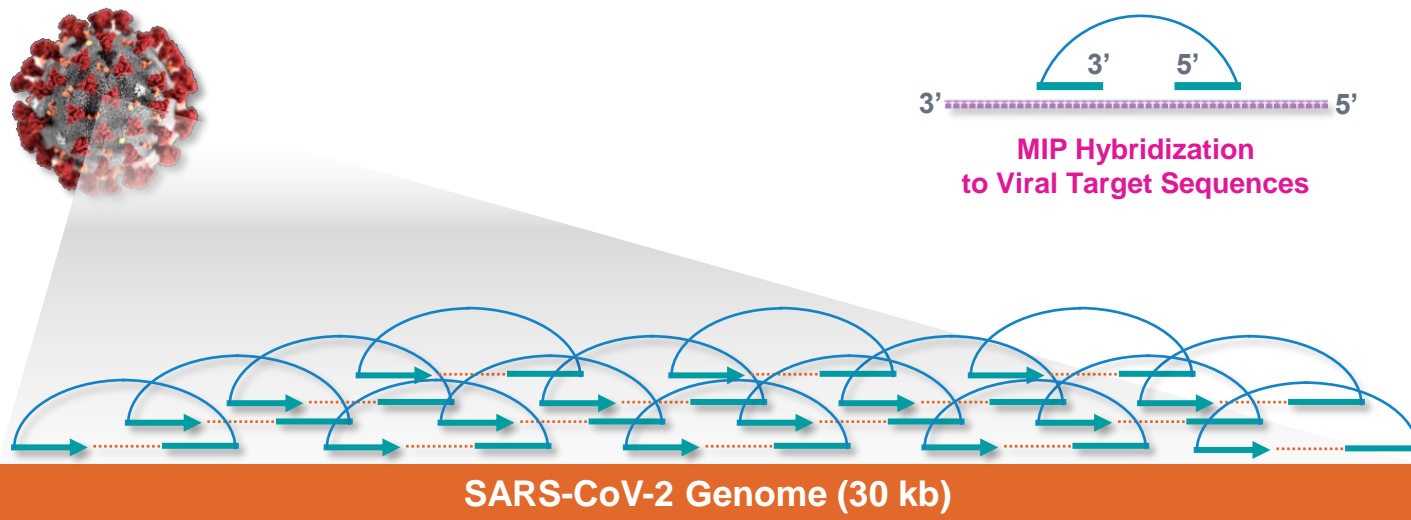
PCR with M13 universal primers to add dual indices for sample multiplexing

| Barcoded M13 Primer (F) | Extension Arm | 675 bp Insert | Ligation Arm | Barcoded M13 Primer (R) |
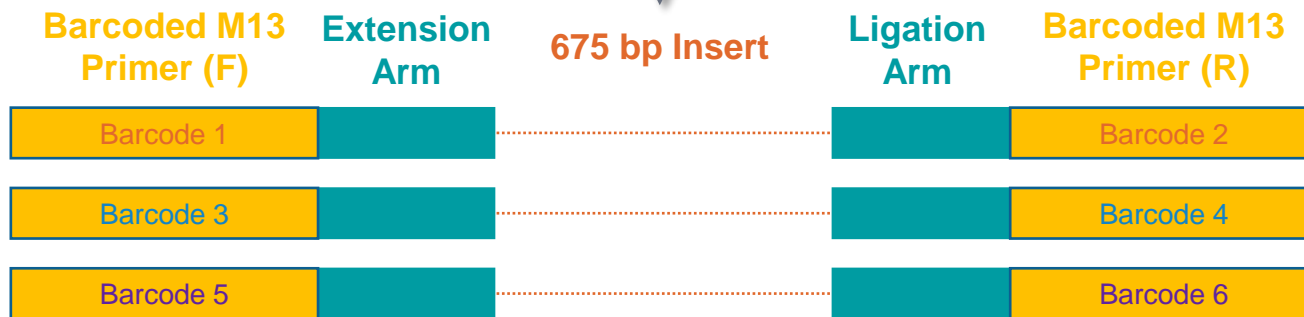
Barcode 1				Barcode 2	Sample 1 (bc1001 – – bc1002)
Barcode 3				Barcode 4	Sample 2 (bc1003 – – bc1004)
Barcode 5				Barcode 6	Sample 3 (bc1005 – – bc1006)

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.

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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

genome cov
- >=95%
- >=90%
- >=70%
- <70%

HiFi Reads (100,000 / 10,000 / 1,000 / 100)

Sample Ct (20 / 25 / 30 / 35)
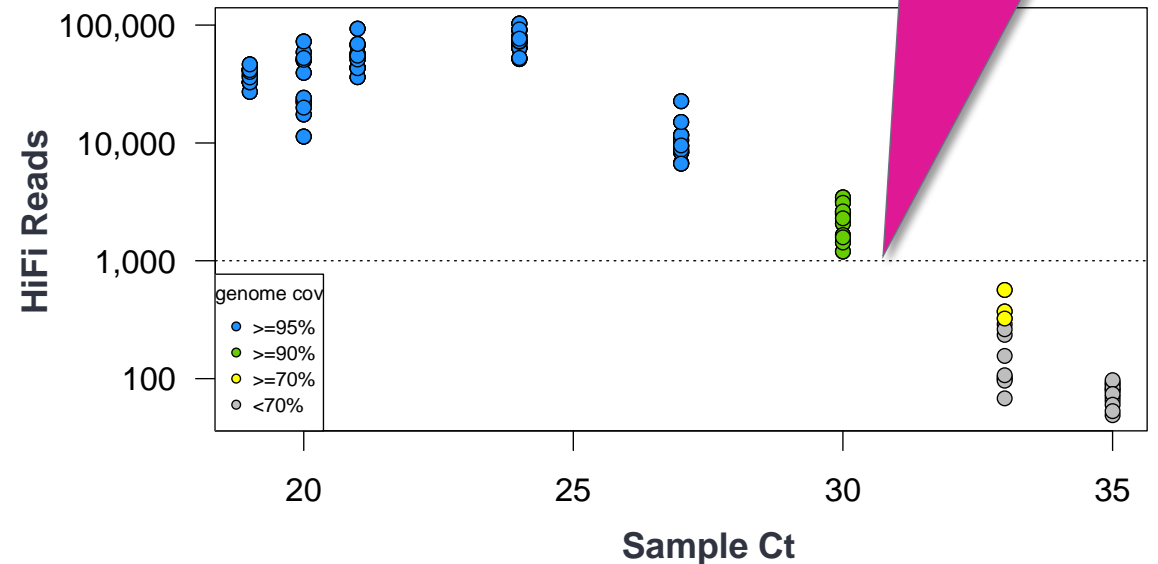
High Viral Copy Number Abundance

Low Viral Copy Number Abundance

Complete = ≥90% genome coverage

HiFiViral SARS-CoV-2 kit workflow overview

HiFiViral SARS-CoV-2 sample preparation procedure description

Procedure & checklist – PacBio HiFiViral high-throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0 (102-396-100) 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 the HiFiViral SARS-CoV-2 kit (102-132-000) and SMRTbell prep kit 3.0 (102-182-700)

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

SMRTbell prep kit 3.0
(102-182-700)

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

Procedure & checklist
April 2022

PacBio Documentation (102-396-100)

Procedure highlights

- 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

RESEARCH FOCUS
PUBLIC HEALTH + SURVEILLANCE
HiFiViral COVID-19 Surveillance
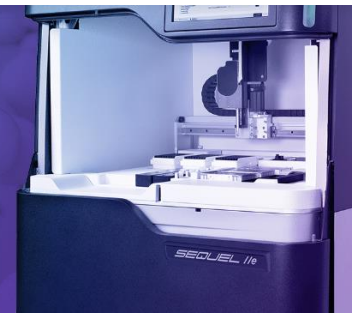
HiFiViral SARS-CoV-2 kit product description

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)

1. 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

2. Barcoded M13 Primer Plate (102-135-500)*

- 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)
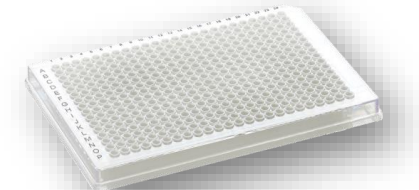
Barcoded M13 Primer Plate

HiFiViral SARS-CoV-2 viral enrichment workflow overview

HiFiViral Viral Enrichment Workflow
(102-396-100)

Prepare assay plate containing extracted RNA samples

Walk-away time

>4 hrs

4 – 16 hrs → cDNA synthesis & probe hybridization*

~4 hrs

1.1 hrs → Fill-in reaction (circularization)

1.3 hrs → Cleanup reaction

1.2 hrs → cDNA amplification with barcoded M13 primers

0.1 hrs → Sample pooling & cleanup

SMRTbell library construction & sequencing preparation
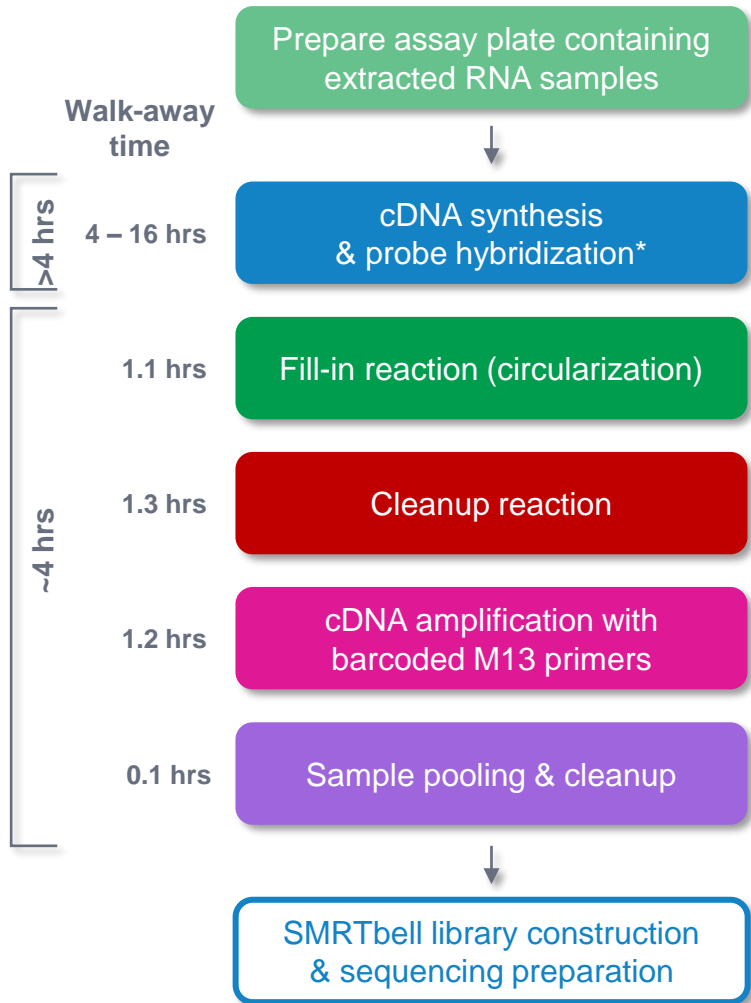
The add-only viral enrichment workflow reduces handling errors with premixed reagents and color-change indicators at every step.

Add extracted RNA samples to the assay plate. The remaining enrichment workflow is add-only.

1. Add **RT reagent + probe mix**

2. Add **fill-In mix** to create circular DNA

3. Add **cleanup mix** to remove non-circular RNA/DNA

4. Add PCR mix and premixed primers to **barcode + amplify**

5. Pool samples and proceed to SMRTbell library construction

Same plate is used for each step of the viral enrichment workflow

MIPs

Fill-In Mix

Cleanup Mix

PCR

Perform **equal-volume pooling** of barcoded SARS-CoV-2 PCR products from up to 384 cohort samples (per SMRT Cell 8M)

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* Perform 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 can increase HiFi data yield for high-Ct SARS-CoV-2 samples.)

