LoopSeq™ dsDNA 3x8-plex Kit

Version 0.1

Contents

Workflow Schematic

TEXT

The kit

Kit Components

Component	Part number	Page #
BC End Prep Mix	LG00B-100	
BC End Prep Enzyme	LG00B-105	
BC Ligation Mix	LG00B-110	6
BC Ligation Additive	LG00B-115	5
Inactivation Enzyme M	LG00B-130	6
BC Adapter 1	LG00B-141	6
BC Adapter 2	LG00B-142	6
BC Adapter 3	LG00B-143	6
BC Adapter 4	LG00B-144	7
BC Adapter 5	LG00B-145	7
BC Adapter 6	LG00B-146	7
BC Adapter 7	LG00B-147	7
BC Adapter 8	LG00B-148	7
Amplification Mix S	LG00B-151	7
Distribution Mix	LG00B-160	7
Distribution Enzyme	LG00B-170	7
Activation Mix	LG00B-190	7
Activation Enzyme	LG00B-210	6
Neutralization Enzyme	LG00B-230	6
Fragmentation Mix	LG00B-250	6
Fragmentation Enzyme	LG00B-270	
Ligation Mix	LG00B-290	
Ligation Enzyme	LG00B-310	
Index Master Mix	LG00B-330	
Index Primer P1	LG00B-341	
Index Primer P2	LG00B-342	
Index Primer P3	LG00B-343	
Index Primer P4	LG00B-344	
Index Primer P5	LG00B-345	
Index Primer P6	LG00B-346	
Index Primer P7	LG00B-347	

Important Parameters

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.

Input DNA Preparation and Quality Guidelines

Information.

Understanding Sample Complexity

More stuff.

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 DNA Assay Kit for DNA quantification**
  Invitrogen etc.
  XXX

Reagents Supplied by User

- **SPRIselect Reagent or Ampure XP**
  Beckman Coulter, Cat No. B23317 for SPRIselect
  Beckman Coulter, Cat No. A63881 for Ampure XP
- **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**
- **Library QC on the Agilent 2100 Bioanalyzer**
  DNA Kit, XXX
- **Library Quantification**
  KAPA Library Quantification Kit for the Illumina platform

Section I: Barcode Assignment

1.1. DNA Quantification and Dilution

A. Determine the concentration of the input DNA using the Qubit dsDNA High Sensitivity (HS) Assay or equivalent fluorometer. Note that Nanodrop is not recommended due to its typical overestimation of 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

B. Dilute template DNA stock to 2 ng/µl with Buffer EB and use immediately in section 1.2 (see below). 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. Fragment End Repair and Barcode Ligation

A. Combine the following for each individual sample

Component	Volume per Sample (µl)
BC End Prep Mix	23.5
BC End Prep Enzyme	1.5
Diluted input DNA (at 2 ng/µl)	5
Total =	30

B. 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	∞

C. Combine the following, mix thoroughly, and dispense 15.5 µl for each sample:

Component	Volume per Sample (µl)
BC Ligation Mix	15
BC Ligation Additive	0.5
Total =	15.5

D. Add 4.5 µl of a **unique BC Adapter** (eight provided) per unique sample well, and mix thoroughly

E. 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	∞

1.3. PCR Inactivation

A. Add 3 µl of **Inactivation Enzyme M** to each unique sample

B. Pipet mix each unique sample thoroughly (do not vortex), 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	∞

1.4. 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

1.5. Barcode Dilution

A. Dilute each unique sample 800-fold using a two-step dilution:

- Take 10 µl of an undiluted sample and add to 90 µl Buffer EB in a new PCR tube (this makes a 10-fold dilution)
- Take 2 µl of the 10-fold diluted sample and add to 158 µl Buffer EB in a new PCR tube (this makes the final 800-fold dilution)

Section II: Barcode Distribution

2.1. Barcode Amplification

A. Dispense 30 µl of **Amplification Mix S** into a new PCR tube, enough tubes for each unique sample

B. Add 15 µl of nuclease-free water to each PCR tube

C. Transfer 5 µl from a diluted sample from section 1.5 to a PCR tube. Do this for each unique sample

D. Pipet mix or vortex each tube thoroughly. Centrifuge briefly

Component	Volume per Sample (µl)
Amplification Mix S	30
Nuclease-free water	15
Diluted DNA	5
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	28 cycles	2 to 3°C/s
60°C	20 seconds		
72°C	2 min		
4°C	HOLD	∞	2 to 3°C/s

2.2. Pooling of Samples

A. From each sample (eight in total), take 15 µl and combine them in a new PCR tube. This is the multiplex pool of eight samples

2.3. Post-amplification SPRIselect Cleanup

B. Add 72 µl of SPRIselect to the multiplex pool (120 µl volume). Pipet mix
C. Incubate at room temperature for 5 min.
D. Place the PCR tube on a magnetic holder for 3 min.
E. Carefully remove and discard the supernatant
F. Keeping the tube on the magnet, add 200 µl of 80% ethanol
G. Incubate 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 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 sample supernatant to 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

2.4. Barcode Distribution

A. To the 15 µl of eluate from section 2.3 in a 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	∞

2.5. 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. 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	∞

2.6. Post-activation SPRIselect Cleanup

A. Add 80 µl of SPRIselect to the reaction. Pipet mix
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on the magnet for 3 min.
D. Carefully remove and discard the supernatant
E. Keeping the 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 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	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 2.6 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 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

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	∞

3.3. Post-ligation SPRIselect Cleanup

A. Add 80 µl of SPRIselect to the reaction. Pipet mix
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on the magnet 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

3.4. 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 P-series** to the reaction tube (chose only one, **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 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	11 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. Add 40 µl of SPRIselect to the reaction. Pipet mix
B. Incubate at room temperature for 5 min.
C. Place the PCR tube on the magnet 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

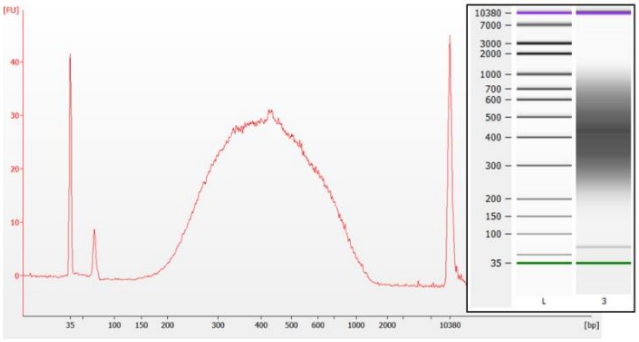
N. Incubate at room temperature for 5 min.
O. Return the PCR tube to the magnet for 3 min.
P. Transfer the supernatant to a new 1.5 mL Eppendorf tube

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.



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
Index Primer P5	D705	
Index Primer P6	D706	
Index Primer P7	D707	

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.