PROTOCOL

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ACCEL-NGS® METHYL-SEQ DNA LIBRARY KIT

Single, Dual Combinatorial and Unique Dual Indexing

Protocol for Cat. Nos. 30024 and 30096 to be used with:

- Methyl-Seq Set A Indexing Kit (Cat. No. 36024)
- Methyl-Seq Dual Combinatorial Indexing Kit (Cat. No. 38096)
- Methyl-Seq Unique Dual Indexing Kit (24 indices, 96 rxns - Cat. No. 39096)
- Methyl-Seq Unique Dual Indexing Kit (96 indices, 384 rxns - Cat.No. 390384)

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About This Guide

This guide provides instructions for the preparation of high complexity NGS libraries from bisulfite-converted DNA using an Accel-NGS Methyl-Seq DNA Library Kit. This kit is suitable for NGS library prep with Single, Dual Combinatorial or Unique Dual Indexing.

IMPORTANT!
Read the entire Protocol before use, especially the sections pertaining to Product Information, Kit Contents, Material and Equipment Not Included, and Input Material Considerations.

Product Information

The Accel-NGS Methyl-Seq DNA Library Kit enables the preparation of high complexity next-generation sequencing (NGS) libraries from bisulfite-converted DNA for sequencing on Illumina® platforms. The Methyl-Seq technology utilizes Illumina-compatible adapter sequences and has been validated for whole genome bisulfite sequencing (WGBS) and targeted sequencing (such as hybridization capture enrichment using NimbleGen™ SeqCap™ Epi Enrichment Systems) with genomic DNA, including formalin-fixed, paraffin-embedded (FFPE) samples, and cell-free DNA samples.

Libraries can be made from as little as 100 pg of high quality DNA starting input DNA. The technology powering the Accel-NGS Methyl-Seq Kit is compatible with single-stranded DNA (ssDNA), making it an ideal choice for NGS library prep from DNA fragments damaged and denatured by bisulfite conversion. By using single-stranded, bisulfite-converted DNA molecules as input, the Accel-NGS Methyl-Seq Kit overcomes the significant library loss associated with alternative library preparations that require double-stranded input DNA and bisulfite conversion of completed library molecules. This empowers users of the Accel-NGS Methyl-Seq DNA Library Kit to use fewer PCR cycles from the same input, and further enables the use of inputs that were formerly prohibitively low.

Applications

The Accel-NGS Methyl-Seq DNA Library Kit is suitable for the following applications:

- WGBS
- Reduced Representation Bisulfite Sequencing (RRBS)*
- Hybridization capture using NimbleGen SeqCap Epi Enrichment Systems†‡
- Bisulfite-converted DNA enriched by ChIP or other methods*
- Sequencing ancient DNA samples when retention of fragments containing uracil nucleotides as a result of damage is desired**

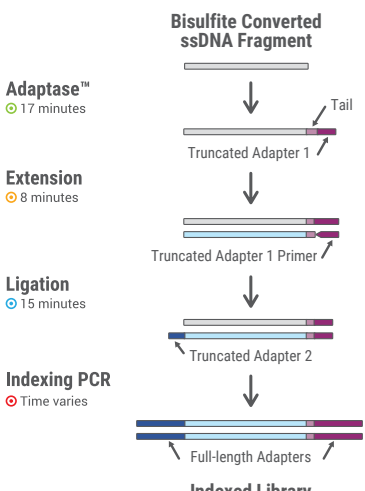
**Enrichment methods generally require higher input quantities.*

† NimbleGen SeqCap Epi hybridization requires the use of single indexing (Swift Cat. No. 36024) for compatibility. Additionally, please use the amplification polymerase provided in the SeqCap Epi Accessory Kit (Roche NimbleGen Cat. No. 07145519001/07185707001) for Pre-Hybridization LM-PCR during the Indexing PCR Step of this protocol. Swift's Accel-NGS Methyl-Seq Kit can support inputs lower than those supported by the recommended NimbleGen library preparation. Please email TechSupport@swiftbiosci.com for more information on validated input quantities and recommended number of PCR cycles. See Appendix, Section A for protocol modifications specific to libraries for NimbleGen SeqCap Epi hybridization.

