PROTOCOL

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ACCEL-NGS® 2S PLUS DNA LIBRARY KITS

Single Indexing and Molecular Identifiers (MIDs)

Protocol for Cat. Nos. 21024 and 21096 to be used with single indexing kits:

- 2S Set A MID (Cat. No. 27148)
- 2S Set B MID (Cat. No. 27248)
- 2S Set A+B MID (Cat. No. 27396)
- 2S Set S1 MID (Cat. No. 27596)
- 2S Set S2 MID (Cat. No. 27696)
- 2S Set S3 MID (Cat. No. 27796)
- 2S Set S4 MID (Cat. No. 27896)
- 2S Set S1-S4 MID (Cat. No. 279384)

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About This Guide

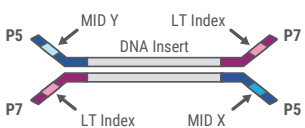
This guide provides instructions for the preparation of high complexity NGS libraries from double-stranded DNA (dsDNA) using an Accel-NGS 2S Plus DNA Library Kit. This kit is suitable for NGS library prep with broad input range and single indexing.

IMPORTANT!
Read the entire Protocol before use, especially the sections pertaining to Product Information, Kit Contents, and Material and Equipment Not Included. Read the Protocol thoroughly to ensure that you absorb all important information needed for success.

Product Information

The Accel-NGS 2S Plus DNA Library Kit offers a unique solution to improve next-generation sequencing (NGS) sample preparation of dsDNA for sequencing on Illumina platforms by including molecular identifiers (MIDs) for more accurate deduplication; thereby improving data retention and substantially greater genomic complexity necessary for more comprehensive sample analysis. The 2S technology utilizes Illumina-compatible adapter sequences and has been validated on the MiniSeq®, MiSeq®, NextSeq®, and HiSeq® platforms.

MID libraries can be prepared with the Accel-NGS 2S Plus Library Kit. The standard Illumina TruSeq® high throughput (HT) P5 adapter is incorporated with a 9-base random N sequence as a MID sequence at the [i5] (index 2 position), and it is used with a standard Illumina TruSeq low throughput (LT) P7 adapter containing a single index for multiplexed sequencing (index 1 position).



Swift MIDs are strand-specific and each dsDNA will receive two independent P5 MID adapters. A schematic of a completed MID-tagged and singly-indexed library molecule is shown below.

Applications

The Accel-NGS 2S Plus DNA Library Kit with MIDs is suitable for accurate deduplication and improved data retention when fragmentation duplicates are present in a DNA sample.

There are several types of duplicate molecules that exist in sequencing data – PCR duplicates, fragmentation duplicates, and complementary sister-strand duplicates derived from input dsDNA. Standard tools, such as Picard Mark Duplicates, cannot distinguish between these three classes of duplicates, each of which have a common aligned map position, so all three types will be removed prior to data analysis.

This pertains to the following applications:

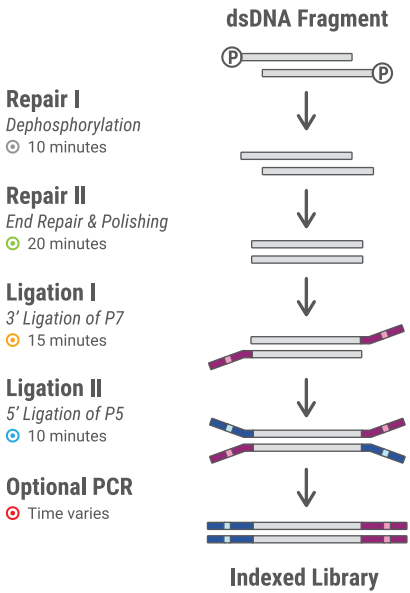
- Non-random fragmentation patterns in DNA samples
  - Nucleosomal fragmentation of cell-free DNA
  - ChIP DNA resulted from chromatin shearing
- Deduplication of single end reads
  - ChIP DNA resulted from chromatin shearing

Accel-NGS 2S Plus DNA Library Kit Workflow

Using four incubations, this protocol repairs both 5' and 3' termini and sequentially attaches Illumina adapter sequences to the ends of fragmented dsDNA.

Bead-based clean-ups are used to remove oligonucleotides and small fragments, and to change enzymatic buffer composition between steps. Different bead-to-sample ratios are utilized for different input quantities and insert sizes.

The P7 adapter added during Ligation I step contains the sample index sequence (i7) at the index 1 position. The P5 adapter added during Ligation II contains the MID index sequence at the index 2 position. Please refer to the table on page 6 for recommended library sizes and input requirements.



Kit Contents

The Accel-NGS 2S Plus DNA Library Kit is available in two sizes with reagents (10% excess volume) for the preparation of either 24 or 96 libraries. Reagent Y2 (indexed adapter), Reagent B2 (MID adapter), and Reagent R1 (PCR primer mix), are provided separately in one of the available Accel-NGS 2S indexing kits (see Appendix, Section B).

