Procedure & Checklist – Amplification of Full-Length SARS-CoV2 Spike Gene with Barcoded Primers for Multiplexed SMRTbell® Library Preparation and Sequencing

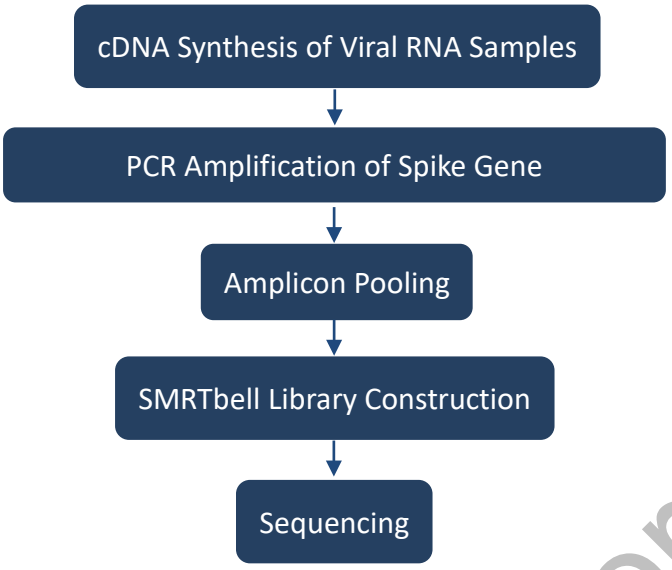
This document contains instructions for 1) synthesis of first-strand cDNA from SARS-CoV2 viral RNA, 2) PCR amplification of the full-length Spike gene, 3) preparation of multiplexed SMRTbell libraries of Spike gene amplicons, and 4) recommendations for sequencing of multiplexed SMRTbell libraries of Spike gene amplicons. We also provide links to the sequences of, and ordering information for, all primers required for this procedure.

Required Materials

Item	Where Used	Vendor	Part Number
SuperScript™ IV First-Strand Synthesis System	cDNA Synthesis	Invitrogen	18091050
Spike Gene-Specific RT Primer (Customer-supplied)	cDNA Synthesis	Oligo Synthesis Company	N/A
Universal Human Reference RNA	cDNA Synthesis	ThermoFisher	QS0639
Spike Gene-Specific F/R Primers (Customer-supplied)	PCR Amplification	Oligo Synthesis Company	N/A
Platinum™ SuperFi™ II PCR Master Mix	PCR Amplification	Thermo Fisher Scientific	12368050
SMRTbell® Express Template Prep Kit 2.0	Library Prep	PacBio	100-938-900
AMPure® PB Beads	Library Purification	PacBio	100-265-900
Sequel® System or Sequel II System Binding and Internal Control Kit (Recommended kits are listed below)			
Sequel® II Bind Kit 2.0 and Int Ctrl 1.0	Sequencing long (>3 kb) amplicon samples on the Sequel II System	PacBio	101-843-000
Sequel® Binding and Internal Ctrl Kit 3.0	Sequencing amplicon samples on the Sequel System	PacBio	101-626-600
Sequel® System or Sequel II System Sequencing Kit (Recommended kits are listed below)			
Sequel® II Sequencing Kit 2.0	Supports 4 sequencing reactions on the Sequel II System	PacBio	101-789-500
Sequel® Sequencing Kit 3.0 (8-rxn)	Supports 8 sequencing reactions on the Sequel System	PacBio	101-597-800
Sequel® Sequencing Kit 3.0 (4-rxn)	Supports 4 sequencing reactions on the Sequel System	PacBio	101-597-900
Sequel® System or Sequel II System SMRT® Cells			
SMRT® Cell 8M Tray	Sequencing on the Sequel II System	PacBio	101-389-001
SMRT® Cell 1M v3 Tray	Sequencing on the Sequel System (Max. 10-hour movie collection time)	PacBio	101-531-000
SMRT® Cell 1M v3 LR Tray	Sequencing on the Sequel System (Max. 20-hour movie collection time)	PacBio	101-531-001
8- or 12-Multichannel Pipettor	High Throughput Pipetting	Any	Vendor-specific
Qubit™ 4 Fluorometer	DNA concentration measurement	ThermoFisher	Q33238
Qubit™ 1x dsDNA HS Assay Kit	DNA concentration measurement	ThermoFisher	Q33230