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HiFiViral SARS-CoV-2 SMRTbell library construction workflow overview

**HiFiViral Library
Construction Workflow**
(102-396-100)

Walk-away time

Pooled barcoded viral cDNA (100 – 1000 ng)

20 min — 1.3X SMRTbell bead cleanup

Safe Stop Point ⬡ → DNA QC (Sizing & Qubit)

~2.3 hrs*

35 min — DNA repair / A-tailing

50 min — Adapter ligation & 1.3X SMRTbell bead cleanup

35 min — Nuclease Tx & 1.3X SMRTbell bead cleanup

Safe Stop Point ⬡ → DNA QC (Qubit)

Sequencing preparation & data collection

Purified, barcoded & pooled SARS-CoV-2 cDNA samples in a single tube

Non-barcoded adapter

Purified multiplexed SARS-CoV-2 SMRTbell library in a single tube

- The amount of total pooled (barcoded) viral cDNA required for SMRTbell library construction is 500 ng – 1000 ng
- SMRTbell Library construction can be completed in ~3 hrs
- Typical library construction yield is ≥40%

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 using SMRTbell prep kit 3.0

Procedure & checklist 102-396-100 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 the HiFiViral SARS-CoV-2 Kit (102-132-000) and SMRTbell prep kit 3.0 (SPK 3.0) (102-182-700)

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

SMRTbell prep kit 3.0 (102-182-700)

Procedure & checklist contents

1. Input viral RNA QC requirements and general 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 the HiFiViral SARS-CoV-2 kit (102-132-000)

3. Instructions for pooling amplified SARS-CoV-2 cDNA products and constructing SMRTbell libraries using SMRTbell prep kit 3.0 (102-182-700)

PacBio

PacBio HiFiViral high throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0
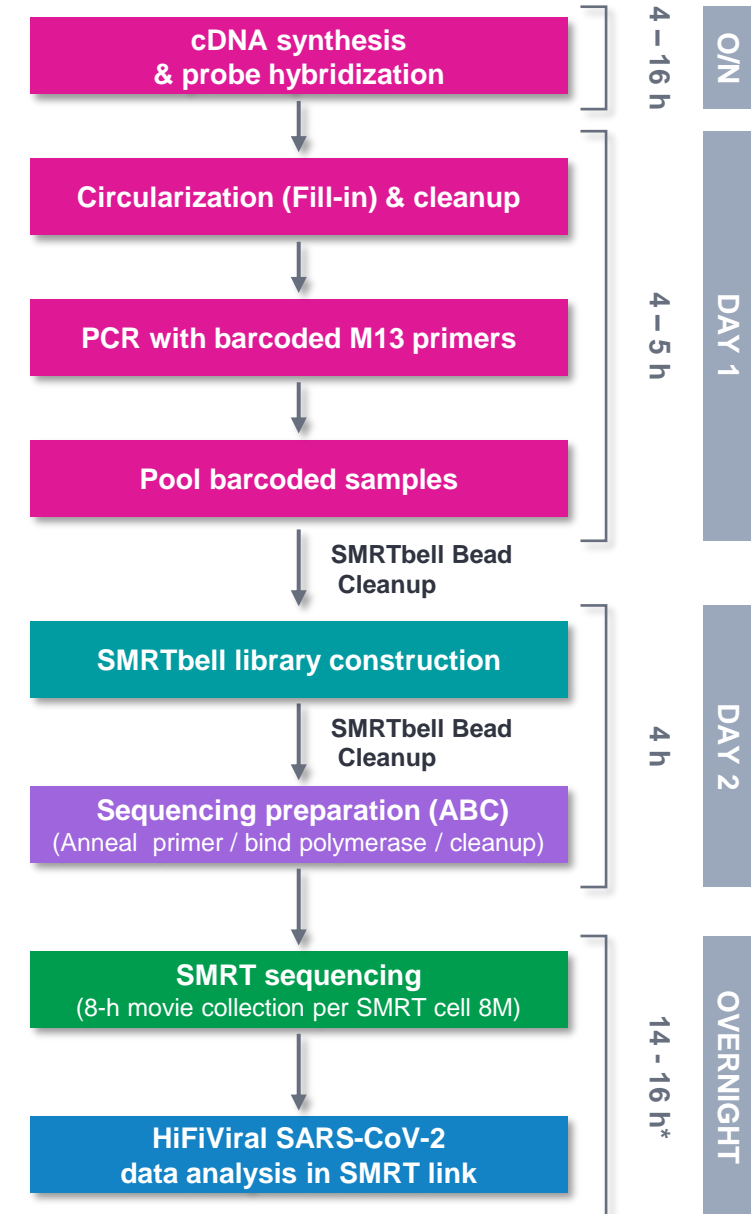
Procedure & checklist
April 2022

PacBio Documentation (102-396-100)

HiFiViral SARS-CoV-2 Kit sample prep workflow timing summary

Efficient workflow with only ~1 – 2.5 hrs 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 SMRTbell bead cleanup + Qubit assay	5 – 10	0.3
Total	**~40 – 110**	**~9.0 – 21.0**
SMRTbell library construction (~2.5 h)		
DNA repair & a-tailing	2 – 4	0.6
Adapter ligation	2 – 4	0.5
1.3X SMRTbell bead cleanup	2 – 4	0.3
Nuclease treatment	2 – 4	0.3
1.3X SMRTbell bead cleanup + qubit assay	5 – 10	0.3
Total	**~15 – 30**	**~2.0**
Sequencing preparation (ABC) (~1.5 h)		
Anneal sequencing primer	2.5 – 5	0.25
Bind polymerase	2.5 – 5	0.25
1.2X SMRTbell 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 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.

Workflow diagram:

- **cDNA synthesis & probe hybridization** — 4 – 16 h — O/N
- **Circularization (Fill-in) & cleanup**
- **PCR with barcoded M13 primers**
- **Pool barcoded samples** — 4 – 5 h — DAY 1
- SMRTbell Bead Cleanup
- **SMRTbell library construction**
- SMRTbell Bead Cleanup
- **Sequencing preparation (ABC)** (Anneal primer / bind polymerase / cleanup) — 4 h — DAY 2
- **SMRT sequencing** (8-h movie collection per SMRT cell 8M)
- **HiFiViral SARS-CoV-2 data analysis in SMRT link** — 14 – 16 h* — OVERNIGHT

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Barcoded M13 Primer Plate

Asymmetric barcode plate map for Barcoded M13 primer plate (102-135-500)

- 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)
- Plate Layout (Excel): Link
- Barcode Sequences (FASTA): Link
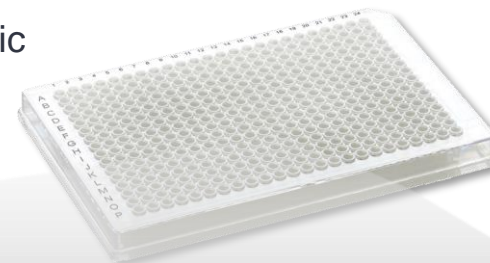
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
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B	1003/1050	1003/1051	1003/1052	1003/1053	1003/1054	1003/1055	1003/1056	1003/1057	1003/1058	1003/1059	1003/1060	1003/1061	1003/1062	1003/1063	1003/1064	1003/1065	1003/1066	1003/1067	1003/1068	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
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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
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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
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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

General best practices recommendations for preparing HiFiViral SARS-CoV-2 SMRTbell libraries

RNA input requirements viral genome enrichment

- 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 preparing HiFiViral SARS-CoV-2 SMRTbell libraries (cont.)

Master mixes

- Prepare master mixes in a PCR workstation
    - The PCR workstation should be UV-irradiated after each setup. If unsure, UV-irradiate the workstation before setting up a master mix.
    - Do not turn on the UV light when reagents are in the workstation
- Master mixes are prepared in 0.5 mL, 1.5 mL or 2 mL microfuge tubes. Briefly vortex to mix and spin down.
- If using a multichannel pipette to transfer master mixes, pre-aliquot appropriate volumes with overage into PCR strip tubes instead of troughs (pipetting reservoirs) to help ensure accurate and efficient pipetting of solutions (i.e., minimize reagent waste so that you do not run out of reagents)
- Use special care when handling small volumes of reagents – Slowly pipette small reaction volumes and viscous reagents

Samples

- RNA samples should be stored at -80⁰C until use and thawed on ice.
- Heavily degraded RNA or RNA samples with many freeze-thaw cycles should be avoided.
- All work surfaces and gloves should be sanitized with RNaseZap (or the equivalent) prior to setup.
- For most consistent performance, all samples included in a batch, including control samples, should be from the same sample type and extracted by the same RNA extraction procedure.
- A no-RNA control is recommended but not required.
- Upon thawing frozen samples, briefly vortex and spin down prior to use.

General best practices recommendations for preparing HiFiViral SARS-CoV-2 SMRTbell libraries (cont.)

Reaction plates for viral genome enrichment

- 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.
  - 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.
  - 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.
  - Proper plate sealing is critical, especially for the overnight probe hybridization step.
- When removing plate seals, a heated plate sealer can be used if desired to briefly warm the seal and loosen the adhesive.
  - Be careful when removing plate seals to avoid cross contamination.
- Always perform a visual check of liquid volumes before and after each incubation step.
  - After centrifugation, inspect the bottom of the plate to ensure the expected volume is present in every well.
  - Centrifuge in an Eppendorf 5810 fitted with a swinging bucket plate rotor at maximum rpm for approximately 30 sec.
- Verify that the liquid solution color at each reach step is correct.

General best practices recommendations for preparing HiFiViral SARS-CoV-2 SMRTbell libraries (cont.)

Reagent handling

HiFiViral SARS-CoV-2 kit reagents

- Have all reagents and other required materials for each step of the MIP assay staged and ready for use to enable you to work quickly

- Set a timer during each step of the MIP assay to enable verification that reagents are added to all samples within 10 minutes
  - Correct timing is important to maximize result quality

- If performing the MIP assay for the first time, we recommend including an appropriate positive control sample in your experimental design

- Be mindful of required temperature conditions for setting up each reaction step of the MIP assay
  - **cDNA synthesis and probe hybridization step:**
    - RNA samples and reagents should be kept on ice while setting up the master mix and while transferring reagents and samples to the assay plate wells.
    - A cold block (e.g., Eppendorf PCR Cooler) is recommended to help keep reagents cold during reaction setup
  - **Fill-in reaction step:**
    - Allow the Fill-in mix to fully thaw and add to samples at room temperature (not on ice)
  - **Cleanup reaction step:**
    - Allow the Cleanup mix to fully thaw and add to samples at room temperature (not on ice)
  - **cDNA amplification step:**
    - Sample plate and reagents should be kept on ice while setting up the master mix and while transferring reagents to the assay plate wells.
    - A cold block (e.g., Eppendorf PCR Cooler) is recommended to help keep reagents cold during reaction setup

- For the Fill-in reaction and Cleanup reaction steps, do not remove the reaction plate from the thermal cycler until the reagent mix is ready.

General best practices recommendations for preparing HiFiViral SARS-CoV-2 SMRTbell libraries (cont.)

Reagent handling

SMRTbell prep kit 3.0 reagents

- Room temperature is defined as any temperature in the range of 18-23°C for this protocol.

- Thaw the repair buffer, nuclease buffer, and elution buffer at room temperature.

- Mix reagent buffers and SMRTbell adapter with a brief vortex prior to use. Enzyme mixes do not require vortexing.

- Quick spin all reagents in microcentrifuge to collect liquid at bottom prior to use.

- Keep all temperature-sensitive reagents on ice.

- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30-60 minutes prior to use.

- Pipette mix all bead binding and elution steps until beads are distributed evenly in solution.

- Pipette mix all SMRTbell prep reactions by pipetting up and down 10 times.

- Samples can be stored at 4°C at all safe stopping points listed in the protocol.

- Puncture the top of the seal on the barcoded M13 primer plate with a clean, empty pipette tip before pipetting the primer mix.

