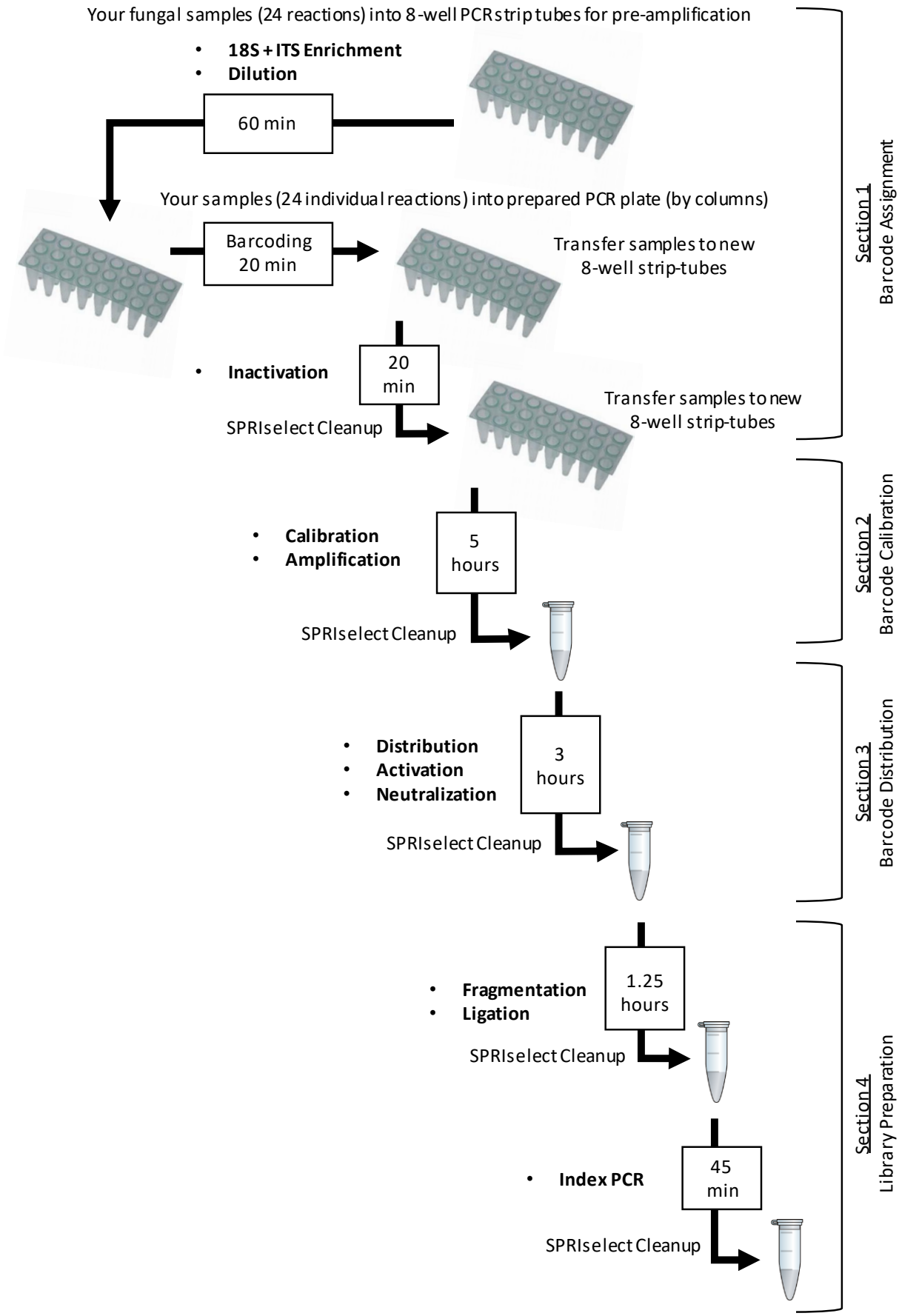
LoopSeq™ 18S – ITS Mycobiome Kit

Version 2.0*

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

Contents

Workflow Schematic



18S - ITS Mycobiome 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.

The **LoopSeq™ 18S - ITS Mycobiome** kit, which was designed to selectively pre-amplify and barcode the locus containing 18S, ITS1, and ITS2 from fungal DNA. The sequence of this extended locus provides a better determination of species identity than 18S or ITS sequence alone. Pre-amplification ensures that even samples with low quantities of DNA or high contamination with non-fungal genomic DNA can be sequenced.

