PACBIO®

PROCEDURE & CHECKLIST

PacBio HiFiViral High Throughput Multiplexing for Full-Viral Genome Sequencing of SARS-CoV-2

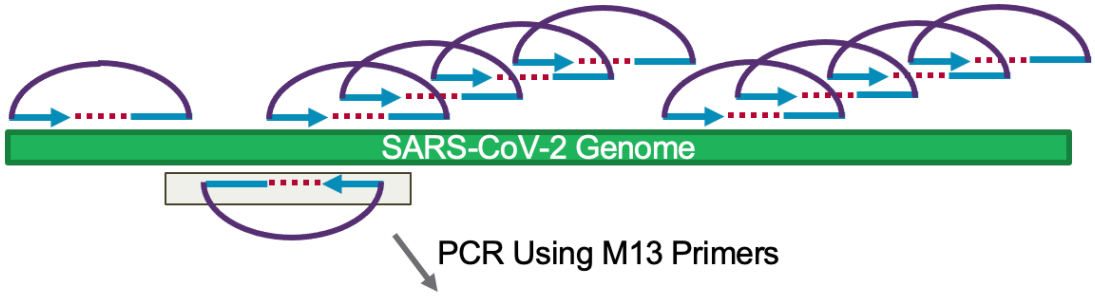
January 2022

Overview

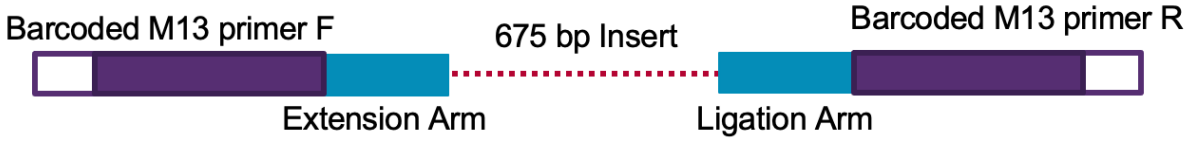
This procedure captures the SARS-CoV-2 genome with tiled molecular inversion probes that create highly-redundant overlapping amplicons resulting in comprehensive sequence coverage on the Sequel® II and Sequel IIe Systems (Sequel II Systems). Target capture uses an addition-only 4-step workflow with color-coded master mixes to simplify setup.

Single-Stranded DNA Probe

Hybridization and Fill-In Reaction

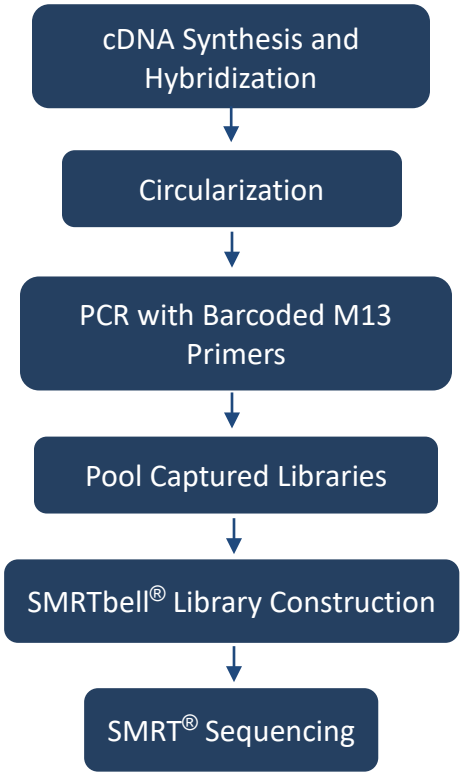


Double Stranded Library Molecule (~800 bp, not to scale)



Workflow

The general workflow described in this procedure is summarized below.



Required Materials

Item	Where Used	Vendor	Part Number
RNA Preparation			
Nuclease-Free Water	RNA Preparation	Any	Vendor-specific
RNaseZap	RNA Preparation	Thermo Fisher Scientific	AM9780
Viral Enrichment with HiFiViral SARS-CoV-2 Kit (Includes 102-135-400 & 102-135-500)		PacBio	102-132-000
SARS-CoV-2 Enrichment Kit	Viral Enrichment	PacBio	102-135-400
Barcoded M13 Primer Plate	Asymmetric Sample Barcoding (Dual Indexing)	PacBio	102-135-500
SMRTbell Library Construction			
SMRTbell® Express Template Prep Kit 2.0	Library Preparation	PacBio	100-938-900
SMRTbell® Enzyme Cleanup Kit 2.0	Library Preparation	PacBio	101-932-600
DynaMag-2 Magnet	Purification	Invitrogen	12321D
100% Ethanol, Molecular Biology Grade	Purification	Any	Vendor-specific
ProNex Beads	Purification	Promega	NG2001-10mL, NG2002-125mL, NG2003-500mL
Sequencing			
Sequel® II Binding Kit 2.1 and Int Ctrl 1.0	Sequencing short (<3 kb) amplicon samples on the Sequel® Systems	PacBio	101-843-000
Sequel® II Sequencing Kit 2.0	Supports 4 sequencing reactions on the Sequel® Systems	PacBio	101-820-200
SMRT® Cell 8M Tray	Sequencing on the Sequel® Systems	PacBio	101-389-001
Sequel® Pipette Tips v2	Sequencing on the Sequel® Systems	PacBio	100-667-601
Sequel® Mixing Plates	Sequencing on the Sequel® Systems	PacBio	100-667-500
Sample Plate	Sequencing on the Sequel® Systems	BioRad	HSP9601
Tube Septa	Sequencing on the Sequel® Systems	PacBio	001-292-541
Foil Heat Seals	Sequencing on the Sequel® Systems	Thermo Fisher Scientific	AB0757
Others			
96-well PCR plates	cDNA Preparation	Bio-Rad	HSP9601
Microseal ‘B’ Film	cDNA Preparation	Bio-Rad	MSB1001
Film sealing roller for PCR plates	cDNA Preparation	Bio-Rad	MSR0001
DNA LoBind Tubes, 1.5 mL	Library Preparation	Eppendorf	22431021

