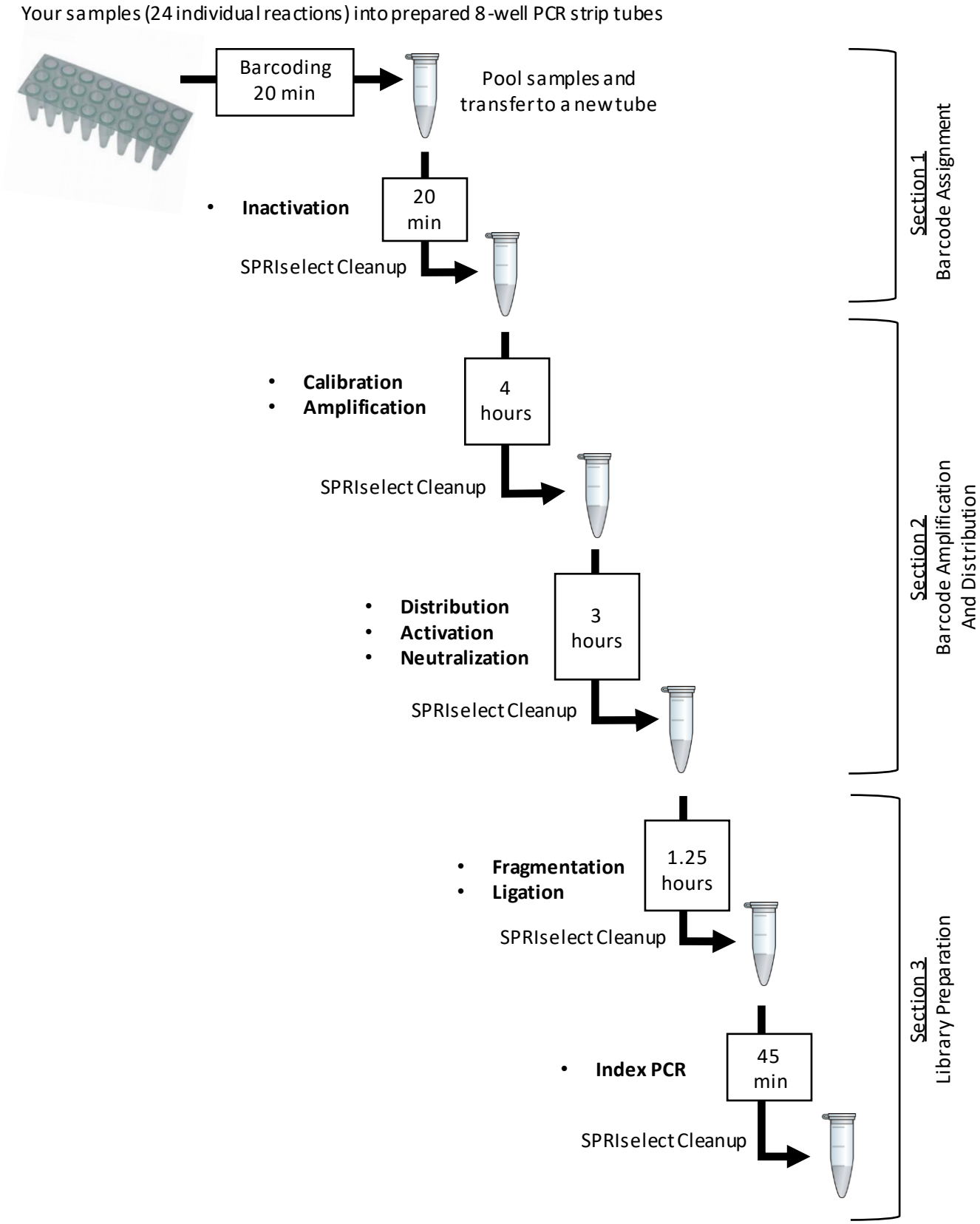
LoopSeq™ 16S Microbiome 24-Plex Kit
LoopSeq™ 16S & 18S Microbiome 24-Plex Kit