Reagents	Quantity (µl) 24 rxn	96 rxn	Storage (°C)
Buffer W1	158	634	-20
Enzyme W2	28	106	-20
Buffer G1	132	528	-20
Reagent G2	344	1374	-20
Enzyme G3	28	106	-20
Enzyme G4	28	106	-20
Buffer Y1	80	316	-20
Reagent Y2	*	*	-20
Enzyme Y3	53	212	-20
Buffer B1	132	528	-20
Reagent B2-MID	*	*	-20
Reagent B3	238	950	-20
Enzyme B4	28	106	-20
Enzyme B5	53	212	-20
Enzyme B6	28	106	-20
Reagent R1	*	*	-20
Reagent R2	106	424	-20
Buffer R3	264	1056	-20
Enzyme R4	28	106	-20

** Provided separately with an indexing kit.*

Reagents	Quantity (mL)	Storage (°C)
PEG NaCl Solution	20	Room Temp
Low EDTA TE	20	Room Temp

IMPORTANT!
Place the enzymes on ice, NOT in a cryocooler, for at least 10 minutes to allow enzymes to reach 4 ˚C prior to pipetting.

Material and Equipment Not Included

- A compatible Accel-NGS 2S Indexing Kit (Reagents Y2, R1, and B2-MID)
- Magnetic beads for clean-up steps, e.g., SPRIselect™ beads (Beckman Coulter, Cat. No. B23317/B23318/B23319)
- Magnetic rack for clean-up steps, e.g., Invitrogen DynaMag™ or Agencourt® SPRIPlate™
- Library quantification kit (qPCR-based for PCR-free)
- Qubit® or other fluorometric-based assays for determining DNA concentration
- Method for fragmentation of input DNA by mechanical or enzymatic shearing
- Microfuge
- Programmable thermocycler
- 0.2 mL PCR tubes
- 1.5 mL low retention microfuge tubes
- Aerosol-resistant, low retention pipettes and tips, 2 to 1000 µL
- 200-proof/absolute ethanol (molecular biology-grade)
- Nuclease-free water (molecular biology-grade)

Storage and Usage Warning

Upon receipt, store the Accel-NGS 2S Plus DNA Library Kit products at -20 ˚C with the exception of PEG and TE solutions, which are stored at room temperature.

To maximize use of enzyme reagents when ready to use, remove enzyme tubes from -20 ˚C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow enzymes to reach 4 ˚C prior to pipetting. Attempting to pipette enzymes at -20 ˚C may result in a shortage of enzyme reagents.

After thawing reagents to 4 ˚C, briefly vortex (except the enzymes) to mix them well. Spin all tubes in a microfuge to collect contents prior to opening.

IMPORTANT!

- Assemble all reagent master mixes ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes, use our **Accel-NGS 2S Master Mix Volume Calculator** and prepare them in advance to ensure the magnetic beads do not over-dry during size selection steps.
- Always add reagents to the master mix *in the specified order* as stated throughout the Protocol. Reagent Y2 (indexed adapter) is the only reagent that is added individually to each sample.

Tips and Techniques

Avoiding Cross-Contamination

To reduce the risk of DNA and library contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed. Follow the instructions below to avoid cross-contamination:

- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.

DNA Fragmentation

If working with cfDNA or samples that have already undergone mechanical or enzymatic fragmentation, such as ChIP-DNA, this step is omitted.

Input Material	Fragmentation	Supported Fragment Size (bp)
High Quality gDNA	✓	200, 350, 450
ChIP DNA	–	200, 350, 450
cfDNA	–	165

Size Selection During Clean-Up Steps

This protocol has been validated with SPRIselect beads (Beckman Coulter), but can be used with Agencourt AMPure® XP (Beckman Coulter). If other beads are used, solutions and conditions for DNA binding may differ. Consider the information below for performing efficient size selection:

- Prior to performing the library preparation workflow, analyze the samples' size distribution by electrophoretic methods to determine the median fragment size of your dsDNA samples.
- The size selections utilized in this protocol perform a Left Side Size Selection, and are designed to produce an average fragment size of 200, 350, or 450 bp. For customizing size selection, please use Beckman Coulter's SPRIselect User Guide for desired conditions not included in this protocol.

Recommended PCR Cycles

Below are examples of recommended PCR cycles for high quality genomic DNA. Yields are approximate and will vary between sample types.

Input Material (ng)	PCR Cycles	Average Yield (nM)
100	3	≥ 4
10	6	≥ 4
1	9	≥ 4
0.1	12	≥ 4
0.01	15	≥ 4

Prepare the DNA Sample

Input DNA Quantification

For high quality samples, it is recommended to determine dsDNA concentration using Qubit, or a similar fluorometric method, as it will accurately represent the double-stranded, adaptable DNA content of your sample. For cfDNA or low quality DNA samples, we recommend quantification by qPCR using Alu primer pairs provided in this kit (see Input DNA Quantification Assay) to accurately assess the usable amount of DNA in the samples and their integrity.

