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

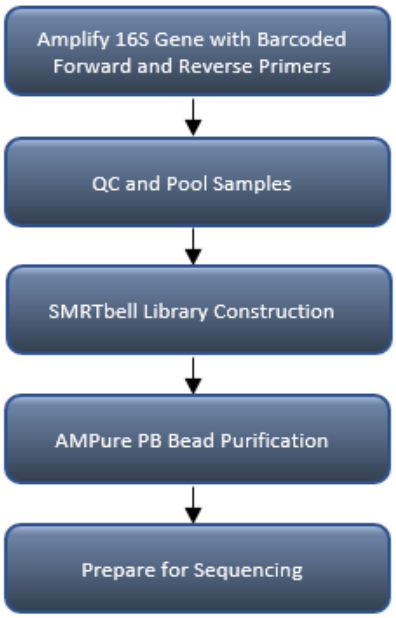
This document contains instructions for:

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 on the Sequel®, Sequel II and Sequel IIe systems (Sequel Systems).

We also provide the sequences of and ordering information for 8 barcoded forward, and 24 barcoded reverse, 16S-specific primers that can be combined for multiplexed analysis of up to 192 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 Kit 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 and Equipment		
DNA LoBind tubes, 2.0 mL	Eppendorf	022431048
DNA LoBind tubes, 5.0 mL	Eppendorf	EP0030108310
Tube Rotator	VWR	10136-084



Workflow

Barcoded 16S Primer Sequences, Ordering and Storage Information

Table 1 in the Appendix lists sequences for thirty-two (32) barcoded,16S gene-specific primers (8 Forward and 24 Reverse) that can be used in all possible asymmetric pairs for multiplexing up to 192 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 a buffered solution (e.g., 100 µM primer in 10 mM Tris-HCl pH 8.0-8.5) at -20°C. Avoid repeated freeze-thaw cycles.

The **Oligo Order Sheet** and **FASTA file** for data analysis are available on our Multiplexing Page.

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 genes from bacterial gDNA extracted from metagenomic samples using barcoded primers in a single round of PCR. The recommended input gDNA amount 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 product yields are ~500 ng. Yields 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 isolated from up to 192 metagenomic samples. For best results, characterize the bacterial gDNA samples thoroughly and normalize gDNA concentrations before use.

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

   Assess sample purity using a Nanodrop system. OD260/280 should be between 1.8 and 2.0 for purified double-stranded DNA.

   To ensure pipetting accuracy, plan to deliver 25 pg – 2.5 ng of gDNA 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 in the Appendix for oligo 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 of 100 µM primer stock to 195 µL of 10 mM Tris-HCl pH 8.0-8.5 buffer. Mix well by pipetting. This volume of diluted oligo is sufficient for running 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. For a 192-plex, each forward primer will be used in 24 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 cycles.

   Prior to use, verify that the concentration of each diluted oligo solution is 2.5 µM by directly measuring the OD260 value using a Nanodrop system.

STEP	✓	PCR Amplification of 16S Genes	Notes
1		This section describes the preparation of a PCR Master Mix and recommended amplification conditions for processing up to 192 metagenomic DNA samples. Prepare the PCR Master Mix of all common components outlined below in a 2.0- or 5.0-mL DNA LoBind tube, including a 25% overage. Ensure that all reagents are thawed and mixed prior to use.	

Component	1 sample	N	For 96-plex*	For 192-plex*
PCR-grade Water	1.5 µL	1.5 x N x 1.25	180.0 µL	360.0 µL
2X KAPA HiFi HotStart ReadyMix	12.5 µL	12.5 x N x 1.25	1500.0 µL	3000.0 µL
Total volume	14.0 µL	14.0 x N x 1.25	1680.0 µL	3360.0 µL

*Includes 25% overage

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 ✓

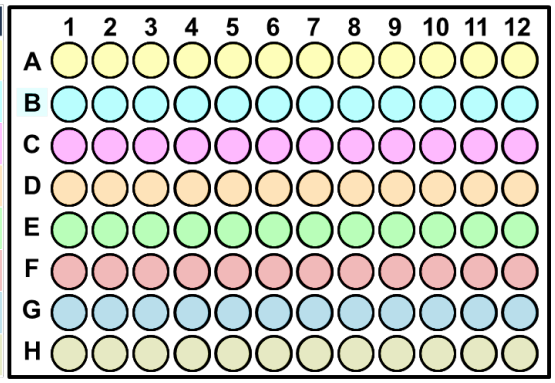
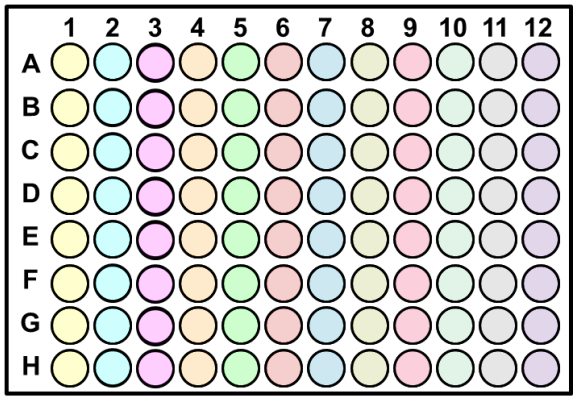
Add **Barcoded Forward Primers** to the PCR Master Mix to generate eight different Forward Primer Master Mix solutions as follows:

- Label 8 LoBind tubes A, B, C, D, E, F, G, and H to assign a specific Barcoded Forward Primer to each tube (see table below).

