

Preparing Kinnex™ libraries from 16S rRNA amplicons

Procedure & checklist

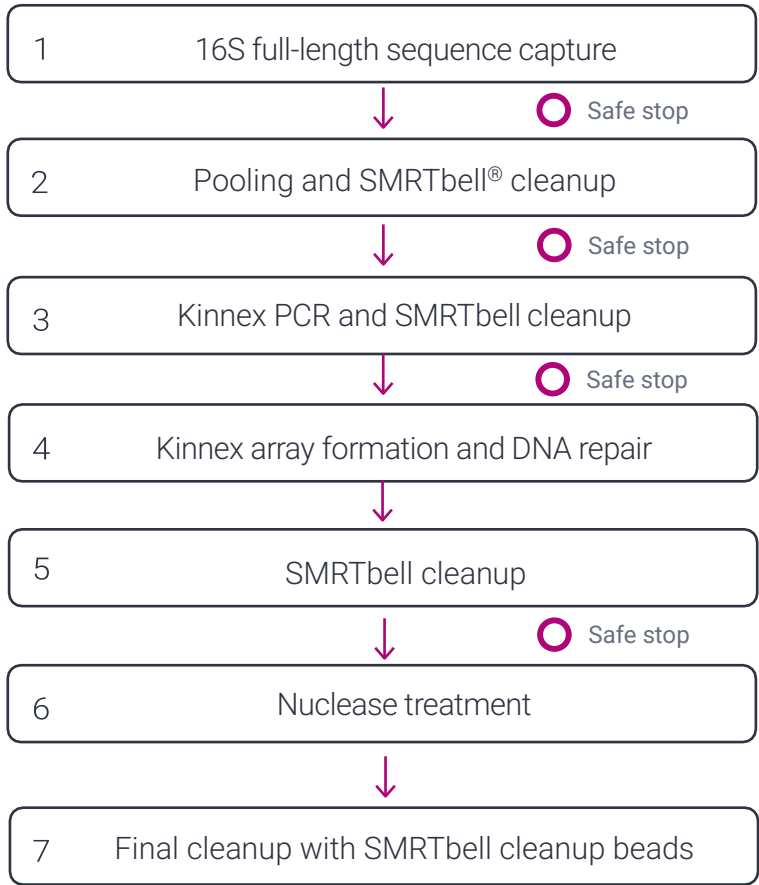
Overview

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.

Workflow



Required materials and equipment

16S amplification	
2X KAPA HiFi HotStart ReadyMix, 6.25mL	Roche KK2602
Barcoded 16S gene-specific forward primers, 12 F primers	Any major lab supplier (MLS) (see appendix)
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*
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)

Reagents 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 with 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. OD_{260/280} 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 μ L volume. Normalize sample gDNA concentration to 0.2–0.4 ng/ μ L 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.

Workflow steps

1. PCR amplification of 16S gene with barcoded primers

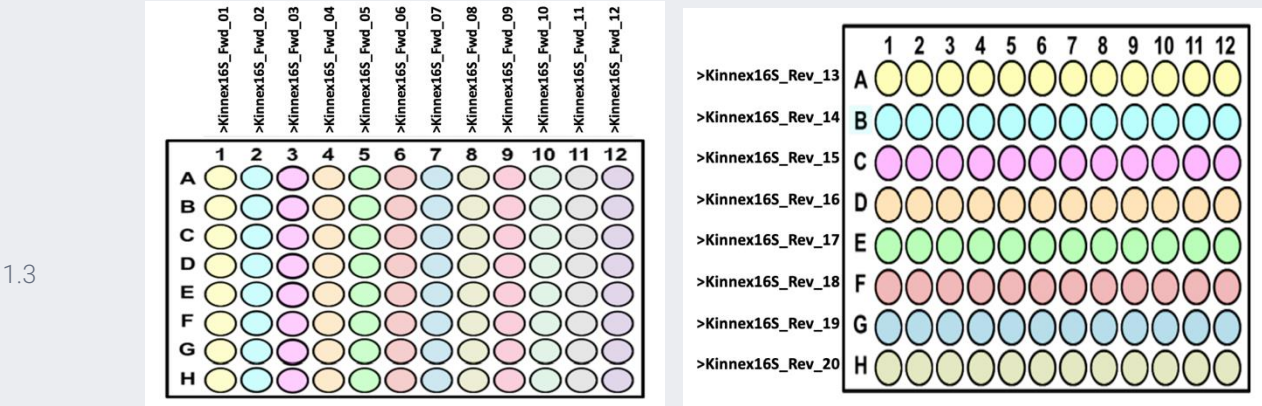
This section describes the preparation of a PCR Master Mix and the recommended amplification conditions for processing up to 384 metagenomic DNA samples. Prepare the PCR Master Mix of all common components outlined below in a 2.0- or 5.0-mL DNA LoBind tube, including a 10% overage. Ensure that all reagents are thawed and mixed prior to use.

