Preparing Kinnex™ libraries from 16S rRNA amplicons

Procedure & checklist

Overview

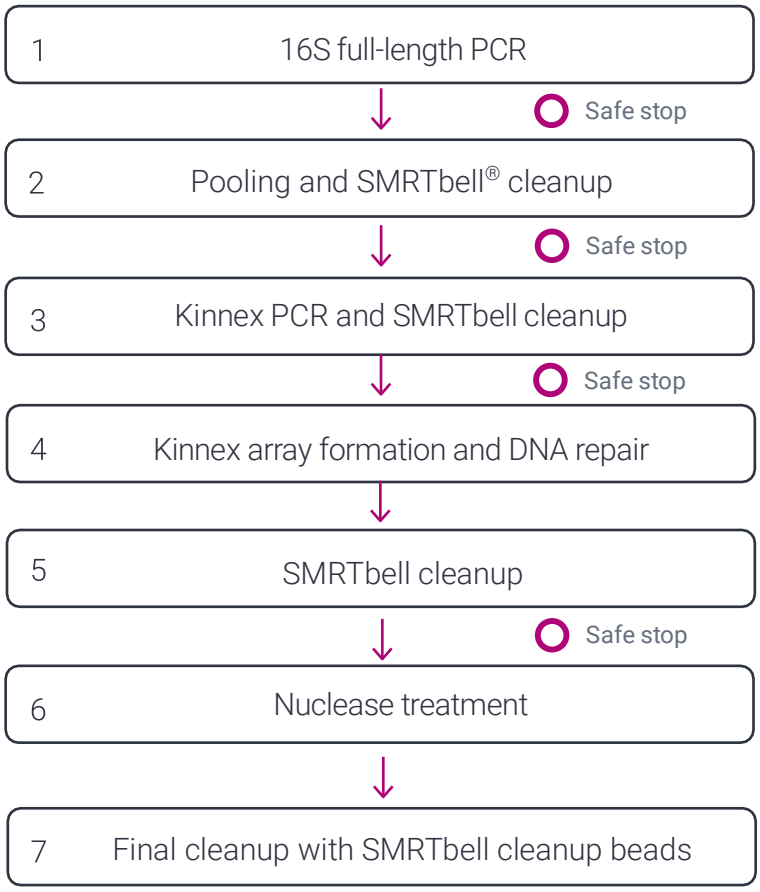
This procedure provides instructions for generating Kinnex libraries from full-length 16S amplicons for sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

1. Amplification of full-length 16S genes (V1–V9 regions) from metagenomic samples using barcoded Forward and Reverse 16S primers
2. Concatenation of 16S amplicons to ~19 kb
3. Multiplexed sequencing on the Sequel II/IIe and Revio systems

Barcoded 16S-specific primers (12 forward and 32 reverse) can be used in different combinations allowing for the multiplexing of up to 384 samples on one SMRT® Cell. If combined with barcoded Kinnex adapters (4-plex), a total of 1536 samples can be sequenced.

103-238-800 REV02 MAR2024

Workflow



Required materials and equipment

16S amplification	
2X KAPA HiFi HotStart ReadyMix, 6.25mL	Roche KK2602
HPLC Purified barcoded 16S gene-specific forward primers, 12 F primers	Any major lab supplier (MLS) (see appendix)
HPLC Purified barcoded 16S gene-specific reverse primers, 32 R primers	Any MLS (see appendix)
Library Preparation	
Kinnex™ concatenation kit	PacBio® 103-071-800*
Kinnex™ PCR 12-fold kit	PacBio® 103-071-700*
SMRTbell® cleanup beads	PacBio® 102-158-300*
Elution Buffer	PacBio® 101-633-500*
AmPure® PB	PacBio® 100-265-900
QC Tools	
2100 BioAnalyzer	Agilent Technologies G2939BA
DNA 12000 Kit	Agilent Technologies 5067-1508
Qubit Fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA High Sensitivity Kit	Thermo Fisher Scientific Q33230
Femto Pulse system	Agilent Technologies M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies FP-1002-0275
General Lab Supplies and Equipment	
DNA LoBind tubes, 2.0 mL	Eppendorf 022431048
DNA LoBind tubes, 5.0 mL	Eppendorf EP0030108310
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS
Thermal cycler (support up to 0.1 mL)	Any MLS

*Sold together as part of Kinnex 16S rRNA kit (103-072-100)

Reagent list

Kinnex PCR 12-fold kit 103-071-700	
Tube color	Reagent
Green	Kinnex PCR mix 103-107-700
Orange	Kinnex primer mix A 103-107-800
	Kinnex primer mix B 103-107-900
	Kinnex primer mix C 103-108-000
	Kinnex primer mix D 103-108-100
	Kinnex primer mix E 103-108-200
	Kinnex primer mix F 103-108-300
	Kinnex primer mix G 103-108-400
	Kinnex primer mix H 103-153-000
	Kinnex primer mix I 103-153-100
	Kinnex primer mix J 103-153-200
	Kinnex primer mix K 103-153-300
	Kinnex primer mix LQ 103-144-000

