

Overview

This procedure describes the workflow for generating Kinnex-compatible full-length 16S rRNA amplicons (V1 – V9 regions). The resulting amplicons are compatible with (1) generation of Kinnex concatenated libraries using Kinnex concatenation kit 2.0 or (2) construction of monomer SMRTbell libraries using the SMRTbell prep kit 3.0. The resulting libraries can be sequenced on PacBio® Vega™ and Revio® systems.

For generating Kinnex concatenated libraries, please refer to one of these protocols:

- Procedure & checklist - Preparing Kinnex libraries using Kinnex 8-fold prep kit
- Procedure & checklist - Preparing Kinnex libraries using Kinnex 12-fold prep kit

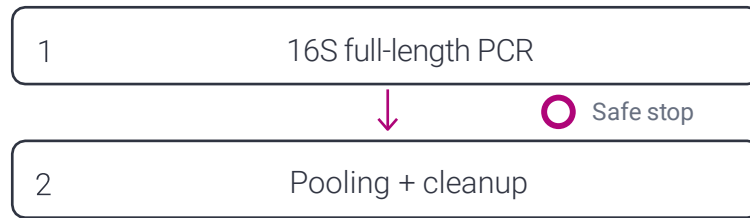
For generating monomer SMRTbell libraries, please refer to the protocol:

- Procedure & checklist - Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0

Barcoded 16S-specific primer sequences are provided (12 forward and 32 reverse). These primers are ordered separately and can be used in different combinations to allow multiplexing up to 384 samples. Combining 384-plex pools with barcoded Kinnex adapters (up to 4-plex) enables up to 1536 samples that can be sequenced on a single SMRTCell acquisition.

Overview	
16S unique dual indexing	Up to 384 samples supported
Workflow time	~4 hours for up to 96 samples (one plate) ~2 days for up to 384 samples (four plates)
Metagenomic DNA mass	Total genomic DNA of 1–2 ng per metagenomic sample
16S (V1-V9) amplicon size	~1.5 kb

Workflow



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 section A1)
HPLC purified barcoded 16S gene-specific reverse primers, 32 R primers	Any MLS (see Appendix section A1)
Recommended QC Tools (Use one of the following sizing system)	
2100 BioAnalyzer	Agilent Technologies G2939BA
DNA 12000 Kit	Agilent Technologies 5067-1508
4150 or 4200 TapeStation System	Agilent Technologies G2992AA/ G2991BA
High Sensitivity D5000 ScreenTape Assay	Agilent Technologies 5067-5592/5593/5594
D5000 ScreenTape Assay	Agilent Technologies 5067-5588/5589/5590
Qubit Fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA High Sensitivity Kit	Thermo Fisher Scientific Q33230
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
96-well PCR plate, 0.2 mL	Any MLS
Microcentrifuge	Any MLS

Before you begin

Kinnex compatible barcoded 16S gene-specific forward and reverse primers

We recommend HPLC purified oligos for best performance. Oligo sequences provided in [Appendix](#).

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

12 Forward Primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
B												
C	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
D												
E	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
F												
G	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
H												

Barcoded 16S gene-specific reverse primers

32 Reverse Primers

	1	2	3	4	5	6	7	8	9	10	11	12
A	R13		R21		R29		R37					
B	R14		R22		R30		R38					
C	R15		R23		R31		R39					
D	R16		R24		R32		R40					
E	R17		R25		R33		R41					
F	R18		R26		R34		R42					
G	R19		R27		R35		R43					
H	R20		R28		R36		R44					

DNA input

The recommended gDNA input amount per sample is 1–2 ng. The expected 16S rRNA amplicon size is approximately 1,500 bp. Typical amplicon product yields are 50–300 ng per sample.

Reagent and sample handling best practices

- Room temperature is defined as any temperature in the range of 18–23°C for this protocol.
- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30 minutes prior to use.
- Quick-spin all reagents in a microcentrifuge to collect liquid at tube bottom prior to use.
- Keep all temperature-sensitive reagents on ice.
- Ensure the SMRTbell cleanup beads are thoroughly mixed to a homogenous suspension before accurately pipetting the required volume.
- Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.
- Mix each reaction by pipetting up and down 10 times or until fully resuspended. Avoid introducing bubbles.
- The workflow takes ~4hr to complete. If a stop is necessary, refer to workflow for safe stopping points.