‡ The Accel-NGS Methyl-Seq Kit cannot be used with hybridization capture technologies that utilize probes which are incompatible with bisulfite-converted DNA (e.g. Agilent SureSelect^{XT} Human Methyl-Seq). Please email TechSupport@swiftbiosci.com for more information and recommendations.

*** For samples containing small DNA fragments, like ancient DNA samples, modified bead ratios can be used to retain fragments as small as 40 bases (see Appendix, Section B).*

Accel-NGS Methyl-Seq DNA Library Kit Workflow



This protocol sequentially attaches adapters to single-stranded DNA fragments.

The Adaptase step is a highly efficient, template-independent reaction that simultaneously performs tailing and ligation of truncated adapter 1 to 3' ends.

The Extension step is used to incorporate truncated adapter 1' by a primer extension reaction.

The Ligation step is used to add truncated adapter 2 to the **bottom strand only**.

The Indexing PCR step increases yield and incorporates full length adapters for single or dual indexing.

Bead-based clean-ups are used to remove both oligonucleotides and small fragments, as well as to change enzymatic buffer composition.

Kit Contents

The Accel-NGS Methyl-Seq DNA Library Kit is available in two sizes with reagents (10% excess volume) for the preparation of either 24 or 96 libraries. Index X (Single Indexing primer mixture), D50X/D7XX (Combinatorial Dual Index primers), or U001-U096 (Unique Dual Index primers), are provided separately in one of the available Accel-NGS Methyl-Seq Indexing Kits (see Appendix, Section D).

Reagents	Quantity (µl)		Storage (°C)
	24 rxn	96 rxn	
Buffer G1	106	423	-20
Reagent G2	106	423	-20
Reagent G3	66	264	-20
Enzyme G4	27	106	-20
Enzyme G5	27	106	-20
Enzyme G6	27	106	-20
Reagent Y1	53	212	-20
Enzyme Y2	1109	4436	-20
Buffer B1	80	317	-20
Reagent B2	264	1056	-20
Enzyme B3	53	212	-20
Buffer R1	264	1056	-20
Reagent R2	106	423	-20
Enzyme R3	27	106	-20

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.

Reagents	Quantity (mL)	Storage (°C)
Low EDTA TE	20	RT

Indexing Reagents Provided Separately

Reagents	Volume per rxn (µl)	Quantity per tube (µl)			Storage (°C)
		24 rxn	96 rxn	384 rxn	
Single Index X	5	11	—	—	-20
Combinatorial Dual Index (D50X/D7XX)	2.5 + 2.5	—	33/22	—	-20
Unique Dual Index (U001-U024)	5	—	22	—	-20
Unique Dual Index (U001-U096)	5	—	—	22	-20

Material and Equipment Not Included

- A compatible Accel-NGS Methyl-Seq Indexing Kit (Reagents Index X, Index D50X/D7XX, or Index U001-U096)
- Bisulfite conversion kit which does not include a nucleic acid carrier, e.g. Zymo Research EZ DNA Methylation-Gold Kit™
- Unmethylated Lambda DNA (Promega Cat. No. D1521)
- PhiX or other high complexity library for loading purposes
- 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
- Qubit® or other fluorometric-based assays for determining double-stranded DNA (dsDNA) concentration
- NanoDrop® or other device for determining ssDNA 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 Methyl-Seq DNA Library Kit products at -20 ˚C with the exception of Low EDTA TE solution, which is 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 Methyl-Seq Master Mixing 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. Index X, or D50X/D7XX (Combinatorial Dual index primers) or U001-U096 (Unique Dual index primer) are the only reagents that are added individually to each sample.

Tips and Techniques

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 to remove small fragments and unused adapter (see Appendix, Section B for modified bead ratios to retain fragments as small as 40 bp). For customizing size selection, please use Beckman Coulter's SPRIselect User Guide for desired conditions not included in this protocol.