Workflow

The general workflow described in this procedure is summarized below:



First-strand cDNA Synthesis with Spike Gene-specific RT Primer

Prior to PCR amplification of the Spike gene, first strand cDNA must be prepared from viral RNA samples. For best results, we recommend using a Spike gene-specific RT primer for cDNA synthesis with the following sequence: AAGCCATCCGAAAGGGAGTGAGGC. Before use, dilute RT primer to 5 µM in 10 mM Tris-HCl pH 7.5.

Anneal primers to RNA

1. Mix the following components on ice:

Reagent	1X volume	✓	Notes
Nuclease Free Water[1]	5 µL		
10mM dNTP Mix[1]	1 µL		
5 µM Spike RT Primer	1 µL		
UHRR[2] (100-200 ng/µL)	1 µL		
Viral RNA Sample	5 µL		
Total Volume	**13 µL**		

[1]These components are provided in Invitrogen's SuperScript™ IV First-Strand Synthesis System, ThermoFisher, Part No. 18091200
[2]Invitrogen Universal Human Reference RNA, ThermoFisher, Part No. QS0639

2. Incubate at 60°C for 5 minutes, then quick chill at 4°C or on ice for ≥ 1 minute.

First strand cDNA synthesis

1. Mix the following components on ice:

Reagent	1X volume	✓	Notes
5x Superscript IV RT buffer	4 µL		
DTT	1 µL		
Ribonuclease Inhibitor	1 µL		
Superscript IV RT	1 µL		
Total Volume	**7 µL**		

2. Add mix to primer-annealed RNA on ice.
3. Incubate at 50°C for 60 minutes, heat kill at 80°C for 10 minutes, then hold at 4°C.
4. Proceed to AMPure PB bead purification and pooling.

STEP	✓	AMPure PB Bead Purification	Notes
1		Add an appropriate volume of 1x Elution Buffer (EB) to bring the sample volume to 100 µL before proceeding. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process. Add **1X** of AMPure PB beads to the sample.	
2		Mix the bead/DNA solution thoroughly by finger tapping or pipetting.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by mixing on an end-over-end rotator for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect the beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Store 80% ethanol in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube. Slowly dispense the 80% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8** above.	
10		Remove residual 80% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 60 seconds.	
13		Add 2**0 µL** of Elution Buffer to the beads to elute the DNA: – Elute the DNA by incubating at room temperature for 2 minutes. – Briefly spin the tube, then place the tube back on the magnetic bead rack and let the beads separate fully. – Without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
14		Proceed to PCR Amplification or store AMPure PB bead-purified cDNA samples at -20°C for future use.	

Preparing Primers for PCR Amplification

Sequences for Spike gene-specific, barcoded forward and reverse PCR primers are available here. This procedure utilizes an asymmetric barcoding strategy for high sample multiplexing. With asymmetric barcoding, different barcodes are used on the forward (F) and reverse (R) PCR primers. The number of PCR primers to order depends on the desired plex level. For example, to multiplex up to 2304 samples in a single SMRTbell library, order 48 forward primers [S_FWD_BC1001 through S_FWD_BC1048] and 48 reverse primers [S_REV_BC1049 through S_REV_BC1096]. Be sure to use different barcodes for all forward and reverse primers.

Barcoded PCR primers may be ordered from any oligo synthesis provider. For PCR, desalted oligos are typically of sufficient purity. For convenience, we order lab-ready oligos (i.e. delivered as 100 µM stocks in 10mM Tris-HCl pH 7.5) arrayed in 96-well plates.

Before proceeding with PCR amplification, dilute and array PCR primers based on the desired sample multiplex level. For each sample to be processed, combine **one forward** and **one reverse** barcoded PCR primer, each at a final concentration of 2.5 µM, in a single well of a 96-well plate. For example, add 5 µL each 100 µM stock primer to 190 µL nuclease-free buffer (10mM Tris-HCl pH 7.5). Mix primer stocks well before dilution.