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

- Depending on the desired level of multiplex (96-plex or 192-plex), transfer the appropriate volume of PCR master mix into each of the eight tubes (see table below).
- Then add the required volume of Barcoded Forward Primer (2.5 µM) to the appropriate tube.
- Mix well by pipetting. The total volume of each tube for the 96-plex Forward Primer Master Mix is 244.8 µL and 489.6 µL for the 192-plex Forward Primer Master Mix.

Component	For 96-plex Forward Primer Master Mix	For 192-plex Forward Primer Master Mix
PCR Master Mix	201.6	403.2
Barcoded Forward Primer (2.5 µM)	43.2	86.4
Total Volume per Tube	244.8	489.6

3		Transfer 17 µL aliquots of the Forward Primer Master Mix (tubes A-H) across rows A-H of a 96-well plate. For a 96-plex experiment design, use one 96-well plate. For a 192-plex experiment design, use two 96-well plates. **The figure below illustrates an example plate layout for setting up a 96-plex PCR design using eight different 16S Barcoded Forward Primer Master Mixes. For a 192-plex design, set up two PCR plates.** **BARCODED FORWARD PRIMER MASTER MIX TO ADD** >16S_For_bc1005 Master Mix (17 µL per well) >16S_For_bc1007 Master Mix (17 µL per well) >16S_For_bc1008 Master Mix (17 µL per well) >16S_For_bc1012 Master Mix (17 µL per well) >16S_For_bc1015 Master Mix (17 µL per well) >16S_For_bc1020 Master Mix (17 µL per well) >16S_For_bc1022 Master Mix (17 µL per well) >16S_For_bc1024 Master Mix (17 µL per well) 	
4		Add **Barcoded Reverse Primers** to appropriate wells containing the 17 µL aliquots of Forward Primer Master Mix dispensed in Step 3 above. To each well of columns 1-12 of the 96-well plate(s), add 3 µL of **Barcoded Reverse Primers** (2.5 µM). For a 96-plex, use the first 12 reverse primers listed in Table 1 in Appendix A. For 192-plex, use all 24 reverse primers listed in Table 1. The total volume in each well is 20 µL. The final concentration of barcoded forward and reverse primers in each well is 0.375 µM. **The figure below illustrates an example plate layout for setting up a 96-plex PCR design using twelve different 16S Barcoded Reverse Primers (2.5 µM). For a 192-plex design, set up two PCR plates using twenty-four different 16S Barcoded Reverse Primers.** **BARCODED REVERSE PRIMER TO ADD (3 µL PER WELL)** >16S_Rev_bc1033 >16S_Rev_bc1035 >16S_Rev_bc1044 >16S_Rev_bc1045 >16S_Rev_bc1054 >16S_Rev_bc1056 >16S_Rev_bc1057 >16S_Rev_bc1059 >16S_Rev_bc1060 >16S_Rev_bc1062 >16S_Rev_bc1065 >16S_Rev_bc1075 	

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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(s) for a total reaction volume of 25 µL. Mix well by pipetting. Seal the plates thoroughly with adhesive seal to prevent evaporation during PCR. Briefly spin the plate in a refrigerated centrifuge (4°C) to ensure that the entire 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 instrument 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 the amount of input gDNA used. 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 25 µL PCR reaction is ~500 ng.

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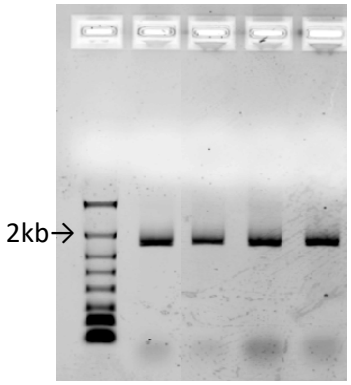
Spot-check amplification results by directly loading 1 µL of one or more PCR reactions onto an agarose gel. A typical result is shown in Figure 1 below.

The expected target amplicon size is ~1500 bp, and the amount of amplicon material generated from each sample should be comparable as assessed by analyzing the relative intensity of the ~1500 bp PCR product on an agarose electrophoresis gel.

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



1 µL from each of 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 their band intensities.