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

HiFiViral SARS-CoV-2 kit procedural notes

cDNA synthesis and probe hybridization

1. cDNA synthesis and probe hybridization

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

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

RT-Hybridization Master Mix

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

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

Hyb Rx Mix = Blue
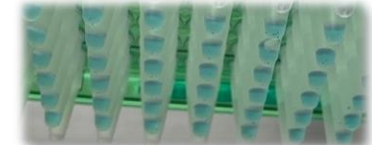
Starting and monitoring hyb reactions

- 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

PacBio

HiFiViral SARS-CoV-2 kit procedural notes (cont.)

Circularization (Fill reaction)

2. Fill reaction

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

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

Fill Rx Mix = Green

Cleanup reaction

3. Cleanup reaction

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

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

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
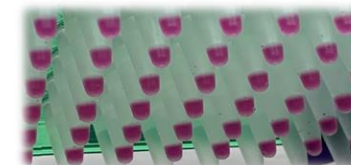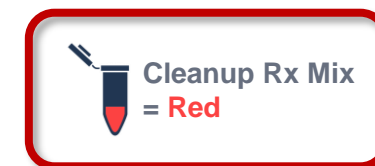
Cleanup Rx Mix = Red

PacBio

HiFiViral SARS-CoV-2 kit procedural notes (cont.)

PCR amplification with barcoded M13 primers

4. cDNA amplification

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

✔	Step	Instructions
	4.1	Remove the sample plate from the thermocycler.
	4.2	Spin down the plate, perform a quick visual check of the liquid level to make sure th... top seal or side walls, and remove the seal carefully to avoid cross contami...
	4.3	Using a multichannel pipette, add **12 µL** of PCR Mix... ...ple on the plate.
	4.4	Add **2.4 µL** of primer from the barcoded M13 primer plate to the corresponding sample wells. Puncture the top of the seal on the barcoded M13 primer plate with a clean, empty pipette tip before pipetting the primer mix.
	4.6	The total reaction volume in each well is approximately **24.0 µL**. See table below:

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

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

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

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

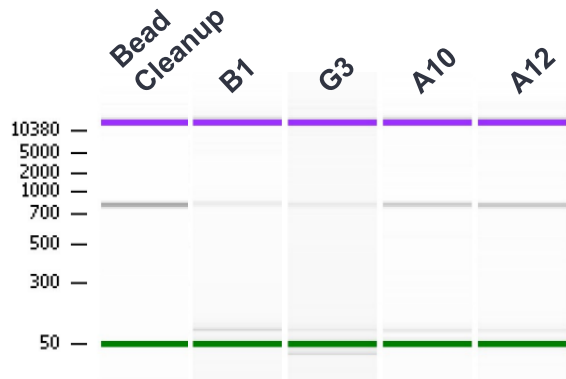**PCR Rx Mix = Magenta**
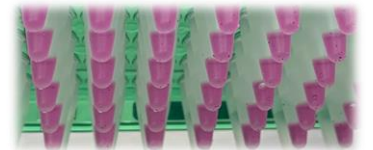
Starting and monitoring PCR reactions

- 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 -20⁰C until further processing

HiFiViral SARS-CoV-2 kit procedural notes (cont.)

PCR amplification with barcoded M13 primers (cont.)

5. Library quantification/QC (optional)

✔	Step	Instructions
	5.1	Remove the reaction plate from the thermocycler.
	5.2	Spin down the reaction plate and perform a quick visual check of the liquid level. Take note of any well with low volume, which indicates excessive evaporation during amplification.
	5.3	Remove the seal carefully to avoid cross contamination.
	5.4	Use 1 μL of sample to quantify with a Qubit fluorometer using the 1x dsDNA HS assay kit.

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
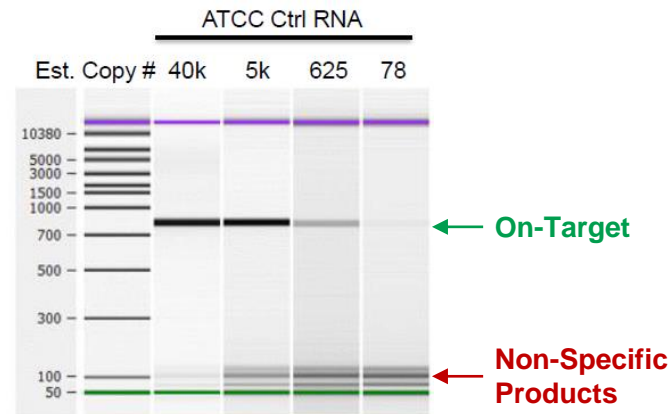

Example post-PCR DNA sizing analysis results for extracted viral RNA samples.

- Spot-checking PCR amplification products prior to pooling is highly recommended when performing the HiFiViral workflow for the first time

- 1.3X SMRTbell cleanup Bead purification can help remove non-specific amplification products

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 SMRTbell cleanup Bead purification can help remove non-specific amplification products

PCR Product Yield vs. Input Control RNA Copy Number
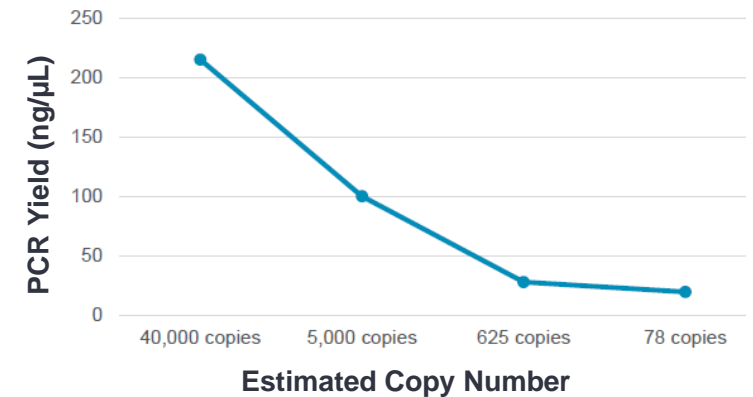
Example post-PCR yield results for ATCC Control RNA samples.

- Higher-copy number samples are generally correlated with higher PCR yields (*via* Qubit dsDNA HS assay quantification)
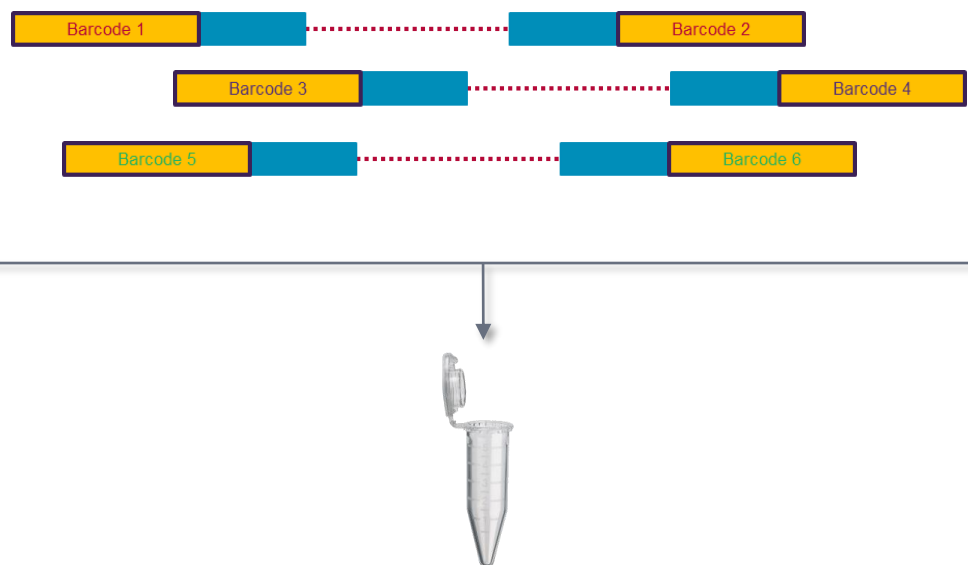
HiFiViral SARS-CoV-2 kit procedural notes (cont.)

Sample pooling for SMRTbell library construction

6. Sample pooling for library construction

✔	Step	Instructions
		Sample pooling
	6.1	Remove the reaction plate from the thermocycler.
	6.2	Spin down the reaction plate and perform a quick visual check of the liquid level. Take note of any well with low volume, which indicates excessive evaporation during amplification.
	6.3	Remove the seal carefully to avoid cross contamination.
	6.4	Transfer a minimum of **5 μL** per reaction into a clean 1.5 or 2.0 mL DNA Lo-bind tube. If pooling **384 reactions**, vortex to mix and transfer no more than **800 μL** to a new 2.0mL Lo-bind tube for purification. Save the rest of the sample pool at -20°C.

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 -20°C.)

Barcode 1 .... Barcode 2

Barcode 3 .... Barcode 4

Barcode 5 .... Barcode 6

HiFiViral SARS-CoV-2 Kit Procedural Notes (Cont.)

Sample pooling for SMRTbell library construction (cont.)

Cleanup with 1.3X SMRTbell cleanup beads

6.5	Add **1.3X** volume over volume (v/v) of resuspended, room-temperature SMRTbell cleanup beads to the pooled library in the 1.5 or 2.0 mL LoBind tube.
6.6	Pipette mix beads until evenly distributed.
6.7	Quick spin the tube in a microcentrifuge to collect liquid.
6.8	Leave at room temperature for **10 minutes** to allow DNA to bind beads.
6.9	Place tube in a magnetic separation rack until beads separate fully from the solution.
6.10	Slowly pipette off the cleared supernatant without disturbing the supernatant.
6.11	Slowly dispense **1400 µL**, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.

|	
6.19	**Recommended**: Evaluate sample quality (concentration and size distribution). • Take **1 µL** of eluted DNA and dilute with **9 µL** of elution buffer or water. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • (Optional): Measure DNA size distribution on the Agilent 2100 Bioanalyzer using the DNA 12000 chip. Follow all manufacturer's instructions. Target peak should be ≥700 bp with minimal non-specific peaks near 170-200 bp.

Purifying pooled samples

- Add 1.3X volume of resuspended, room-temperature SMRTbell cleanup beads to the pooled library.
  - Bead incubation: 10 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
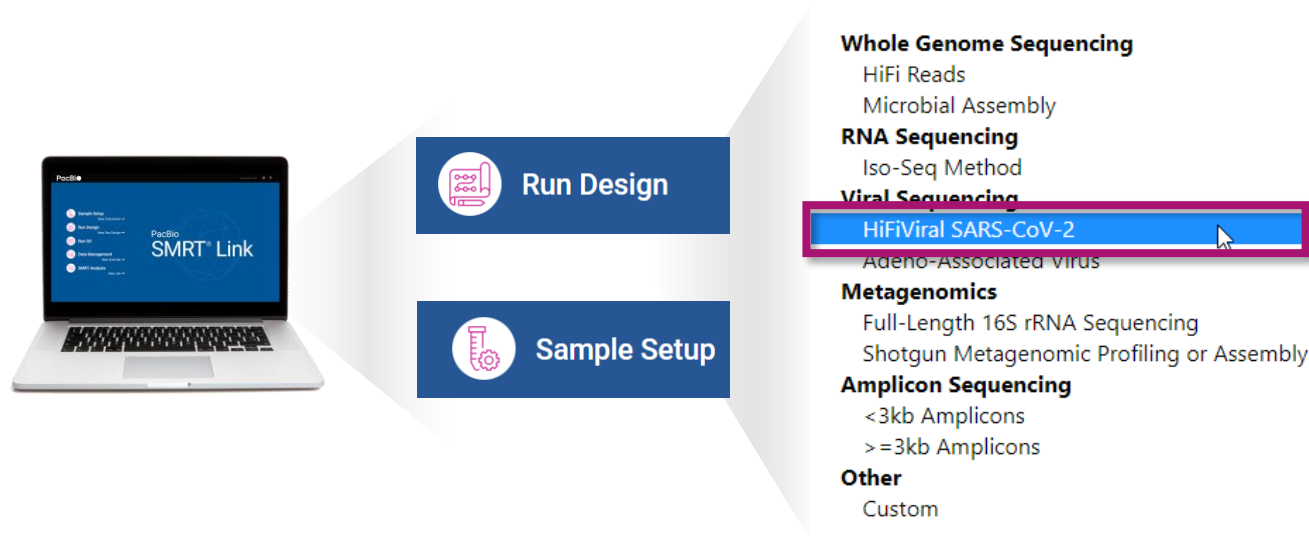 - Non-specific amplicons (~170-200 bp) should be removed completely.

