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

With Single Sample Calibration
Version 1.7*

*Content may have been updated. To download the latest version, please visit https://www.loopgenomics.com/start

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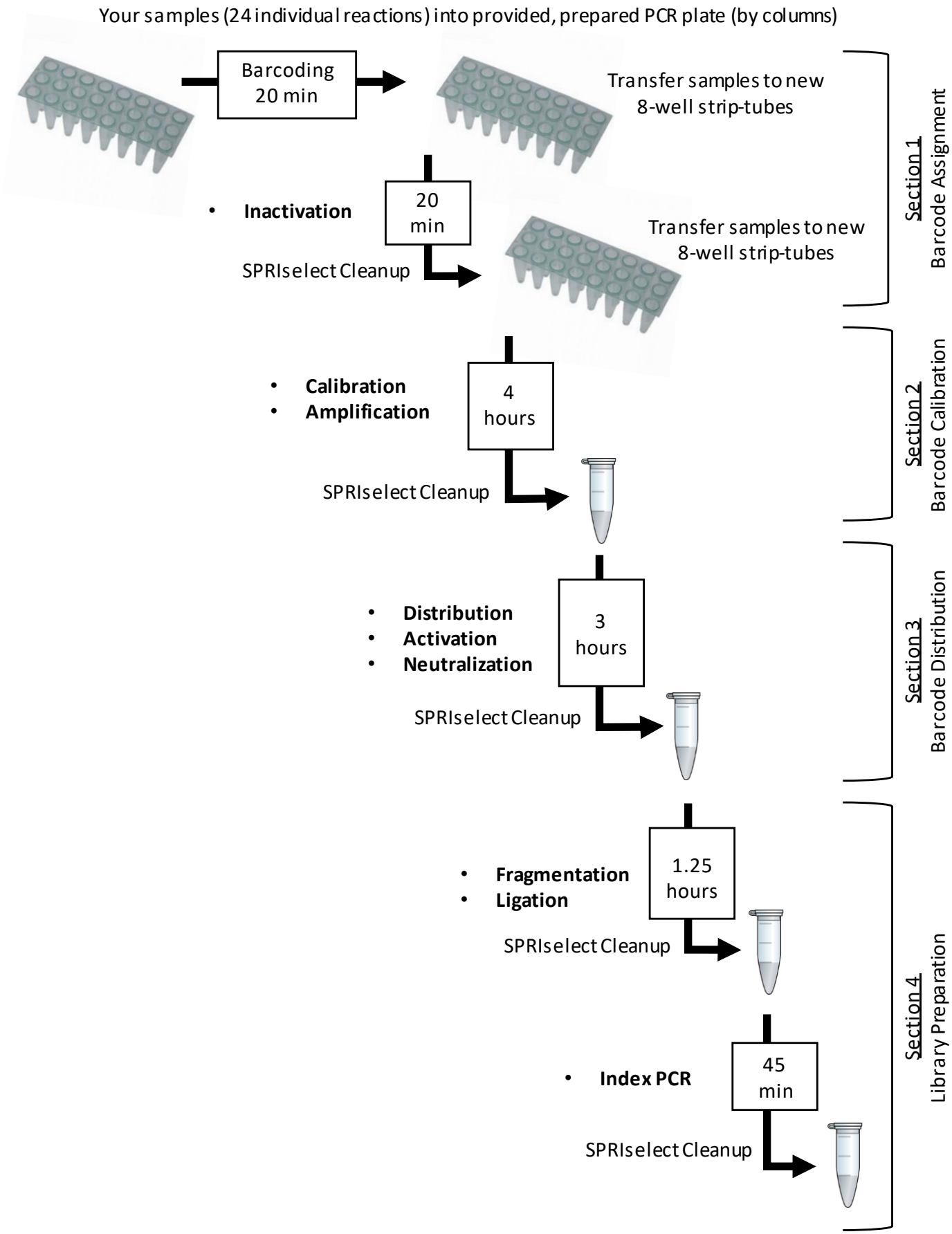
Workflow Schematic



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 SSC 24-Plex** kit and the **LoopSeq™ 16S & 18S Microbiome SSC 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.