High Quality gDNA	Quantify with Qubit or similar fluorometric method
ChIP DNA	(*Optional*) Quantify with Qubit or similar fluorometric method
cfDNA	Quantify by qPCR with Alu primer pairs (see Input DNA Quantification Assay)

- We recommend using between 10 pg–250 ng input DNA per library preparation.
- Input DNA should be re-suspended in 40 µl of Low EDTA TE buffer. Contact TechSupport@swiftbiosci.com if you would like to work with larger volumes.
- Library amplification by PCR: minimum input of 10 pg DNA.

Input Material	Supported Input With PCR
High Quality gDNA	10 pg–250 ng
ChIP DNA	≥ 10 pg
cfDNA	≥ 1 ng

IMPORTANT!
Input quantities referenced in this Protocol refer to total DNA quantified prior to DNA fragmentation.

DNA Fragmentation

When working with high molecular weight genomic DNA, the DNA must be fragmented prior to library preparation. Fragmentation may be performed via mechanical shearing, such as sonication, or through enzymatic digestion methods to produce DNA fragments in the range of 150–450 bp. If using larger fragments, please contact TechSupport@swiftbiosci.com. This kit has been specifically validated on Covaris®-fragmented DNA.

Please note that fragmentation through enzymatic methods produces a wider size distribution of fragments than that produced through mechanical shearing. For this reason, we recommend performing a 2-sided size selection prior to library preparation to narrow the size distribution.

Note: The size selection may result in loss of a portion of your samples. Consider starting with a larger amount of DNA to compensate for the DNA loss.

We recommend analyzing the sheared DNA samples prior to library preparation. The analysis can be done using electrophoretic methods.

Prepare the Reagent Master Mixes and Ethanol

1. To create the master mix, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes based on the number of reactions of choice, use our Accel-NGS 2S Master Mix Volume Calculator. This tool automatically incorporates 5% excess volume to compensate for pipetting loss.

2. To assemble reagent master mixes for the Repair I, Repair II, Ligation I, Ligation II, and Optional PCR steps, ensure the reagent vials are at room temperature and enzymes are at 4 °C. After thawing reagents, briefly vortex (except the enzymes) to mix them well. Spin tubes in a microfuge to collect contents prior to opening. Add reagents in order listed when preparing master mix. Once prepared, master mixes should be stored ON ICE until used.

IMPORTANT!

Prepare the reagents in advance to ensure the magnetic beads do not dry out during size selection steps. Always add reagents in specified order. This applies to all reagents except for Reagent Y2, a unique indexed adapter provided separately in the indexing kit that should be added individually to uniquely index each library.

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3. Prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Approximately 2 mL of 80% ethanol solution will be used per sample.

Prepare the DNA Libraries

Repair I

1. Transfer the fragmented dsDNA sample to a 0.2 mL PCR tube and adjust the volume of the sample to a final volume of 40 µl using Low EDTA TE, if necessary.

2. Add 20 µl of pre-mixed Repair I Master Mix (listed in the table below) to each sample containing the 40 µl DNA sample.

Reagents	Volume per Sample
Low EDTA TE	13 µl
Buffer W1	6 µl
Enzyme W2	1 µl
Total Volume	**20 µl**

3. Mix by gently pipetting, place in the thermocycler, and run the Repair I Thermocycler Program in the order described below. For cfDNA inputs, please follow the Repair I Thermocycler Program specific to cfDNA.

Sample Type	Thermocycler Program
cfDNA	1. 37 ˚C, 5 min, lid heating ON 2. 65 ˚C, 2 min, lid heating ON 3. 37 ˚C, 5 min, lid heating ON
All Other Inputs	37 ˚C, 10 min, lid heating OFF*

* Alternatively, the thermocycler lid may be left open.

4. Clean up the Repair I reaction using a magnetic rack, magnetic beads (see Material and Equipment Not Included), and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the clean-up instructions below.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
Less than 10 ng gDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
10 ng–250 ng gDNA	200 bp	60 µl	60 µl (ratio: 1.0)	—
	350 bp	60 µl	54 µl (ratio: 0.9)	—
	450 bp	60 µl	42 µl (ratio: 0.7)	—

5. Carefully remove and discard the supernatant without removing any beads.

Repair II

6. Add 50 µl of pre-mixed Repair II Master Mix (listed in the table below) to the beads for each sample and mix by pipetting until homogeneous.