Version 3.3

Contents

Workflow Schematic



16S & 18S Microbiome Long Reads

At Loop Genomics, our mission is to build genomics tools that bring clarity to genetic data. In pursuit of that goal, we developed a technology that reconstructs long molecules from short-read sequences. This is the backbone of the **LoopSeq™ 16S Microbiome 24-Plex** kit and the **LoopSeq™ 16S & 18S Microbiome 24-Plex** kit. The 16S kit selectively barcodes full length 16S molecules from bacterial genomic DNA (excluding 18S), while the 16S & 18S kit barcodes both 16S and 18S full length molecules from bacterial, archaeal, and eukaryotic genomic DNA. The choice of kits offers flexibility for when the exclusive targets of interest in a sample are bacterial, or for when the identification from all kingdoms of life is desired.

LoopSeq™ kits are designed with a simple workflow in mind; to take 24 individual microbiome samples and multiplex them into a single reaction tube after the samples have been tagged. Processing one tube means less hands-on time, reduced chance of error, and more reliable output when all the samples are pooled and processed in the same reaction. Because projects are never isolated experiments, we rigorously optimized our kit for reproducibility so that samples processed on different days can still be compared with each other with confidence.

Kit Components

Component	Part number	Page #
Barcoding Adapters, 24 wells	LG00A-001/LG00A-002	5
Inactivation Enzyme A	LG00A-130	5
Amplification Mix B	LG00A-150	6
Distribution Mix	LG00A-160	5
Distribution Enzyme	LG00A-170	6
Activation Mix	LG00A-190	6
Activation Enzyme	LG00A-210	6
Neutralization Enzyme	LG00A-230	6
Fragmentation Mix	LG00A-250	7
Fragmentation Enzyme	LG00A-270	7
Ligation Mix	LG00A-290	7
Ligation Enzyme	LG00A-310	7
Index Master Mix	LG00A-330	7
Index Primer P1	LG00A-341	7
Index Primer P2	LG00A-342	7
Index Primer P3	LG00A-343	7
Index Primer P4	LG00A-344	7
Calibration Standard 1	LG00A-361	6
Calibration Standard 2	LG00A-362	6
Calibration Standard 3	LG00A-363	6
Calibration Standard 4	LG00A-364	6
Calibration Reaction	LG00A-375	6

Important Parameters

Sample Type Guidelines

This protocol is optimized for sequencing 16S and/or 18S molecules from genomic DNA purified from complex, high-diversity sample sources. For multiplexing these prepared samples, the protocol pools equal volumes of barcoded samples across the 24 samples processed within a single kit after samples are individually barcoded. To ensure even representation of each sample in the final sequencing data, care should be taken to ensure that the 24 pooled samples processed within a single kit have comparable numbers of 16S and/or 18S molecules. Different sample types typically contain different concentrations of 16S and/or 18S molecules.

It is not recommended to combine samples derived from different sources within the same kit as this may lead to under/over-representation of some samples within the pool of 24 samples. For example, 24 individual samples from marine soil and isolated using the same extraction method can all be processed in the same LoopSeq™ 24-Plex kit. However, 12 samples from marine soil and 12 samples from animal stool should not be grouped into the same kit run. Mixing unlike sample sources can bias the results in favor of one sample source over the other. Examples of sample source variation include: use of different extraction methods or kits used for genomic DNA isolation, samples extracted from different physical sites (e.g. soil versus water), and samples extracted from different host organisms (e.g. human gut microbiome versus rumen microbiome).

For the LoopSeq™ 16S Microbiome 24-Plex kit, sequencing 16S molecules from samples that have different concentrations of bacterial versus non-bacterial DNA can lead to a biased number of 16S molecules across the 24 samples. For example, pooling human gut microbiome samples with low versus high levels of human genomic DNA contamination would lead to fewer than expected 16S molecules in the sample that had high levels of human genomic DNA in the extracted sample.

Note that it is not recommended to process skin microbiome or low-mass samples with the LoopSeq™ 16S Microbiome 24-Plex kit or the LoopSeq™ 16S & 18S Microbiome 24-Plex kit.