NOTE: Any forward primer may be combined with any **different** reverse primer to create asymmetrically barcoded pairs.

An example of the PCR primer plate layout for a 96-plex assay utilizing 8 Forward and 12 Reverse Barcoded Spike Gene PCR Primers is shown in the table below. Each well contains one forward and one reverse primer, each at a concentration of 2.5 µM.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FWD_1001 & REV_1049	FWD_1001 & REV_1050	FWD_1001 & REV_1051	FWD_1001 & REV_1052	FWD_1001 & REV_1053	FWD_1001 & REV_1054	FWD_1001 & REV_1055	FWD_1001 & REV_1056	FWD_1001 & REV_1057	FWD_1001 & REV_1058	FWD_1001 & REV_1059	FWD_1001 & REV_1060
B	FWD_1002 & REV_1049	FWD_1002 & REV_1050	FWD_1002 & REV_1051	FWD_1002 & REV_1052	FWD_1002 & REV_1053	FWD_1002 & REV_1054	FWD_1002 & REV_1055	FWD_1002 & REV_1056	FWD_1002 & REV_1057	FWD_1002 & REV_1058	FWD_1002 & REV_1059	FWD_1002 & REV_1060
C	FWD_1003 & REV_1049	FWD_1003 & REV_1050	FWD_1003 & REV_1051	FWD_1003 & REV_1052	FWD_1003 & REV_1053	FWD_1003 & REV_1054	FWD_1003 & REV_1055	FWD_1003 & REV_1056	FWD_1003 & REV_1057	FWD_1003 & REV_1058	FWD_1003 & REV_1059	FWD_1003 & REV_1060
D	FWD_1004 & REV_1049	FWD_1004 & REV_1050	FWD_1004 & REV_1051	FWD_1004 & REV_1052	FWD_1004 & REV_1053	FWD_1004 & REV_1054	FWD_1004 & REV_1055	FWD_1004 & REV_1056	FWD_1004 & REV_1057	FWD_1004 & REV_1058	FWD_1004 & REV_1059	FWD_1004 & REV_1060
E	FWD_1005 & REV_1049	FWD_1005 & REV_1050	FWD_1005 & REV_1051	FWD_1005 & REV_1052	FWD_1005 & REV_1053	FWD_1005 & REV_1054	FWD_1005 & REV_1055	FWD_1005 & REV_1056	FWD_1005 & REV_1057	FWD_1005 & REV_1058	FWD_1005 & REV_1059	FWD_1005 & REV_1060
F	FWD_1006 & REV_1049	FWD_1006 & REV_1050	FWD_1006 & REV_1051	FWD_1006 & REV_1052	FWD_1006 & REV_1053	FWD_1006 & REV_1054	FWD_1006 & REV_1055	FWD_1006 & REV_1056	FWD_1006 & REV_1057	FWD_1006 & REV_1058	FWD_1006 & REV_1059	FWD_1006 & REV_1060
G	FWD_1007 & REV_1049	FWD_1007 & REV_1050	FWD_1007 & REV_1051	FWD_1007 & REV_1052	FWD_1007 & REV_1053	FWD_1007 & REV_1054	FWD_1007 & REV_1055	FWD_1007 & REV_1056	FWD_1007 & REV_1057	FWD_1007 & REV_1058	FWD_1007 & REV_1059	FWD_1007 & REV_1060
H	FWD_1008 & REV_1049	FWD_1008 & REV_1050	FWD_1008 & REV_1051	FWD_1008 & REV_1052	FWD_1008 & REV_1053	FWD_1008 & REV_1054	FWD_1008 & REV_1055	FWD_1008 & REV_1056	FWD_1008 & REV_1057	FWD_1008 & REV_1058	FWD_1008 & REV_1059	FWD_1008 & REV_1060