Reagents	Volume per Sample
Low EDTA TE	30 µl
Buffer G1	5 µl
Reagent G2	13 µl
Enzyme G3	1 µl
Enzyme G4	1 µl
Total Volume	**50 µl**

7. Place the samples in the thermocycler, programmed at 20 ˚C for 20 minutes with lid heating OFF.

8. Clean up the Repair II reaction using a magnetic rack, PEG NaCl solution, and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the clean-up instructions below.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
Less than 10 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
10 ng–250 ng gDNA	200 bp	50 µl	—	42.5 µl (ratio: 0.85)
	350 bp	50 µl	—	37.5 µl (ratio: 0.75)
	450 bp	50 µl	—	27.5 µl (ratio: 0.55)

9. Carefully remove and discard the supernatant without removing any beads.

Ligation I

10. Add 25 µl of pre-mixed Ligation I Master Mix (listed in the table below) to the beads for each sample.

Reagents	Volume per Sample
Low EDTA TE	20 µl
Buffer Y1	3 µl
Enzyme Y3	2 µl
Total Volume	**25 µl**

11. Add 5 µl of the appropriate indexed P7 adapter (Reagent Y2) to each sample and re-suspend by pipetting.

Reagents	Volume per Sample
Master Mix	25 µl
Sample	Beads
⊙ Reagent Y2	5 µl
Total Volume	**30 µl**

12. Place the samples in the thermocycler, programmed at 25 ˚C for 15 minutes with lid heating OFF. Alternatively, the thermocycler lid may be left open.

13. Clean up the Ligation I reaction using a magnetic rack, PEG NaCl solution, and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the clean-up instructions below.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	165 bp	30 µl	—	31.5 µl (ratio: 1.05)
Less than 10 ng gDNA	All Sizes	30 µl	—	25.5 µl (ratio: 0.85)
10 ng–250 ng gDNA	All Sizes	30 µl	—	36.0 µl (ratio: 1.2)

14. Carefully remove and discard the supernatant without removing any beads.

⊙ Ligation II

15. Add 50 µl of pre-mixed Ligation II Master Mix (listed in the table below) to the beads for each sample and re-suspend by pipetting.

Reagents	Volume per Sample
Low EDTA TE	30 µl
⊙ Buffer B1	5 µl
⊙ Reagent B2-MID	2 µl
⊙ Reagent B3	9 µl
⊙ Enzyme B4	1 µl
⊙ Enzyme B5	2 µl
⊙ Enzyme B6	1 µl
Total Volume	**50 µl**

Note: Reagent B2-MID (MID adapter) is provided separately in the indexing kit.

16. Place the samples in the thermocycler, programmed at 40 ˚C for 10 minutes with lid heating OFF (25 ˚C hold). Alternatively, the thermocycler lid may be left open.

17. Clean up the Ligation II Reaction using a magnetic rack, PEG NaCl solution, and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the size selection instructions below.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	50 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	50 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	50 µl

18. At the end of the clean-up, resuspend the beads in 50 µl of Low EDTA TE buffer and incubate for 1-2 minutes. DO NOT transfer elute to a new tube, but proceed directly to second clean-up by addition of PEG NaCL to the bead elute.

19. To ensure optimal removal of the unincorporated MID adapter, perform a second clean-up using PEG NaCl solution and freshly prepared 80% ethanol.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	20 µl

20. At the end of the clean-up, resuspend the beads in 20 µl of Low EDTA TE buffer.

21. Place the sample tubes on a magnetic rack and wait 2 minutes.

22. Carefully transfer the supernatant containing the final library to a clean tube without carrying any beads.

Safe Stopping Point

Store freshly prepared libraries at 4 ˚C (or long term at -20 ˚C). The library is now ready for quantification, which must be performed by qPCR to ensure accuracy. PCR-free libraries cannot be accurately quantified or assessed for library size by electrophoretic methods (see Appendix, Section C).

PCR-Library Amplification

23. Add 30 µl of the pre-mixed PCR Master Mix (listed in the table below) to the entire eluted library (20 µl). Mix by pipetting. If analysis of the PCR-free library by qPCR is desired before PCR amplification, set aside 3 µl of the final eluate for this analysis and instead add 17 µl of eluate plus 3 µl of Low EDTA TE buffer to the 30 µl of PCR Master Mix.

Reagents	Volume per Sample
Low EDTA TE	10 µl
⊙ Reagent R1	5 µl
⊙ Reagent R2	4 µl
⊙ Buffer R3	10 µl
⊙ Enzyme R4	1 µl
Total Volume	**30 µl**

Note: Reagent R1 (PCR primer mix) is provided separately as part of the indexing kit.

24. Place the sample tubes in the thermocycler and run the Optional PCR Thermocycler Program in the order listed below.

! IMPORTANT!

The number of cycles required to produce enough library for sequencing will depend on input quantity and quality. In the case of low quality samples including FFPE, the number of cycles required may vary based on the quality of the sample and amount of usable DNA present. Approximate guidelines for high quality DNA are as indicated above, but the exact number of cycles required must be determined by the user.