Input DNA Quality and Quantification Guidelines

The quality of the input genomic DNA significantly impacts the number of 16S and/or 18S molecules per sample that can be successfully sequenced and assembled into synthetic long reads. Differential sample quality and purity, arising from different extraction methods, carryover from sample extraction, and sample storage conditions, may impact kit performance.

Only full length 16S and/or 18S molecules can be barcoded and amplified in this protocol. Therefore, extraction methods that perform adequately for short 16S variable regions may not be suitable for this protocol. If the input DNA sample is partially or highly degraded, the number of reported synthetic long reads may be low. We recommend performing a calibration PCR whenever changes are made to the sample types being processed or the extraction method used to obtain samples. For more information on sample calibration, please refer to the “Guidelines for Estimating Sample Complexity” section of this manual.

Accurate quantification of extracted DNA samples is important for placing the samples within the dynamic range of this kit. Quantification of the extracted DNA samples using the Qubit™ Fluorometer is the preferred method for accurately determining the concentration of DNA that will be used as input material. Other DNA-Binding Fluorescent Dye quantification methods may be suitable alternatives to using a Qubit Fluorometer. DNA quantification by NanoDrop™ is not recommended as the concentration estimate by that method can be adversely impacted by salt concentration, presence of free nucleotides, RNA, and other contaminants that absorb at similar wavelengths to double stranded DNA.

Guidelines for Estimating Sample Complexity

Accurate estimate of the 16S and/or 18S molecules are important in the success of the library preparation and for ensuring the level of sequencing depth is adequate for synthetic long read assembly. This protocol aims to generate long-read sequences for ~300,000 molecules. The number of full length 16S and/or 18S molecules available for sequencing in a sample can vary based on sample types, sources, and extraction methods. Samples obtained using different extraction methods, kits, samples extracted from different sources, and samples extracted from different host organisms, can lead to different 16S and/or 18S molecules in the samples. Examples of different sample sources include water, soil, human gut, rumen, stool, and pure cultures.

Accurate quantification of the input DNA does not differentiate between bacterial and eukaryotic DNA. The presence of non-bacterial genomic material can lead to an overestimation of the bacterial input material when sequencing 16S molecules only. Differences in sample recovery from sample cleanup due to variability between users can also alter the number of barcoded molecule available for sequencing. Accurate counts

of the 16S and/or 18S molecules in samples can only be achieved by using a calibration PCR.

The table below provides examples of the "pooled sample" volume to use in the Barcode Amplification reaction (section 3) to achieve the target complexity of ~300,000 molecules from the 24-plex pool.

Sample type	Volume to carry forward (µl)
Lake microbiomes	10 to 12
Soil microbiomes	6 to 8
Gut microbiomes	3 to 4
Pure cultures	0.5 to 2
ZymoBIOMICS™ microbial community (D6300)	0.5 to 2

Safe Stopping Points

When necessary, this protocol can be paused at multiple steps along the way as indicated by the "stop sign" symbol or maintained at 4°C overnight at the end of a PCR-based step as indicated by the word "HOLD". Always follow the recommended storage temperature and duration indicated at each safe stopping point.

Prolonged Sample Storage

Prolonged storage (>3 days at 4°C) of the library pool prior to completing the Index PCR reaction in section 4 is not recommended. Amplified library product after the Index PCR reaction can be stored at 4°C for up to 1 week or -20°C for up to 1 month.