Kinnex concatenation kit 103-071-800	
Tube color	Reagent
Red	Kinnex enzyme 103-110-400
Yellow	Kinnex ligase 103-110-500
White	Kinnex array and repair buffer 103-110-300
Green	DNA repair mix 103-110-000
Light Purple	Nuclease buffer 103-110-200
Light Green	Nuclease mix 103-110-100
Blue	Kinnex adapter bc01 mix 103-109-600
	Kinnex adapter bc02 mix 103-109-700
	Kinnex adapter bc03 mix 103-109-800
	Kinnex adapter bc04 mix 103-109-900

Important notes

Barcoded 16S gene-specific forward and reverse primers

We recommend resuspending stock oligos to a target concentration of 100 µM in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) or low TE (10 mM Tris-HCl with 0.1 mM EDTA). Dilute each primer individually to 2.5 µM in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) or low TE. For example, add 5 µL of 100 µM primer stock to 195 µL of 10 mM Tris-HCl pH 8.0–8.5 buffer. This volume of diluted oligo is sufficient for running more than 50 PCR reactions.

Always mix primer stocks well before preparing dilutions. 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.

Aliquot the diluted oligos in 96-well plates in the format provided below.

Plate Map

Barcoded 16S gene-specific forward primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	Fwd_01	Fwd_02	Fwd_03	Fwd_04	Fwd_05	Fwd_06	Fwd_07	Fwd_08	Fwd_09	Fwd_10	Fwd_11	Fwd_12
B												
C	Fwd_01	Fwd_02	Fwd_03	Fwd_04	Fwd_05	Fwd_06	Fwd_07	Fwd_08	Fwd_09	Fwd_10	Fwd_11	Fwd_12
D												
E	Fwd_01	Fwd_02	Fwd_03	Fwd_04	Fwd_05	Fwd_06	Fwd_07	Fwd_08	Fwd_09	Fwd_10	Fwd_11	Fwd_12
F												
G	Fwd_01	Fwd_02	Fwd_03	Fwd_04	Fwd_05	Fwd_06	Fwd_07	Fwd_08	Fwd_09	Fwd_10	Fwd_11	Fwd_12
H												

Barcoded 16S gene-specific reverse primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	Rev_13		Rev_21		Rev_29		Rev_37					
B	Rev_14		Rev_22		Rev_30		Rev_38					
C	Rev_15		Rev_23		Rev_31		Rev_39					
D	Rev_16		Rev_24		Rev_32		Rev_40					
E	Rev_17		Rev_25		Rev_33		Rev_41					
F	Rev_18		Rev_26		Rev_34		Rev_42					
G	Rev_19		Rev_27		Rev_35		Rev_43					
H	Rev_20		Rev_28		Rev_36		Rev_44					

General best practices

DNA input

The recommended input gDNA amount per sample is 1–2 ng. The expected amplicon size is approximately 1500 bases. Typical amplicon product yields are 50–300 ng per sample.

Reagent and sample handling

PCR Ready Mix

Thaw on ice and mix well before use.

All PCR reactions described in this procedure must be set up and kept on ice until PCR; the high proofreading activity of the enzyme in the PCR Ready Mix will rapidly degrade primers at room temperature.

Bacterial gDNA isolated 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 1–2 ng of gDNA to each individual PCR reaction in a constant 5 µL volume. Normalize sample gDNA concentration to 0.2–0.4 ng/µL in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) prior to setting up PCR reactions. The recommended total input gDNA per reaction is 1–2 ng.

Note: Nuclease-free water and Elution buffer from PacBio can be used in place of 10mM Tris-HCl pH 8.0–8.5 for gDNA normalization.

Based on prior PacBio experience, QIAgen Powerfecal Pro kit extracts DNA of sufficient quality for this workflow.