PCR Amplification of Full-length Spike Gene

1. For each sample to be processed, prepare the following PCR master mix on ice, including 15% overage:

Reagent	Stock Conc.	Final Conc.	1X volume	✓	Notes
Nuclease Free Water			7.5 µL		
Platinum SuperFi II Master Mix (2X)	2X	1X	12.5 µL		
Total Volume			**20 µL**		

2. Mix well by pipetting, then aliquot 20 µL PCR master mix to each well of destination PCR plate on ice.
3. Add 2.5 µL each individual cDNA sample to a single well of PCR destination plate.
4. Add 2.5 µL diluted and arrayed forward and reverse barcoded primers at 2.5 µM each (as prepared above) per well of destination PCR plate.
5. Mix reactions well using a multichannel pipettor, seal PCR plate thoroughly, then quickly spin down contents in 4°C refrigerated centrifuge.
6. Place PCR plate into a thermocycler and run the following program (lid 105°C).

Step	Temperature	Time
1	98ºC	3 minutes
2	98ºC	30 seconds
3	60ºC	30 seconds
4	72ºC	4 minutes
5	Repeat steps 2 to 4*	30-40 cylces[1]
6	72ºC	4 minutes
7	4ºC	Hold

[1]Optimization is highly recommended

7. After amplification, perform visual inspection of the PCR products on an agarose gel to ensure 4 kb products are visible in all wells.
8. Proceed to Pooling Barcoded Amplicons.

STEP	✓	Pooling Barcoded Amplicons	Notes
1		If PCR products are of the expected size and comparable quantity as determined visually on an agarose gel, pool equal volumes of each PCR reaction in a clean 2.0 mL DNA LoBind microcentrifuge tube. We recommend pooling 15 µL from each well.	
2		Proceed to AMPure PB bead purification below. If desired, any remaining un-pooled PCR reactions may be stored at -20°C for future use.	

STEP	✓	AMPure PB Bead Purification	Notes
1		Determine the sample volume. If pooled sample volume is < 100 µL, add an appropriate volume of 1x Elution Buffer (EB) to bring the sample volume to 100 µL before proceeding. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process. Add **0.45X** of AMPure PB beads to the sample.	
2		Mix the bead/DNA solution thoroughly by finger tapping or pipetting.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by mixing on an end-over-end rotator for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect the beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Store 80% ethanol in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube. Slowly dispense the 80% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8** above.	
10		Remove residual 80% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 60 seconds.	
13		Add **50 µL** of Elution Buffer to the beads to elute the DNA: – Elute the DNA by incubating at room temperature for 2 minutes. – Briefly spin the tube, then place the tube back on the magnetic bead rack and let the beads separate fully. – Without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
14		Measure DNA concentration using the Qubit Fluorometer. – Using 1 µL of the sample, make a 1:10 dilution in 1x EB. – Using dsDNA High Sensitivity Kit reagents, measure the DNA concentration of 1 µL of the diluted sample. – Use the remaining 9 µL of diluted sample to verify the size of the final SMRTbell library on an agarose gel.	
15		Proceed to SMRTbell library preparation.	

SMRTbell Library Construction

DNA Damage Repair

1. Prepare the following reaction, using 2000 ng pooled sample. Dilute pooled PCR product to 42.6 ng/µL in 47 µL 1X Elution Buffer before proceeding.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Pooled and Purified PCR Product (2000 ng)		47.0 µL		
NAD		1.0 µL		
DNA Damage Repair Mix v2		2.0 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 at 37°C for 30 minutes, then return the reaction to 4°C. 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 at 20°C for 30 minutes.
5. Incubate at 65°C for 30 minutes, then return the reaction to 4°C. 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 at 20°C for 60 minutes, then return the reaction to 4°C. Proceed to the next step.

Purification of SMRTbell Templates

STEP	✓	First AMPure PB Bead Purification	Notes
1		Bring volume of ligation reaction to 200 µL using 1x Elution Buffer, then add **90 µL** (0.45X) of AMPure PB beads to the 200 µL diluted ligation reaction. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by finger tapping or pipetting.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate samples on bench top for 10 minutes at room temperature on an end-over-end rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **100 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by incubating at room temperature for 2 minutes. – Briefly spin the tube, then place the tube back on the magnetic bead rack and let the beads separate fully. – Without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
13		Proceed to the final AMPure PB bead purification.	