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

Step	Instructions																								
1.1	<p>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.</p> <p>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.</p> <table border="1"> <thead> <tr> <th>16S PCR Master Mix 1 components</th> <th>1 sample</th> <th>N</th> <th>For 96-plex</th> <th>For 192-plex</th> <th>For 384-plex</th> </tr> </thead> <tbody> <tr> <td>PCR-grade Water</td> <td>1.5 µL</td> <td>1.5 x N x 1.1</td> <td>158 µL</td> <td>317 µL</td> <td>634 µL</td> </tr> <tr> <td>2X KAPA HiFi HotStart ReadyMix</td> <td>12.5 µL</td> <td>12.5 x N x 1.1</td> <td>1320 µL</td> <td>2640 µL</td> <td>5280 µL</td> </tr> <tr> <td>Total</td> <td>14 µL</td> <td>14 x N x 1.1</td> <td>1478 µL</td> <td>2957 µL</td> <td>5914 µL</td> </tr> </tbody> </table>	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
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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 (1ng) of each diluted gDNA sample to each well containing 16S PCR Master Mix 1 on ice.

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.



- 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.

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.

1.5

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	<p>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:</p> <ul style="list-style-type: none"> 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. <p>Typical total yield from each 25 µL PCR reaction is ~50-300 ng. If doing less than 96-plex, pool 20µL from each PCR reaction into subsequent steps, but sure there is at least 35ng into the Kinnex PCR step.</p> <p>Store unused PCR reactions at -20°C for future use if desired.</p>
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 amplicon. Note: Please 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	<p>Split the sample evenly into two or three new 1.5 mL tubes:</p> <ul style="list-style-type: none"> 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. <p>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.</p>
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.

Remove residual 80% ethanol:

- 2.2.9
- 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																																							
		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.																																							
		<table border="1"> <thead> <tr> <th>12X concatenation</th> <th>Tube color</th> <th>P/N</th> </tr> </thead> <tbody> <tr> <td>1 Kinnex primer mix A</td> <td>Orange</td> <td>103-107-800</td> </tr> <tr> <td>2 Kinnex primer mix B</td> <td>Orange</td> <td>103-107-900</td> </tr> <tr> <td>3 Kinnex primer mix C</td> <td>Orange</td> <td>103-108-000</td> </tr> <tr> <td>4 Kinnex primer mix D</td> <td>Orange</td> <td>103-108-100</td> </tr> <tr> <td>5 Kinnex primer mix E</td> <td>Orange</td> <td>103-108-200</td> </tr> <tr> <td>6 Kinnex primer mix F</td> <td>Orange</td> <td>103-108-300</td> </tr> <tr> <td>7 Kinnex primer mix G</td> <td>Orange</td> <td>103-108-400</td> </tr> <tr> <td>8 Kinnex primer mix H</td> <td>Orange</td> <td>103-153-000</td> </tr> <tr> <td>9 Kinnex primer mix I</td> <td>Orange</td> <td>103-153-100</td> </tr> <tr> <td>10 Kinnex primer mix J</td> <td>Orange</td> <td>103-153-200</td> </tr> <tr> <td>11 Kinnex primer mix K</td> <td>Orange</td> <td>103-153-300</td> </tr> <tr> <td>12 Kinnex primer mix LQ</td> <td>Orange</td> <td>103-144-000</td> </tr> </tbody> </table>	12X concatenation	Tube color	P/N	1 Kinnex primer mix A	Orange	103-107-800	2 Kinnex primer mix B	Orange	103-107-900	3 Kinnex primer mix C	Orange	103-108-000	4 Kinnex primer mix D	Orange	103-108-100	5 Kinnex primer mix E	Orange	103-108-200	6 Kinnex primer mix F	Orange	103-108-300	7 Kinnex primer mix G	Orange	103-108-400	8 Kinnex primer mix H	Orange	103-153-000	9 Kinnex primer mix I	Orange	103-153-100	10 Kinnex primer mix J	Orange	103-153-200	11 Kinnex primer mix K	Orange	103-153-300	12 Kinnex primer mix LQ	Orange	103-144-000
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	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										
	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.										
3.2.1	<table border="1"> <thead> <tr> <th>Master mix components</th> <th>Volume for 12X concatenation*</th> </tr> </thead> <tbody> <tr> <td>PCR-grade water</td> <td>132-X μL</td> </tr> <tr> <td>Kinnex PCR Mix (103-107-700)</td> <td>165 μL</td> </tr> <tr> <td>35 ng of purified amplicons from Step 2.2.14*</td> <td>X μL</td> </tr> <tr> <td>Total volume</td> <td>297 μL</td> </tr> </tbody> </table> <p>X = 35 (ng) / purified pooled DNA concentration from step 2.2.14</p> <p>*10% overage included.</p>	Master mix components	Volume for 12X concatenation*	PCR-grade water	132-X μ L	Kinnex PCR Mix (103-107-700)	165 μ L	35 ng of purified amplicons from Step 2.2.14 *	X μ L	Total volume	297 μ L
Master mix components	Volume for 12X concatenation*										
PCR-grade water	132-X μ L										
Kinnex PCR Mix (103-107-700)	165 μ L										
35 ng of purified amplicons from Step 2.2.14 *	X μ L										
Total volume	297 μ L										
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.										

