Procedure & Checklist – Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell® Library Preparation and Sequencing

This document contains instructions for:

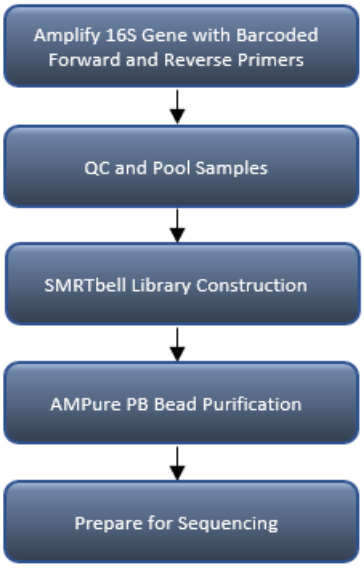
1) PCR amplification of full-length 16S genes (V1-V9 regions) from bacterial DNA isolated from metagenomic samples.
2) Multiplexed SMRTbell library preparation and sequencing of 16S amplicons.

We also provide the sequences of and ordering information for 8 barcoded forward, and 12 barcoded reverse, 16S-specific primers that can be combined for multiplexed analysis of up to 96 samples using the asymmetric barcoding strategy described in this procedure.

Materials and Kits Needed

Item	Vendor	Part Number
16S Amplification		
KAPA HiFi HotStart ReadyMix PCR Kit	KAPA Biosystems	KK2600 (or KK2601 or KK2602)
Barcoded 16S Primers	Any Oligo vendor	See Table 1 for ordering information
Library Preparation		
SMRTbell Express Template Prep 2.0	PacBio	100-938-900
AMPure® PB beads	PacBio	100-265-900
QC Tools		
Qubit Fluorometer	Thermo-Fisher	Q33238
Qubit 1X dsDNA High Sensitivity Kit	Thermo-Fisher	Q33231
NanoDrop Technologies ND-2000 UV/Vis Spectrophotometer or equivalent	Thermo-Fisher	ND-2000
BioAnalyzer	Agilent Technologies, Inc.	
General Lab Supplies		
DNA LoBind tubes, 2.0 mL	Eppendorf	022431048

Workflow



Barcoded 16S Primer Sequences, Ordering and Storage Information

Table 1 below lists sequences for twenty (20) barcoded,16S gene-specific primers (8 Forward and 12 Reverse) that can be used in all possible asymmetric pairs for multiplexing up to 96 samples.

Oligos must contain 5' phosphates. HPLC-purification is recommended, but not required. Each oligo contains a 5' buffer sequence (GCATC), a 16-base barcode (in bold), and degenerate 16S gene-specific forward or reverse primer sequences. Degenerate base identities are: R = A,G; Y = C,T; M = A,C. Primers should be stored at high concentration in buffered solution (e.g., 100 µM primer in 10 mM Tris-HCl pH 8.0-8.5) at -20°C. Avoid repeated freeze thaws.

Table 1. Recommended sequences for barcoded 16S gene-specific primers.

Barcoded Forward Primer	
>16S_For_bc1005	/5Phos/GCATC**CACTCGACTCTCGCGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1007	/5Phos/GCATC**TCTGTATCTCTATGTG**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1008	/5Phos/GCATC**ACAGTCGAGCGCTGCG**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1012	/5Phos/GCATC**ACACTAGATCGCGTGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1015	/5Phos/GCATC**CGCATGACACGTGTGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1020	/5Phos/GCATC**CACGACACGACGATGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1022	/5Phos/GCATC**CACTCACGTGTGATAT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1024	/5Phos/GCATC**CATGTAGAGCAGAGAG**AGRGTTYGATYMTGGCTCAG
Barcoded Reverse Primer	
>16S_Rev_bc1033	/5Phos/GCATC**AGAGACTGCGACGAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1035	/5Phos/GCATC**CAGAGAGTGCGCGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1044	/5Phos/GCATC**CGCGCGTCGTCTCAGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1045	/5Phos/GCATC**AGAGAGTACGATATGT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1054	/5Phos/GCATC**TCTGTAGTGCGTGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1056	/5Phos/GCATC**ATGTGCGTGTGTGTCT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1057	/5Phos/GCATC**CTCTCAGACGCTCGTC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1059	/5Phos/GCATC**TATCTCAGTGCGTGTG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1060	/5Phos/GCATC**TGTGTCTATACTCATC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1062	/5Phos/GCATC**TATAGACTATCTGAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1065	/5Phos/GCATC**GTATGTGAGAGAGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1075	/5Phos/GCATC**CACGCGACGCTCTCTA**RGYTACCTTGTTACGACTT

Extracting Genomic DNA from Metagenomic Samples

Due to the harsh lysis methods required for some organisms, it may be difficult to extract large quantities of high quality, intact genomic DNA (gDNA) from metagenomic samples. However, for most metagenomic samples, gDNA quality and quantity are likely sufficient for full-length 16S amplification. It is important to note that the relative abundance of gDNA may be impacted by the extraction method used.