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 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, QIAamp 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

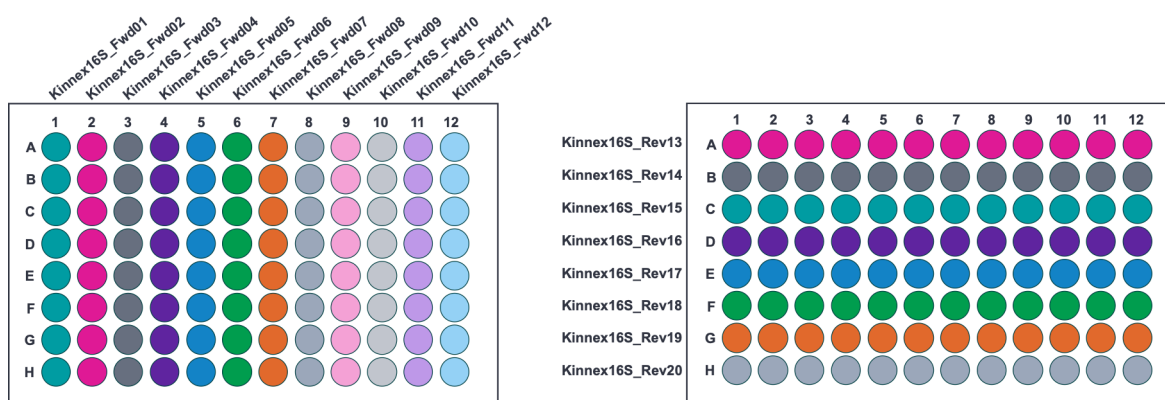
This section describes the preparation of PCR Master Mix and the recommended amplification conditions for processing up to 384 metagenomic DNA samples. Prepare the PCR Master Mix of all common components outlined below in a 2.0- or 5.0-mL DNA LoBind tube, including a 10% overage. Ensure that all reagents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

- 1.2 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 section A2](#) - 384 barcodes layout for all supported 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 and run a thermal cycler with the program shown below. **Keep the 96-well PCR plates on ice until the lid has heated to 105°C.** The duration of PCR is a 1 hour.

Heated lid set at 105°C. Reaction volume set to 25 μL

- 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 with Agilent Bioanalyzer or TapeStation systems.

- 1.6 The expected target amplicon size is ~1,500 bp, and the amount of amplicon material generated from each sample should be comparable as assessed by analyzing the relative intensity of the ~1,500 bp PCR product. See Figure 2 in the Appendix.

- 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. If preparing less than 96-plex, pool 20 µL from each PCR reaction into subsequent steps. A total mass of 35 ng is required for Kinnex library preparation, while 100 ng is required for monomer SMrtbell library preparation.
2.1.2	Proceed to the next step of the protocol.

2.2 Cleanup of pooled 16S PCR amplicons 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 remove the cleared supernatant without disturbing the beads. Discard the supernatant.

2.2.7 Slowly dispense **1,000 μL** or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. Remove the 80% ethanol and discard.

2.2.8 Repeat the previous step.

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.
- Remove 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 Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer the eluate 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.

PROTOCOL COMPLETE – Store at 4°C or -20°C for long-term storage

Appendix

A1. 16S forward and reverse primer sequences

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

Name	Sequence
Kinnex16S_Fwd_01	<u>CTACACGACGCTCTTCCGATCT</u> <u>GATCGAGTCA</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_02	<u>CTACACGACGCTCTTCCGATCT</u> <u>AGATCGCATG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_03	<u>CTACACGACGCTCTTCCGATCT</u> <u>AGACTAGCGT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_04	<u>CTACACGACGCTCTTCCGATCT</u> <u>GTACTGTCAG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_05	<u>CTACACGACGCTCTTCCGATCT</u> <u>ACGTGCAGAT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_06	<u>CTACACGACGCTCTTCCGATCT</u> <u>GACTATGACG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_07	<u>CTACACGACGCTCTTCCGATCT</u> <u>GACGCATAGT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_08	<u>CTACACGACGCTCTTCCGATCT</u> <u>AGCATGTACG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_09	<u>CTACACGACGCTCTTCCGATCT</u> <u>GTCGCACGAT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_10	<u>CTACACGACGCTCTTCCGATCT</u> <u>AGTGCGATCG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_11	<u>CTACACGACGCTCTTCCGATCT</u> <u>ACGCTCAGT</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Fwd_12	<u>CTACACGACGCTCTTCCGATCT</u> <u>GTAGACGCTG</u> AGRGTTYGATYMTGGCTCAG
Kinnex16S_Rev_13	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CATCGACG</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_14	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TCGCATGACT</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_15	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CATGATCGAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_16	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>GACTGTAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_17	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CGACTCGTAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_18	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CAGCTGACAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_19	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CAGTATGAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_20	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>ACGATGACG</u> TRGYTACCTTGTTACGACTT
Kinnex16S_Rev_21	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CGATGATGCT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_22	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TACGACAGT</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_23	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TGCATACTG</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_24	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CAGACTAGT</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_25	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CTCAGCATAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_26	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TAGCACGCAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_27	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TACTGACGCT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_28	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>ATACGAGCT</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_29	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TGAGCTATG</u> CRGYTACCTTGTTACGACTT
Kinnex16S_Rev_30	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CTGTCTAGT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_31	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TACTGCTCAC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_32	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CTCGTCAGAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_33	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CTCACTGAGT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_34	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>TACTAGCAGC</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_35	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>CGTAGCAGAT</u> RGYTACCTTGTTACGACTT
Kinnex16S_Rev_36	<u>AAGCAGTGGTATCAACGCAGAGT</u> <u>ACAGCTGTAC</u> RGYTACCTTGTTACGACTT