Our LoopSeq™ SSC kits are designed around a simple workflow while still retaining the complexity needed to generate a robust dataset. After the samples are individually tagged with a sample ID and molecular barcodes, each sample is normalized using single sample calibration and amplified individually. The samples are then multiplexed into a single reaction tube for downstream sample prep. Processing one tube means less hands-on time, reduced chance of error, and more reliable output because all the samples are pooled and processed in the same reaction. Because projects are never isolated experiments, we have rigorously optimized our kits for reproducibility so that samples processed on different days can still be compared to one another with confidence.

Kit Components

Component	Part number	Page #
Barcoding Adapters, 24 wells	LG00A-001/LG00A-002	6
Inactivation Enzyme SSC	LG00A-132	6
Amplification Mix SSC	LG00A-152	7
Distribution Mix	LG00A-160	7
Distribution Enzyme	LG00A-170	7
Activation Mix	LG00A-190	7
Activation Enzyme	LG00A-210	7
Neutralization Enzyme	LG00A-230	8
Fragmentation Mix	LG00A-250	8
Fragmentation Enzyme	LG00A-270	8
Ligation Mix	LG00A-290	8
Ligation Enzyme	LG00A-310	8
Index Master Mix	LG00A-330	8
Index Primer P1	LG00A-341	8
Index Primer P2	LG00A-342	8
Index Primer P3	LG00A-343	8
Index Primer P4	LG00A-344	8
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 SSC	LG00A-376	6

Important Parameters

Sample Type Guidelines

Not all samples are created equal. LoopSeq SSC technology takes this into account, allowing different sample types to be processed in the same sequencing preparation. 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 amplified products from 24 individually barcoded samples that have been normalized and amplified within a single kit. Different sample types typically contain different concentration of 16S and/or 18S molecules. To ensure uniform representation of each sample in the final sequencing data, care should be taken to ensure that the pooled samples processed within a single kit have comparable numbers of 16S and/or 18S molecules. The most accurate estimation of molecule counts per sample is with calibration PCR. For more information on sample calibration, refer to the "Guidelines for Estimating Sample Complexity" section of this manual.

Sample types that have been processed successfully with LoopSeq™ 16S Microbiome SSC 24-Plex kits and LoopSeq™ 16S & 18S Microbiome SSC 24-Plex kits include gut, stool, soil, and water microbiome samples. It is not recommended to process skin microbiome or other low-mass samples with the LoopSeq™ 16S Microbiome SSC 24-Plex kit or the LoopSeq™ 16S & 18S Microbiome SSC 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 captured, sequenced, and assembled into synthetic long reads. Differential sample quality and purity may result in sample source variation that impacts kit performance.

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), sample storage conditions, and samples extracted from different host organisms (e.g. human gut versus rumen). Different concentrations of bacterial versus non-bacterial DNA in the input sample may also lead to variable abundances of 16S and/or 18S molecules sequenced and assembled across the 24 samples.

Only full length 16S and/or 18S molecules can be captured and amplified with this protocol. Therefore, methods that perform adequately for short 16S variable regions (e.g. V3-V4 region only) may not be suitable for this protocol. Note that if the input DNA sample is partially or highly degraded, the number of reported synthetic long-reads may be low.

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

An accurate estimation of molecule counts is important for successful library preparation, and for ensuring that the level of sequencing depth is adequate for generating synthetic long-read sequences. If the intended sequencer will be a HiSeq/NextSeq/NovaSeq (PE150 reads), this protocol aims to generate long-read sequences for ~12,500 molecules per sample, or ~300,000 molecules per 24-plex kit. If the intended sequencer will be a MiSeq (PE300 reads), the protocol aims to generate long-read sequences for ~8,000 molecules per sample, or ~200,000 molecules per 24-plex kit.

The number of full-length molecules available for sequencing in a sample can vary based on sample type, sources, and extraction methods. Samples obtained using different extraction methods, prep kits, samples from different sources, and samples from different host organisms, can all lead to different amounts of 16S and/or 18S molecules that can be detected in each sample. Examples of different sample sources include water, soil, 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 an estimate of molecule counts per sample type (i.e. sample complexity) based on Calibration qPCR in section 2.