Equipment Supplied by User

- **Liquid-handling supplies**
  1.5 ml microcentrifuge tubes, PCR plates, PCR microcentrifuge tubes, or PCR strips, pipettes. Perform PCR reactions in vessels suitable for sealing and cycling in PCR conditions
- **Thermal cycler**
  Suitable for PCR with a heated lid and ramp rate adjustable
- **Magnetic stand**
  Permagen, Cat No. MSR812, or equivalent product
- **Qubit dsDNA HS Assay Kit for DNA quantification**
  Invitrogen™, Cat No. Q23851 or Q23854. Qubit™ Fluorometer has been validated for quantifying dsDNA in this protocol

Reagents Supplied by User

- **SPRIselect Reagent or Ampure XP**
  Beckman Coulter, Cat No. B23317 for SPRIselect
  Beckman Coulter, Cat No. A63881 for Ampure XP
  Substitution is not recommended
- **Nuclease-free water**
  VWR, Cat No. 97062-794, or equivalent
- **Buffer EB**
  Qiagen, Cat No. 19086, or lab-made 10mM Tris Buffer, pH 8.5
- **80% Ethanol in Nuclease-free water**
- **Barcoded Sample Quantification**
  KAPA SYBR FAST qPCR Kit Master Mix, Cat No. KK4600 (Roche Cat No. 07959362001)
  BioRad iQ SYBR Green Supermix, Cat No. 1708880
  Substitution is not recommended
- **Library QC on the Agilent 2100 Bioanalyzer**
  Agilent High Sensitivity DNA Kit, Cat No. 5067-4626, or equivalent
- **Library Quantification**
  KAPA Library Quantification Kit for the Illumina platform, Cat No. KK4824 (Roche Cat No. 07960140001), or equivalent

Section I: Barcode Assignment

1.1. DNA Quantification and Dilution

A. Quantify the concentration of template genomic DNA using a Qubit dsDNA High Sensitivity Assay or equivalent fluorometer. Nanodrop is not recommended due to its overestimation of genomic DNA concentration in prepared samples

Note: It is recommended that at least 2 µl of the genomic DNA stock is used for quantification to decrease pipetting error

B. Dilute template genomic DNA stock to 2 ng/µl with Buffer EB and use immediately in Barcode Assignment PCR (section 1.2). Diluted stocks can be stored at -20°C for up to 2 weeks

Note: It is recommended that freeze-thaw cycles of the template DNA and the working stocks are minimized

1.2. Barcode Assignment PCR

24 Barcoding Adapters are provided pre-dispensed into the first three columns of a 96-well plate (24 wells in total). Each adapter has a unique sample index to identify the genomic DNA templates that will be barcoded in that well.

A. Briefly thaw the Barcoding Adapter plate at room temperature and centrifuge to collect liquid to the bottom of each well
B. Carefully remove and discard the foil plate cover

Note: Take care not to cross-contaminate the sample indices. Use a fresh pipet tip for loading each well

C. Add 5 µl of genomic DNA sample (2 ng/µl) per well, noting the column and row position for downstream identification of the sample
D. Pipet mix the tube thoroughly (do not vortex) and centrifuge briefly

Component	Volume per Sample (µl)
Barcoding Adapters (in plate)	15
Diluted Genomic DNA (at 2 ng/µl)	5
Total =	20

E. Seal the plate wells with fresh caps or a PCR-certified adhesive sticker
F. Initiate the following PCR program in a thermal cycler:

Heated lid at 100°C

Temperature	Duration	STEP	Ramp speed
95°C	3 min	1 cycle	2 to 3°C/s
98°C	15 seconds	2 cycles	2 to 3°C/s
48°C	20 seconds		
72°C	2 min		
4°C	HOLD	∞	2 to 3°C/s

1.3. PCR Inactivation

A. Combine 5 µl from each of the 24 reaction wells into a 1.5 mL Eppendorf tube. Pipet mix the tube thoroughly (do not vortex)
B. Transfer 95 µl from this large pool into a new PCR tube
C. Add 15 µl of **Inactivation Enzyme A** to the PCR tube
D. Pipet mix the tube thoroughly (do not vortex) and centrifuge briefly
E. Incubate in a thermal cycler using the following conditions:

Heated lid at 100°C

Temperature	Duration	STEP
37°C	10 min	1 cycle
80°C	5 min	1 cycle
4°C	HOLD	∞

1.4. Post-barcoding SPRIselect Cleanup

A. Add 66 µl of SPRIselect to the reaction PCR tube. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on the magnetic stand for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
F. Incubate the beads on the magnet for 30 seconds
G. Carefully remove and discard the ethanol wash
H. Repeat the wash once by adding 200 µl of 80% ethanol
I. Incubate the beads on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube from the magnet and immediately resuspend the beads in 20 µl of Buffer EB

