# LoopSeq™ Bacterial Genome 3x 8-plex Kit

Version 1.0*


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## Single Molecule 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™ Bacterial Genome 3x 8-plex** kit. Each kit supplies enough reagent to process up to 24 single bacteria species (as 3 groups of 8 single samples) with genomic DNA fragment sizes ranging from 3kb to 6kb. During Barcode Assignment, double-stranded DNA fragments from the same bacterial genomic DNA sample are assigned the same sample barcode, as well as a molecular barcode that is unique to every fragment in that sample. After sequencing, the barcoded short reads are first binned by sample barcode, and then by the molecule barcode and used for long DNA sequence reconstruction. The reconstructed long reads can be used to elucidate sequence variations separated by a distance, which cannot be accomplished using stand-alone sequencing short reads. In addition, the reconstructed long reads can be used for *de novo* genome assembly, with or without a separate short read library as input.

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 sample barcodes and molecular barcodes, each sample is amplified individually before being multiplexed into single reaction tubes as groups of 8 for downstream sample prep. Processing one tube means less hands-on time, reduced chance of error, and more reliable output because 8 samples are pooled and processed in the same reaction. At the conclusion of the workflow, each multiplexed sample library is ready for short read sequencing on the Illumina sequencing platform. Using Loop Genomics' data informatics pipeline, short reads are reconstructed into long reads that represent molecules of tagged DNA in each sample. 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 # |
|---|---|---|
| BC End Prep Mix | LG00B-100 | 6 |
| BC End Prep Enzyme | LG00B-105 | 6 |
| BC Ligation Mix | LG00B-110 | 6 |
| BC Ligation Additive | LG00B-115 | 6 |
| BC Adapter 1 | LG00B-141 | 6 |
| BC Adapter 2 | LG00B-142 | 6 |
| BC Adapter 3 | LG00B-143 | 6 |
| BC Adapter 4 | LG00B-144 | 6 |
| BC Adapter 5 | LG00B-145 | 6 |
| BC Adapter 6 | LG00B-146 | 6 |
| BC Adapter 7 | LG00B-147 | 6 |
| BC Adapter 8 | LG00B-148 | 6 |
| Inactivation Enzyme M | LG00B-130 | 6 |
| Amplification Mix S | LG00B-151 | 6 |
| Amplification Additive | LG00C-154 | 6 |
| Distribution Mix | LG00B-160 | 7 |
| Distribution Enzyme | LG00B-170 | 7 |
| Activation Mix | LG00B-190 | 7 |
| Activation Enzyme | LG00B-210 | 7 |
| Neutralization Enzyme | LG00B-230 | 8 |
| Fragmentation Mix | LG00B-250 | 8 |
| Fragmentation Enzyme | LG00B-270 | 8 |
| Ligation Mix | LG00B-290 | 8 |
| Ligation Enzyme | LG00B-310 | 8 |
| Index Master Mix | LG00B-330 | 8 |
| Index Primer P1 | LG00B-341 | 8 |
| Index Primer P2 | LG00B-342 | 8 |
| Index Primer P3 | LG00B-343 | 8 |
| Index Primer P4 | LG00B-344 | 8 |
| Index Primer P5 | LG00B-345 | 8 |
| Index Primer P6 | LG00B-346 | 8 |
| Index Primer P7 | LG00B-347 | 8 |

## Important Parameters

### Input DNA Size and Sequence

This protocol has been optimized for sequencing bacterial genome fragment sizes in the range of 3kb to 6kb. Ideally, this is achieved by taking high molecular (HMW) bacterial genomic DNA and shearing it to the relevant range using Covaris g-Tube. A good starting point is following the Covaris recommendation for a target size of 8kb, followed by confirming the sheared gDNA size on an agarose gel. Typically, the sheared gDNA fragment size is observed to be in the 5kb range following the 8kb shearing protocol, though the sheared gDNA fragment size can vary due to the fragment lengths of the starting genomic material as well as the instrument used. After g-Tube shearing, concentrate the sheared gDNA using SPRIselect cleanup.