Figure 1. Example 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 DNA LoBind microcentrifuge tube according to the recommendations below: • For a 96-plex experiment design, we recommend pooling 10 µL from each PCR reaction. • For a 192-plex experiment design, we recommend pooling 5 µL from each PCR reaction. Typical total yield from each 25 µL PCR reaction is ~500 ng. At least 500 ng of total pooled PCR product is required for SMRTbell library preparation. Store unpooled PCR reactions at -20ºC for future use if desired.	
2		Proceed to AMPure PB Bead Purification of Pooled Barcoded Amplicons below.	

STEP	✓	AMPure PB Bead Purification of Pooled Barcoded Amplicons	Notes
1		Determine the pooled sample volume. If the 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 AMPure PB 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 rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic rack, slowly pipette off the 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 the 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 the tube from the magnetic rack and spin to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic 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 rack and allow beads to air-dry (with the tube cap 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 rack and let the beads separate fully. – Without disturbing the beads, transfer the supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
14		Measure DNA concentration using a Qubit Fluorometer. – Using 1 µL of the sample, make a 1:10 dilution in 1X EB. – Using Qubit 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 agarose gel analysis of a typical pooled sample.	
15		Proceed to SMRTbell library preparation.	

SMRTbell Library Construction

DNA Damage Repair

1. Prepare the following reaction using 500 – 1000 ng of pooled amplicon sample in 47 µL of 1X Elution Buffer. Do not exceed 1000 ng of input pooled PCR product per 47 µL of sample volume. If using >1000 ng of input pooled sample, scale up all reaction volumes proportionally.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Pooled and Purified PCR Product (500-1000 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.

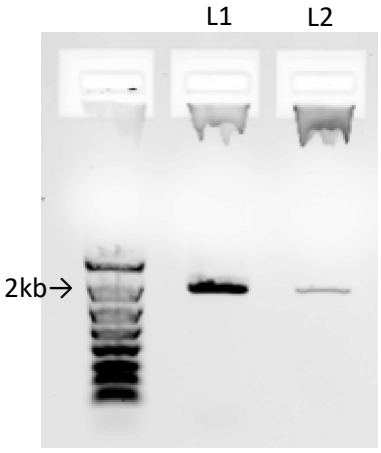
Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

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** (0.6X) of AMPure PB beads to the 97 µL ligation reaction from the previous step. 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 the beads by mixing end-over-end on a 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 rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared the supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash the 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 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 the 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 the beads to elute the DNA. 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 rack and let the beads separate fully. – Without disturbing the beads, transfer the supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
13		Proceed to the final AMPure PB bead purification step below.	

STEP	✓	Final AMPure PB Bead Purification	Notes
1		Bring the AMPure PB 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 the 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 rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic rack, slowly pipette off the 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 the 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 the tube from magnetic rack and spin to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic 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 rack and allow beads to air-dry (with the tube cap 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 rack and let the beads separate fully. – Without disturbing the beads, transfer the supernatant to a new 1.5 ml DNA Lo-Bind tube. – Discard the beads.	
14		Measure the DNA concentration using a Qubit Fluorimeter. – Using 1 µL of the sample, make a 1:10 dilution in 1X EB. – Using Qubit 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 agarose gel analysis 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 16S SMRTbell library (Lane 2) are of the expected size (~1.5 kb).

Figure 2. Example agarose gel QC of pooled PCR reactions and the final 16S 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. For Sequel II and IIe Systems, use Sequel II Binding Kit 2.1.

Sequencing

For more information, see *Quick Reference Card - Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System* and *Quick Reference Card - Loading and Pre-Extension Recommendations for the Sequel II and IIe Systems*.

Appendix

Table 1. Barcoded forward and reverse16S gene-specific primers recommended for use with this procedure (PacBio barcode sequences are **bolded**).

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
>16S_Rev_bc1076	/5Phos/GCATC**GAGAGCGCGAGTGCAC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1082	/5Phos/GCATC**GTGCTCTGTGTGTCAC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1083	/5Phos/GCATC**TGCGTGTATGTCATAT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1089	/5Phos/GCATC**ACGAGATACTCGCGCG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1096	/5Phos/GCATC**CTGTGTAGAGAGCACA**RGYTACCTTGTTACGACTT
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>16S_Rev_bc1100	/5Phos/GCATC**ACTACTGAGACATAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1101	/5Phos/GCATC**TATATCGCGTCGCTAT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1105	/5Phos/GCATC**GCGTACTGCGACTGTG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1107	/5Phos/GCATC**ATATATGCACGCTCTA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1110	/5Phos/GCATC**CGCTGTATACACGCTC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1112	/5Phos/GCATC**AGAGACTGTAGCGCAC**RGYTACCTTGTTACGACTT

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
Added 12 more Reverse Primers for a total of 24, allowing multiplexing of up to 192 samples.	04	January 2021