Additionally, our Mycobiome kit excels at capturing rDNA material from samples that may contain trace amounts of enzymatic inhibitors, which otherwise block the PCR and enzyme reactions necessary for NGS library construction. In this context, the presence of inhibitors makes a sample effectively "low biomass" for how many molecules per sample can be processed into a library.

Our LoopSeq™ 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 #
18S ITS Enrichment	LG00A-128	7
Barcoding Adapters, 24 wells	LG00A-001	7
Inactivation Mix A	LG00A-132	8
Amplification Mix B	LG00A-152	8
Distribution Mix	LG00A-160	9
Distribution Enzyme	LG00A-170	9
Activation Mix	LG00A-190	9
Activation Enzyme	LG00A-210	9
Neutralization Enzyme	LG00A-230	9
Fragmentation Mix	LG00A-250	10
Fragmentation Enzyme	LG00A-270	10
Ligation Mix	LG00A-290	10
Ligation Enzyme	LG00A-310	10
Index Master Mix	LG00A-330	10
Index Primer P1	LG00A-341	10
Index Primer P2	LG00A-342	10
Index Primer P3	LG00A-343	10
Index Primer P4	LG00A-344	10
Calibration Standard 1	LG00A-361	8
Calibration Standard 2	LG00A-362	8
Calibration Standard 3	LG00A-363	8
Calibration Standard 4	LG00A-364	8
Calibration Primer Mix	LG00A-376	8

Important Parameters

Sample Type Guidelines

Not all sample types are created equal. LoopSeq™ technology takes this into account, allowing different sample types to be processed in the same sequencing library preparation as part of a single multiplex pool. This protocol is optimized for capturing 18S - ITS1 molecules from high or low biomass samples, which may have picogram amounts of DNA and/or may include contaminating 18S content, such as human genome from skin swabs or biopsies. As such, the most accurate estimation of molecule counts per sample requires a calibration PCR because different sample types contain different abundances of 18S and ITS molecules. For more information on sample calibration, please refer to the "Guidelines for Estimating Sample Complexity" section of this manual.

Working with high and low mass samples in the same kit

If your 24 samples are a combination of high and low biomass inputs, both can be processed within the same low biomass kit. For example, low mass samples require an enrichment step (as described in the protocol) before loading samples into the barcoding reaction plate. However, for high mass samples, the enrichment step is skipped, and samples are instead directly loaded into the barcoding reaction plate. From that point, all 24 samples follow the same process (i.e. barcoding, calibration, amplification, etc.) as described staring from section 1.3 in the protocol.

Input DNA Quality and Quantification Guidelines

The quality of the input genomic DNA significantly impacts the number of 18S and ITS1/2 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.

Only full-length rDNA molecules can be captured and amplified with this protocol. Therefore, extraction methods that perform well for short-read regions (e.g. ITS1 or ITS2 only) may not be suitable for this protocol. If the input sample is partially or highly degraded, the number of reported synthetic long-reads may be low.

Quantification using a 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 methods are suitable alternatives to using a Qubit Fluorometer. Quantification by NanoDrop™ is not recommended as the concentration estimate by that method can be adversely impacted by buffer salt concentrations, the presence of free nucleotides, and other contaminants that absorb at similar wavelengths to double stranded DNA.

Because the 18S - ITS protocol includes a pre-barcoding enrichment step that is PCR based, the final output may be subject to a PCR bias, such that the reported distribution of species within a sample may slightly vary from expected ratios.

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 ~5,000 molecules per sample, or ~120,000 molecules per 24-plex kit.

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
- **Sample Enrichment PCR**
  Q5® Hot Start High-Fidelity 2X Master Mix from New England Biolabs (Cat No. 102500-140)
- **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
2.0	Apr 2020	• Updated enrichment section with new guidelines • Updated new names and part numbers for reagents • Updated section numbering
1.1	Aug 2019	• Updated cycle number for amplification PCR • Modified molecule complexity per sample for MiSeq applications
1.0	June 2019	• Initial release

Section I: Sample Enrichment

1.1: Input DNA quantification

A. Quantify the DNA concentration of microbiome samples using a Qubit dsDNA High Sensitivity Assay or an equivalent DNA-binding fluorescence dye-based method. Nanodrop is not recommend due to the potential signal interference from protein or salt.

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

A. For high quality samples of fungal DNA with little to no non-fungal DNA (e.g. from a pure culture), dilute template genomic DNA stocks to 2 ng/µl with Buffer EB. Diluted stocks can be stored at -20°C for up to 2 weeks. Otherwise use genomic DNA stocks undiluted.