IMPORTANT!
For optimal removal of undesirable small fragments and unused adapter, users should perform clean-up steps in 1.5 mL tubes using 500 µl of 80% ethanol solution to wash the beads. However, for inputs ≥ 1 ng, adequate small fragment and unused adapter removal can be achieved by clean-up steps performed in 0.2 mL PCR tubes using 180 µl of 80% ethanol solution to wash the beads.

Recommended PCR Cycles

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

Input Material	Input Quantity (ng)	PCR Cycles	Average Yield (nM)
gDNA	100	4-6	≥ 4
	10	7-9	≥ 4
	1	11-13	≥ 4
	0.1	14-16	≥ 4
cfDNA	5	7-9	≥ 4

Input Material Considerations

The Accel-NGS Methyl-Seq DNA Library Kit designed for Illumina platforms enables the preparation of high complexity NGS libraries from bisulfite-converted input DNA.

For direct sequencing applications, the Accel-NGS Methyl-Seq Kit has been validated for an input range of 100 pg-100 ng gDNA and ≥ 5 ng cfDNA. Please use the recommended PCR cycles for 5 ng cfDNA to estimate the number of PCR cycles needed when using larger cfDNA input quantities.

Enrichment methods generally require higher input quantities. The Accel-NGS Methyl-Seq Kit has been validated for an input range of 1 ng-100 ng DNA for hybridization capture with NimbleGen SeqCap Epi (see Appendix, Section A for modifications to the standard protocol). For RRBS, we recommend using at least 50 ng of input gDNA, as a significant amount of sample loss is expected during the enzymatic digestion and size selection steps.

IMPORTANT!

Please consider genome complexity and sample quality when choosing input DNA quantity. Although libraries may be successfully prepared from ultra-low inputs, reduced representation of genome complexity may occur.

It is recommended to use the highest input available for best results. The Accel-NGS Methyl-Seq Kit has been validated with up to 100 ng of starting input material. If using more than 100 ng of input material, please contact Technical Support for recommended modifications to the Protocol.

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 the sample. For cfDNA or low quality DNA samples, we recommend quantification by qPCR using the 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)
FFPE DNA	Quantify by qPCR with Alu primer pairs (see Input DNA Quantification Assay)

- We recommend using between 100 pg-100 ng input DNA per library preparation.
- After determining DNA concentration, proceed directly to the DNA fragmentation step.

IMPORTANT!

- Input quantities referenced in this Protocol refer to total DNA quantified prior to DNA fragmentation.
- Unmethylated lambda gDNA must be spiked into the sample gDNA prior to fragmentation. For samples that do not require fragmentation (cfDNA), lambda gDNA may be separately fragmented to a size similar to that of the fragments in the sample. We recommend a spike in level of 0.5% (w/w), as specified in the Standards and Guidelines for Whole Genome Shotgun Bisulfite Sequencing (roadmapepigenomics.org/protocols).

DNA Fragmentation

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

Input Material	Fragmentation	Supported Fragment Size (bp)
High Quality gDNA	✓	200, 350, 650
ChIP DNA	–	200, 350, 650
cfDNA	–	165
FFPE DNA	✓	200, 350, 650

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 sonica-

tion, or through enzymatic digestion methods to produce 650 or 350 bp DNA fragments (direct sequencing) or 200 bp DNA fragments (NimbleGen SeqCap Epi hybridization capture; see Appendix, Section A for modifications to standard protocol). If using other fragment size, please contact TechSupport@swiftbiosci.com for clean-up bead ratio recommendations. This kit has been specifically validated using Covaris®-fragmented DNA.

For RRBS applications, MspI will be used to enzymatically fragment gDNA, followed by DNA purification and isolation of small fragments (100-220 bp).

For cfDNA samples, fragmentation is not necessary. Proceed directly to the bisulfite conversion step.