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Workflow steps

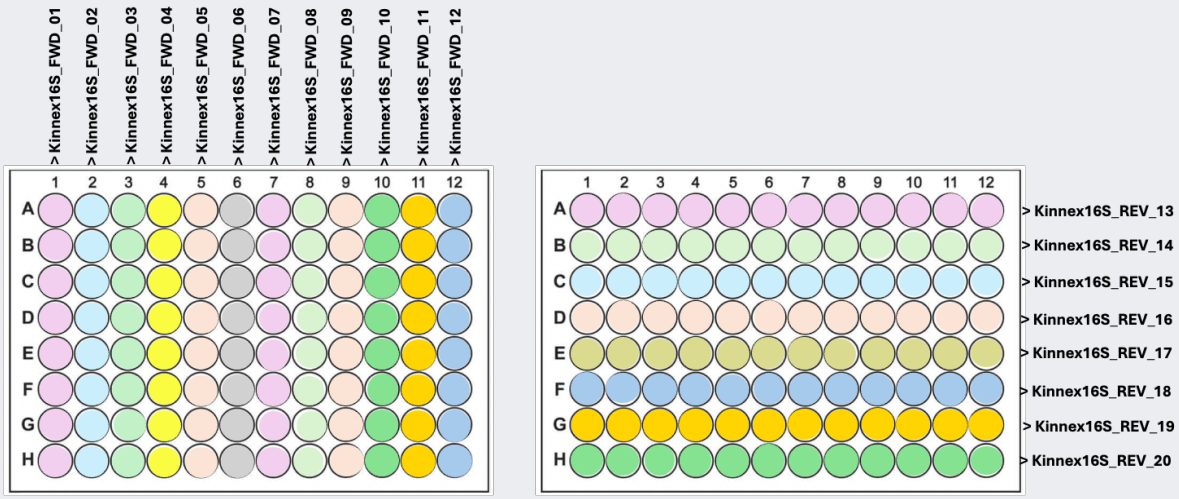
1. PCR amplification of 16S gene with barcoded primers

This section describes the preparation of a PCR Master Mix and the recommended amplification conditions for processing up to 384 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 10% overage. Ensure that all reagents are thawed and mixed prior to use.

Amplicon QC is recommended for size check and amplicon input calculation before pooling.

✔ Step	Instructions
1.1	Thaw the PCR Ready Mix, briefly vortex to mix, and place on ice. Note that all PCR reactions must be set up on ice; the high proofreading activity of the enzyme will result in rapid primer degradation at room temperature. **Thaw plates containing the diluted forward and reverse primers.** Briefly spin the plate to ensure that the entire volume is at the bottom of each well.
1.2	Transfer 14 µL of the prepared 16S PCR Master Mix 1 into a 96-well PCR plate for each 96-plex. For a 96-plex experiment design, use one 96-well plate. For a 384-plex experiment design, use four 96-well plates. Add 5 µL (1–2 ng) of each diluted gDNA sample to each well containing 16S PCR Master Mix 1 on ice.
1.3	The figures below illustrate an example plate layout for setting up a 96-plex PCR design using twelve different 16S Barcoded Forward Primers and eight different 16S Barcoded Reverse Primers. Please refer to Appendix - 384 barcodes layout for all of the plate designs.

16S PCR Master Mix 1 components	1 sample	N	For 96-plex	For 192-plex	For 384-plex
PCR-grade Water	1.5 µL	1.5 x N x 1.1	158 µL	317 µL	634 µL
2X KAPA HiFi HotStart ReadyMix	12.5 µL	12.5 x N x 1.1	1320 µL	2640 µL	5280 µL
Total	14 µL	14 x N x 1.1	1478 µL	2957 µL	5914 µL



1.4 On ice, add 3 µL of the **Barcoded Forward Primers (2.5 µM)** to wells containing 19 µL of gDNA and 16S PCR Master Mix followed by 3 µL of the **Barcoded Reverse Primers (2.5 µM).** The final concentration of the barcoded forward and reverse primers in each well is 0.3 µM. The final reaction volume in each well is 25 µL. Mix well by pipetting. Seal the plates 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.

1.5 Set up a thermal cycler with the program shown below. **Set the lid temperature to 105˚C and pre-heat the thermal cycler until the lid temperature reaches 105˚C and before adding the 96-well PCR plate. Keep the 96-well PCR plates on ice until the lid is pre-heated.**

The duration of PCR is around 1 hour.

Step	Temperature	Duration	Cycle
Initial Denaturation	95 ˚C	3 min	1
Denaturation	98 ˚C	20 s	20
Annealing	57 ˚C	30 s	
Extension	72 ˚C	75 s	
Final Extension	72 ˚C	5 min	1
Hold	4 ˚C	Hold	

Spot-check amplification results by directly loading 1 µL of one or more PCR products onto an Agilent Bioanalyzer Chip.

1.6 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. (Figure 1)

1.7 Proceed to pooling and SMRTbell cleanup in the next step.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

2. Pooling

2.1 Pooling of barcoded 16S PCR amplicons

✔	Step	Instructions
	2.1.1	If PCR products are of the expected size and comparable quantity, 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 or higher-plex experiment design, we recommend pooling **5 µL** from each PCR reaction. Typical total yield from each 25 µL PCR reaction is ~50-300 ng. If doing less than 96-plex, pool **20uL from each PCR reaction into subsequent steps. A total mass of 35ng is required for the Kinnex PCR step.** Store unused PCR reactions at -20ºC for future use.
	2.1.2	Proceed to SMRTbell cleanup in the next step.