Caution! do not allow the beads to dry as it significantly decreases elution efficiency

N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new PCR tube

Proceed immediately to the next step or store at 4°C for up to 24 hours before proceeding to the next step

Section II: Barcode Calibration

2.1. Sample Pool Dilution

A. Add 18 µl EB buffer to a 1.5 mL tube
B. To this tube add 2 µl from the eluted reaction from section 1.4; this is the "sample pool dilution"

2.2. Calibration by Real-Time qPCR

A. Use an 8-strip tube or well plate to set up the calibration
B. Thaw on ice a chosen 2x qPCR Master Mix reagent

Validated 2x SYBR-based qPCR Master Mix reagents	
KAPA SYBR FAST qPCR Kit Master Mix	Cat. # KK4600
BioRad iQ SYBR Green Supermix	Cat. # 1708880

C. Combine 140 µl of a chosen 2x qPCR Master Mix reagent with 70 µl of **Calibration Reaction** in a 1.5 mL Eppendorf tube
D. Vortex mix the tube thoroughly

Prepare the following as duplicate or triplicate reactions

E. Dispense 15 µl of the above combined qPCR Master Mix plus Calibration Reaction into:
    a. well #1, then add 5 µl of **Calibration Standard 1**
    b. well #2, then add 5 µl of **Calibration Standard 2**
    c. well #3, then add 5 µl of **Calibration Standard 3**
    d. well #4, then add 5 µl of **Calibration Standard 4**
    e. well #5, then add 5 µl of EB buffer (neg. control)
    f. well #6, then add 5 µl of the "sample pool dilution"
F. Cap strip tubes or seal plate and vortex mix. Briefly centrifuge

G. Initiate the following PCR program in a machine suitable for real-time SYBR-based qPCR:

Heated lid at 100°C

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

** collect SYBR signal during the 2 minute elongation step

2.3. Calibration Analysis

Go to the Loop Genomics website (see below) to calculate the number of molecules per microliter in your undiluted SPRI cleaned pooled sample.

www.loopgenomics.com/start

This number is the **molecule count per microliter** for the **undiluted pool** sample. Carry forward the volume of your pool sample appropriate for **HiSeq/NextSeq/NovaSeq** into the Barcode Amplification (see section 3.1 below) step.

Section III: Barcode Distribution

3.1. Barcode Amplification

A. Dispense 30 µl of **Amplification Mix B** into a new PCR tube

The value of X (table below) is based on either prior experience using this kit and knowing the volume of your sample pool that needs to be used, or by performing a calibration as described in section 2 above.

B. Add 20 minus X µl of nuclease-free water to the reaction
C. Transfer X µl from the cleaned sample pool from section 1.4 as calculated by the calibration PCR
D. Pipet mix or vortex the tube thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
Amplification Mix B	30
Nuclease-free water	20 - X
DNA from previous step	X
Total =	50

E. Initiate the following PCR program in a thermal cycler:

Heated lid at 100°C

Temperature	Duration	STEP	Ramp speed
95°C	3 min	1 cycle	2 to 3°C/s
98°C	15 seconds	20 cycles	2 to 3°C/s
60°C	20 seconds		
72°C	2 min		
4°C	HOLD	∞	2 to 3°C/s

3.2. Post-amplification SPRIselect Cleanup

A. Add 30 µl of SPRIselect to the reaction. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on a magnetic stand for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
F. Incubate the beads on the magnet for 30 seconds
G. Carefully remove and discard the ethanol wash
H. Repeat the wash once by adding 200 µl of 80% ethanol
I. Incubate the beads on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube from the magnet and immediately resuspend the beads in 15 µl of Buffer EB

Caution! do not allow the beads to dry as it significantly decreases elution efficiency