We do not recommend omitting the fragmentation step. Fragmentation of gDNA using bisulfite treatment alone produces a wider size distribution of fragments than that produced with mechanical or enzymatic shearing. This relatively wide size distribution constitutes some library molecules that are too large for efficient cluster generation on the flow cell, resulting in reduced cluster density. For this reason, if the fragmentation step is omitted, please consider 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 using electrophoretic methods.

Optional Concentration Step

If you have performed enzymatic fragmentation or your fragmented DNA concentration is too low to provide sufficient quantity in the starting volume specified in the BS conversion kit, concentrate with Zymo Research DNA Clean & Concentrator™ or other method and elute in appropriate volume of Low EDTA TE buffer. Otherwise, proceed directly to the bisulfite conversion step.

Bisulfite Conversion

Our Accel-NGS Methyl-Seq product has been validated using the EZ DNA Methylation-Gold Kit following the manufacturer's instructions. Accel-NGS Methyl-Seq **has not been validated** with the QIAGEN EpiTect® Bisulfite kit or other conversion kits that contain nucleic acid carriers which may interfere with the Accel-NGS Methyl-Seq protocol.

Input quantities and PCR cycling recommendations are based on ≥ 50% recovery of input DNA from the EZ DNA Methylation-Gold Kit. If using another bisulfite conversion kit, become familiar with percent DNA recovery to ensure a sufficient quantity of bisulfite-converted DNA for library synthesis. We recommend quantifying the amount of DNA recovered using a NanoDrop on the RNA setting after bisulfite conversion. Low input quantities (less than 100 ng) may not be detectable on the NanoDrop. It is important to note that DNA will be single-stranded following bisulfite conversion.

For cfDNA samples, quantification after bisulfite conversion is not required. Proceed with the entire bisulfite-converted cfDNA sample into the denaturation step below.

IMPORTANT!
The first step of the Accel-NGS Methyl-Seq library prep requires a DNA volume of 15 µl. Be sure to note the volume for final elution of bisulfite-converted DNA in Low EDTA TE solution to prevent sample over-dilution.

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 Methyl-Seq Master Mixing Volume Calculator. This tool automatically incorporates 5% excess volume to compensate for pipetting loss.
2. To assemble reagent master mixes for the Adaptase, Extension, Ligation, and Indexing 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.
3. Prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Approximately 2.0 mL of 80% ethanol solution will be used per sample.

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 the indexed adapter primers provided separately in the indexed adapter kit that should be added individually to uniquely index each library.

Prepare the DNA Libraries

Denaturation

1. Due to the short incubation time of the Denaturation step, pre-assemble all of the reagents of the Adaptase Reaction Mix (see next step for recipe), and place on ice.
2. Pre-heat the thermocycler to 95 °C.
3. Transfer the fragmented, bisulfite-converted DNA sample to a 0.2 mL PCR tube and adjust the volume of the sample to a final volume of 15 µl using Low EDTA TE, if necessary.
4. Place the samples in the thermocycler, programmed at 95 °C for 2 minutes with lid heating ON.
5. Upon completion, place tube(s) on ice immediately for 2 minutes. Proceed directly to the Adaptase step to preserve the maximum amount of ssDNA substrate.

Adaptase

6. Load the Adaptase Thermocycler Program on the thermocycler and pause it at the first step to pre-heat to 37 °C until all samples are loaded.

Assembly Order	Reagents	Volume per Sample
Pre-assemble	Low EDTA TE	11.5 µl
	Buffer G1	4.0 µl
	Reagent G2	4.0 µl
	Reagent G3	2.5 µl
	Enzyme G4	1.0 µl
	Enzyme G5	1.0 µl
	Enzyme G6	1.0 µl
	Total Volume	**25.0 µl**

7. Add 25 µl of the pre-assembled Adaptase Reaction Mix to each PCR tube containing a 15 µl DNA sample and mix by pipetting or gentle vortexing until homogeneous. Spin down.
8. Place the samples in the thermocycler and run the program, with **lid heating ON**.