Input	Recommended PCR Cycles	Thermocycler Program
100 ng	3	98 °C for 30 seconds PCR Cycles: 98 °C for 10 seconds 60 °C for 30 seconds 68 °C for 60 seconds Hold at 4 °C — proceed immediately to clean-up step
10 ng	6	
1 ng	9	
100 pg	12	
10 pg	15	
10 ng cfDNA	0–2	
1 ng cfDNA	5–6	

25. Clean up the PCR Reaction using magnetic beads and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the size selection instructions below.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	All Sizes	50 µl	37.5 µl (ratio: 0.75)	—	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	37.5 µl (ratio: 0.75)	—	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	70.0 µl (ratio: 1.4)	—	20 µl

26. At the end of the clean-up, resuspend the beads in 20 µl of Low EDTA TE buffer.

27. Place the sample tubes on a magnetic rack and wait 2 minutes.

28. Carefully transfer the supernatant containing the final library to a clean tube without carrying any beads.

Appendix

Section A: Size Selection/Clean-Up Protocol

Please use the following protocol for each clean-up step, substituting the correct **Bead Volume**, **PEG NaCl Volume**, and **Elution Volume** based on the table provided for each section.

1. Ensure the magnetic beads are at room temperature and vortex beads to homogenize the suspension before use.
2. Add the specified Bead Volume or PEG NaCl Volume to each sample. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
3. Incubate the samples for 5 minutes at room temperature.
4. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
5. Remove and discard the supernatant without disturbing the pellet (less than 5 µl may be left behind).
6. Add 180 µl of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
7. Repeat step 6 once more for a second wash with the 80% ethanol solution.
8. Quick spin the samples in a tabletop microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube.
9. Add the specified volume of each reaction mix (Repair II, Ligation I, and Ligation II) or elution volume (Post-Ligation II and Post-Library PCR) of Low EDTA TE buffer and re-suspend the pellet. Mix well by pipetting up and down until homogenous.

Post-Repair I Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
Less than 10 ng gDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
10 ng–250 ng gDNA	200 bp	60 µl	60 µl (ratio: 1.0)	—
	350 bp	60 µl	54 µl (ratio: 0.9)	—
	450 bp	60 µl	42 µl (ratio: 0.7)	—

Post-Repair II Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
Less than 10 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
10 ng–250 ng gDNA	200 bp	50 µl	—	42.5 µl (ratio: 0.85)
	350 bp	50 µl	—	37.5 µl (ratio: 0.75)
	450 bp	50 µl	—	27.5 µl (ratio: 0.55)

Post-Ligation I Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	165 bp	30 µl	—	31.5 µl (ratio: 1.05)
Less than 10 ng gDNA	All Sizes	30 µl	—	25.5 µl (ratio: 0.85)
10 ng–250 ng gDNA	All Sizes	30 µl	—	36.0 µl (ratio: 1.2)

Post-Ligation II Clean-Up (1)

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	50 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	50 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	50 µl

Post-Ligation II Clean-Up (2)

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	20 µl

Post-PCR Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	All Sizes	50 µl	37.5 µl (ratio: 0.75)	—	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	37.5 µl (ratio: 0.75)	—	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	70.0 µl (ratio: 1.4)	—	20 µl

Section B: Indexing Kits

Set A, B, and A+B Indexing Kits (Cat. Nos. 27148, 27248, and 27396)

During the Ligation I step, you must use a unique indexed adapter (Reagent Y2) to label each library. If no multiplex sequencing is being performed, all libraries may be labeled with Index 1 only. Libraries made with uniquely indexed adapters may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell. The full-length adapter sequences where 9 random N bases is inserted in place of the standard Index 2, 8 bp D501-508 index sequence representing each MID unique sequence, and X is replaced by the index sequences in the tables below are as follows:

P5 TruSeq HT Adapter:

5' AATGATACGGCGACCACCGAGATCTACAC*NNNNNNNNN*CACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

P7 TruSeq LT Adapter (Index 1-12):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXXCTCGTATGCCGTCTTCTGCTTG 3'

P7 TruSeq LT Adapter (Index 13-27):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG 3'

The number on the product tube label indicates which indexed adapter is provided in the tube. The bases in parentheses are not considered part of the six bp index sequences, but can be used for 8 bp index reads.

Table 1

Set A Adapters	Sequence	27148	27248	27396
Reagent Y2 (Index 2), A002	CGATGT(AT)	22 µl	—	22 µl
Reagent Y2 (Index 4), A004	TGACCA(AT)	22 µl	—	22 µl
Reagent Y2 (Index 5), A005	ACAGTG(AT)	22 µl	—	22 µl
Reagent Y2 (Index 6), A006	GCCAAT(AT)	22 µl	—	22 µl
Reagent Y2 (Index 7), A007	CAGATC(AT)	22 µl	—	22 µl
Reagent Y2 (Index 12), A012	CTTGTA(AT)	22 µl	—	22 µl
Reagent Y2 (Index 13), A013	AGTCAA(CA)	22 µl	—	22 µl
Reagent Y2 (Index 14), A014	AGTTCC(GT)	22 µl	—	22 µl
Reagent Y2 (Index 15), A015	ATGTCA(GA)	22 µl	—	22 µl
Reagent Y2 (Index 16), A016	CCGTCC(CG)	22 µl	—	22 µl
Reagent Y2 (Index 18), A018	GTCCGC(AC)	22 µl	—	22 µl
Reagent Y2 (Index 19), A019	GTGAAA(CG)	22 µl	—	22 µl