Pooled Amplicons [835 bp]

SMRTbell Library [928 bp]
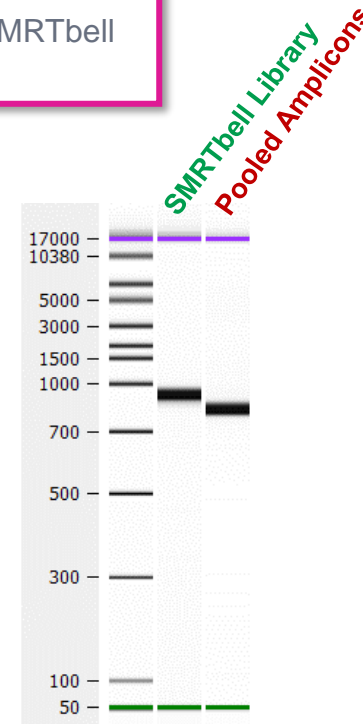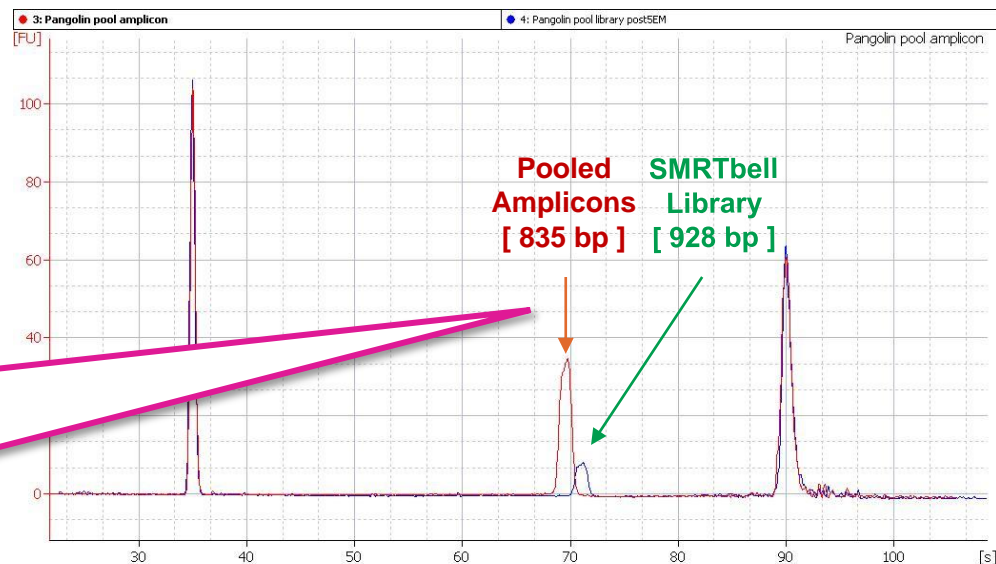
Multiplexed SARS-CoV-2 library sequencing workflow recommendations

Sample Setup & Run Design recommendations for HiFiViral SARS-CoV-2 libraries

In SMRT Link Sample Setup & Run Design, select 'Viral Sequencing' / 'HiFiViral SARS-Cov-2' for application type

Whole Genome Sequencing
  HiFi Reads
  Microbial Assembly
RNA Sequencing
  Iso-Seq Method
Viral Sequencing
  HiFiViral SARS-CoV-2
  Adeno-Associated Virus
Metagenomics
  Full-Length 16S rRNA Sequencing
  Shotgun Metagenomic Profiling or Assembly
Amplicon Sequencing
  <3kb Amplicons
  >=3kb Amplicons
Other
  Custom

Binding kit 3.1 & cleanup beads (102-333-400) is recommended for preparing AAV samples for sequencing.

- We recommend using Sequel II binding kit 3.1 & cleanup beads (102-333-400) to perform ABC (anneal primer / bind polymerase / clean up complex) with HiFiViral SARS-CoV-2 samples

- Refer to Quick reference card – Loading and pre-extension time recommendations for the Sequel II and IIe systems (101-769-100) for updates to ABC workflow for specific applications

Sequel II binding kit 3.1 & cleanup beads (102-333-400) includes the following components:

- Sequencing primer 3.1
- Sequel II polymerase 2.1
- SMRTbell cleanup beads for complex cleanup
- DNA internal control 3.1 (defined 2 kb template bound to Polymerase 2.1)
- Supports ≥24 binding reactions, and up to 4 SMRT Cells 8M per binding reaction (96 cells total), depending on use case, sample size and concentration

HiFiViral SARS-CoV-2 Sample Setup guidance

Use SMRT Link Sample Setup High-Throughput (HT) mode and follow instructions to perform ABC (anneal primer / bind polymerase / clean up complex) using recommended settings for HiFiViral SARS-CoV-2 samples

By default, **Insert Size** is automatically specified to be 800 bp for HiFiViral SARS-CoV-2 samples.

- **Sample Setup High-Throughput** mode provides a simplified, streamlined workflow to efficiently process either one sample or multiple samples with similar library properties (such as mean insert size and DNA concentration) in parallel
- You can also export the calculated values to a CSV file for laboratory automation

Note: We recommend starting with an on-plate loading concentration (OPLC) of **200 pM** for HiFiViral SARS-CoV-2 samples and adjusting higher or lower if needed to achieve optimal *P1* loading.

Example Sample Setup HT mode worksheet for a batch comprised of two HiFiViral SARS-CoV-2 samples.

HiFiViral SARS-CoV-2 Run Design guidance

Follow SMRT Link Run Design instructions to set up a sequencing run using recommended settings for HiFiViral SARS-CoV-2 samples

- Select HiFiViral SARS-CoV-2 from the Application field drop-down menu in SMRT Link Run Design

- The following fields are auto-populated and high-lighted in green:
    - Template Prep Kit
    - Binding Kit
    - Sequencing Kit
    - DNA Control Complex
    - Insert Size
    - Movie Time Per SMRT Cell

By default, **Automatic Launch of SARS-CoV-2 analysis** is specified to be '**YES**'

Note: By default, all newly created run designs (regardless of application type) will specify to **automatically** perform CCS analysis and output **only** HiFi reads

Run Information

System Type
- ○ SEQUEL II
- ● SEQUEL IIe

Run Name
Run 04.18.2022 15:47

Run Comments

Experiment Name

Experiment ID

Estimated Run Duration (hours): 11.3

Run Reagents / Consumables
1 SMRT Cell
1 sequencing reagent plate
1 mineral oil tube
3 boxes of tips
1 mixing plate
1 sample plate

Sample Information

SAMPLE 1: Pooled_SARS_CoV_2_Sample_01, A01, 8 hour movie, 800 bp insert

Import from Sample Setup — Select Sample

Application — HiFiViral SARS-CoV-2
Required

Well Sample Name — Pooled_SARS_CoV_2_Sample_01
Required

Bio Sample Name

Sample Comment

Sample Well — A01

Template Prep Kit — SMRTbell® Prep Kit 3.0
Required

Binding Kit — Sequel® II Binding Kit 3.1
Required

Sequencing Kit — Sequel® II Sequencing Plate 2.0 (4 rxn)
Required

DNA Control Complex — Sequel® II DNA Internal Control Complex 3.1

Insert Size (bp) — 800
Required

Recommended Concentration on Plate (pM) — 100-300 pM

On-Plate Loading Concentration (pM) — 200
Required

Movie Time per SMRT Cell (hours) — 8

Use Pre-Extension — ○ YES ● NO

Auto Analysis

Automatic Launch of SARS-CoV-2 Analysis — ● YES ○ NO

Analysis Name — SARS_CoV_2_Sample_01_Analysis
Required

CCS Analysis will be performed on-instrument to produce HiFi .bam files.

Example sample information entered into Run Design for sequencing a HiFiViral SARS-CoV-2 sample.

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.

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 Auto Analysis Workflow

HiFiViral SARS-CoV-2 Manual Analysis Workflow
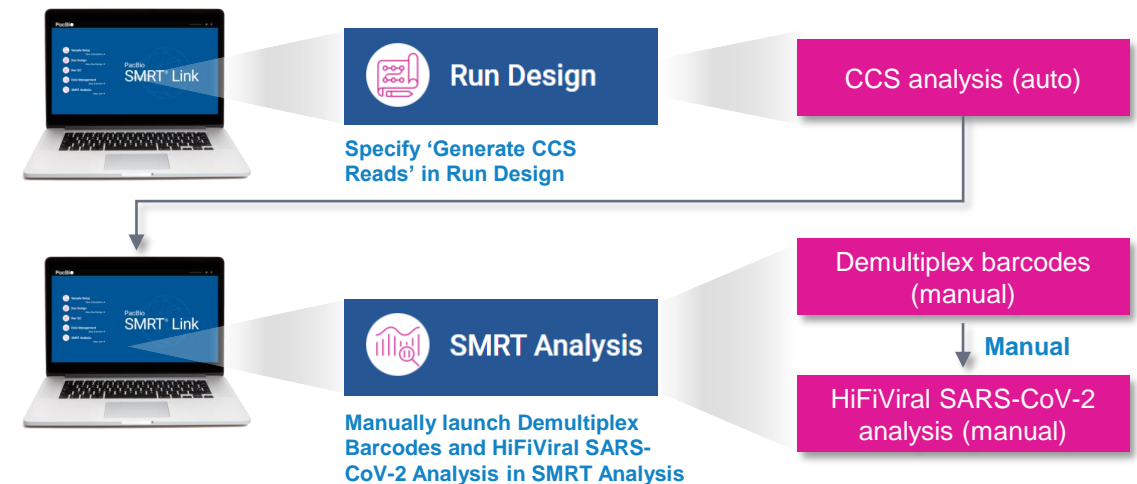
HiFiViral SARS-CoV-2 analysis setup – Auto analysis

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**

Auto Analysis

(1) Automatic Launch of SARS-CoV-2 Analysis   ● YES   ○ NO

(2) Analysis Name
Required
`HiFiViral_SARS-CoV-2_Library_01_Analysis`

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**

Barcoded Sample Options

(1) Sample Is Barcoded   ● YES   ○ NO

(2) Barcode Set
Required
`HiFiViral_SARS-CoV-2_M13barcodes`

(3) Same Barcodes on Both Ends of Sequence ⓘ   ○ YES   ● NO

HiFiViral SARS-CoV-2 analysis setup – 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.

8. Specify to perform barcode demultiplexing on-instrument (Sequel IIe system only) or in SMRT Link.

Refer to the "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

| Assign Bio Sample Names to Barcodes Required | Interactively | From a File | ④ |

| Autofilled Barcoded Sample File | ⬇ Download File |

⑤

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		
M13_bc1017_F--M13_bc1070_R	HiFiViral_SARS-CoV-2_Sample_381	D	
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

⑥

| Barcoded Sample Name File | Choose file Required | Browse |

⑦

| Barcoded Sample Name File | Barcode_Names_HiFiViral_384_Samples.csv | Browse |
| Required | | |

Upload was successful

⑧

| Demultiplex Barcodes | ● ON INSTRUMENT ○ IN SMRT LINK ○ DO NOT GENERATE |

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** data utility, 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 `Barcoded M13 Primer Plate`

3. Barcodes on Both Ends of Sequence: Select **No**

SMRT Analysis / Create New Analysis

1. Select Data   2. Select Analysis

Data Utility Required

① Demultiplex Barcodes

⬆ Import Analysis Settings   ⬇ Export   ⓘ

Associated Inputs

Barcode Set Required

② Barcoded M13 Primer Plate

Same Barcodes on Both Ends of Sequence ⓘ

③ ○ YES   ● NO

HiFiViral SARS-CoV-2 analysis setup – 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 the "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

Assign Bio Sample Names to Barcodes Required   Interactively   From a File   ④

Autofilled Barcoded Sample File 🛈   ⬇ Download File

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

⑤

Barcoded Sample Name File 🛈   Choose file   Browse
Required

⑥

Barcoded Sample Name File 🛈   Barcode_Names_HiFiViral_384_Samples.csv   Browse
Required

Upload was successful

⑦

Demultiplexed Output Data Set Name Required 🛈

HiFiViral_SARS-CoV-2_Sample_Plate_01_CCS_Demux

⑧

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

HiFiViral SARS-CoV-2 analysis setup – 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**'

6. Click **Next**.

HiFiViral SARS-CoV-2 analysis setup – Manual analysis (cont.)