1.2: Enrichment PCR cycle conditions

The supplied **18S ITS Enrichment** reagent tube contains a primer mix at a concentration of 2 µM. Draw from this tube what volume is needed for an enrichment run, and then recap and store the tube at -20°C. Use 5 µl from the **Enrichment** tube for every 20 µl of PCR reaction (final volume).

EXAMPLE

Component	Volume per Sample (µl)
Enrichment oligos from tube	5
DNA sample (diluted to ~1 ng/µl)	5
Q5® Hot Start High-Fidelity 2X Master Mix	10
Total =	20

We recommend using Q5® Hot Start High-Fidelity 2X Master Mix from New England Biolabs (Cat No. 102500-140) for this amplification. The following thermocycling program was designed specifically for this master mix. If using a different master mix, it may be necessary to experimentally optimize the annealing temperature, number of PCR cycles and extension times in order to produce a clean amplicon product of the correct size (typically between 2-3kb for full-length 18S-ITS targets).

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	5-30* cycles	2 to 3°C/s
52°C	20 seconds		
72°C	3 min		
4°C	HOLD	∞	2 to 3°C/s

*Determined experimentally for each sample type (see section 2.3)

Always confirm that the amplicons made are of the correct size using a gel electrophoresis method (on agarose or Bioanalyzer) that is sensitive enough to detect amplicon at the concentration made during PCR.

1.3: The dangers of over-cycling

Without proper control, over-cycling in PCR amplification can create chimeric amplicons and non-specific amplification products. Therefore, determining the correct number of cycles for PCR and not cycling up to or past the amplification plateau is important and cannot be ignored.

Using a real-time PCR machine

For the most accurate method of avoiding over cycling, we recommend monitoring the PCR in a real-time qPCR machine to visualize amplification. For example, in a test run, cycle samples for 30 cycles. Based on the test run, determine the ideal number of cycles needed to amplify your samples such that the amplification is detectable but does not cycle pass plateau. Alternatively, input gDNA concentrations can be adjusted so all samples reach the same RFU roughly at the same cycle. The goal is to limit over cycling and for samples to make a clean product of the correct size.

Using a standard PCR machine

To limit over-cycling, it may be necessary to run samples over a range of cycle numbers (e.g. 5 cycles, 10 cycles, 20 cycles, etc.) and then visualize products on agarose gel to determine which cycle condition gives a clean, single band AND is not overly concentrated. As a guideline, choose the number of PCR cycles for a sample such that only the correct size amplicon is made and:

- The final amplicon ng/ul concentration is >= 2 ng/µl
- The final amplicon ng/ul concentration is < 15 ng/µl

On a gel, a single, tight peak at the correct size for 18S-ITS (~2.5kb) should be detected per sample, with no other bands, signal smearing, or peak-noise anywhere on the trace.

If a sample is not clean, do **not** use gel extraction to excise the target size region. Instead, optimize PCR conditions (see section 1.4 below) to make a clean band.

If a sample remains undetectable after 30 PCR cycles, that sample is not usable as an input (i.e. may be degraded, contains inhibitors, etc.).

1.4: Troubleshooting suggestions

If PCR amplicons appear dirty, smeared, or too short on the gel, consider the following tips to improve the PCR:

- Use Q5® Hot Start High-Fidelity 2X Master Mix
- Perform an anneal temperature sweep
- Perform an elongation temperature sweep
- Increase the elongation time
- Redo the sample prep to improve sample quality
- Test the process on a control sample of high quality gDNA

Section II: Barcode Assignment

2.1: Post-enrichment DNA Dilution

B. Quantify the concentration of each PCR enriched sample using Qubit dsDNA High Sensitivity Assay or equivalent fluorometer. Nanodrop is not recommended due to its over-estimation of DNA concentration in prepared samples

C. Dilute samples **AT LEAST** 10-fold **PLUS** any additional factor such that each enriched sample concentration confirms to the following table:

DNA concentration of the 10-fold diluted sample	Dilution
>0.3 ng/µl	Dilute further to ~0.3 ng/µl
<0.3 ng/ul	No further dilution is needed
Undetected	No further dilution is needed

2.2: Barcode Assignment PCR

Twenty-four (24) Barcoding Adapters are provided pre-dispensed into the first three columns of a 96-well plate. Each adapter has a unique sample index used to identify the sample 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 seal

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

C. For each diluted/enriched sample, add 5 µl to a well in the Barcoding Adapter plate, noting the position (column and row)