Note that working with non-sheared, partially degraded bacterial gDNA versus high molecular weight (HMW) bacterial gDNA sheared using g-Tube to the desired 3kb to 6kb range will likely yield different results. In the former sample, there will likely be many fragments smaller than 3kb, which will interfere in the amplification of longer fragments as well as having nicks in the barcoded DNA that will reduce the number of any amplifiable long fragments. It is advantageous and recommended to use only high-quality HMW bacterial gDNA as input that is freshly sheared using g-Tube.

### Input DNA Quality and Quantification Guidelines

The quality of input bacterial gDNA significantly impacts the number of molecules per sample that can be successfully barcoded, sequenced, and assembled into synthetic long reads. Issues leading to poor sample quality and purity may arise from differences in preparation methods, reagent carryover during preparation (e.g. residual ethanol), and sample storage conditions. If an input sample is of poor quality, such as partially or highly degraded, the reconstructed long reads may be short and/or the number of reported long reads may be low. We recommend checking the fragmentation size pattern by gel or Bioanalyzer for each sample prior to processing them with this kit.

Furthermore, accurate determination of input bacterial gDNA sample concentration is important for placing samples within the dynamic range of this kit. Quantification of 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.

### Sample Complexity Considerations

Different samples have different sequence diversity within the sample and can impact the sequencing depth needed for surveying the sequence diversity. Depending on the sample type processed, a specific sequencing depth is required to reconstruct enough full-length sequences suitable for your application. For example, if the sample is composed of a collection of 4kb fragments that come from a purified single genome of 3 million bases in total length (and broken into 4kb fragments), there are roughly 750 effective "variants" to identify per genome. Sampling the genome at 10x requires 7,500 molecules of 4kb length, while sampling the genome at 30x requires 22,500 molecules of 4kb length. We refer to this concept as "setting sample complexity", which should be taken into consideration when planning an experiment and deciding how much sequencing depth will be required. The higher the complexity, the greater the sequencing depth needed to reconstruct full-length sequences. This protocol aims to generate long-read sequences for roughly 40,000 barcoded molecules per sample. With the 3x 8-plex kit design, this translates to 320,000 molecules per 8-plex library, or 1.28 million molecules per kit. The sequencing depth

may need to be adjusted higher or lower depending on the actual sample complexities of the tagged samples.

The table below provides examples of sample complexity concentration estimates for different DNA sources:

| DNA source | Sample integrity | BC molecules per µl |
|---|---|---|
| g-Tube sheared HMW genome | High | 1,000,000 – 4,000,000 |
| Partially degraded genome | Low | 100,000 – 200,000 |

## Guidelines on Sample Multiplexing

After groups of 8 prepared gDNA samples have been individually tagged with sample barcodes and molecular barcodes, they are amplified before being multiplexed into single reaction tubes for downstream sample prep. Eight sample barcodes are supplied in the kit. Only individual samples that have been tagged with different sample barcodes can be multiplexed into the same reaction group.

If multiplexing fewer than 8 samples per multiplexed library, it is highly recommended that the following groupings of sample barcodes be used to ensure optimal base-balanced signal on Illumina short read sequencers. Additionally, a higher percent of PhiX spike-in can help to ensure optimal sequencer performance.

| Group size | Sample barcode(s) to use | Sample barcode(s) omitted |
|---|---|---|
| 7 | BC Adapter 1, 3, 4, 5, 6, 7, 8 | BC Adapter 2 |
| | BC Adapter 1, 2, 3, 4, 5, 6, 8 | BC Adapter 7 |
| 6 | BC Adapter 1, 3, 4, 5, 6, 8 | BC Adapter 2, 7 |
| 5 | BC Adapter 3, 4, 5, 6, 8 | BC Adapter 1, 2, 7 |
| | BC Adapter 1, 3, 5, 6, 8 | BC Adapter 2, 4, 7 |
| | BC Adapter 1, 3, 4, 5, 8 | BC Adapter 2, 6, 7 |
| | BC Adapter 1, 3, 4, 5, 6 | BC Adapter 2, 7, 8 |
| 4 | BC Adapter 3, 4, 5, 6 | BC Adapter 1, 2, 7, 8 |
| | BC Adapter 1, 3, 5, 6 | BC Adapter 2, 4, 7, 8 |
| 1-3 | Not recommended | NA |

## Reagent Handling

All reagents must be thawed and well mixed prior to use. Throughout the kit processing, remove reagent tubes needed for a given step, thaw on ice, and briefly centrifuge to collect all the reagents at the bottom of the tubes. Vortex mix the tubes, briefly centrifuge again, and return to ice. Do this for all reagents unless otherwise noted in the manual.