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

8. Under **Associated Inputs**, SARS-CoV-2 Genome NC_045512.2 (the Wuhan reference genome) is automatically loaded as the reference genome; advanced users may select a different reference if desired.

9. 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.

SMRT Analysis / Create New Analysis

1. Select Data | 2. Select Analysis

Analysis Application Required

⑦ HiFiViral SARS-CoV-2 Analysis

⬆ Import Analysis Settings | ⬇ Export ⓘ

Associated Inputs

Reference Genome Required

⑧ SARS-CoV-2 Genome NC_045512.2 ☰

⑨ Advanced Parameters

PacBio

HiFiViral SARS-CoV-2 analysis setup – Manual analysis (cont.)

10. 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.

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

Advanced Analysis Parameters

Plate QC CSV ⓘ
Choose file | Browse
⬇ Download Template

Minimum Base Coverage ⓘ
4

Minimum Variant Frequency ⓘ
0.5

Minimum Barcode Score ⓘ
80

Advanced Processing Options ⓘ

Compute Settings ⓘ
-- select --

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_bc1002_F--M13_bc1054_R	HiFiViral_SARS-CoV-2_Sample_5	A	E01
M13_bc1002_F--M13_bc1055_R	HiFiViral_SARS-CoV-2_Sample_6	A	F01
M13_bc1002_F--M13_bc1056_R	HiFiViral_SARS-CoV-2_Sample_7	A	G01
M13_bc1002_F--M13_bc1057_R	HiFiViral_SARS-CoV-2_Sample_8	A	H01
M13_bc1002_F--M13_bc1058_R	HiFiViral_SARS-CoV-2_Sample_9	A	A02
M13_bc1002_F--M13_bc1059_R	HiFiViral_SARS-CoV-2_Sample_10		
M13_bc1002_F--M13_bc1060_R	HiFiViral_SARS-CoV-2_Sample_11		
M13_bc1002_F--M13_bc1061_R	HiFiViral_SARS-CoV-2_Sample_12		
M13_bc1002_F--M13_bc1062_R	HiFiViral_SARS-CoV-2_Sample_13		

Plate QC CSV ⓘ
assayPlateQC_template_4by96_HiFiV | Browse
⬇ Download Template
Upload was successful

Start ▶

HiFiViral SARS-CoV-2 analysis setup – Manual analysis (cont.)

Comparison of CSV templates for demultiplex barcodes analysis and HiFiViral SARS-CoV-2 assay plate QC analysis

Demultiplex barcodes CSV

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_bc____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 CSV

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	
M13_bc1017_F--M13_bc____R	HiFiViral_SARS-CoV-2_Sample_381	D	
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

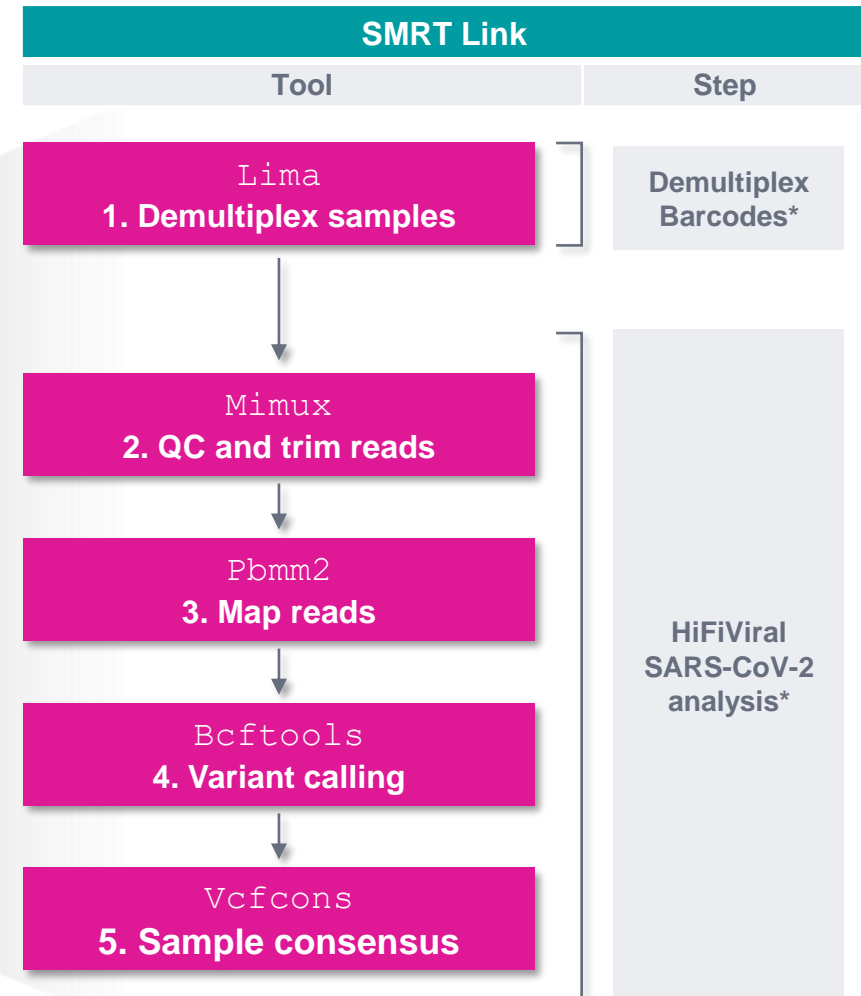When editing CSV templates for Demultiplex Barcodes analysis and HiFiViral SARS-CoV-2 Assay Plate QC analysis:

❑ 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.

| SMRT Link	
Tool	**Step**
Lima 1. Demultiplex samples	Demultiplex Barcodes*
Mimux 2. QC and trim reads	
Pbmm2 3. Map reads	HiFiViral SARS-CoV-2 analysis*
Bcftools 4. Variant calling	
Vcfcons 5. Sample consensus	

HiFiViral SARS-CoV-2 analysis 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

Sample Summary

Bio Sample Name	Substitutions	Insertions	Deletions	Reads	Read Coverage	On-Target Rate	Multiple Strains (Probability)	Ns	Genome Coverage
4655747	36	0	3	12,964	288	99.99%	No (0.00)	156	99.47%
4657055	Sample 1	0	3	1,075	24	99.81%	No (0.00)	761	97.45%
4656469	Sample 2	0	3	2,289	51	99.91%	No (0.00)	219	99.26%