16S Amplification

Below are instructions for amplification of full-length 16S gene from bacterial gDNA extracted from metagenomic samples using barcoded primers in a single round of PCR. The recommended input gDNA per sample is 1-2 ng; however, as little as 25 pg may be used. The expected amplicon size is approximately 1500 bases. Typical amplicon yields are ~500 ng. Yield may be lower for samples that contain a significant amount of contaminating non-bacterial gDNA.

Before You Begin

For 16S amplification, you will need the following:

1) KAPA HiFi HotStart 2x ReadyMix PCR Reagent. Thaw on ice and mix well before use.

   Note: All KAPA HiFi hot start reagents and reactions must be set up and kept on ice until PCR; the high proofreading activity of the enzyme will rapidly degrade primers at room temperature.

2) Bacterial gDNA from up to 96 metagenomic samples. For best results, characterize the bacterial gDNA samples thoroughly and normalize gDNA concentration before use.

   Bring gDNA to room temperature and mix well by pipetting to ensure sample homogeneity, then measure gDNA concentration using Qubit dsDNA reagents.

   Assess sample purity by Nanodrop. OD260/280 should be between 1.8 and 2.0.

   To ensure pipetting accuracy, plan to deliver 25 pg – 2.5 ng to each individual PCR reaction in a constant 5 µL volume. Normalize sample gDNA concentration to 5-500 pg/µL in 10 mM Tris-HCl pH 8.0-8.5 prior to setting up PCR reactions. The recommended total input gDNA per reaction is 1-2 ng.

3) Barcoded, 16S gene-specific forward and reverse primers (see Table 1 above for sequences and ordering information) diluted to 2.5 µM in 10 mM Tris-HCl pH 8.0-8.5.

   If necessary, resuspend oligos at 100 µM in 10 mM Tris-HCl pH 8.0-8.5.

   Mix well by pipetting or vortexing, then dilute each primer individually to 2.5 µM in 10 mM Tris-HCl pH 8.0-8.5. For example, add 5 µL 100 µM primer to 195 µL 10 mM Tris-HCl pH 8.0-8.5. Mix well by pipetting. This volume of diluted oligo is sufficient for more than 50 PCR reactions. For a 96-plex, each forward primer will be used in 12 separate reactions, and each reverse oligo will be used in 8 separate reactions.

   Note: Always mix primer stocks well before preparing dilutions, as gradients may form during freeze-thaw.

   Prior to use, verify diluted oligo concentration by directly measuring OD260 of 2.5 µM primer dilution using the Nanodrop system.

STEP	✓	PCR Amplification of 16S Genes	Notes
1		This section describes preparation of PCR Master Mix and amplification for 96 samples. First, prepare the PCR Master Mix of all common components outlined below in a 2.0 mL LoBind tube, including a 25% overage. Ensure that reagents are thawed and mixed prior to use. **Reminder: All KAPA HiFi hot start reactions must be set up on ice; the high proofreading activity of the enzyme will result in rapid primer degradation at room temperature.**	
2		Second, add **Barcoded Forward Primer** to the master mix. Transfer 201.6 µL aliquots of the above Master Mix to 8 LoBind tubes labeled A, B, C, D, E, F, G, H. Add 43.2 µL of the appropriate **Barcoded Forward Primer** (2.5 µM) in the order below. Mix well by pipetting.	
3		Transfer 17 µL aliquots of the Forward Primer Master Mix (tubes A-H) across rows A-H of the 96-well plate.	

Component	1 sample	For 96-plex*
PCR-grade Water	1.5 µL	180.0 µL
2X KAPA HiFi HotStart ReadyMix	12.5 µL	1500.0 µL
Total volume	14.0 µL	1680.0 µL

*Includes 25% overage

Component	Volume (µL)
Master Mix	201.6
Barcoded Forward Primer*	43.2
Total	244.8

Tube	*Barcoded Forward Primer
A	>16S_For_bc1005
B	>16S_For_bc1007
C	>16S_For_bc1008
D	>16S_For_bc1012
E	>16S_For_bc1015
F	>16S_For_bc1020
G	>16S_For_bc1022
H	>16S_For_bc1024

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Finally, add **Barcoded Reverse Primers.**

To each well of columns 1-12 of the 96-well plate, add 3 µL of 2.5 µM **Barcoded ReversePprimers** (see table below).

Primer	Column
>16S_Rev_bc1033	1
>16S_Rev_bc1035	2
>16S_Rev_bc1044	3
>16S_Rev_bc1045	4
>16S_Rev_bc1054	5
>16S_Rev_bc1056	6
>16S_Rev_bc1057	7
>16S_Rev_bc1059	8
>16S_Rev_bc1060	9
>16S_Rev_bc1062	10
>16S_Rev_bc1065	11
>16S_Rev_bc1075	12

The final concentration of barcoded forward and reverse primers is 0.375 µM.