2.2 Cleanup of pooled 16S PCR amplicon using 1.1X SMRTbell Cleanup beads

✔	Step	Instructions
	2.2.1	Add **1.1X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to the tube of pooled 16S amplicons. Note: use a 5 mL LoBind tube if the volume is more than 2mL.
	2.2.2	Pipette-mix the beads until evenly distributed.
	2.2.3	Quick-spin the tube in a microcentrifuge to collect liquid.
	2.2.4	Incubate at **room temperature** for **10 minutes** to allow DNA to bind the beads
	2.2.5	Split the sample evenly into two or three new 1.5 mL tubes: • For 96-plex and 192-plex experiment design, transfer 960 µL of sample into two 1.5 mL tubes respectively. • For 384-plex experiment design, transfer 1280 µL of sample into three 1.5 mL tubes respectively. Quick-spin the tube in a microcentrifuge to collect liquid. Place those tubes in a magnetic separation rack until the beads separate fully from the solution.
	2.2.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

2.2.7	Slowly dispense **1000 µL** or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.
2.2.8	Repeat the previous step.
2.2.9	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in a magnetic separation rack until the beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
2.2.10	Remove the tube from the magnetic rack. **Immediately** add **100 µL** of **Elution buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.
2.2.11	Quick-spin the tube in a microcentrifuge to collect liquid.
2.2.12	Leave at **room temperature** for **5 minutes** to elute DNA.
2.2.13	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
2.2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer the supernatant to a **single new 1.5mL LoBind tube or tube strip**. Discard the old tube with beads.
2.2.15	Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit using **1 µL** aliquot from the LoBind tube. Typical total yield from each 25 µL PCR reaction is ~50–300 ng.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

3. Kinnex PCR

This step adds Kinnex adapters to the ends of barcoded 16S full-length amplicons, which enables the concatenation of 16S PCR products to ~19 kb.

3.1 Prepare Kinnex primers premix

✔	Step	Instructions
	3.1.1	Thaw the following components. The entire volume of primers can be transferred to an 8-strip tube for ease of use with a multi-channel pipette.

12X concatenation		Tube color	P/N
1	Kinnex primer mix A	Orange	103-107-800
2	Kinnex primer mix B		103-107-900
3	Kinnex primer mix C		103-108-000
4	Kinnex primer mix D		103-108-100
5	Kinnex primer mix E		103-108-200
6	Kinnex primer mix F		103-108-300
7	Kinnex primer mix G		103-108-400
8	Kinnex primer mix H		103-153-000
9	Kinnex primer mix I		103-153-100
10	Kinnex primer mix J		103-153-200
11	Kinnex primer mix K		103-153-300
12	Kinnex primer mix LQ		103-144-000

	3.1.2	Briefly vortex to mix, and quick-spin to collect liquid. Place the primer mixes on ice and proceed to the preparation of the Kinnex PCR master mix.

3.2 Kinnex PCR

✔	Step	Instructions
	3.2.1	Thaw the following components on ice, briefly vortex to mix, and quick-spin to collect liquid. Place reagents on ice. Add the below components to a LoBind tube, pulse vortex to mix and quick-spin. Place master mix on ice.

Master mix components	Volume for 12X concatenation*
PCR-grade water	132-X µL
Kinnex PCR Mix (103-107-700)	165 µL
35 ng of purified amplicons from Step 2.2.14*	X µL
Total volume	297 µL

X= 35 (ng)/ purified pooled DNA concentration from step 2.2.14

*10% overage included.

	3.2.2	Distribute **22.5 µL** of Master Mix 2 into each 12 PCR tubes (for 12X concatenation) on ice.

3.2.3	Add **2.5 µL** of Kinnex primers premix into each of 12 PCR tubes of Step 3.2.2 on ice.

3.2.4 Set up the thermal cycler as shown below with lid temperature set to 105°C. **Keep samples on ice and do not add samples to thermal cycler until the lid has reached 105°C.** The duration of PCR is approximately 35 minutes.

Step	Temperature	Duration	Cycle
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	20 s	9
Annealing	68°C	30 s	
Extension	72°C	90 s	
Final Extension	72°C	5min	1
Hold	4°C	Hold	

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

3.3 Pooling of 12 Kinnex PCR products and 1.1x SMRTbell cleanup

✔	Step	Instructions
	3.3.1	Add **23 µL** from each of the 12 PCR reactions into a 1.5 mL tube for a total volume of 276 µL.
	3.3.2	Add **304 µL** (1.1X v/v) of resuspended, room-temperature SMRTbell cleanup beads to the tube of pooled Kinnex PCR amplicon.
	3.3.3	Pipette-mix the beads or invert the tube until evenly distributed.
	3.3.4	Quick-spin the tube in a microcentrifuge to collect liquid.
	3.3.5	Incubate at **room temperature** for **10 minutes** to allow DNA to bind the beads.
	3.3.6	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
	3.3.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.3.8	Slowly dispense **1000 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, remove the 80% ethanol and discard.
	3.3.9	Repeat the previous step.
	3.3.10	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in a magnetic separation rack until the beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	3.3.11	Remove the tube from the magnetic rack. **Immediately** add **40 µL** of **Elution buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.