Sample 3

Genome Coverage
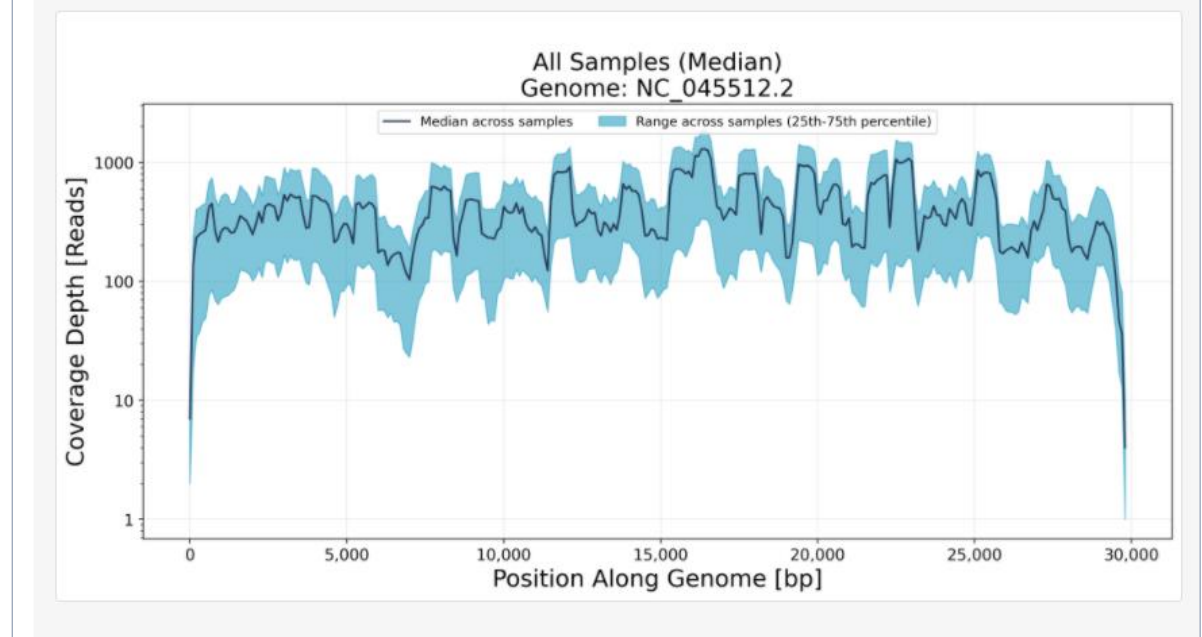
File Downloads

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

HiFiViral SARS-CoV-2 analysis 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
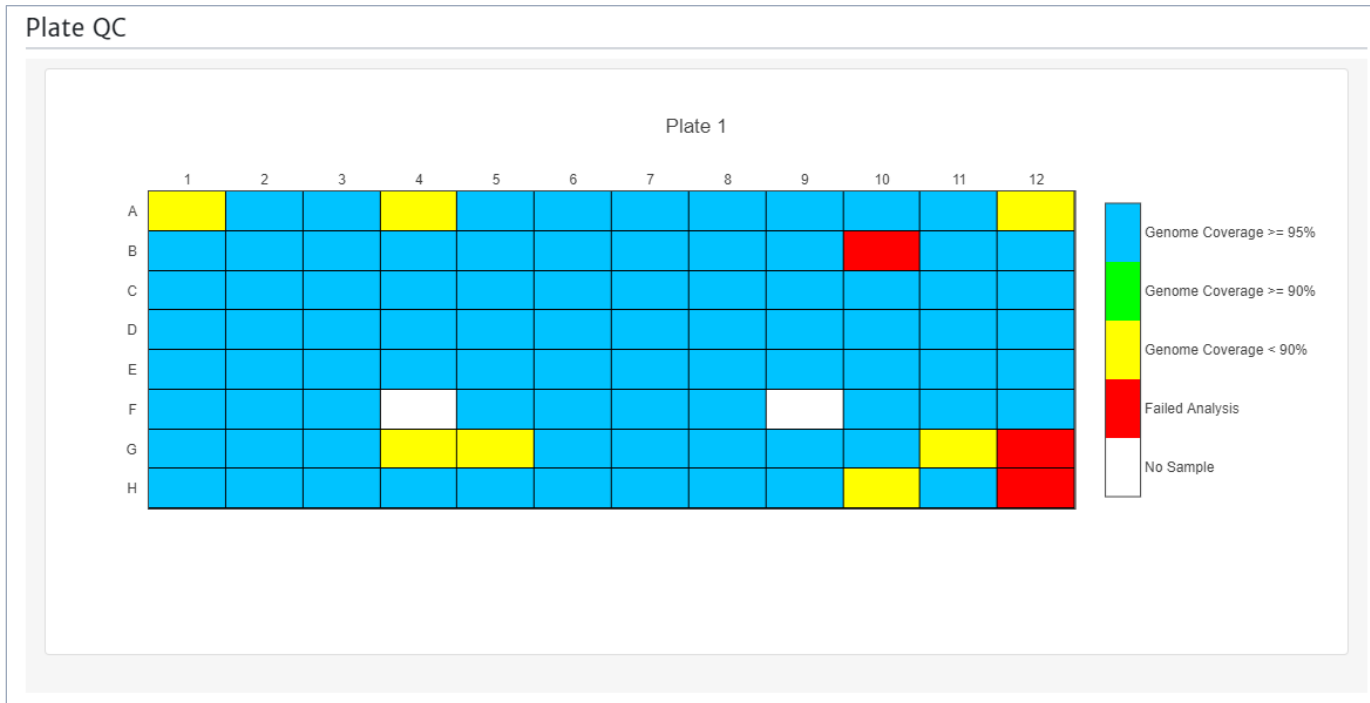
Lower HiFi read counts due to evaporation-induced edge-effects during viral enrichment
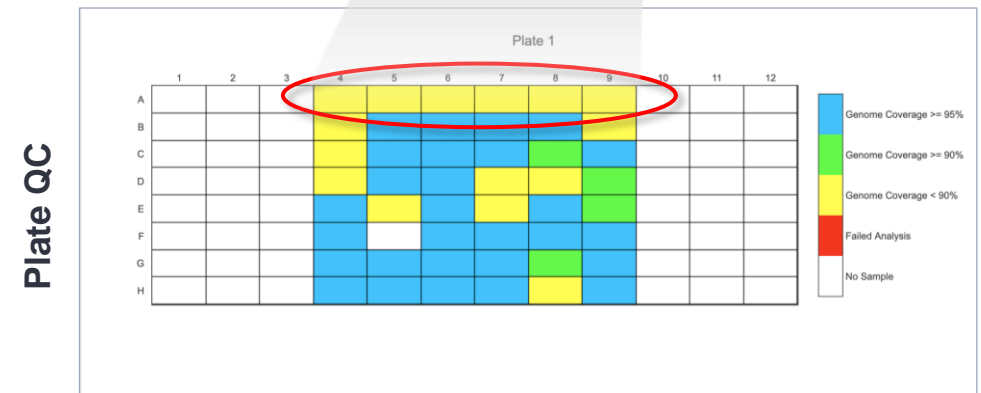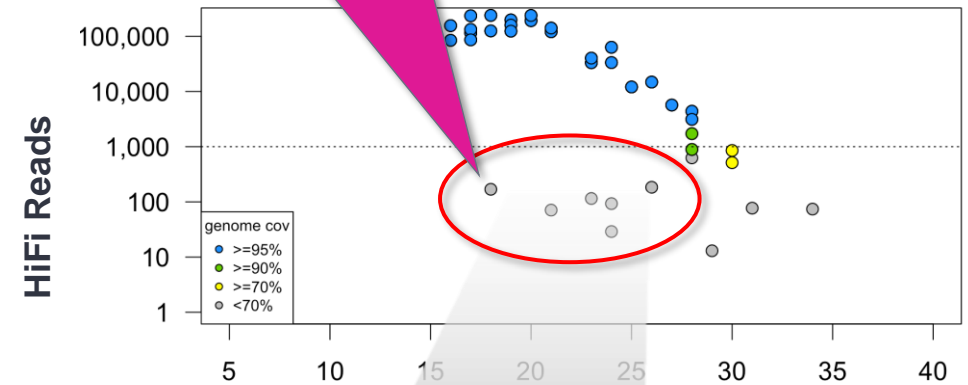
Downloading HiFiViral SARS-CoV-2 analysis results in SMRT Link

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 (cont.)

⌄ 📁 analysis-Twist_RNA_23_Ct29p8_rep2-45495-samples.consensus.fasta	--	Folder
Twist_RNA_13_Ct19p1_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct19p1_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct19p1_rep3.consensus.fasta		
Twist_RNA_13_Ct21p9_rep1.consensus.fasta		
Twist_RNA_13_Ct21p9_rep2.consensus.fasta		
Twist_RNA_13_Ct21p9_rep3.consensus.fasta		
Twist_RNA_13_Ct22p6_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct22p6_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct22p6_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct24p4_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct24p4_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct24p4_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct26p2_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct26p2_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct26p2_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct28_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct28_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct28_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct29p8_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct29p8_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct29p8_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct31p5_rep1.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct31p5_rep2.consensus.fasta	30 KB	FASTA File
Twist_RNA_13_Ct31p5_rep3.consensus.fasta	30 KB	FASTA File
Twist_RNA_14_Ct19p1_rep1.consensus.fasta	30 KB	FASTA File

For each sample, HiFiViral analysis application outputs **a single SARS-CoV-2 consensus sequence**
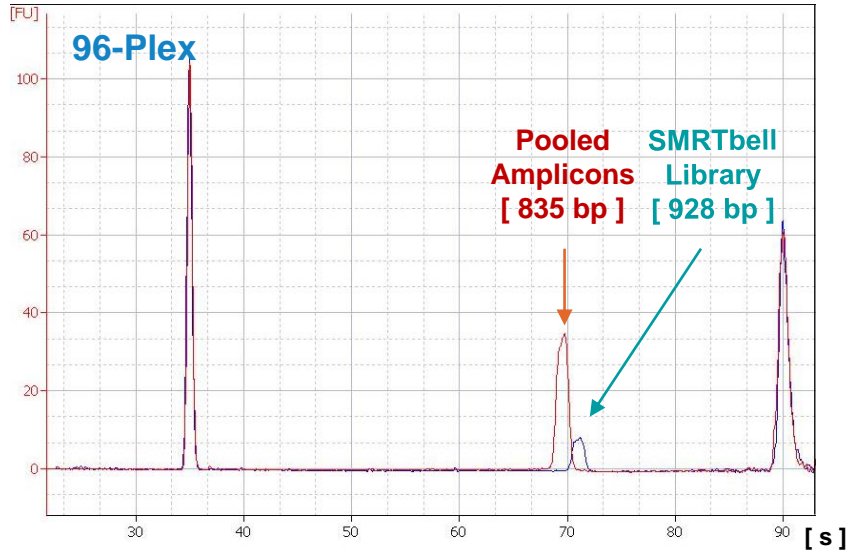
PacBio

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

96-Plex

Pooled Amplicons [835 bp]
SMRTbell Library [928 bp]

Raw Data Report

96-Plex

Most CCS read lengths are ~800 bp

1 kb
500 bp
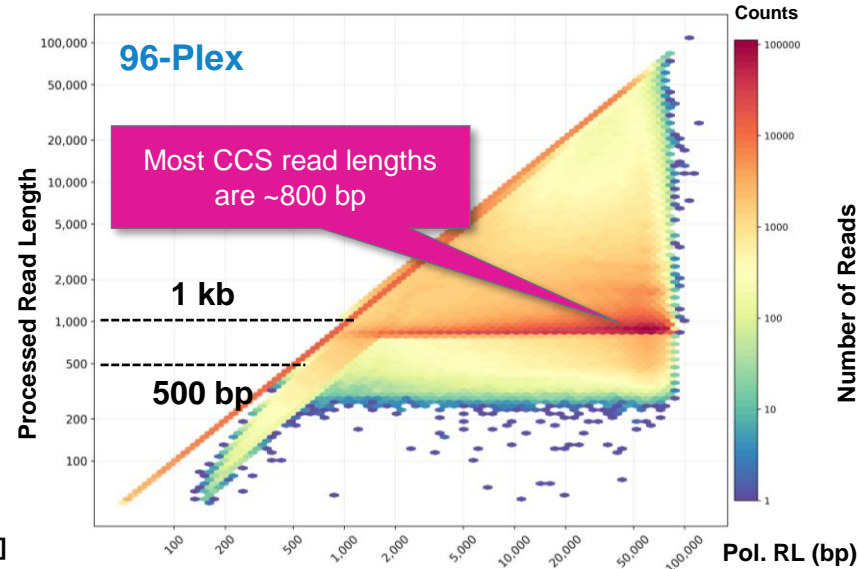
CCS Analysis Report

96-Plex

772 bp Mean HiFi Read Length

HiFi read lengths are consistent with expected insert size (~800 bp) for this SARS-CoV-2 library
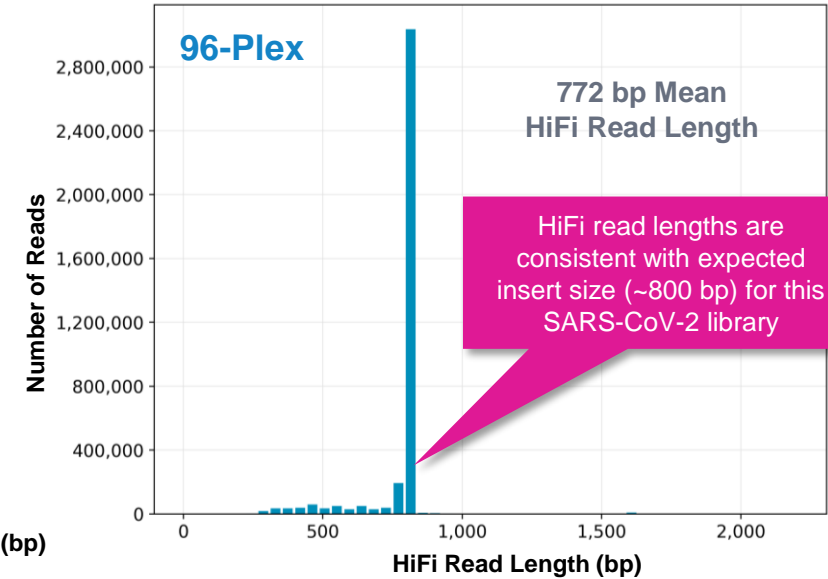
	96-Plex	384-Plex
Yield of Pooled Barcoded PCR Products	2049 ng	12,400 ng
Pooled DNA Input for Library Construction	1000 ng	1000 ng
Final Yield of SMRTbell cleanup Bead Purified Library (%)	142 ng (14.2%)	408 ng (40.8%)

Twist 14 and 17 controls were enriched with the HiFiViral SARS-CoV-2 Kit. Pooled barcoded PCR products were purified with 1.3X SMRTbell cleanup 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 RL	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

PacBio

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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

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%

- 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)

Plate QC
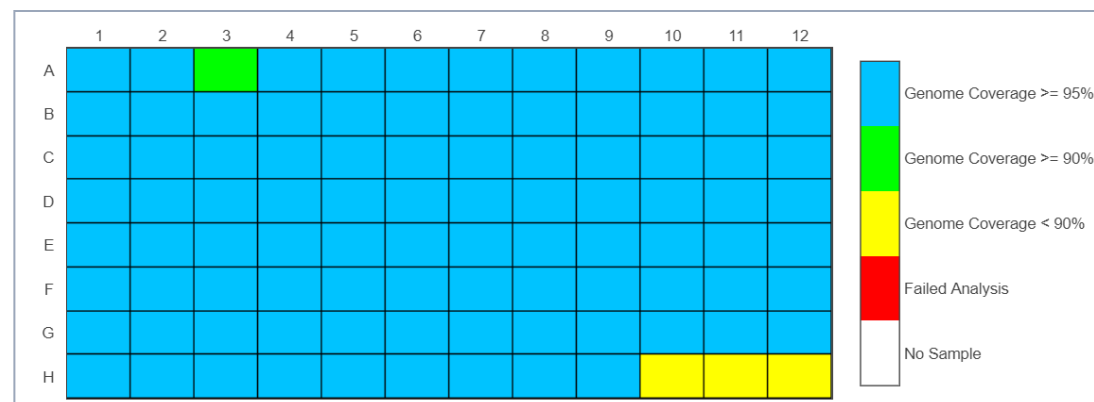
Genome Coverage >= 95%
Genome Coverage >= 90%
Genome Coverage < 90%
Failed Analysis
No Sample