N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new PCR tube

3.3. Barcode Distribution

A. To the 15 µl of eluate in the new PCR tube, add 5 µl of **Distribution Mix** to the reaction
B. Add 2 µl of **Distribution Enzyme** to the reaction
C. Pipet mix or vortex the tube thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
DNA from previous step	15
Distribution Mix	5
Distribution Enzyme	2
Total =	22

D. Incubate in a thermal cycler using the following conditions:

Heated lid at 100°C

Temperature	Duration	STEP
20°C	15 min	1 cycle
75°C	5 min	1 cycle
4°C	HOLD	∞

3.4. Barcode Activation and Neutralization

A. Add 75.5 µl of **Activation Mix** to the reaction
B. Add 2.5 µl of **Activation Enzyme** to the reaction
C. Pipet mix or vortex the tube thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
DNA from previous step	22
Activation Mix	75.5
Activation Enzyme	2.5
Total =	100

D. Incubate in a thermal cycler using the following conditions:

Heated lid turned off or not in direct contact with sample tube

Temperature	Duration	STEP
20°C	2 hours	1 cycle
4°C	HOLD	∞

E. Add 6 ul of **Neutralization Enzyme**
F. Incubate in a thermal cycler using the following conditions:

Heated lid at 100°C

Temperature	Duration	STEP
37°C	15 min	1 cycle
4°C	HOLD	∞

3.5. Post-activation SPRIselect Cleanup

A. Add 80 µl of SPRIselect to the reaction. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on a magnetic stand for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
F. Incubate the beads on the magnet for 30 seconds
G. Carefully remove and discard the ethanol wash
H. Repeat the wash once by adding 200 µl of 80% ethanol
I. Incubate the beads on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube and return to the magnet

L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube from the magnet and immediately resuspend the beads in 20 µl of Buffer EB

Caution! do not allow the beads to dry as it significantly decreases elution efficiency

N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new PCR tube

Proceed immediately to the next step or store at 4°C for up to 72 hours before proceeding to the next step

Section IV: Library Preparation

Assemble the following reaction on ice

4.1. Fragmentation, End Repair, and A-tailing

A. Program a thermal cycler with the following conditions:

Heated lid at 100°C

Temperature	Duration	STEP
4°C	1 min	Paused
32°C	12 min	1 cycle
65°C	30 min	1 cycle
4°C	HOLD	∞

B. Start the program but pause the PCR machine during the initial 4°C step
C. To the volume of clean sample in the PCR tube from section 3.5 add 20 µl of **Fragmentation Mix** and keep on ice
D. Add 10 µl of **Fragmentation Enzyme** to the reaction on ice
E. Mix the reaction thoroughly by pipet or finger flick (do not vortex), centrifuge briefly, and immediately return to ice

Component	Volume per Sample (µl)
DNA from previous step	20
Fragmentation Mix	20
Fragmentation Enzyme	10
Total =	50

F. Place the reaction in the thermal cycler and resume the program

4.2. Adapter Ligation

A. Add 40 µl of **Ligation Mix** to the reaction
B. Add 10 µl of **Ligation Enzyme** to the reaction
C. Pipet mix or vortex the reaction thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
DNA from previous step	50
Ligation Mix	40
Ligation Enzyme	10
Total =	100

D. Incubate in a thermal cycler according to the following program with the heated lid turned off:

Heated lid turned off or not in direct contact with sample tube

Temperature	Duration	STEP
20°C	15 min	1 cycle
4°C	HOLD	∞

4.3. Post-ligation SPRIselect Cleanup

A. Add 80 µl of SPRIselect to the reaction. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on a magnetic stand for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
F. Incubate the beads on the magnet for 30 seconds
G. Carefully remove and discard the ethanol wash
H. Repeat the wash once by adding 200 µl of 80% ethanol
I. Incubate the beads on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube from the magnet and immediately resuspend the beads in 20 µl of Buffer EB

Caution! do not allow the beads to dry as it significantly decreases elution efficiency

N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new PCR tube

Proceed immediately to the next step or store at 4°C for up to 72 hours before proceeding to the next step