D. As you add each sample, pipet mix into the well thoroughly (do not vortex the plate)

Component	Volume per Sample (µl)
Barcoding Adapter (in plate)	15
Diluted/Enriched Sample	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	3 min		
4°C	HOLD	∞	2 to 3°C/s

2.3: PCR Inactivation

A. Add 2 µl of **Inactivation Mix A** 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	∞

2.4: Post-barcoding SPRIselect Cleanup

A. Add 30 µl of EB Buffer to each reaction
B. Add 30 µl of SPRIselect to each reaction and pipet mix or briefly vortex to fully mix beads into the sample
C. Transfer the samples to 8-well strip-tubes
D. Incubate at room temperature for 5 min
E. Place the PCR tube on a magnetic stand for 3 min
F. Carefully remove and discard the supernatant
G. Keep the PCR tube 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 and return to the magnet
N. Remove any remaining ethanol with a P10 pipet
O. 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

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 III: Barcode Calibration

3.1: Calibration by Real-Time qPCR

A. Dilute 10-fold each cleaned barcoded sample from section 1.6 by taking 4 µl of sample into 36 µl of EB Buffer. This diluted set is referred to as **10FD**, and is used in all subsequent steps
B. Use a new PCR plate suitable for real-time SYBR-based qPCR reaction to set up the calibration

If your qPCR machine requires ROX as a passive reference, please refer to the information on ROX Passive Reference Dye reagent from BioRad (Cat. # 1725858)

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

C. Combine 600 µl of the BioRad qPCR Master Mix reagent with 300 µl of the **Calibration Primer Mix** with 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 **10FD** 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	3 min**	
4°C	HOLD	∞

** collect SYBR signal during the 3 minute elongation step

3.2: 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 4.1 below) depending on the intended sequencer.

Section IV: Barcode Distribution

4.1: Barcode Amplification

A. Using a new PCR plate or three 8-tube strips, dispense 15 µl of **Amplification Mix B** into 24 wells
B. Take each **10FD** sample from section 3.1 and dilute it further to a final dilution based on calibration worksheet instructions
C. Add 5 µl of this final dilution to an **Amplification Mix B** well
D. Pipet mix or vortex the tubes thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
Amplification Mix B	15
Diluted 10FD sample 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	3 min		
4°C	HOLD	∞	2 to 3°C/s

4.2: Post-amplification sample pooling

A. Combine enough volume from each barcoding sample to add up to 120 µl, taking equal volumes from each sample.

For example:

- Take 5 µl from each of 24 samples = 120 µl pool volume
- Take 15 µl from each of 8 samples = 120 µl pool volume

4.3: Post-pooling SPRIselect Cleanup

A. Add 72 µl of SPRIselect to the pool. 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

4.4: Barcode Distribution

A. To the 15 µl of eluate (from section 4.3) 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	∞

4.5: 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. Pipet mix or vortex the tube thoroughly. Centrifuge briefly
G. 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	∞

4.6: 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 V: Library Preparation

Assemble the following reaction on ice

5.1: 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 tube in the thermal cycler and resume the program

5.2: 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 using the following conditions:

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

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

5.3: 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

5.4: 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	11 cycles
60°C	30 seconds	
72°C	30 seconds	
4°C	HOLD	∞

5.5: 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

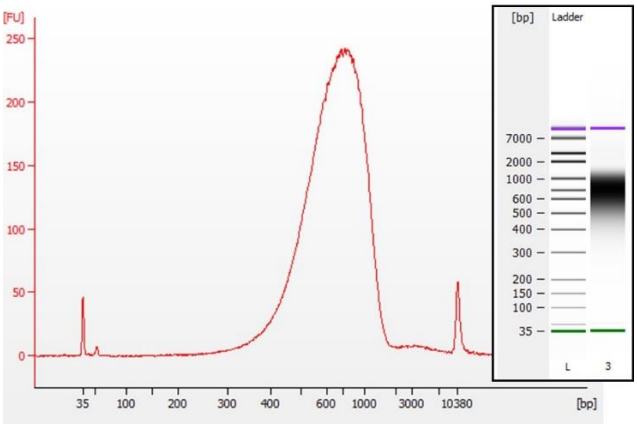
N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new 1.5 mL Eppendorf tube

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 **undiluted** of the final library on an Agilent Bioanalyzer High Sensitivity (HS) 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	175-250M PE reads (87.5-125M 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

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The LoopSeq™ kits 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. Refer to the 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.