PacBio

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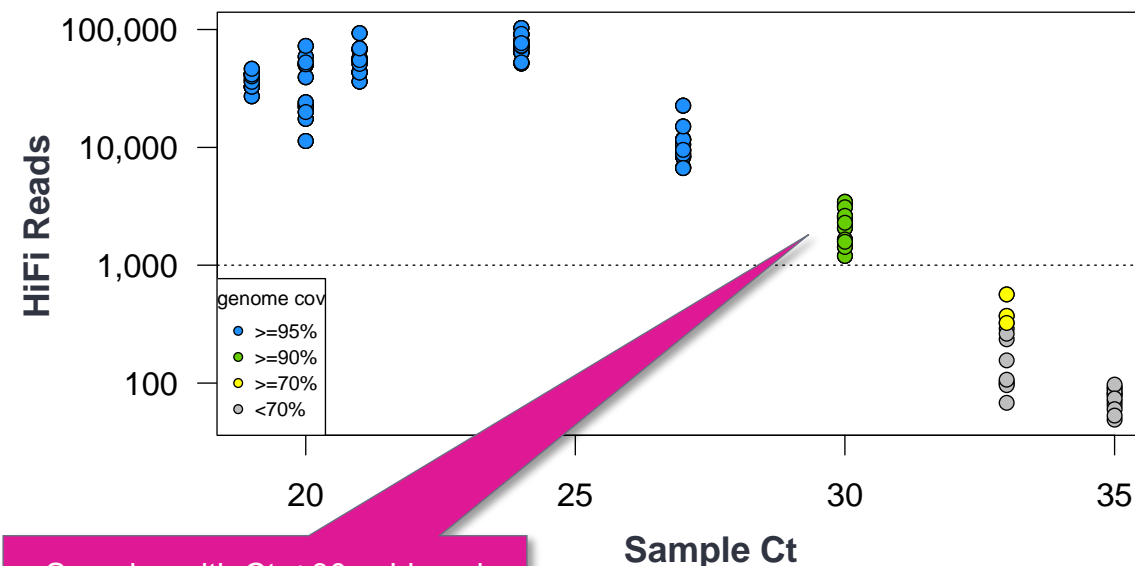
Samples with Ct ≤ 30 achieved **complete genome coverage**** with 1000 HiFi reads

- 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
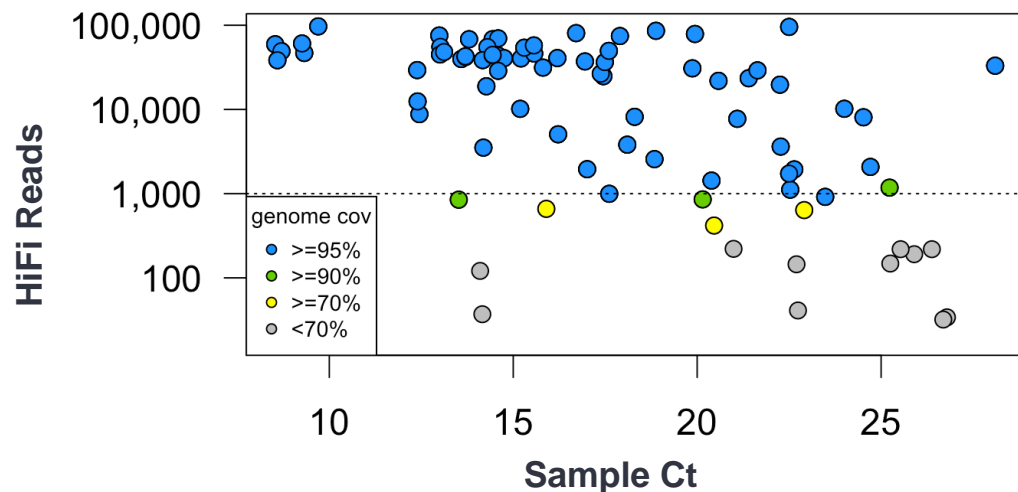
Genome completeness in surveillance samples

SAMPLE INPUT	NO. OF SAMPLES	> 90% GENOME COVERAGE
Known Ct	84	83%
Unknown Ct	9	44%
Twist Controls	2	100%
Negative Control	1	0
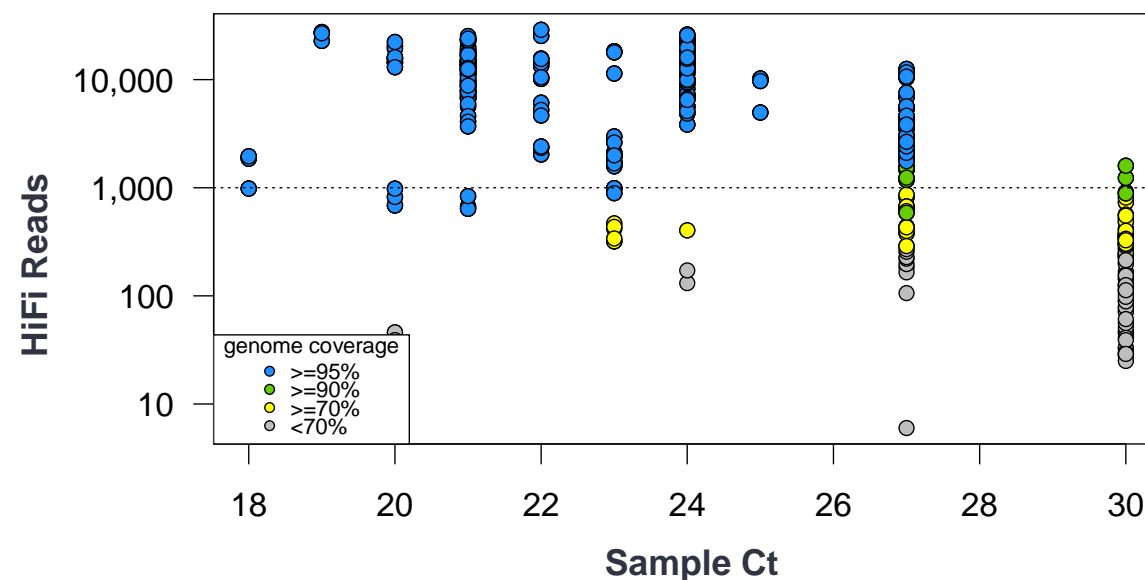
Genome completeness in 384-plex

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

PacBio

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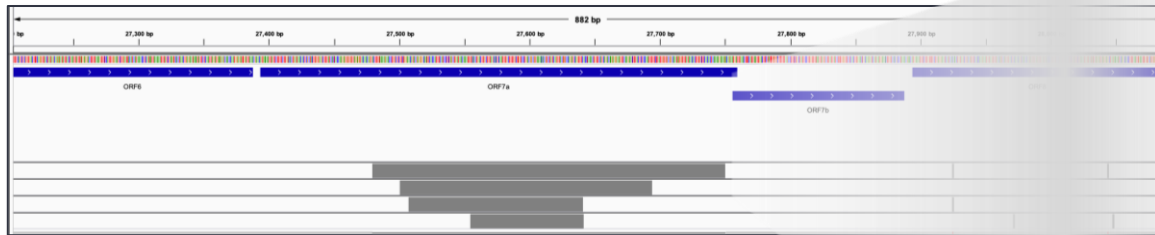
Deletions (87 – 271 bp) are called in VCF and consensus sequence.

194 bp del

271 bp del

Example visualizations of HiFi reads spanning around large deletions.

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%

- 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**

**Multi-strain calling performance
For mixed controls**
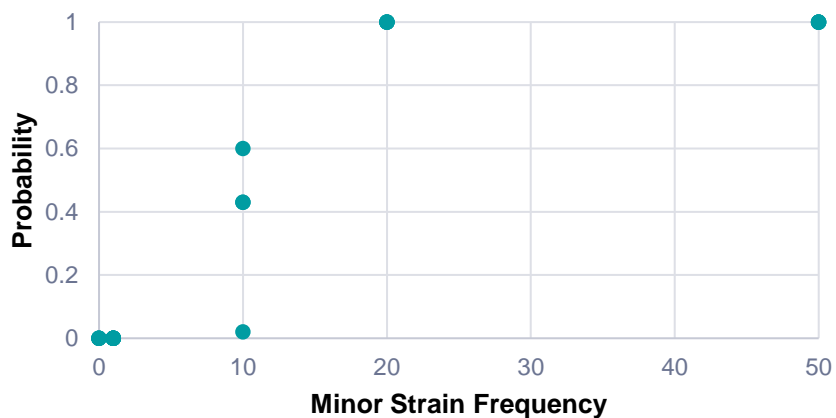
Detection of Minor Variants in Surveillance Samples

Surveillance samples flagged as containing multiple strains at Spike protein gene
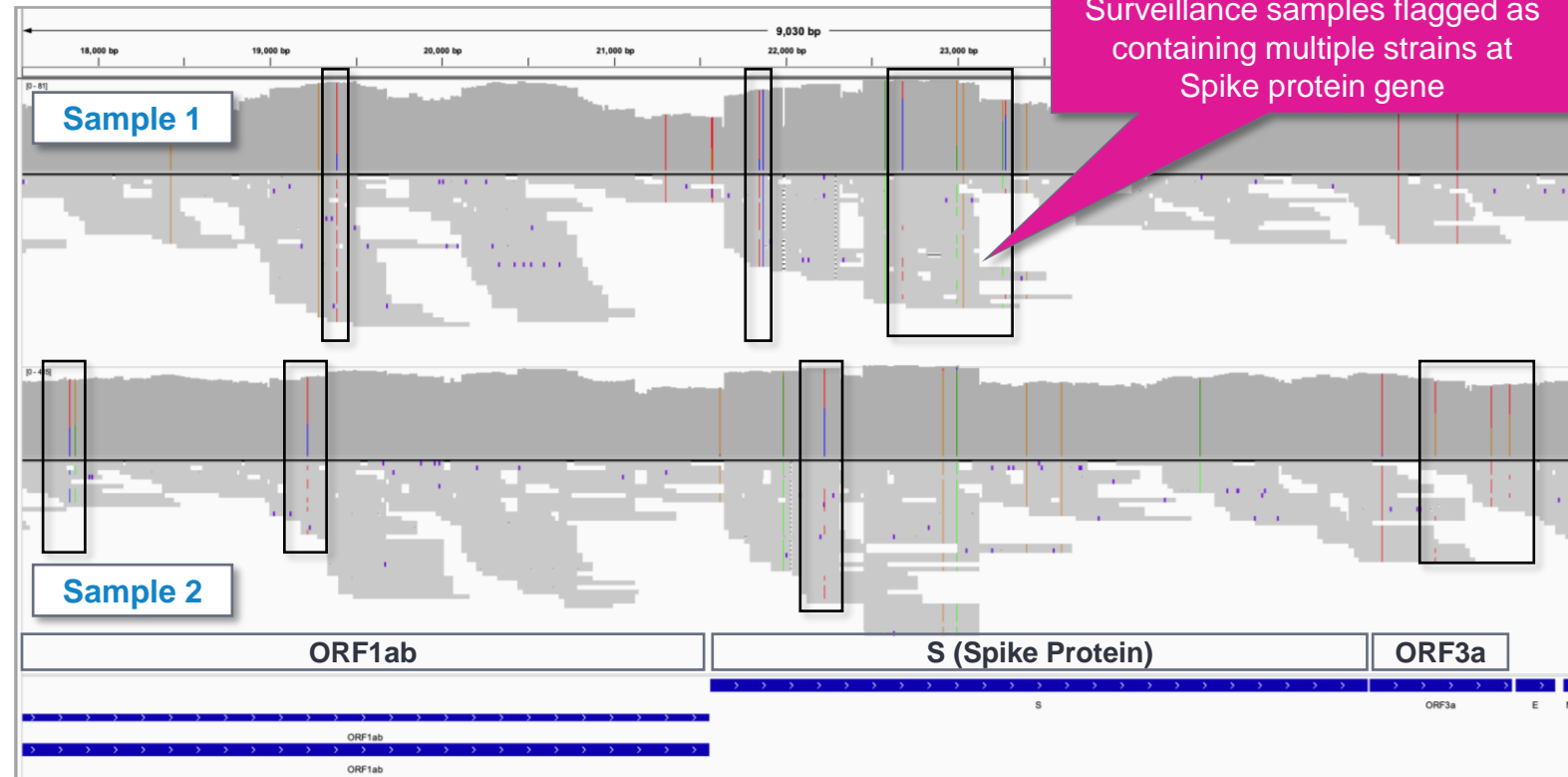
Possible Sources of Multiple Strains in Sample

- 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

Sample Preparation Literature

- Procedure & checklist – PacBio HiFiViral high-throughput multiplexing for full-viral genome sequencing of SARS-CoV-2 using SMRTbell prep kit 3.0 (102-396-100)

- 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 (102-193-692)

- 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 (102-399-300)

FAQ

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

Visit PacBio's *HiFiViral COVID-19 surveillance* website for HiFiViral SARS-CoV-2 workflow updates and other resources

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

Technical resources for SARS-CoV-2 library preparation, sequencing & data analysis (cont.)