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	
Annealing	68°C	30 s	9
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 μ L from each of the 12 PCR reactions into a 1.5 mL tube for a total volume of 276 μ L.
3.3.2	Add 1.1X v/v (volume over volume, 304 μ L) 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.

Remove residual 80% ethanol:

- 3.3.10
- 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 4–8 μg of sample from Step 3.3.15 , in 39 μL of volume (102–205 ng/ μL). Dilute with Elution buffer going into this step if the sample is too concentrated. Add 2 μL of Kinnex adapter bc01–04 mix (select a single barcode per sample). Note: if not barcoding, select any Kinnex adapter barcode for use.															
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.															
	<table border="1"> <thead> <tr> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td>White</td> <td>Kinnex array and repair buffer (103-110-300)</td> <td>7.0 μL</td> </tr> <tr> <td>Red</td> <td>Kinnex enzyme (103-110-400)</td> <td>4.0 μL</td> </tr> <tr> <td>Yellow</td> <td>Kinnex ligase (103-110-500)</td> <td>6.0 μL</td> </tr> <tr> <td colspan="2">Total RM1 volume</td> <td>17 μL</td> </tr> </tbody> </table>	Tube	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
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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 39 μL of sample (102–205 ng/ μL) and 2 μL of Kinnex 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.

4.1.5 Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.

Run the DNA Damage Repair Program with the lid set to $>55^\circ\text{C}$.

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: <ul style="list-style-type: none"> • 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.

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

6. Nuclease treatment

✓ Step	Instructions												
6.1	<p>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.</p> <p>Nuclease master mix</p> <table border="1"> <thead> <tr> <th>Tube</th> <th>Component</th> <th>Volume</th> </tr> </thead> <tbody> <tr> <td>Light purple</td> <td>Nuclease buffer (103-110-200)</td> <td>5 μL</td> </tr> <tr> <td>Light green</td> <td>Nuclease mix (103-110-100)</td> <td>5 μL</td> </tr> <tr> <td colspan="2">Total volume</td> <td>10 μL</td> </tr> </tbody> </table>	Tube	Component	Volume	Light purple	Nuclease buffer (103-110-200)	5 μ L	Light green	Nuclease mix (103-110-100)	5 μ L	Total volume		10 μ L
Tube	Component	Volume											
Light purple	Nuclease buffer (103-110-200)	5 μ L											
Light green	Nuclease mix (103-110-100)	5 μ L											
Total volume		10 μ L											
6.2	Add 10 μ L of Nuclease Master mix to each sample. The total volume should be 50 μ L.												
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.												
	<table border="1"> <thead> <tr> <th>Step</th> <th>Temperature</th> <th>Duration</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>37°C</td> <td>15 min</td> </tr> <tr> <td>2</td> <td>4°C</td> <td>Hold</td> </tr> </tbody> </table>	Step	Temperature	Duration	1	37°C	15 min	2	4°C	Hold			
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.
	Remove residual 80% ethanol:
7.8	<ul style="list-style-type: none"> 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	<p>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.</p> <p>Recommended: Further dilute each aliquot to 250 $\text{pg}/\mu\text{L}$ with EB. Measure the final SMRTbell library size distribution with a Femto Pulse system (Figure 1).</p>
7.15	Proceed to the SMRT® Link Sample Setup to prepare the SMRTbell library for sequencing.
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.