DNA LoBind Tubes, 2.0 mL	Library Preparation	Eppendorf	22431048
8- or 12-Multichannel Pipette	High Throughput Pipetting	Any	Vendor-specific
Qubit™ 4 Fluorometer	Quantification	Thermo Fisher Scientific	Q33238
Qubit™ 1x dsDNA HS Assay Kit	Quantification	Thermo Fisher Scientific	Q33230
Bioanalyzer 2100	Library QC (Optional)	Agilent	G2939A
Agilent DNA 12000 Kit	Library QC (Optional)	Agilent	5067-1508
VeritiPro Thermal Cycler, 96 well	PCR Amplification	Thermo Fisher Scientific	A48141
ProFlex PCR System	PCR Amplification	Thermo Fisher Scientific	4483636
PCR Tube Strips, 0.2 mL	PCR Amplification (Optional)	USA Scientific	1402-4708
96-welll Plate Centrifuge		Any MLS	

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 microfuge tubes. Briefly vortex to mix and spin 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 batch, including control samples, should 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 can be used if desired to briefly warm the seal and 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.

RNA Input Recommendations

Best results will be achieved if reactions contain at least 10,000 copies of RNA. Higher amounts will generally produce superior results. 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 (followed by full and complete inactivation/removal) 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.

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 down.
    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 slowly.
5. Seal the plate tightly with a film. Poor sealing could result in significant sample loss due to evaporation.
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	1 minute
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).

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 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 **pale green** color.
6. Place the reaction plate in the thermal cycler and continue the program for another 60 minutes.
7. Record the time the reaction plate was returned to the thermal cycler; correct timing is important to maximize result quality.

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
3	4°C	Hold

7. The program will take approximately 65 minutes to run; proceed immediately to the cDNA amplification step when finished.

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.

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	✓	Notes
Cleanup reaction		9.6 µL*		
PCR Mix		12 µL		
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 primers from the Barcoded M13 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 seconds
3	55°C	15 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.

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.

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. Pooling fewer than 8 reactions is not recommended.
    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.

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 prepared 80% ethanol. After removal of the second 1400 µL ethanol wash, spin the tube briefly, return to the magnetic stand, and remove residual ethanol with a P20 pipette. Do not let the beads dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add 50 µL of EB 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.

SMRTbell Library Construction

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

DNA Damage Repair

1. Prepare the following reaction.

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

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick-spin in a microfuge.
4. Incubate in a thermocycler with the following program. Set the lid temperature to 47°C.

Temperature	Time
37°C	30 minutes
4°C	Hold

5. Proceed to the next step.

End-Repair/A-tailing

1. Prepare the following reaction.

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

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick-spin in a microfuge.
4. Incubate in a thermocycler with the following program. Set the lid temperature to 75°C.

Temperature	Time
20°C	30 minutes
65°C	30 minutes
4°C	Hold

5. Proceed to the next step.

Adapter Ligation

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

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

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick-spin in a microfuge.
4. Incubate in a thermocycler with the following program. Set the lid temperature to 30°C.

Temperature	Time
20°C	60 minutes
4°C	Hold

5. Proceed to the next step.

Purification of SMRTbell Templates

STEP	✓	Purification with ProNex Beads	Notes
1		Add **126 µL** (1.3X) of resuspended, room-temperature ProNex beads to the 97 µL Reaction. 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 700 µL (250ul if using PCR tube) of freshly prepared 80% ethanol. After removal of the second ethanol wash, spin the tube briefly, return to the magnetic stand and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add 40 µL of EB 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.	

Nuclease Treatment of SMRTbell Libraries

Use the following table to prepare a reaction to remove damaged SMRTbell templates.

1. Prepare a Nuclease Treatment Master Mix as follows.

Reagent	Tube Cap Color	Volume	✓	Notes
Purified SMRTbell library		40 µL		
SMRTbell Enzyme Clean Up Mix		5.0 µL		
SMRTbell Enzyme Clean Up Buffer 2.0		5.0 µL		
Total Volume		50.0 µL		

2. Mix the reaction well by pipetting up and down 10 times. It is important to mix well.
3. Incubate in a thermocycler with the following program. Set the lid temperature to 47°C.

Temperature	Time
37°C	30 minutes
4°C	Hold

4. Proceed immediately to “Purification of SMRTbell Templates”.

Purification of SMRTbell Templates

STEP	✓	Purification with ProNex Beads	Notes
1		Add **65 µL** (1.3X) of resuspended, room-temperature ProNex beads to the 50 µL Reaction. 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 700 µL (250ul if using PCR tube) of freshly prepared 80% ethanol. After removal of the second ethanol wash, spin the tube strip briefly, return to the magnetic stand, and remove residual ethanol with a P20 pipette. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add 20 µL of EB and pipette 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.	

Sequencing Preparation

Proceed to Sample Setup in SMRT® Link to prepare the SMRTbell library for sequencing.

Revision History (Description)	Version	Date
Initial Release.	01	November 2021
Added cover page. Removed some instances of multichannel pipette. Updated some recommended reactions.	02	November 2021
Clarification of SARS-CoV-2 kit contents and removed references to asymmetric barcodes.	03	January 2022