4.4. Library Index PCR

A. To the 20 µl of eluate in the new PCR tube, add 25 µl of **Index Master Mix** to the reaction
B. Add 5 µl of **Index Primer P1, P2, P3, or P4** to the reaction tube (choose only one)
C. Pipet mix or vortex the reaction thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
Index Master Mix	25
Index Primer P1 to P4 (choose one)	5
DNA from previous step	20
Total =	50

D. Initiate PCR in a thermal cycler according to the following program:

Heated lid at 100°C

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

4.5. Post-indexing SPRIselect Cleanup

A. Add 40 µl of SPRIselect to the reaction. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on a magnetic stand for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
F. Incubate the beads on the magnet for 30 seconds
G. Carefully remove and discard the ethanol wash
H. Repeat the wash once by adding 200 µl of 80% ethanol
I. Incubate the beads on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube from the magnet and immediately resuspend the beads in 20 µl of Buffer EB

Caution! do not allow the beads to dry as it significantly decreases elution efficiency

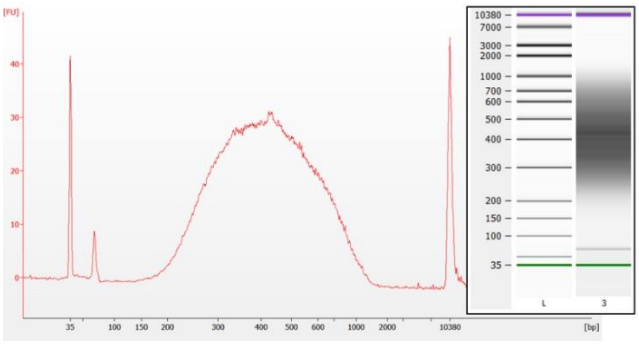
N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new 1.5 mL Eppendorf tube

Proceed immediately to the QC step, or store at 4°C for up to 1 week, or store at -20°C for up to 1 month before proceeding

Sample QC

Library Size Distribution Determination

Evaluate 1 µl of the final library on an Agilent Bioanalyzer High Sensitivity chip or equivalent machine to determine the insert size range. Below is an example trace of a correct final library. The peak height and fragment size range might vary slightly from this example depending on the source of the DNA samples used in this protocol.



Quantify the final library concentration using a KAPA library quantification kit for Illumina libraries or equivalent.

Sequencing

The final library contains Illumina universal sequencing adapters, namely P5 and P7, for library cluster generation on the flow cell, as well as "Read 1" and "Read 2" sequences. Due to the nature of the library, at least 3% PhiX should be included to achieve optimal read quality and to minimize error rates during the run.

Each final library should receive at least the following sequencing depth for optimal phasing performance:

Read Length	Sequencing depth
2 x 150 bp	100-150M PE reads (~50M clusters passing filter)

However, the sequencing depth required may vary based on sample type, quantification method, or extraction method, and can be adjusted based on the observed phasing performance.

Illumina Index Table

Primer	Illumina ID	Indexing Sequence
Index Primer P1	D701	ATTACTCG
Index Primer P2	D702	TCCGGAGA
Index Primer P3	D703	CGCTCATT
Index Primer P4	D704	GAGATTCC

Legal

Use Restrictions

The LoopSeq™ Complete kit and its components are designed, developed, and sold for research use only. They are suitable for *in vitro* research and are not recommended or intended to diagnose or treat disease in humans or animals. Loop Genomics makes no claims or representations for clinical use (diagnostic, prognostic, or therapeutic). Please do not use internally or externally in or on humans or animals.

Handling, Safety Warnings and Precautions

This product and its components should be handled by persons trained in laboratory techniques and used in accordance with the principles of good laboratory practice. All chemicals are potentially hazardous. Therefore, when handling chemical reagents, it is advisable that suitable protective clothing, such as laboratory coats, safety glasses and gloves be worn. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash the affected area immediately with water. Refer to appropriate Safety Data Sheets for more specific recommendations.

Storage Conditions

Store the entire kit at -20°C. Thaw components on ice and maintain on ice during all reaction setup steps. Return components to -20°C after setup is completed.