3.3.12	Quick-spin the tube in a microcentrifuge to collect liquid.
3.3.13	Incubate at **room temperature** for **5 minutes** to elute DNA.
3.3.14	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
3.3.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer the supernatant to a PCR **tube strip**. Discard the old tube with beads.
3.3.16	Make a 1:10 dilution of the sample and measure the DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Typical yield is 5–9 µg.

4. Kinnex array formation

4.1 Kinnex array formation

In this step, treat PCR-amplified 16S fragments from Step 3 with Kinnex enzyme, ligase, and barcoded Kinnex terminal adapters to assemble 16S segments into a linear array.

✔	Step	Instructions
	4.1.1	In a 0.2 mL PCR tube, add 5 µg of sample from Step 3.3.15, in 39 µL of volume (128 ng/µL). Dilute with Elution buffer going into this step if the sample is too concentrated. Add 2 µL of Kinnex adapter barcode 01–04 mix (select a single barcode per sample). Note: if not barcoding, select any Kinnex adapter barcode for use.

Tube color	Component	Volume
Blue	Kinnex adapter	2.0 µL

	4.1.2	Add the following components in the listed order. If processing multiple samples, make a master mix with 10% overage. Pipette to mix.

Tube color	Component	Volume
White	Kinnex array and repair buffer (103-110-300)	7.0 µL
Red	Kinnex enzyme (103-110-400)	4.0 µL
Yellow	Kinnex ligase (103-110-500)	6.0 µL
	Total RM1 volume	17 µL

	4.1.3	Add 17 µL of master mix to the PCR tube containing sample and Kinnex barcode adapter. Pipette-mix and run the Kinnex primer digestion/ligation program with the lid set to 55°C.

Step	Temperature	Duration
1	45°C	60 min
2	4°C	Hold

4.1.4	After running the Kinnex primer digestion/ligation program, add 2 µL of DNA repair mix directly to the Kinnex primer digestion/ligation sample. **Tube color** / **Component** / **Volume** Green / DNA repair mix (103-110-000) / 2 µL
4.1.5	Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.
4.1.6	Run the DNA Damage Repair Program with the lid set to >55°C. **Step** / **Temperature** / **Duration** 1 / 45°C / 30 min 2 / 4°C / Hold

5. 1X SMRTbell bead cleanup

Cleanup with 1X SMRTbell cleanup beads

✔	Step	Instructions
	5.1	Add 60 µL (1X v/v) of resuspended, room temperature SMRTbell cleanup beads to each sample.
	5.2	Pipette-mix the beads until evenly distributed and quick-spin in a microcentrifuge to collect liquid.
	5.3	Incubate at room temperature for 10 minutes to allow the DNA to bind the beads.
	5.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	5.5	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, remove the 80% ethanol and discard.
	5.7	Repeat the previous step.
	5.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until the beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	5.9	Remove the tube strip from the magnetic rack. Immediately add 40 µL of Elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed. Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.10	Leave at room temperature for 5 minutes to elute DNA.
	5.11	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

	5.12	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new PCR strip tube**. Discard old tube with beads.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

6. Nuclease treatment

✔	Step	Instructions
	6.1	Add the following components to a new microcentrifuge tube. Adjust the component volumes for the number of samples being prepared, plus 10% overage. Pipette-mix the master mix. For individual preps, add components directly to each sample from the previous step in the order and volume listed below. Nuclease master mix

Tube	Component	Volume
Light purple	Nuclease buffer (103-110-200)	5 µL
Light green	Nuclease mix (103-110-100)	5 µL
Total volume		10 µL

	6.2	Add 10 µL of Nuclease Master mix to each sample. The total volume should be 50 µL.
	6.3	Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.
	6.4	Run the nuclease treatment program with the lid set to >47°C.

Step	Temperature	Duration
1	37°C	15 min
2	4°C	Hold

7. Final cleanup with SMRTbell cleanup beads

✔	Step	Instructions
	7.1	Add 50 µL (1X v/v) of resuspended, room temperature SMRTbell cleanup beads to each sample from the previous step. Pipette-mix the beads until evenly distributed.
	7.2	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
	7.3	Incubate at room temperature for 10 minutes to allow DNA to the bind beads.
	7.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	7.5	Slowly pipette off the cleared supernatant without disturbing the beads.