Table 2

Set B Adapters	Sequence	27148	27248	27396
Reagent Y2 (Index 1), A001	ATCACG(AT)	—	22 µl	22 µl
Reagent Y2 (Index 3), A003	TTAGGC(AT)	—	22 µl	22 µl
Reagent Y2 (Index 8), A008	ACTTGA(AT)	—	22 µl	22 µl
Reagent Y2 (Index 9), A009	GATCAG(AT)	—	22 µl	22 µl
Reagent Y2 (Index 10), A010	TAGCTT(AT)	—	22 µl	22 µl
Reagent Y2 (Index 11), A011	GGCTAC(AT)	—	22 µl	22 µl
Reagent Y2 (Index 20), A020	GTGGCC(TT)	—	22 µl	22 µl
Reagent Y2 (Index 21), A021	GTTTCG(GA)	—	22 µl	22 µl
Reagent Y2 (Index 22), A022	CGTACG(TA)	—	22 µl	22 µl
Reagent Y2 (Index 23), A023	GAGTGG(AT)	—	22 µl	22 µl
Reagent Y2 (Index 25), A025	ACTGAT(AT)	—	22 µl	22 µl
Reagent Y2 (Index 27), A027	ATTCCT(TT)	—	22 µl	22 µl

During library prep, make sure to note which indexed adapter you are using with your sample and do not use the same indexed adapter on two different samples you plan to multiplex together.

Reagents	27148	27248	27396
Reagent B2-MID	106 µl	106 µl	212 µl
Reagent R1	264 µl	264 µl	528 µl

Set S1, S2, S3, S4, and S1-S4 Indexed Adapters (Cat. Nos. 27596, 27696, 27796, 27896, and 279384)

The Accel-NGS 2S Single Indexed Adapters with MIDs are validated for sets of 12 plexing. If a lower plexing is desired, contact TechSupport@swiftbiosci.com.

Libraries indexed with the new indices **CANNOT** be used in combination with libraries indexed with our standard indexing kits, including Set A, Set B, or Dual (D701-D712).

Sample Sheet Creation

- In Illumina Experiment Manager software, select your sequencer of choice.
- On the Application Selection page, select other – FASTQ Only.
- Choose TruSeq HT. By selecting HT, you can alter the index sequences read in the sample sheet.
- On the next step, select placeholder index sequences from the menu until sample sheet status is valid.
- Click Finish to generate the .CSV file.
- Alter your sample sheet (by editing the .CSV file in Excel) to specify the MID as 9 random N bases for index 2, and the 96 unique index sequences provided in Table 3 for index 1.
- Be sure to re-save your edits as a .CSV file.

The full length adapter sequences where 9 random N bases are inserted in place of the standard index 2, 8 bp D501-D508 index sequence representing each MID unique sequence, and X is replaced by the indexed sequences in the tables below are as follows:

P5 TruSeq HT Adapter:

5´AATGATACGGCGACCACCGAGATCTACAC*NNNNNNNNN*ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3´

P7 TruSeq LT Indexed Adapter:

5´GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG 3´

Table 3

Set S1 Adapters	Sequence	27596
Reagent Y2 (Index S701)	CAACACAG	24 µl
Reagent Y2 (Index S702)	ACACCTCA	24 µl
Reagent Y2 (Index S703)	ACCATAGG	24 µl
Reagent Y2 (Index S704)	CAGGTAAG	24 µl
Reagent Y2 (Index S705)	AACGCACA	24 µl
Reagent Y2 (Index S706)	TAGTCTCG	24 µl
Reagent Y2 (Index S707)	CAGTCACA	24 µl
Reagent Y2 (Index S708)	CCAACACT	24 µl
Reagent Y2 (Index S709)	ACATGCCA	24 µl
Reagent Y2 (Index S710)	ATTCCGCT	24 µl
Reagent Y2 (Index S711)	CAAGGTAC	24 µl

Set S1 Adapters	Sequence	27596
Reagent Y2 (Index S712)	CCATGAAC	24 µl
Reagent Y2 (Index S713)	TCAGCCTT	24 µl
Reagent Y2 (Index S714)	CAGTGCTT	24 µl
Reagent Y2 (Index S715)	CTCGAACA	24 µl
Reagent Y2 (Index S716)	ACAGTTCG	24 µl
Reagent Y2 (Index S717)	ATCCTTCC	24 µl
Reagent Y2 (Index S718)	CGAAGTCA	24 µl
Reagent Y2 (Index S719)	CTCTATCG	24 µl
Reagent Y2 (Index S720)	ACTCTCCA	24 µl
Reagent Y2 (Index S721)	TCCTCATG	24 µl
Reagent Y2 (Index S722)	AACAACCG	24 µl
Reagent Y2 (Index S723)	CTCGTTCT	24 µl
Reagent Y2 (Index S724)	TCAGTAGG	24 µl