Sample type	BC molecules per µl
Lake microbiomes	5,000 to 6,000
Soil microbiomes	7,500 to 10,000
Gut microbiomes	15,000 to 20,000
Pure cultures	30,000 to 120,000
ZymoBIOMICS™ microbial community (D6300)	80,000 to 120,000

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, PCR tube strips, and 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**
  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

Revision History

Version	Date	Description of Change
1.7	July 2019	• Removed KAPA SYBR FAST mix as a validated qPCR master mix • Updated language describing sequencing on an Illumina platform
1.6	Apr 2019	• Updated Library PCR cycle conditions • Clarified text in “Sample Type Guidelines” regarding mixing samples of different types • Changed calibration cycles from 35 to 40 cycles in section 2.1 • Updated calibration worksheet • Updated workflow diagram • Updated storage times and temperature at the pause point in section 1.4 • Minor text changes for clarity and consistency
1.5	Mar 2019	• Updated calibration worksheet
1.4	Feb 2019	• Included product web address on the cover page • Combined the HiSeq/NextSeq/NovaSeq and the MiSeq protocol into a single consolidated workflow • Updated the Library Fragmentation time to 5 min • Updated the SPRIselect stringency on the final library to 0.6x (30 uL of SPRIselect reagent) • Updated the final library Bioanalyzer trace
1.3	Feb 2019	• Updated the BC/uL calculation in the Calibration worksheet • Added the warning of “do not dry beads” during SPRIselect cleanup in the Quick Guide
1.2	Jan 2019	• Updated the time estimate in Workflow Schematics • Added recommendations on “Sample Type Guidelines” • Updated recommendations on “Input DNA Quality and Quantification Guidelines” • Updated recommendation on “Guidelines for Estimating Sample Complexity” • Updated the example sample complexity table from volumes used in Barcode Amplification to BC molecules per µl calculated using Calibration qPCR • Added a list of validated qPCR master mix reagents in the Quick Guide • Updated the volume used in Single Sample Calibration to 2 uL • Updated the volume used in Barcode Amplification to a maximum of 5 uL
1.1	Dec 2018	• Combined the 16S and the 16S & 18S documentation
1.0	Dec 2018	• Initial release

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 over-estimation of genomic DNA concentration in prepared samples

Note: To decrease pipetting error, it is recommended to use at least 2 µl of the genomic DNA stock for quantification

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 to minimize freeze-thaw cycles of the template DNA and the working stocks

1.2. Barcode Assignment PCR

Twenty-four (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 used to identify the genomic DNA 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 well position (column and row) for downstream identification
D. As you add each sample, pipet mix into the well thoroughly (do not vortex the plate)

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

E. Seal the plate with caps or a PCR-certified adhesive sticker and centrifuge the plate briefly
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. Add 2 µl of **Inactivation Enzyme SSC** to each reaction well for all 24 sample wells

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

B. As you add enzyme, pipet mix thoroughly (do not vortex)
C. Seal the plate with caps or a PCR-certified adhesive sticker and centrifuge the plate briefly
D. 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 30 µl of EB Buffer to each PCR well
B. Add 30 µl of SPRIselect to each PCR well and pipet mix thoroughly or briefly vortex to fully mix beads with sample
C. Transfer the samples to 8-well strip-tubes if using PCR Strip Magnetic Separators
D. Incubate at room temperature for 5 min.
E. Place the PCR tube strips on the magnetic stand for 3 min.
F. Carefully remove and discard the supernatant
G. Keep the PCR tube strips on the magnet; add 200 µl of 80% ethanol
H. Incubate the beads on the magnet for 30 seconds
I. Carefully remove and discard the ethanol wash
J. Repeat the wash once by adding 200 µl of 80% ethanol
K. Incubate the beads on the magnet for 30 seconds
L. Carefully remove and discard the supernatant
M. Briefly centrifuge the PCR tube strips and return to the magnet
N. Remove any remaining ethanol with a P10 pipet
O. Remove the PCR tube strips 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

P. Incubate at room temperature for 5 min.
Q. Return the PCR tube to the magnet for 3 min.
R. Transfer the supernatant to new PCR tube strips

Proceed immediately to the next step or store at 4°C for up to 24 hours before proceeding to the next step, or long-term storage at -20°C for up to two weeks

Section II: Barcode Calibration

2.1. Calibration by Real-Time qPCR

A. Use a new PCR plate suitable for real-time SYBR-based qPCR reaction to set up the calibration
B. Thaw on ice a chosen 2x qPCR Master Mix reagent