PROTOCOL COMPLETE

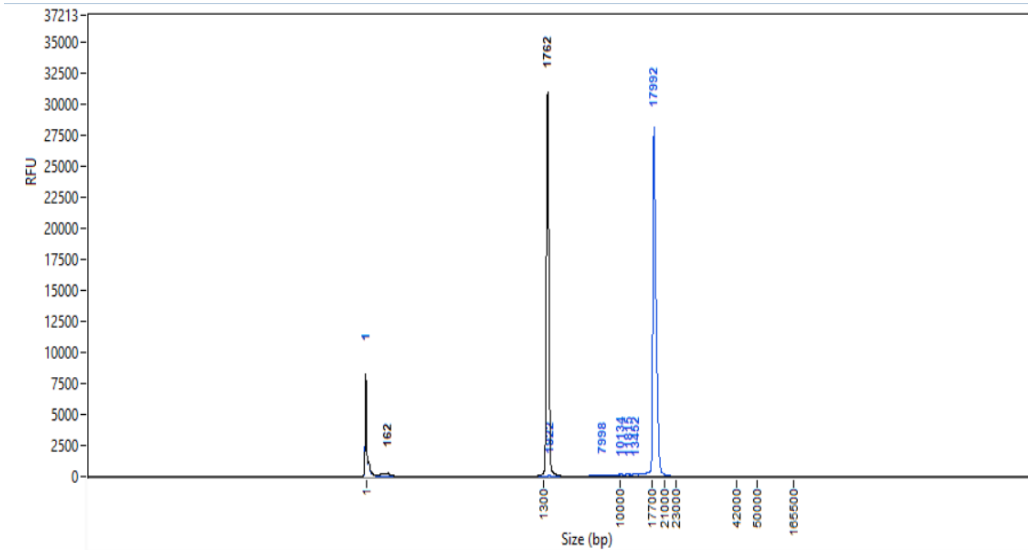


Figure 1. Example Femto pulse QC of the 16S amplicon monomer (~1.5 kb) and 12X concatemer (~18 kb) final SMRTbell libraries.

Samples from Step 1 and Step 7 were loaded in the Femto pulse system using 165kb analysis kit. 16S amplicon monomer (Black) showed the peak at the size of 1762 bp; Concatenated SMRTbell library post -12X concatenation (Blue) at 17992 bp.