Set S2 Adapters	Sequence	27696
Reagent Y2 (Index S725)	GCTTCACA	24 µl
Reagent Y2 (Index S726)	CGATGTTT	24 µl
Reagent Y2 (Index S727)	TTAGGCAT	24 µl
Reagent Y2 (Index S728)	ACAGTGGT	24 µl
Reagent Y2 (Index S729)	GCCAATGT	24 µl
Reagent Y2 (Index S730)	CAGATCTG	24 µl
Reagent Y2 (Index S731)	ACTTGATG	24 µl
Reagent Y2 (Index S732)	TAGCTTGT	24 µl
Reagent Y2 (Index S733)	TGGTTGTT	24 µl
Reagent Y2 (Index S734)	TGTACCTT	24 µl
Reagent Y2 (Index S735)	TCTGCTGT	24 µl
Reagent Y2 (Index S736)	TTGGAGGT	24 µl
Reagent Y2 (Index S737)	TCGAGCGT	24 µl
Reagent Y2 (Index S738)	TGATACGT	24 µl
Reagent Y2 (Index S739)	TGCATAGT	24 µl
Reagent Y2 (Index S740)	TGCGATCT	24 µl
Reagent Y2 (Index S741)	TTCCTGCT	24 µl
Reagent Y2 (Index S742)	TACAGGAT	24 µl
Reagent Y2 (Index S743)	TGTGGTTG	24 µl
Reagent Y2 (Index S744)	TTCCATTG	24 µl
Reagent Y2 (Index S745)	TAACGCTG	24 µl
Reagent Y2 (Index S746)	TTGGTATG	24 µl

Set S2 Adapters	Sequence	27696
Reagent Y2 (Index S747)	TGAACTGG	24 µl
Reagent Y2 (Index S748)	TACTTCGG	24 µl

Set S3 Adapters	Sequence	27796
Reagent Y2 (Index S749)	TCCAGTCG	24 µl
Reagent Y2 (Index S750)	TGTATGCG	24 µl
Reagent Y2 (Index S751)	TCATTGAG	24 µl
Reagent Y2 (Index S752)	TGGCTCAG	24 µl
Reagent Y2 (Index S753)	TATGCCAG	24 µl
Reagent Y2 (Index S754)	TCAGATTC	24 µl
Reagent Y2 (Index S755)	GGTTGGAC	24 µl
Reagent Y2 (Index S756)	GACACTTA	24 µl
Reagent Y2 (Index S757)	GCTATGGA	24 µl
Reagent Y2 (Index S758)	GTAACCGA	24 µl
Reagent Y2 (Index S759)	GGCAAGCA	24 µl
Reagent Y2 (Index S760)	GAACGACA	24 µl
Reagent Y2 (Index S761)	GCGTCGAA	24 µl
Reagent Y2 (Index S762)	AAGGCGAT	24 µl
Reagent Y2 (Index S763)	CAGGCATT	24 µl
Reagent Y2 (Index S764)	AACTGTAT	24 µl
Reagent Y2 (Index S765)	ATGCTTGA	24 µl
Reagent Y2 (Index S766)	AGTATCTG	24 µl
Reagent Y2 (Index S767)	ATGTAATG	24 µl
Reagent Y2 (Index S768)	ACACATGT	24 µl
Reagent Y2 (Index S769)	ATAGCACG	24 µl
Reagent Y2 (Index S770)	ATATTGTA	24 µl
Reagent Y2 (Index S771)	CAATTGAT	24 µl
Reagent Y2 (Index S772)	CACGTCGT	24 µl

Set S4 Adapters	Sequence	27896
Reagent Y2 (Index S773)	AGTCTGTA	24 µl
Reagent Y2 (Index S774)	CCGTATCT	24 µl
Reagent Y2 (Index S775)	CGCTTCCT	24 µl
Reagent Y2 (Index S776)	CAAGACCT	24 µl
Reagent Y2 (Index S777)	CCTAGTAT	24 µl
Reagent Y2 (Index S778)	CCACCGAT	24 µl
Reagent Y2 (Index S779)	CTATCATG	24 µl