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Add 5 µL (1-2 ng) of each diluted gDNA sample to a single well of the 96-well PCR plate. Mix well by pipetting. Seal plates thoroughly with adhesive seal to prevent evaporation during PCR. Briefly spin plate in refrigerated centrifuge (4°C) to ensure sample volume is at the bottom of each well.

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Perform PCR using the cycling parameters indicated in the Table below:

	Step	Temperature	Time
1	Initial Denature	95 °C	3 minutes
2	Denature	95 °C	30 seconds
3	Anneal*	57 °C	30 seconds
4	Extend	72 °C	60 seconds
		Repeat steps 2 to 4 for a total of 20-27	

***Refer to manufacturer's recommendations for your thermocycler to set the ramp rate for the annealing step to <= 3ºC per second.**

**Refer to the Table below to determine the number of cycles of PCR to perform based on input gDNA amount. A higher number of cycles may be required for samples contaminated with significant amounts of non-bacterial DNA.

Input gDNA	N Cycles
25-100 pg	27
100-500 pg	23
500-2500 pg	20

Typical yield from each PCR reaction is ~500 ng.

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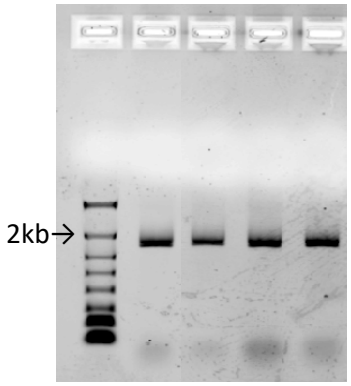
Spot check amplification by directly loading 1 µL of one or more PCR reactions onto an agarose gel. A typical result is shown in the example in Figure 1 below.

The expected amplicon size is ~1500 bp, and the amount of amplicon from each sample should be comparable as assessed by relative intensity of the ~1500 bp PCR product.

If available, you may also use an Agilent Bioanalyzer or TapeStation to spot check PCR product size and quantity.



1 µL from four independent PCR reactions was analyzed per lane of a 1.2% agarose Lonza DNA Flash Gel according to the manufacturer's recommendations. The PCR products were of the expected size (~1.5 kb) and of comparable quantity as determined by visual inspection of band intensity.

Figure 1. Agarose gel spot check of individual PCR reactions.

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 10 µL from each well. Typical total yield from each 25 µL PCR reaction is ~500 ng. At least 500 ng total pooled PCR product is required for SMRTbell library preparation.	
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 **0.60X** 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 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 **100 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. – See Figure 2 below for an example of a typical pooled sample.	
15		Proceed to SMRTbell library preparation.	

SMRTbell Library Construction

DNA Damage Repair

1. Prepare the following reaction, using 500 ng pooled sample. Dilute pooled PCR product to 10.6 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 (500 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.

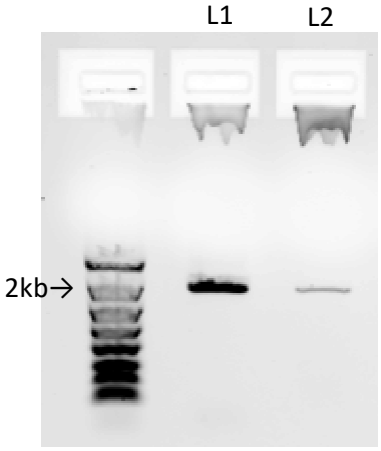
Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

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		Add **58.2 µL** of AMPure PB beads to the 97 µL ligation reaction (0.6X). 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		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 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 **0.60X** 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. – See Figure 2 below for an example of a typical 16S SMRTbell library.	



Samples were loaded and run on a 1.2% Agarose Lonza DNA Flash Gel Cassette according to the manufacturer's recommendations. The pooled PCR products (Lane 1) and final SMRTbell library (Lane 2) are of the expected size (~1.5 kb).

Figure 2. Agarose gel QC of pooled PCR reactions and final SMRTbell library.

Anneal and Bind SMRTbell Templates

For primer annealing, follow the instructions in SMRT Link Sample Setup. Use Sequencing Primer v4 for primer annealing.

For polymerase binding, follow the instructions in SMRT Link Sample Setup. Use Sequel II 2.1 Binding Kit.

Sequencing

For more information, refer to *Quick Reference Card - Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System.*

Revision History (Description)	Version	Date
Initial release.	01	June 2018
Updates throughout based on KAPA HiFi HotStart ReadyMix PCR Kit.	02	June 2019
Updated to provide the sequences (and ordering information) for 8 barcoded forward, and 12 barcoded reverse, 16S-specific primers that can be combined for multiplexed analysis for up to 96 samples using an asymmetric barcoding strategy. Clarify preparation of PCR master mixes for the amplification of 96 samples.	03	February 2020