## 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, single-channel pipettes, and multi-channel pipettes. Perform PCR reactions in vessels suitable for sealing and cycling in PCR conditions
- **Minicentrifuge for gDNA shearing**
  Covaris g-Tube, Cat No. 520079, coupled with an Eppendorf 5424 or 5415 R or MiniSpin Plus centrifuge
- **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. Q32851 or Q32854. 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**
- **Input DNA and 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 |
|---|---|---|
| 1.0 | Jul 2019 | • Initial release |

## Section I: Barcode Assignment

### 1.1. DNA Quantification

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

### 1.2. DNA Shearing using Covaris g-Tube and Post-shearing SPRIselect Cleanup

A. Dilute 150 ng of each individual single genome gDNA stock to 1 ng/ µl with Buffer EB.
B. Shear the diluted gDNA with Covaris g-Tube following the 8kb protocol as outlined in the Covaris manual.
C. Transfer the sheared gDNA to a new 1.5 mL Eppendorf tube
D. Add 75 µl of SPRIselect to each sample. Mix thoroughly
E. Incubate at room temperature for 5 min.
F. Place each sample on a magnetic holder for 3 min.
G. Carefully remove and discard the supernatant
H. Keeping samples on the magnet, add 200 µl of 80% ethanol
I. Incubate on the magnet for 30 seconds
J. Carefully remove and discard the ethanol wash
K. Repeat the wash once by adding 200 µl of 80% ethanol
L. Incubate on the magnet for 30 seconds
M. Carefully remove and discard the supernatant
N. Briefly centrifuge the PCR tubes and return to the magnet
O. Remove any remaining ethanol with a P10 pipet
P. Remove the PCR tubes 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***

Q. Incubate at room temperature for 5 min.
R. Return the PCR tubes to the magnet for 3 min.
S. Transfer sample supernatant to new PCR tube. Do this for each individual sample

*The purified sheared gDNA can be stored at -20°C for 2 weeks before proceeding to the next step*

### 1.3. Post-SPRIselect DNA Quantification and Dilution

A. Determine the concentration of the sheared gDNA using the Qubit dsDNA High Sensitivity Assay or equivalent fluorometer
B. Dilute sheared gDNA to 2 ng/µl with Buffer EB). 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*

### 1.4. Fragment End Repair and Barcode Ligation

A. Combine the following, mix thoroughly, and dispense 25ul for each individual sample in new PCR strip-tubes or a plate

| Component | Volume per Sample (µl) | Volume for 8 samples (µl) | Volume for 24 samples (µl) |
|---|---|---|---|
| BC End Prep Mix | 23.5 | 206.8 | 620.4 |
| BC End Prep Enzyme | 1.5 | 13.2 | 39.6 |
| Total = | 25 | 220 | 660 |

B. Add 5 µl of each diluted sample to its own reaction tube, and pipet mix thoroughly
C. Incubate in a thermal cycler using the following conditions:

Heated lid at 100°C

| Temperature | Duration | STEP |
|---|---|---|
| 20°C | 10 min | 1 cycle |
| 65°C | 30 min | 1 cycle |
| 4°C | HOLD | ∞ |

D. Combine the following, pipet mix, and dispense 15.5 µl to each individual sample tube:

| Component | Volume per Sample (µl) | Volume for 8 samples (µl) | Volume for 24 samples (µl) |
|---|---|---|---|
| BC Ligation Mix | 15 | 132 | 396 |
| BC Ligation Additive | 0.5 | 4.4 | 13.2 |
| Total = | 15.5 | 136.4 | 409.2 |