STEP	✓	Final AMPure PB Bead Purification	Notes
1		Bring AMPure beads to room temperature and mix well by vortexing for 30 seconds before use. Add **0.45X** of AMPure PB beads to the sample.	
2		Mix the bead/DNA solution thoroughly by finger tapping or pipetting.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by mixing on an end-over-end rotator for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect the beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8** above.	
10		Remove residual 80% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 60 seconds.	
13		Add **20 µL** of Elution Buffer to the beads to elute the DNA: – Elute the DNA by incubating at room temperature for 2 minutes. – Briefly spin the tube, then place the tube back on the magnetic bead rack and let the beads separate fully. – Without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Measure DNA concentration using the Qubit Fluorimeter. – Using 1 µL of the sample, make a 1:10 dilution in 1x EB. – Using dsDNA High Sensitivity Kit reagents, measure the DNA concentration of 1 µL of the diluted sample. – Use the remaining 9 µL of diluted sample to verify the size of the final SMRTbell library on an agarose gel.	

Prepare for Sequencing

Sequel System:

- For primer annealing and polymerase binding, follow the instructions in SMRT Link Sample Setup (SMRT Link v8.0). Select the following options:

Sample Setup	Select	
Sequencing Primer	Sequencing Primer v4	Enable 20:1 Polymerase:Template 30:1 Polymerase:Template
Binding Kit	Sequel Binding Kit 3.0	
Sequencing Mode	CLR	

- In **Run Design**, select sequencing mode = CCS.
- For detailed recommendations for sequencing specific amplicon library insert size ranges, refer to the Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System here.

Sequel II System:

- For primer annealing and polymerase binding, follow the instruction in SMRT Link Sample Setup (SMRT Link 8.0). Select the following options:

Sample Setup	Select	
Sequencing Primer	Sequencing Primer v4	Enable 20:1 Polymerase:Template 30:1 Polymerase:Template
Binding Kit	Sequel II Binding Kit 2.0	
Sequencing Mode	CLR	

- In **Run Design**, select sequencing mode = CCS
- Recommended Run parameters:

On-Plate Loading Conc (pM)	50 pM
Movie Time (hours)	20
Pre-Extension Time	0.8 Hours

Sequencing Data Analysis on the Sequel II System

The SMRT Link v8.0 user interface can support a maximum number of 384 barcodes. To analyze more than 384 barcoded samples containing different barcodes on either end of the template, follow the instructions below.

Alternatively, data can be analyzed on the command line using SMRT Tools or PacBio Developers tools in Bioconda. If you need additional information, please contact support@pacb.com.

CCS Analysis:

- When you are designing your sequencing run in the Run Design module of SMRT Link v8.0 or higher use the Auto Analysis option to set up CCS analysis. Data will be automatically analyzed with the CCS analysis application following the sequence acquisition.
- If CCS analysis was not performed automatically, manually run CCS analysis in SMRT Link with default parameters

Demultiplexing Analysis:

- Use the Demultiplex Barcodes analysis application in SMRT Link v8.0 or higher to demultiplex the CCS reads.
- Use PacBio Barcode Set "Sequel_96_barcodes_v1" for demultiplexing
  For details on the Demultiplex Barcodes analysis application, see pg. 62 in the SMRT Link User Guide

Analysis steps:

At the SMRT Analysis module in SMRT Link, click on Create New Analysis:

1. Enter an analysis name
2. Select Data Type "CCS Data"
3. Select your dataset for analysis and click Next
4. Select Analysis Application "Demultiplex Barcodes"
5. Select Barcode Set "Sequel_96_barcodes_v1"
6. Provide the output a New Dataset Name
7. Set Same Barcodes on Both Ends of Sequence to "Off"
8. Set Infer Barcodes Used to "Off"* (Note: Analysis will fail if turned on.)
9. Optionally, set Minimum Barcode Score to 70

Revision History (Description)	Version	Date
Customer Collaboration.	DRAFT	July 14, 2020