Thermocycler Program
37 °C, 15 min
95 °C, 2 min
4 °C hold

Extension

9. Load the Extension Thermocycler Program on the thermocycler and pause it at the first step to pre-heat to 98 °C until all samples are loaded.
10. Add 44 µl of the already pre-mixed Extension Reaction Mix (listed in the table below) to each PCR tube containing 40 µl of the Adaptase Reaction, using reagents in the order listed.

Reagents	Volume per Sample
Reagent Y1	2 µl
Enzyme Y2	42 µl
Total Volume	**44 µl**

11. Mix by pipetting or gentle vortexing until homogenous. Spin down.
12. Place the samples in the thermocycler and run the program, with **lid heating ON**.

Thermocycler Program
98 °C, 1 min
62 °C, 2 min
65 °C, 5 min
4 °C hold

13. Transfer each sample to a 1.5 mL tube and clean up the Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Input	Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
cfDNA ≥ 10 ng gDNA	Single clean-up	84 µl	101 µl (ratio: 1.2)	15 µl
< 10 ng gDNA	1st clean-up	84 µl	101 µl (ratio: 1.2)	50 µl
	2nd clean-up	50 µl	60 µl (ratio: 1.2)	15 µl

Ligation

14. Add 15 µl of the pre-mixed Ligation Reaction Mix (listed in the table below) to a new PCR tube containing 15 µl of the Post-Extension eluate. For the Ligation Reaction Mix, use reagents in the order listed below and note **Enzyme B3 should be added to the master mix just before use**.

Assembly Order	Reagents	Volume per Sample
Pre-asemble	Buffer B1	3 µl
	Reagent B2	10 µl
Add just before use	Enzyme B3	2 µl
	Total Volume	**15 µl**

15. Mix by pipetting or gentle vortexing until homogenous. Spin down.

16. Place the samples in the thermocycler programmed at 25 °C for 15 minutes with **lid heating ON,** followed by a 4 °C hold

17. Transfer each sample to a 1.5 mL tube and clean up the Ligation Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Input	Sample Volume	Bead Volume	Elution Volume
cfDNA	30 µl	36 µl (ratio: 1.2)	20 µl
gDNA	30 µl	30 µl (ratio: 1.0)	20 µl

Indexing PCR

18. Add 5 µl of the appropriate indexed adapter primer(s) directly to each sample.

Reagents	Volume Added to Each Sample (Using Cat. No. 36024)	Volume Added to Each Sample (Using Cat. No. 38096)	Volume added to each sample (Using Cat. No 39096 & 390384)
Index X	5 µl	—	—
Index D50X	—	2.5 µl	—
Index D7XX	—	2.5 µl	—
Index U001-U096	—	—	5 µl

IMPORTANT!
The indexed adapter primers are provided separately as part of the Indexed Adapter Kit.

19. Add 25 µl of the already pre-mixed Indexing PCR Reaction Mix (listed in the table below) to each PCR tube containing 25 µl of sample, using reagents in the order listed below. **Enzyme R3 should be added to the master mix just before use.**

Assembly Order	Reagents	Volume per Sample
Pre-assemble	Low EDTA TE	10 µl
	Buffer R1	10 µl
	Reagent R2	4 µl
Add just before use	Enzyme R3	1 µl
	Total Volume	**25 µl**

20. Mix by pipetting or gentle vortexing until homogenous. Spin down.

21. Place the samples in the thermocycler and run the program, with **lid heating ON**.

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 below, but the exact number of cycles required must be determined by the user.

Input	Recommended PCR Cycles	Thermocycler Program
5 ng cfDNA	7-9	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
100 ng gDNA	4-6	
10 ng gDNA	7-9	
1 ng gDNA	11-13	
100 pg gDNA	14-16	

22. Transfer each sample to a 1.5 mL tube and clean up the Indexing PCR Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Input	Sample Volume	Bead Volume	Elution Volume
cfDNA	50 µl	40.0 µl (ratio: 0.8)	20 µl
gDNA	50 µl	42.5 µl (ratio: 0.85)	20 µl

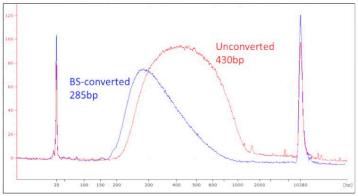
Perform two SPRI clean ups instead of one if sequencing on patterned flow cells, according to the following specifications.