E. Add 4.5 µl of a **unique BC Adapter** (eight provided) per unique sample tube, and pipet mix thoroughly
F. Incubate in a thermal cycler using the following conditions:

Heated lid at 100°C

| Temperature | Duration | STEP |
|---|---|---|
| 20°C | 15 min | 1 cycle |
| 65°C | 10 min | 1 cycle |
| 4°C | HOLD | ∞ |

### 1.5. Barcode Inactivation

A. Add 3 µl of **Inactivation Enzyme M** to each unique sample tube
B. Pipet mix each individual sample and centrifuge briefly
C. 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 | ∞ |

### 1.6. Post-barcoding SPRIselect Cleanup

A. Add 32 µl of SPRIselect to each sample. Mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place each sample on a magnetic holder for 3 min.
D. Carefully remove and discard the supernatant
E. Keeping samples on the magnet, add 200 µl of 80% ethanol
F. Incubate 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 on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tubes and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tubes 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 tubes to the magnet for 3 min.
P. Transfer sample supernatant to new PCR tube. Do this for each unique sample

*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 Distribution

### 2.1. Barcode Dilution

A. Perform the following 125-fold dilution:
   - Combining 2 µl of undiluted sample and 248 µl Buffer EB in a 1.5-mL Eppendorf tube
   - Note that above dilution assumes each individual sample is high quality gDNA. Otherwise, the sample dilution may need to be adjusted depending on the sample quality of the starting samples

### 2.2. Barcode Amplification

A. Dispense 15ul of **Amplification Mix S** for each individual sample in new PCR strip-tubes or a plate
B. Add 3ul of **Amplification Additive** per sample tube
C. Add 2 µl of a diluted individual sample from section 2.1 such that each well contains one unique sample
D. Pipet mix or vortex each tube thoroughly. Centrifuge briefly
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 | 20 seconds | 20 cycles | 2 to 3°C/s |
| 60°C | 20 seconds | | |
| 72°C | 10 min | | |
| 4°C | HOLD | ∞ | 2 to 3°C/s |

### 2.3. Pooling of Samples

A. Combine 15 µl from each of the 8 samples with unique sample barcodes in a new PCR tube. This is one multiplexed pool
B. If processing more than 8 samples, repeat the pooling for the remaining samples as pools of 8 for a total of 3 pools from 24 samples, each pool containing samples with different sample barcodes
C. When pooling fewer than 8 samples into a pool, please consult *Guidelines on Sample Multiplexing* for optimal grouping setup. Add 15 µl of Buffer EB for every sample barcode omitted from a pool.

### 2.4. Post-amplification SPRIselect Cleanup

A. Per pool add 72 µl of SPRIselect reagent. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube(s) on a magnetic holder for 3 min.
D. Carefully remove and discard the supernatant
E. Keeping the tube(s) on the magnet, add 200 µl of 80% ethanol
F. Incubate 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 on the magnet for 30 seconds
J. Carefully remove and discard the supernatant
K. Briefly centrifuge the PCR tube(s) and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube(s) from the magnet, and immediately resuspend the beads in 18 µl of Buffer EB per pool

***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(s) to the magnet for 3 min.
P. Transfer sample supernatant to new PCR tube(s)


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

### 2.5. Barcode Distribution

A. For each elution pool of samples from section 2.4, transfer 15 µl of the cleaned pool to a new PCR tube
B. Add 5 µl of **Distribution Mix** to the reaction tube
C. Add 2 µl of **Distribution Enzyme** to the reaction tube
D. 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 |

E. 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 | ∞ |

### 2.6. Barcode Activation and Neutralization

A. Add 75.5 µl of **Activation Mix** to the reaction tube
B. Add 2.5 µl of **Activation Enzyme** to the reaction tube
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 | 16 hours | 1 cycle |
| 65°C | 10 min | 1 cycle |
| 4°C | HOLD | ∞ |

E. Add 6 ul of **Neutralization Enzyme** to the reaction tube
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 | ∞ |