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

C. Combine 600 µl of a chosen 2x qPCR Master Mix reagent with 300 µl of **Calibration Reaction SSC** and 180 µl of nuclease-free water in a 1.5 mL Eppendorf tube
D. Vortex mix the tube thoroughly
E. Dispense 18 µl of the combined Master Mix into two sets of 29 wells, for a total of 58 wells
F. Prepare the following as duplicate reactions:
    a. In well #1, add 2 µl of **Calibration Standard 1**
    b. In well #2, add 2 µl of **Calibration Standard 2**
    c. In well #3, add 2 µl of **Calibration Standard 3**
    d. In well #4, add 2 µl of **Calibration Standard 4**
    e. In well #5, add 2 µl of Buffer EB (neg. control)
    f. In well #6 to #29, add 2 µl of each cleaned sample
G. Pipet mix the tubes thoroughly and seal plate with optically clear PCR-certified adhesive sticker. Briefly centrifuge
H. 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	40 cycles
60°C	20 seconds	
72°C	2 min**	
4°C	HOLD	∞

** collect SYBR signal during the 2 minute elongation step

2.2. Calibration Analysis

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

www.loopgenomics.com/start

This number is the **molecule count per microliter** for the samples. Carry forward the volume of sample appropriate for **HiSeq/NextSeq/NovaSeq** or **MiSeq** into the single sample Barcode Amplification step (see section 3.1 below) depending on the intended sequencer.

Section III: Barcode Distribution

3.1. Barcode Amplification

A. Use a new PCR plate or 8-tube strips, dispense 15 µl of **Amplification Mix SSC** per reaction into 24 wells
B. Dilute the cleaned samples from section 1.4 based on the calibration qPCR
C. Add 5 µl of the **diluted sample** as calculated by the calibration qPCR per well
D. Pipet mix or vortex the tubes thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
Amplification Mix SSC	15
Diluted DNA based on calibration	5
Total =	20

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	22 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. Combine 5 µl from each of the 24 reaction wells into a new PCR tube
B. Add 72 µl of SPRIselect to the reaction. Pipet mix thoroughly
C. Incubate at room temperature for 5 min
D. Place the PCR tube on a magnetic stand for 3 min
E. Carefully remove and discard the supernatant
F. Keep the PCR tube on the magnet; add 200 µl of 80% ethanol
G. Incubate the beads on the magnet for 30 seconds
H. Carefully remove and discard the ethanol wash
I. Repeat the wash once by adding 200 µl of 80% ethanol
J. Incubate the beads on the magnet for 30 seconds
K. Carefully remove and discard the supernatant
L. Briefly centrifuge the PCR tube and return to the magnet
M. Remove any remaining ethanol with a P10 pipet
N. 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

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

3.3. Barcode Distribution

A. To the 15 µl of eluate in a new PCR tube from section 3.2 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	5 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 the following PCR program in a thermal cycler:

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	
4°C	HOLD	∞

4.5. Post-indexing 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 20 µl of Buffer EB

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

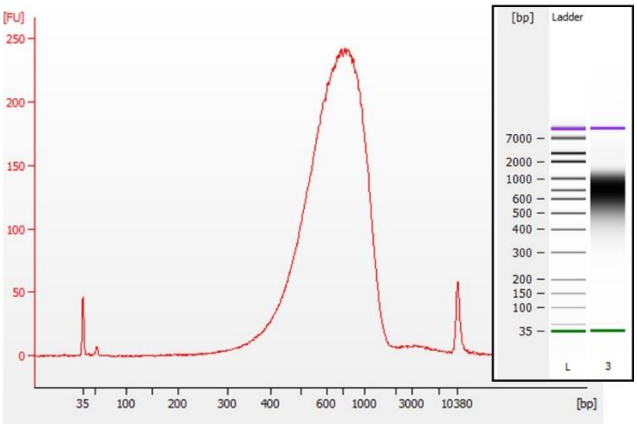
N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new 1.5 mL Eppendorf tube

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

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% Phi X 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:

Sequencer	Read Length	Sequencing depth
HiSeq/NextSeq/NovaSeq	2 x 150 bp	100-150M PE reads (50-75M clusters passing filter)
MiSeq	2 x 300 bp	50M PE reads (25M 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.

For sequencing the final library on an Illumina platform machine, please consult the instructions in the machine-appropriate Illumina users guide. Documentation for Illumina sequencer load guidelines can be found at:

https://support.illumina.com/documentation.html

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.