LoopSeq™ 16S Microbiome 24-Plex
LoopSeq™ 16S & 18S Microbiome 24-Plex
Quick Start Guide

Version 3.3

Sample Dilution

- Quantify template DNA by Qubit
- Dilute DNA stock to **2 ng/ul** using Buffer EB

Barcode Assignment PCR

- Spin down PCR plate
- Add **5ul of diluted DNA** sample to their respective wells (one sample per well) and mix by pipetting (do not vortex)
- Seal the plate with fresh caps or sealing film, and spin down the PCR plate
- Initiate the following PCR program:

Temperature	Duration	STEP
95°C	3 min	1 cycle
98°C	15 seconds	
48°C	20 seconds	2 cycles
72°C	2 min	
4°C	HOLD	

Set the heated lid at 100°C
Ramp rate = 2 to 3°C/second

Barcode Distribution

- Pool **5ul** from each of the 24 reactions into a single tube (ideally 1.5 mL) and mix by pipet (do not vortex)

- Transfer **95ul** of this pool to a new PCR tube
- Add **15ul** of **Inactivation Enzyme A** to the reaction
- Incubate at 37°C for 10 minutes, then 80°C for 5 minutes, then HOLD at 4°C

- Perform a cleanup reaction using **66ul** SPRI reagent
- Wash twice in 200ul 80% ethanol
- Elute in **20ul** Buffer EB

Pause Point. Store sample at 4°C for up to 24 hours if needed

STOP

Calibration

- Add 18ul EB buffer to a 1.5 mL tube
- To this tube, add 2ul of the eluted sample (after 24-plex pooling and cleanup)
- This is the "diluted sample pool"

- Thaw on ice a chosen 2x qPCR Master Mix. Only use the following qPCR Master Mix reagent validated for this kit:

  KAPA SYBR FAST qPCR Kit Master Mix, Catalog No. KK4600
  BioRad iQ SYBR Green Supermix, Catalog No. 1708880

- Using a 96-well plate suitable for real-time SYBR-based qPCR, the following samples will be set up in the following reactions as duplicates:
  - 4 calibration standards (provided)
  - 1 sample (your samples, inactivated)
  - 1 NTC (no template control, your Buffer EB)

- Prepare a Master Mix for the above 6 reactions in duplicate with overage:
  - To a 1.5mL tube, add **140ul** of a chosen 2x qPCR Master Mix
  - Add **70ul** of **Calibration Reaction**

- Mix by vortex and briefly centrifuge
- Aliquot **15ul** of the Master Mix into 12 wells on a 96-well plate

A. Column 1: Fill two wells with 5ul of **Calibration Standard 1**
B. Column 2: Fill two wells with 5ul of **Calibration Standard 2**
C. Column 3: Fill two wells with 5ul of **Calibration Standard 3**
D. Column 4: Fill two wells with 5ul of **Calibration Standard 4**
E. Column 5: Fill two wells with 5ul of Buffer EB (this is the negative control)
F. Column 6: Fill two wells with 5ul of "diluted sample pool"

- Seal the plate using optically clear film or caps
- Briefly centrifuge the sealed plate for at least 20 seconds

- Initiate the following real-time qPCR program:

Temperature	Duration	STEP
95°C	3 min	1 cycle
98°C	15 seconds	
60°C	20 seconds	35 cycles
72°C	**2 min**	
4°C	HOLD	

Set the heated lid at 100°C

SYBR signal is collected during the 2 min elongation

- Analyze the data on the Loop Genomics website at:

  www.loopgenomics.com/start

- Calculate the volume of your sample pool to be carried forward into the next step of Barcode Distribution. Choose the volume appropriate for **HiSeq/NextSeq/NovaSeq**

Barcode Distribution

- Using a new PCR tube, combine the following to a final volume of 50 ul:

Amplification Mix B	**30ul**
Nuclease-free water	**20 - Xul**
DNA from previous step	**Xul***

** Volume may vary based on result of the calibration*

- Initiate the following PCR program:

Temperature	Duration	STEP
95°C	3 min	1 cycle
98°C	15 seconds	
60°C	20 seconds	20 cycles
72°C	2 min	
4°C	HOLD	

Set the heated lid at 100°C
Ramp rate = 2 to 3°C/second

- Perform a cleanup reaction using **30ul** SPRI reagent
- Wash twice in 200ul 80% ethanol
- Elute in **15ul** Buffer EB

- To the eluted sample, add **5ul** of **Distribution Mix**
- Add **2ul** of **Distribution Enzyme** the reaction
- Incubate at 20°C for 15 minutes, then 75°C for 5 minutes, then HOLD at 4°C

- Add **75.5ul** of **Activation Mix** to the reaction
- Add **2.5ul** of **Activation Enzyme** to the reaction
- Incubate at 20°C for 2 hours (heated lid off), then HOLD at 4°C

- Add **6ul** of **Neutralization Enzyme** to the reaction
- Incubate at 37°C for 15 minutes, then HOLD at 4°C

- Perform a cleanup reaction using **80ul** SPRI reagent
- Wash twice in 200ul 80% ethanol
- Elute in **20ul** Buffer EB

Pause Point. Store sample at 4°C for up to 24 hours if needed

STOP

Library Preparation

- Using a new PCR tube, combine **on ice** the following:

Fragmentation Mix	**20ul**
Fragmentation Enzyme	**10ul**
DNA from previous step	**20ul**

- Finger-flick to mix (do not vortex)
- Centrifuge for 2 seconds and immediately put back on ice
- Set up the following PCR program:

Temperature	Duration	STEP
4°C	1 min	**Pause**
32°C	12 min	1 cycle
65°C	30 min	1 cycle
4°C	HOLD	

Set the heated lid at 100°C

- Initiate the empty PCR machine (no tubes yet) then **pause** during the first 4°C step

- Place the assembled reaction in the PCR tube into the paused PCR machine and **resume** the program

- Take out the PCR tube once the program is completed
- Add **40ul** of **Ligation Mix** to the reaction
- Add **10ul** of **Ligation Enzyme** to the reaction
- Incubate at 20°C for 15 minutes (heated lid off), then HOLD at 4°C

- Perform a cleanup reaction using **80ul** SPRI reagent
- Wash twice in 200ul 80% ethanol
- Elute in **20ul** Buffer EB

Pause Point. Store sample at 4°C for up to 24 hours if needed

STOP

- Using a new PCR tube, combine the following:

Index Master Mix	**25ul**
Index Primer P1 to P4	**5ul (choose one Index Primer)**
DNA from most previous elution	**20ul**

- Initiate the following PCR program:

Temperature	Duration	STEP
95°C	3 min	1 cycle
98°C	15 seconds	
60°C	30 seconds	12 cycles
72°C	30 seconds	
72°C	1 min	1 cycle
4°C	HOLD	

Set the heated lid at 100°C

- Perform a cleanup reaction using **40ul** SPRI reagent
- Wash twice in 200ul 80% ethanol
- Elute in **20ul** Buffer EB

Process Complete: Ready for sample QC and sequencing