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]

Example PacBio data sets

Viral sequencing application	Dataset	Data type	PacBio system
SARS-CoV-2 surveillance	Omicron samples	HiFi Reads	Sequel II System
SARS-CoV-2 surveillance	Synthetic RNA controls	HiFi Reads	Sequel II System
SARS-CoV-2 surveillance	Surveillance samples	HiFi Reads	Sequel II System

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Technical resources for SARS-CoV-2 library preparation, sequencing & data analysis (cont.)

Posters, videos & webinars

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Ordering Information

Consumable product	Part number
HiFiViral SARS-CoV-2 kit (384 rxn)	102-132-000
SMRTbell prep kit 3.0 (24 rxn)	102-182-700
SMRT Cell 8M tray	101-389-001
Sequel II binding kit 3.1 & cleanup beads (24 rxn)	102-333-400
Sequel II sequencing kit 2.0 (4 rxn)	101-820-200

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	Supported automation platform
Thermo Fisher Scientific	MagMAX Viral/Pathogen II (MVP II) 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)

- **Notes:**
  - PacBio users have generally reported good success when using the MagMAX Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit [Link] for extracting RNA samples for the HiFiViral SARS-CoV-2 sample preparation workflow
  - Other PacBio users have reported poor results when using the Perkin Elmer chemagic Viral DNA/RNA 300 Kit H96 [Link] for extracting RNA samples for the HiFiViral SARS-CoV-2 sample preparation workflow – thus, this chemagic kit product should be avoided when preparing HiFiViral SARS-CoV-2 samples for SMRT sequencing
  - Superior performance of the HiFiViral SARS-CoV-2 workflow is typically observed for samples obtained from nasopharyngeal extracts
  - Suboptimal performance has been reported by PacBio users for samples obtained from saliva extracts
  - The HiFiViral SARS-CoV-2 workflow is not recommended for analysis of wastewater samples and may lead to failure to produce any high-quality data

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.

Bravo liquid handler (Agilent)
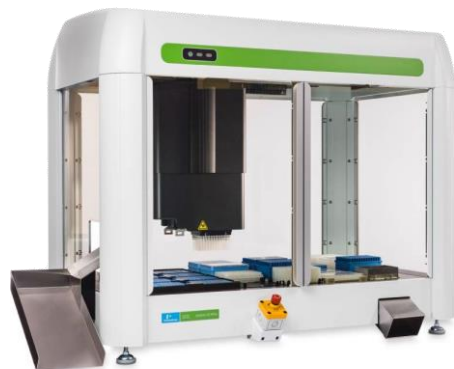
Sciclone G3 / Zephyr G3 NGS workstations (Perkin Elmer)

Biomek 4000 workstation (Beckman Coulter)

Microlab VANTAGE liquid handler (Hamilton)

Dragonfly discovery (SPT Labtech)

Infinite F-series plate reader (Tecan)

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

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Recommended steps to automate for viral genome enrichment workflow using HiFiViral SARS-CoV-2 kit

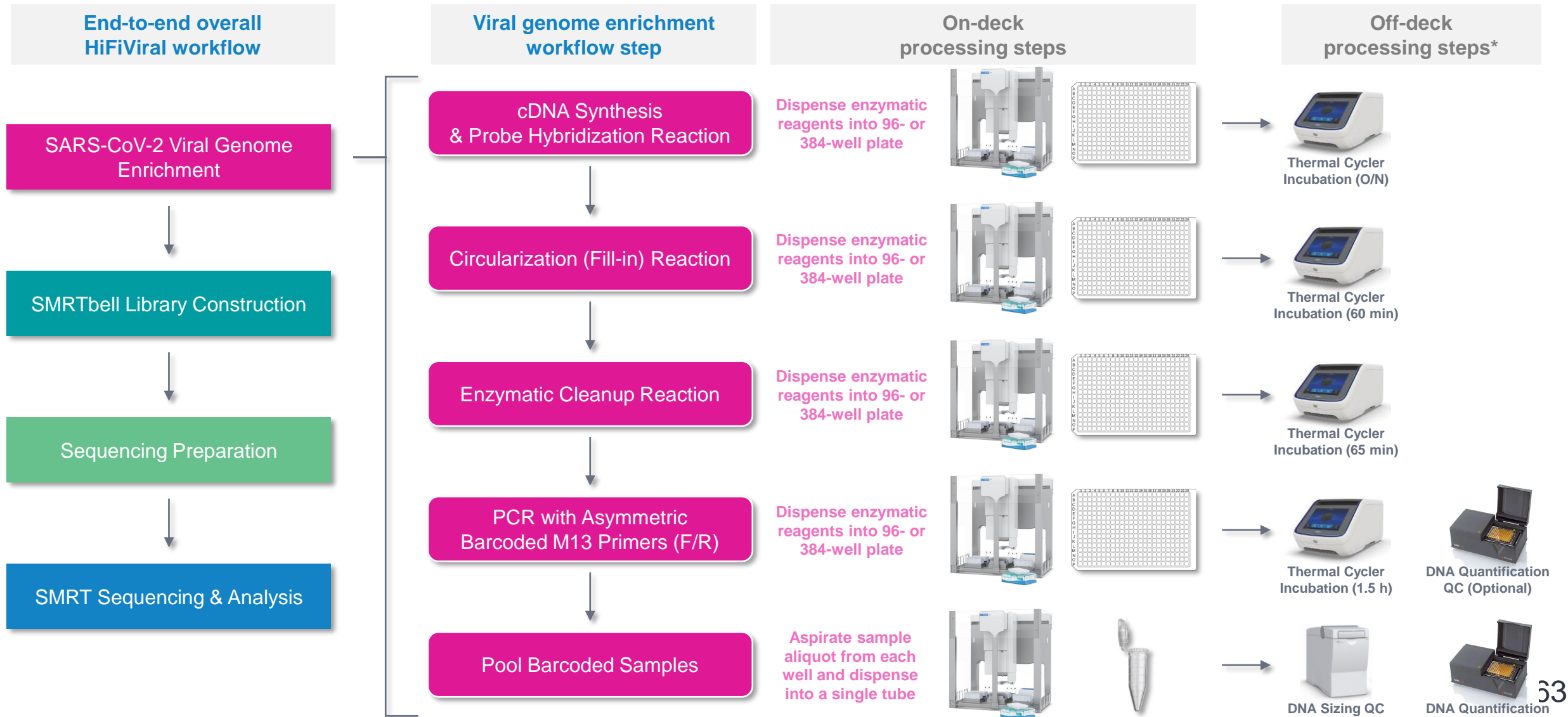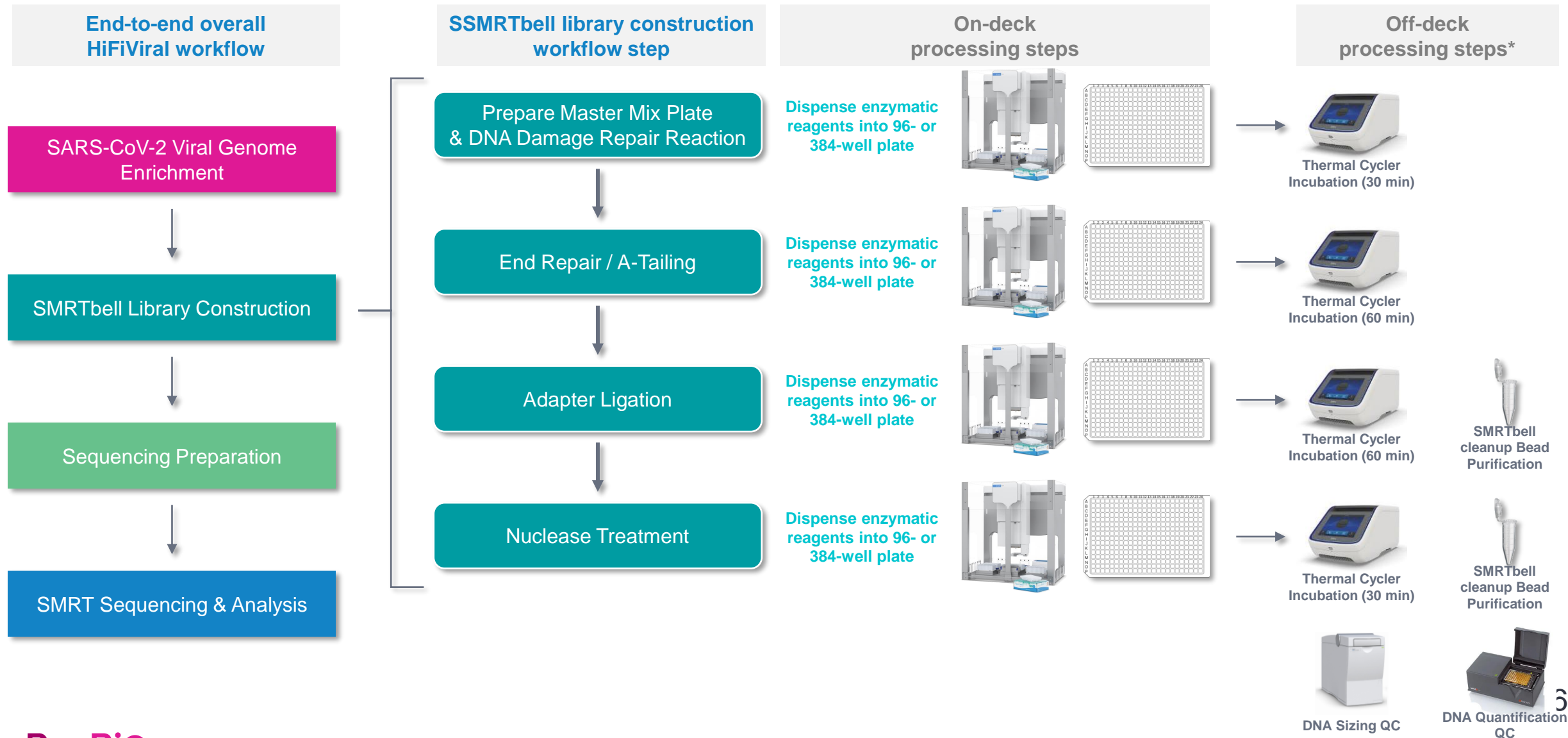| End-to-end overall HiFiViral workflow	Viral genome enrichment workflow step	On-deck processing steps	Off-deck processing steps*
SARS-CoV-2 Viral Genome Enrichment	**cDNA Synthesis & Probe Hybridization Reaction**	Dispense enzymatic reagents into 96- or 384-well plate	Thermal Cycler Incubation (O/N)
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)

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PacBi ** 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 using SMRTbell prep kit 3.0

End-to-end overall HiFiViral workflow	SSMRTbell library construction workflow step	On-deck processing steps	Off-deck processing steps*

SARS-CoV-2 Viral Genome Enrichment

SMRTbell Library Construction

Sequencing Preparation

SMRT Sequencing & Analysis

Prepare Master Mix Plate & DNA Damage Repair Reaction

Dispense enzymatic reagents into 96- or 384-well plate

Thermal Cycler Incubation (30 min)

End Repair / A-Tailing

Dispense enzymatic reagents into 96- or 384-well plate

Thermal Cycler Incubation (60 min)

Adapter Ligation

Dispense enzymatic reagents into 96- or 384-well plate

Thermal Cycler Incubation (60 min)

SMRTbell cleanup Bead Purification

Nuclease Treatment

Dispense enzymatic reagents into 96- or 384-well plate

Thermal Cycler Incubation (30 min)

SMRTbell cleanup Bead Purification

DNA Sizing QC

DNA Quantification QC

www.pacb.com