### 2.7. Post-activation SPRIselect Cleanup

A. Per pool add 80 µl of SPRIselect reagent. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube(s) on the magnet for 3 min.
D. Carefully remove and discard the supernatant
E. Keeping the tube(s) 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(s) and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube(s) from the magnet, and immediately resuspend the beads in 20 µl of Buffer EB per pool

***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(s) to the magnet for 3 min.
P. Transfer the supernatant to new PCR tube(s)

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

## Section III: Library Preparation

***Assemble the following reaction on ice***

### 3.1. 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 each elution pool of samples from section 2.7, add 20 µl of **Fragmentation Mix** and keep on ice
D. Add 10 µl of **Fragmentation Enzyme** to the reaction on ice
E. Pipet mix the reaction thoroughly (do not vortex), centrifuge briefly, and immediately return to the 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

### 3.2. Adapter Ligation

A. Add 40 µl of **Ligation Mix** to the reaction tube
B. Add 10 µl of **Ligation Enzyme** to the reaction tube
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 | ∞ |

### 3.3. Post-ligation SPRIselect Cleanup

A. Per pool add 80 µl of SPRIselect reagent. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube(s) on the magnet for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube(s) 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(s) and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube(s) 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(s) to the magnet for 3 min.
P. Transfer the supernatant to new PCR tube(s)

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

### 3.4. Library Index PCR

A. Per pool of cleaned sample from section 3.3, add 25 µl of **Index Master Mix** to the reaction tube
B. Add 5 µl of **Index Primer "P-series"** to the reaction tube (chose only one from **Index Primer P1 through P7**)
C. Pipet mix or vortex the reaction thoroughly. Centrifuge briefly

| Component | Volume per Sample (µl) |
|---|---|
| Index Master Mix | 25 |
| Index Primer P1 to P7 (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 | |
| 72°C | 1 min | 1 cycle |
| 4°C | HOLD | ∞ |

### 3.5. Post-indexing SPRIselect Cleanup

A. Per pool add 30 µl of SPRIselect reagent. Pipet mix thoroughly
B. Incubate at room temperature for 5 min.
C. Place the PCR tube(s) on the magnet for 3 min.
D. Carefully remove and discard the supernatant
E. Keep the PCR tube(s) 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(s) and return to the magnet
L. Remove any remaining ethanol with a P10 pipet
M. Remove the PCR tube(s) 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.

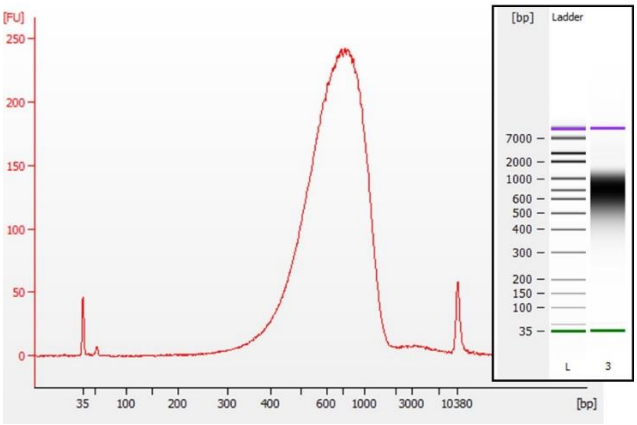
O. Return the PCR tube(s) to the magnet for 3 min.
P. Transfer the supernatant to new 1.5 mL Eppendorf tube(s)

*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.




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 5% Phi X should be included to achieve optimal read quality and to minimize error rates during the run. A higher percentage of PhiX may be needed if a subset of the 8 Loop sample barcodes is used during the sample prep.

Each final library (comprised of 8 unique samples) should receive at least the following sequencing depth for optimal phasing performance:

| Read Length | Sequencing depth |
|---|---|
| 2 x 150 bp | 320M PE reads (160M 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 |
| Index Primer P5 | D705 | ATTCAGAA |
| Index Primer P6 | D706 | GAATTCGT |
| Index Primer P7 | D707 | CTGAAGCT |

## Legal



### Use Restrictions

The LoopSeq™ 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.