Appendix

384 barcodes layout

Plate 1

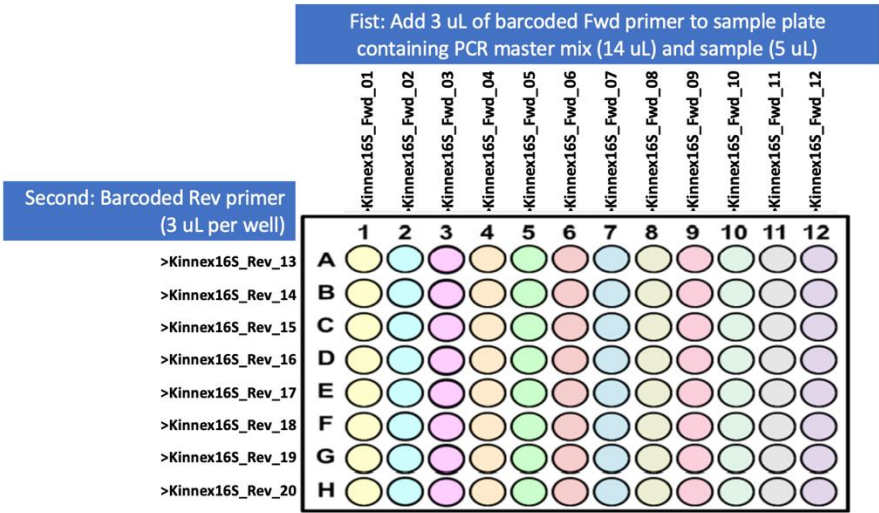


Plate 2

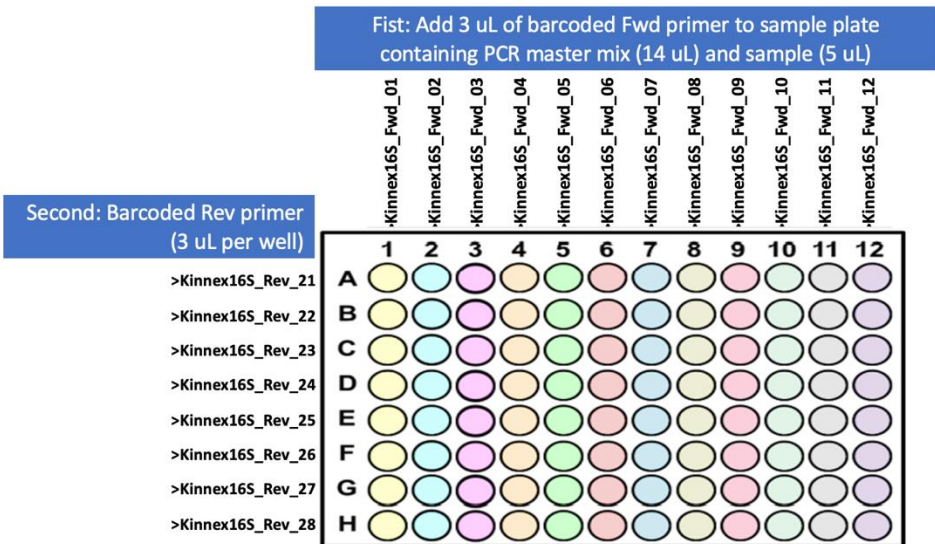


Plate 3

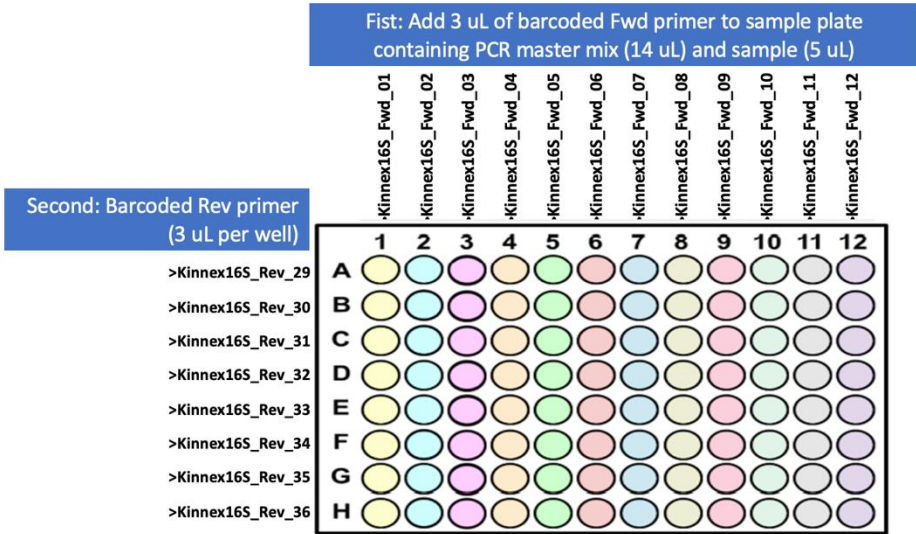


Plate 4

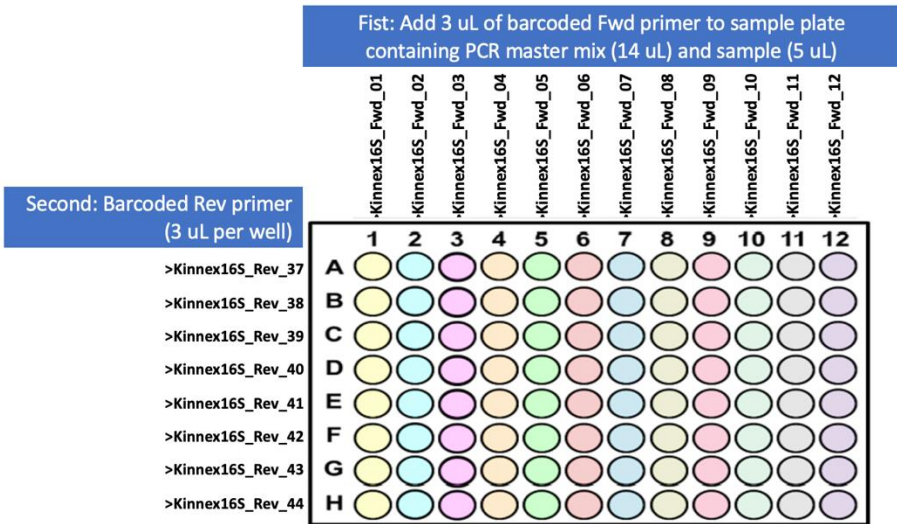


Table 1. List of Kinnex16S forward and reverse primers.