7.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, remove the 80% ethanol and discard.
7.7	Repeat the previous step.
7.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
7.9	Remove the tube strip from the magnetic rack. Immediately add 20 µL of Elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
7.10	Quick-spin the tube strip in a microcentrifuge to collect liquid.
7.11	Incubate at room temperature for 5 minutes to elute DNA.
7.12	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
7.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer the supernatant to a new 0.5 mL LoBind tube or a PCR tube strip. Discard the old tube strip with beads.
7.14	Take a 1 µL aliquot from each tube. Make a 1:5 dilution of the sample in Elution buffer and measure the DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. Expect 10–20% recovery of the starting Kinnex-PCR product. Recommended: Further dilute each aliquot to 250 pg/µL with EB. Measure the final SMRTbell library size distribution with a Femto Pulse system (see Figure 1).
7.15	Proceed to the SMRT® Link Sample Setup to prepare the SMRTbell library for sequencing. **DNA concentration must be less than 60 ng/µL to proceed to ABC.** Using a concentration >60 ng/µL will result in low loading on the sequencer.
7.16	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

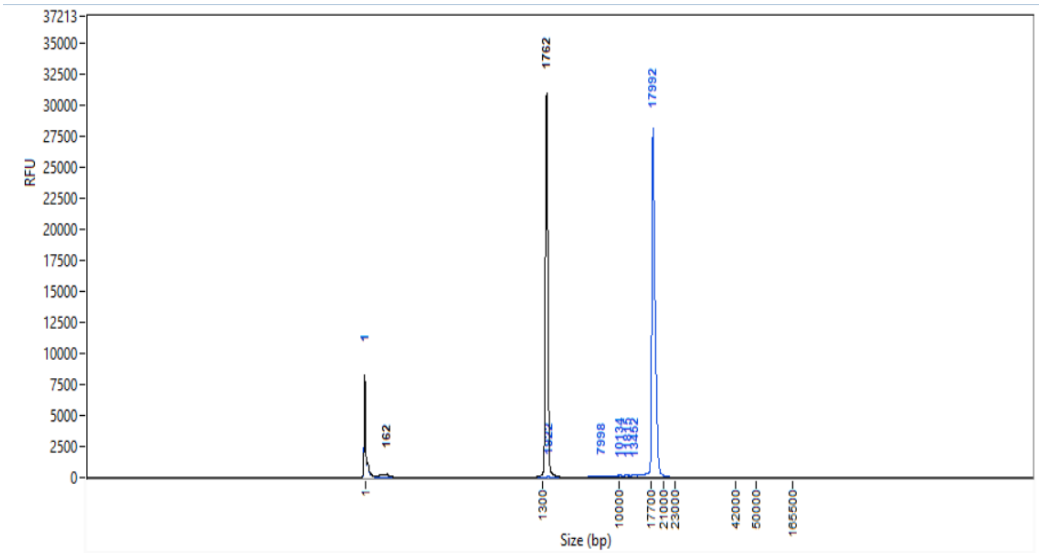


Figure 1. Example Femto pulse QC of a 16S amplicon PCR product and a 12X concatemer final SMRTbell library. Samples from Step 1 and Step 7 were loaded onto the Femto Pulse System using the Femto Pulse gDNA 165kb analysis kit. 16S amplicon monomer (black) peaks at a size of 1762 bp; concatenated SMRTbell library (blue) peaks at 17992 bp.

Note: Due to diverse sources of bacterial genomic DNA, there might be contaminants that affect the sequencing performance. An additional clean-up of final SMRTbell library using 3.1X diluted AmPure® PB (35% v/v, part number 100-265-900) or Monarch Genomic DNA Purification Kit (#T3010S) has been shown to remove contaminants effectively.