Set S4 Adapters	Sequence	27896
Reagent Y2 (Index S780)	CATGAATG	24 µl
Reagent Y2 (Index S781)	CTGTACGG	24 µl
Reagent Y2 (Index S782)	CACTCGAG	24 µl
Reagent Y2 (Index S783)	CCGACAAG	24 µl
Reagent Y2 (Index S784)	CTTGCTTC	24 µl
Reagent Y2 (Index S785)	CGCCTTAT	24 µl
Reagent Y2 (Index S786)	GCAACCAT	24 µl
Reagent Y2 (Index S787)	TGACCGTT	24 µl
Reagent Y2 (Index S788)	TTGAGCTC	24 µl
Reagent Y2 (Index S789)	CCACATTG	24 µl
Reagent Y2 (Index S790)	AGCCAACT	24 µl
Reagent Y2 (Index S791)	ATCACGTT	24 µl
Reagent Y2 (Index S792)	TCTCGGTT	24 µl
Reagent Y2 (Index S793)	TTGACTCT	24 µl
Reagent Y2 (Index S794)	TCGAAGTG	24 µl
Reagent Y2 (Index S795)	CACCCAAA	24 µl
Reagent Y2 (Index S796)	CTTCACAT	24 µl

During library prep, make sure to note which indexed adapter you are using with your sample and do not use the same indexed adapter on two different samples you plan to multiplex together.

Reagents	27596	27696	27796	27896	279384
Reagent B2-MID	211 µl	211 µl	211 µl	211 µl	844 µl
Reagent R1	528 µl	528 µl	528 µl	528 µl	2,112 µl

For questions, please contact TechSupport@swiftbiosci.com.

Section C: Sequencing MID Libraries

NextSeq Instruments

Set up the run in Stand-Alone mode and use bcl2fastq for downstream analysis.

Setting up a run in Stand-Alone mode does not require a sample sheet, and the final files produced by the instrument are base call files (.bcl). Analysis with bcl2fastq will need to be performed, which will require a sample sheet.

- After completing the sequencing run, use the Illumina Experiment Manager software to set up the sample sheet.
- In the CSV file, specify the MID index and the sample index sequences. Samples will be demultiplexed based only on their sample index.
- Using a custom script, join MIDs to their respective fastq read headers, align these fastq, and analyze the reads with a common genomic coordinate.

MiSeq and HiSeq Instruments

- Modify the config file to create a fastq for index reads.
- Using the Illumina Experiment Manager software, specify two index reads for the run.
- In the CSV file, specify the MID index and the sample index sequences. Samples will be demultiplexed based only on their sample index.
- Using a custom script, join MIDs to their respective fastq read headers, align these fastq, and analyze the reads with a common genomic coordinate.

Instrument Set-up and Sample Sheet Preparation

- For Index 2, enter a random index number on the pull-down menu.
- Specify the MID as 9 random N bases (*NNNNNNNNN*) in the sample sheet.
- Click Finish to generate the CSV file.
- Alter your sample sheet to represent the real index sequences.
- When using a low throughput index, include the index (6 bp) and the next two base pairs. For example, with Index 2: Enter **CGATGT**AT. Bold represents the index whereas the underlined bases represent the next two bases on the adapter.
- For Index 2, enter the MID sequence: *NNNNNNNNN*.
- During the sequencing run, the samples are separated based only on their Index 1 sequences, since any Index 2 reads will be valid.

Sample Sheet

- Upon completion of the sequencing run, all reads are separated based on Index 1 reads. Non-identified (undetermined) reads are due to either poor quality or reads containing absent index sequences.

Options for Retrieving Index Sequence Files

Instrument	bcl2fastq	MISeq Reporter	BaseSpace
MiniSeq	✓		
MiSeq	✓	✓	
NextSeq 500	✓		
HiSeq 2500	✓		
HiSeq 4000	✓		

If for any reason the data is not extracted correctly by the Sample Sheet set-up, bcl2fastq Conversion Software can be used to correctly extract the data.

Section D: Helpful Information and Troubleshooting

Problem	Possible Cause	Suggested Remedy
Library migrates unexpectedly on Bioanalyzer.	When analyzed on the Agilent High Sensitivity chip, migration behavior overestimates library size of PCR-free libraries made from DNA fragmented to the 200–300 base range (as required in this protocol).	Consult the Expected Results section and the application note released by Covaris titled "Analysis of DNA Fragments Using the Agilent 2100 Bioanalyzer" to ensure proper analysis of library size.
	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally.	• Quantify library by qPCR, as other quantification methods will not accurately detect heteroduplex library molecules. • Perform the minimum number of PCR cycles necessary to avoid over-amplification.
DNA does not fragment properly: broad or lopsided (high molecular weight) sonication profile of fragmented DNA.	Impure DNA or fragmentation device malfunction.	• Isopropanol purification, bead clean-up, column purification, or other method before fragmentation. • Ensure fragmentation device is functioning within manufacturer's parameters.
Incomplete resuspension of beads after ethanol wash during SPRI™ steps.	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
Shortage of enzyme reagents.	Pipetting enzymes at -20 °C instead of 0-4 °C.	Allow enzyme reagents to equilibrate to 0-4 °C for 10 minutes prior to pipetting.
Retention of liquid in pipette tip	Viscous reagents may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to ensure all liquid and/or beads are released from the pipette tip.

If you experience problems with your library prep, please contact us at TechSupport@swiftbiosci.com, or by phone at 734.330.2568 (9:00 am-5:00 pm ET, Monday-Friday).