Name	Sequence
Kinnex16S_Fwd_01	<u>CTACACGACGCTCTTCCGATCTGATCGAGTCA</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_02	<u>CTACACGACGCTCTTCCGATCTAGATCGCATG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_03	<u>CTACACGACGCTCTTCCGATCTAGACTAGCGT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_04	<u>CTACACGACGCTCTTCCGATCTGTACTGTCAG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_05	<u>CTACACGACGCTCTTCCGATCTACGTGCAGAT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_06	<u>CTACACGACGCTCTTCCGATCTGACTATGACG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_07	<u>CTACACGACGCTCTTCCGATCTGACGCATAGT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_08	<u>CTACACGACGCTCTTCCGATCTAGCATGTACG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_09	<u>CTACACGACGCTCTTCCGATCTGTCGCACGAT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_10	<u>CTACACGACGCTCTTCCGATCTAGTGGCATCG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_11	<u>CTACACGACGCTCTTCCGATCTACGCTCAGTG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_12	<u>CTACACGACGCTCTTCCGATCTGTAGACGCTG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Rev_13	<u>AAGCAGTGGTATCAACGCAGAGTCATCGACG</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_14	<u>AAGCAGTGGTATCAACGCAGAGTCGCATGACT</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_15	<u>AAGCAGTGGTATCAACGCAGAGCATGATCGAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_16	<u>AAGCAGTGGTATCAACGCAGAGTGACTGTAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_17	<u>AAGCAGTGGTATCAACGCAGAGCGACTCGTAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_18	<u>AAGCAGTGGTATCAACGCAGAGCAGCTGACAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_19	<u>AAGCAGTGGTATCAACGCAGAGCAGTATGAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_20	<u>AAGCAGTGGTATCAACGCAGAGACGATGACG</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_21	<u>AAGCAGTGGTATCAACGCAGAGCGATGATGCT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_22	<u>AAGCAGTGGTATCAACGCAGAGTACGACAGTC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_23	<u>AAGCAGTGGTATCAACGCAGAGTGCATACTGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_24	<u>AAGCAGTGGTATCAACGCAGAGCAGACTAGTC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_25	<u>AAGCAGTGGTATCAACGCAGAGCTCAGCATAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_26	<u>AAGCAGTGGTATCAACGCAGAGTAGCACGCAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_27	<u>AAGCAGTGGTATCAACGCAGAGTACTGACGCT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_28	<u>AAGCAGTGGTATCAACGCAGAGATACGAGCTC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_29	<u>AAGCAGTGGTATCAACGCAGAGTGAGCTATGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_30	<u>AAGCAGTGGTATCAACGCAGAGCTGTCGTAGT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_31	<u>AAGCAGTGGTATCAACGCAGAGTACTGCTCAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_32	<u>AAGCAGTGGTATCAACGCAGAGCTCGTCAGAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_33	<u>AAGCAGTGGTATCAACGCAGAGCTCACTGAGT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_34	<u>AAGCAGTGGTATCAACGCAGAGTACTAGCAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_35	<u>AAGCAGTGGTATCAACGCAGAGCGTAGCAGAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_36	<u>AAGCAGTGGTATCAACGCAGAGACAGCTGTAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_37	<u>AAGCAGTGGTATCAACGCAGAGTCGATGCTAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_38	<u>AAGCAGTGGTATCAACGCAGAGCGATCAGTG</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_39	<u>AAGCAGTGGTATCAACGCAGAGACGCACGTAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_40	<u>AAGCAGTGGTATCAACGCAGAGCAGTAGCGT</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_41	<u>AAGCAGTGGTATCAACGCAGAGACTGCAGCAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_42	<u>AAGCAGTGGTATCAACGCAGAGTCACGACGAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_43	<u>AAGCAGTGGTATCAACGCAGAGCGAGTCTAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_44	<u>AAGCAGTGGTATCAACGCAGAGCAGCAGTGAC</u> RGYTACCTTGTTACGACTT

Revision history (description)	Version	Date
Initial release.	01	October 2023

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