PROTOCOL COMPLETE

Appendix

384 barcodes layout

Plate 1

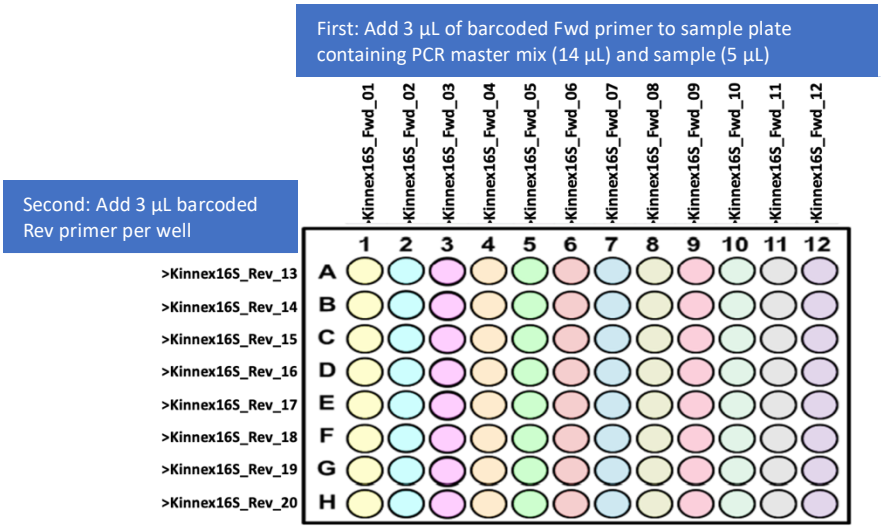


Plate 2

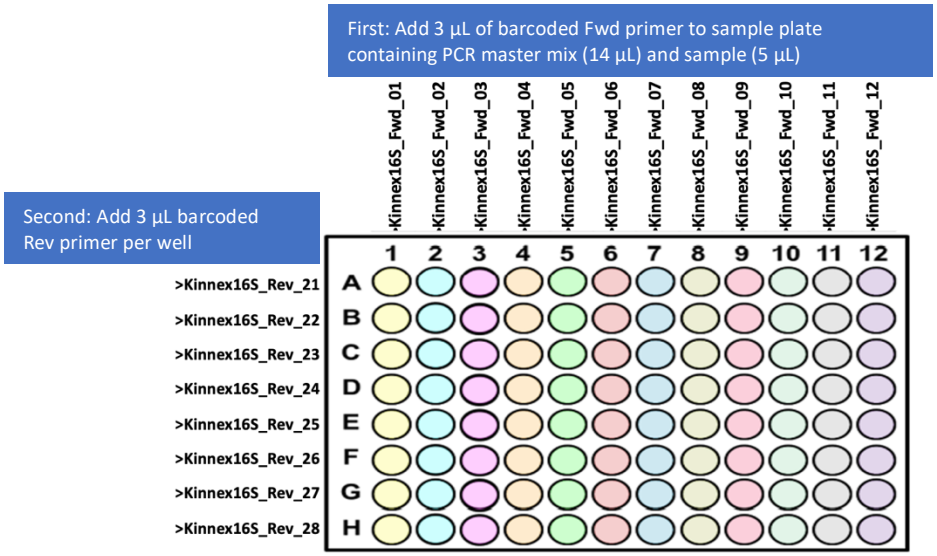


Plate 3

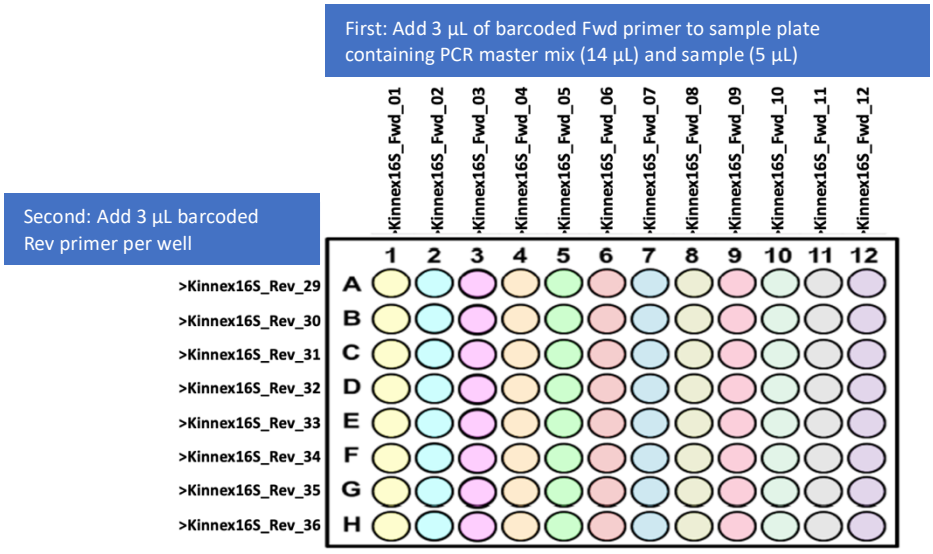


Plate 4

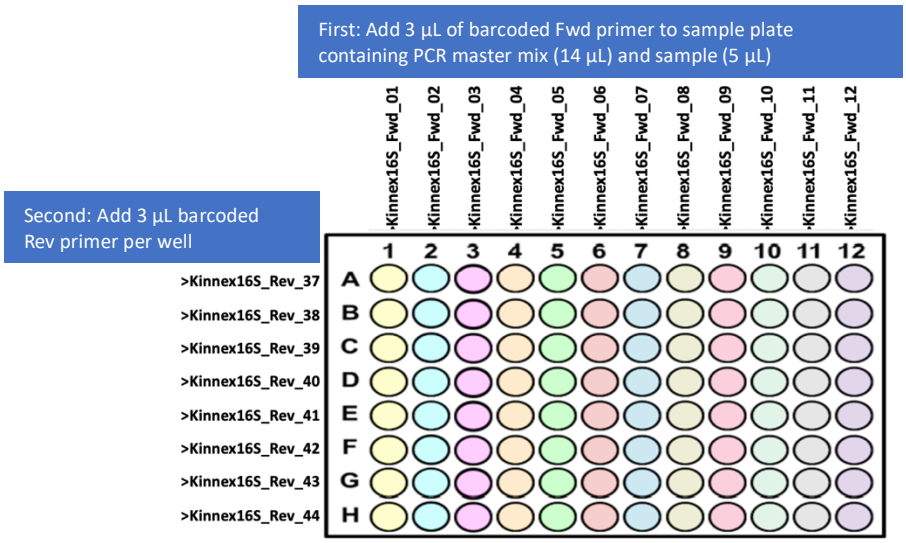


Table 1. List of Kinnex16S forward and reverse primers. HPLC purification is required.