Kinnex16S_Rev_37	<u>AAGCAGTGGTATCAACGCAGAGTCGATGCTACRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_38	<u>AAGCAGTGGTATCAACGCAGAGCGATCAGTGCRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_39	<u>AAGCAGTGGTATCAACGCAGAGACGCACGTACRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_40	<u>AAGCAGTGGTATCAACGCAGAGCAGTAGCGTCRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_41	<u>AAGCAGTGGTATCAACGCAGAGACTGCAGCACRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_42	<u>AAGCAGTGGTATCAACGCAGAGTCACGACGACRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_43	<u>AAGCAGTGGTATCAACGCAGAGCGAGTCTAGCRGYTACCTTGTTACGACTT</u>
Kinnex16S_Rev_44	<u>AAGCAGTGGTATCAACGCAGAGCAGCAGTGACRGYTACCTTGTTACGACTT</u>

A2. 384 barcodes layout

Plate 1

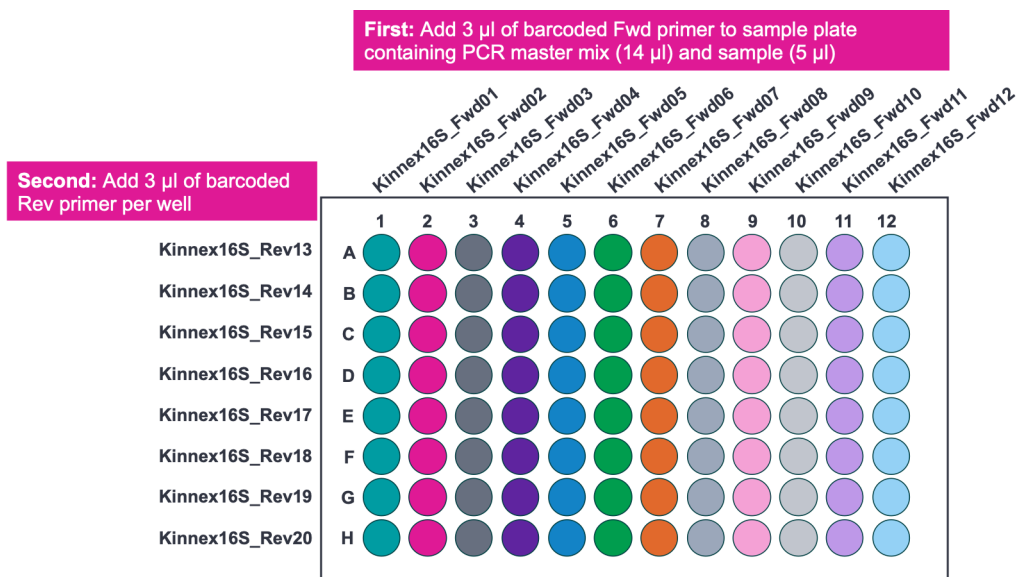


Plate 2

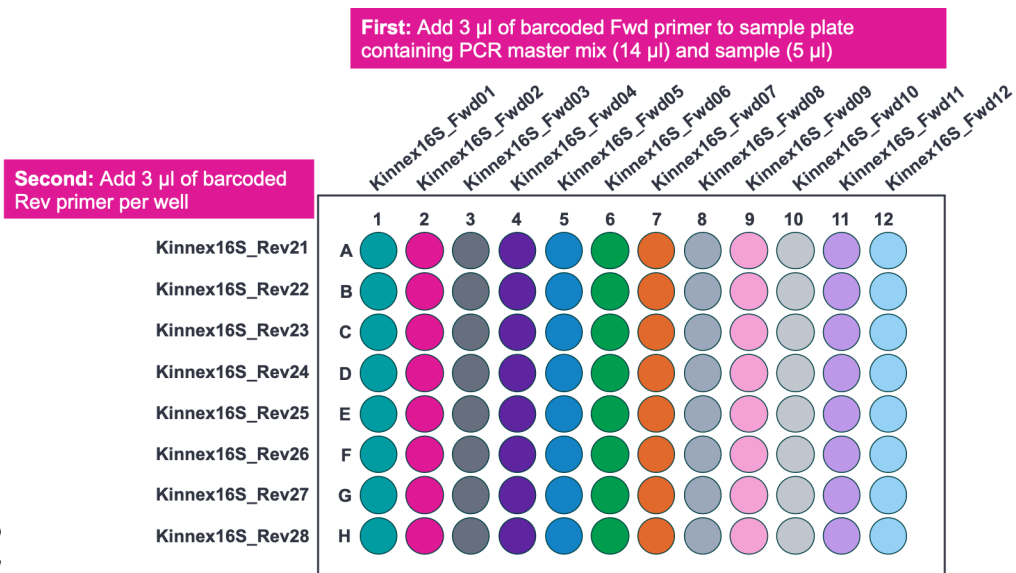


Plate 3

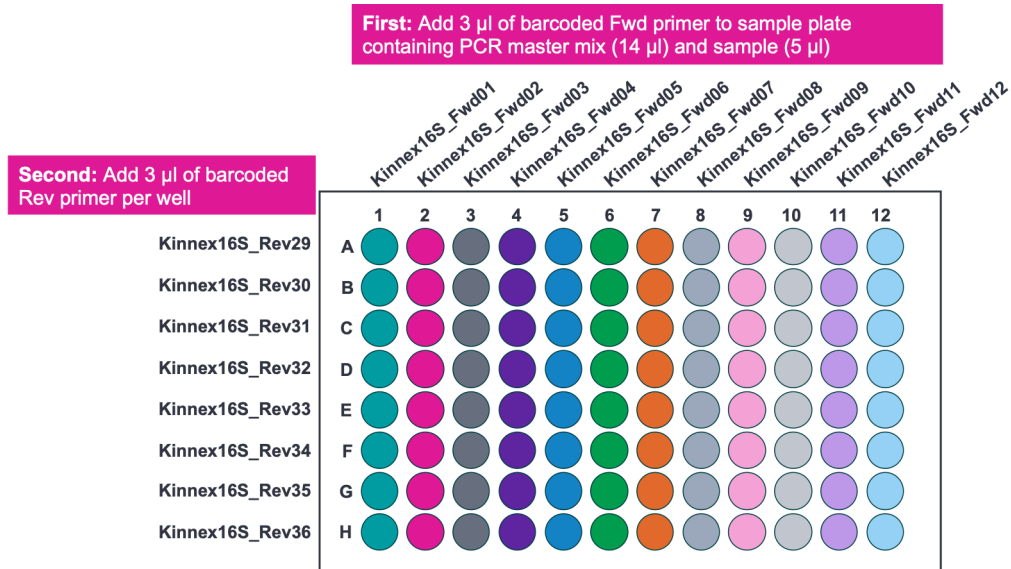
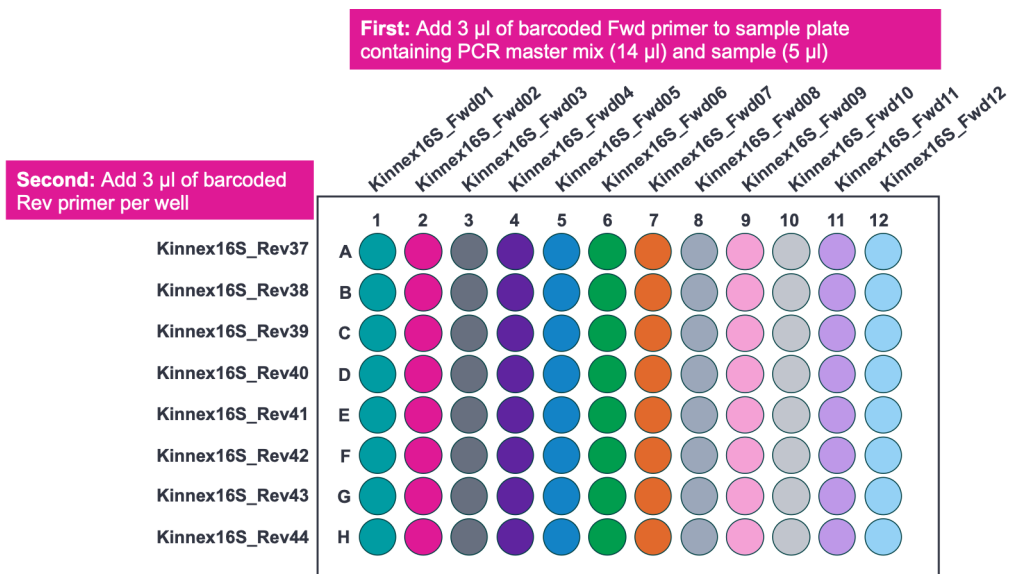