Name	Sequence
Kinnex16S_Fwd_01	CTACACGACGCTCTTCCGATCTGATCGAGTCAAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_02	CTACACGACGCTCTTCCGATCTAGATCGCATGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_03	CTACACGACGCTCTTCCGATCTAGACTAGCGTAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_04	CTACACGACGCTCTTCCGATCTGTACTGTCAGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_05	CTACACGACGCTCTTCCGATCTACGTGCAGATAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_06	CTACACGACGCTCTTCCGATCTGACTATGACGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_07	CTACACGACGCTCTTCCGATCTGACGCATAGTAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_08	CTACACGACGCTCTTCCGATCTAGCATGTACGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_09	CTACACGACGCTCTTCCGATCTGTCGCACGATAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_10	CTACACGACGCTCTTCCGATCTAGTGCGATCGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_11	CTACACGACGCTCTTCCGATCTACGCTCAGTGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_12	CTACACGACGCTCTTCCGATCTGTAGACGCTGAGRGTTYGATYMTGGCTCAG
Kinnex16S_Rev_13	AAGCAGTGGTATCAACGCAGAGTCATCGACGTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_14	AAGCAGTGGTATCAACGCAGAGTCGCATGACTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_15	AAGCAGTGGTATCAACGCAGAGCATGATCGACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_16	AAGCAGTGGTATCAACGCAGAGTGACTGTAGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_17	AAGCAGTGGTATCAACGCAGAGCGACTCGTATRGYTACCTTGTTACGACTT
Kinnex16S_Rev_18	AAGCAGTGGTATCAACGCAGAGCAGCTGACATRGYTACCTTGTTACGACTT
Kinnex16S_Rev_19	AAGCAGTGGTATCAACGCAGAGCAGTATGAGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_20	AAGCAGTGGTATCAACGCAGAGACGATGACGTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_21	AAGCAGTGGTATCAACGCAGAGCGATGATGCTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_22	AAGCAGTGGTATCAACGCAGAGTACGACAGTCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_23	AAGCAGTGGTATCAACGCAGAGTGCATACTGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_24	AAGCAGTGGTATCAACGCAGAGCAGACTAGTCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_25	AAGCAGTGGTATCAACGCAGAGCTCAGCATACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_26	AAGCAGTGGTATCAACGCAGAGTAGCACGCATRGYTACCTTGTTACGACTT
Kinnex16S_Rev_27	AAGCAGTGGTATCAACGCAGAGTACTGACGCTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_28	AAGCAGTGGTATCAACGCAGAGATACGAGCTCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_29	AAGCAGTGGTATCAACGCAGAGTGAGCTATGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_30	AAGCAGTGGTATCAACGCAGAGCTGTCGTAGTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_31	AAGCAGTGGTATCAACGCAGAGTACTGCTCACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_32	AAGCAGTGGTATCAACGCAGAGCTCGTCAGATRGYTACCTTGTTACGACTT
Kinnex16S_Rev_33	AAGCAGTGGTATCAACGCAGAGCTCACTGAGTRGYTACCTTGTTACGACTT
Kinnex16S_Rev_34	AAGCAGTGGTATCAACGCAGAGTACTAGCAGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_35	AAGCAGTGGTATCAACGCAGAGCGTAGCAGATRGYTACCTTGTTACGACTT
Kinnex16S_Rev_36	AAGCAGTGGTATCAACGCAGAGACAGCTGTACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_37	AAGCAGTGGTATCAACGCAGAGTCGATGCTACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_38	AAGCAGTGGTATCAACGCAGAGCGATCAGTGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_39	AAGCAGTGGTATCAACGCAGAGACGCACGTACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_40	AAGCAGTGGTATCAACGCAGAGCAGTAGCGTCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_41	AAGCAGTGGTATCAACGCAGAGACTGCAGCACRGYTACCTTGTTACGACTT

Kinnex16S_Rev_42	AAGCAGTGGTATCAACGCAGAGTCACGACGACRGYTACCTTGTTACGACTT
Kinnex16S_Rev_43	AAGCAGTGGTATCAACGCAGAGCGAGTCTAGCRGYTACCTTGTTACGACTT
Kinnex16S_Rev_44	AAGCAGTGGTATCAACGCAGAGCAGCAGTGACRGYTACCTTGTTACGACTT

Revision history (description)	Version	Date
Initial release	01	October 2023
Minor updates throughout	02	March 2024