Plate 4



A3. Kinnex 16S SMRTbell and amplicon QC

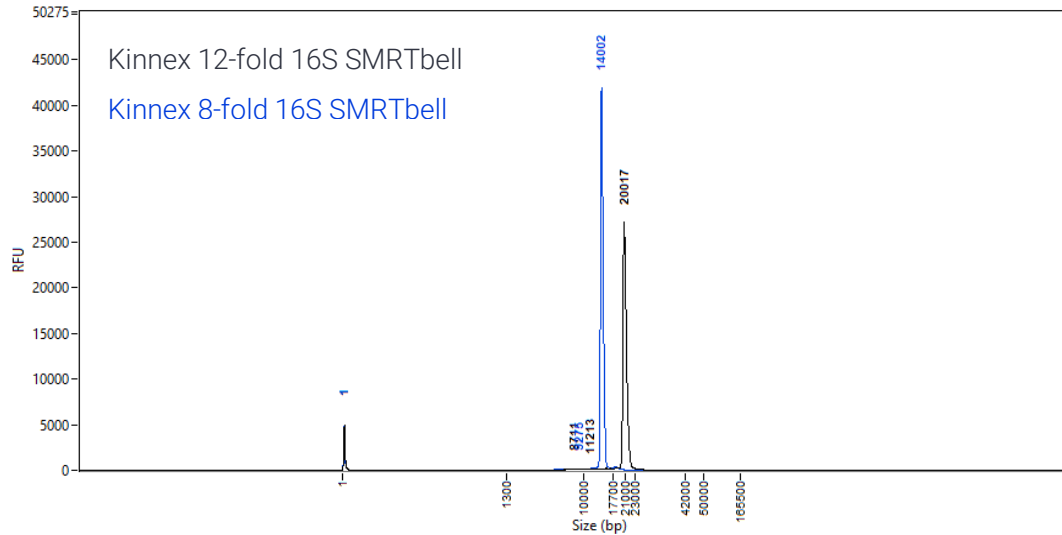


Figure 1. Example of a 16S rRNA Kinnex library quality control using the Femto Pulse system with Femto Pulse gNDA 165kb analysis kit. Kinnex 12-fold 16S SMRTbell library (black) peaks at a size of 20,017 bp; Kinnex 8-fold 16S SMRTbell library (blue) peaks at 14,002 bp.

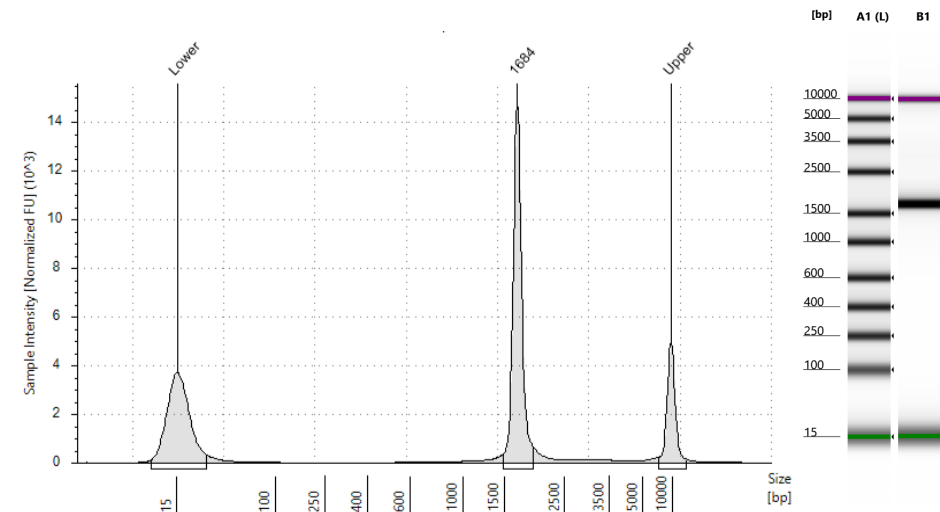


Figure 1. Example of a PCR amplified 16S rRNA amplicon quality control using TapeStation system with D5000 ScreenTape Assay. A single peak at 1,684 bp indicates successful amplification.

APPENDIX COMPLETE

Revision history (description)	Version	Date
Initial release	01	September 2026

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