Input	Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
cfDNA	1st clean-up	50 µl	40.0 µl (ratio: 0.8)	50 µl
	2nd clean-up	50 µl	40.0 µl (ratio: 0.8)	20 µl
gDNA	1st clean-up	50 µl	42.5 µl (ratio: 0.85)	50 µl
	2nd clean-up	50 µl	42.5 µl (ratio: 0.85)	20 µl

This modification is recommended only if sequencing on patterned flow cells because reduction of indexing PCR primer carryover into the ExAmp clustering reaction reduces index hopping overall. This modification is not required if sequencing on non-patterned flow cells.

Sequence the DNA Libraries

Expected Results



It is normal to observe a size shift for converted libraries relative to unconverted libraries. This is due to further fragmentation of input DNA during the bisulfite conversion process. Representative Bioanalyzer traces of libraries produced by Accel-NGS are shown here. We recommend using the High Sensitivity Chip or similar.

Library Quantification

Accurate library quantification is essential to properly load the sequencing instrument. Libraries can be quantified using electrophoresis or qPCR-based methods. Electrophoresis-based methods also allow examination of library molecule size distribution. There are many commercially-available qPCR kits available for library quantification. Following the recommended PCR cycles will result in a library concentration of at least 4 nM.

Instrument Compatibility

Libraries prepared with the Accel-NGS Methyl-Seq DNA Library Kit should be sequenced as follows. These precautions consistently lead to highly successful sequencing runs with Q-scores above 90%:

- **MiniSeq:** Use a 20-40% spike in of PhiX or a balanced, high complexity library.
- **MiSeq:** Use a 10% spike in of PhiX or a balanced, high complexity library.
- **NextSeq 500:** Use a 20-40% spike in of PhiX or a balanced, high complexity library.
- **HiSeq 2500:** Use a 10% spike in of PhiX or a balanced, high complexity library.
- **HiSeq 4000/X Ten:** This instrument utilizes a patterned flow cell with a fixed read length of 150 bp PE. For proper cluster formation, we recommend utilizing libraries with a final insert size of approximately 350 bp. Use a 10-20% spike in of PhiX or a balanced, high complexity library. Depending on the RTA version, users may experience low quality scores for Read 2. Please contact Illumina technical support for recommendations.
- **Novaseq:** Use a 25% spike-in of Phix, or a balanced, high complexity library.

These recommendations are subject to change, depending on the version of sequencer software. Please contact Illumina technical support for recommendations.

IMPORTANT!
To ensure optimal mapping efficiency and precise methylation information, bioinformatic trimming of the low complexity "Adaptase tail" from these libraries is required. See Appendix, Section E.

Appendix

Section A: NimbleGen SeqCap Epi Hybridization Capture

Modifications to the standard Accel-NGS Methyl-Seq protocol are necessary when constructing libraries for enrichment by NimbleGen SeqCap Epi. In addition to specific input fragmentation and bead volumes, please use the amplification polymerase provided in the SeqCap Epi Accessory Kit (Cat. No. 07145519001/07185707001) for Pre-Hybridization LM-PCR during the Indexing PCR Step of this protocol. NimbleGen SeqCap Epi hybridization requires the use of single indexing (Swift Cat. No. 36024) for compatibility.

1-12. For steps 1-12, follow the instructions outlined in the standard protocol.

13. Transfer each sample to a 1.5 mL tube and clean up the Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Insert Size	Sample Volume	Bead Volume	Elution Volume
200 bp (SeqCap Epi)	84 µl	101 µl (ratio: 1.2)	15 µl

14-16. For steps 14-16, follow the instructions outlined in the standard protocol.

17. Transfer each sample to a 1.5 mL tube and clean up the Ligation Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Insert Size	Sample Volume	Bead Volume	Elution Volume
200 bp (SeqCap Epi)	30 µl	36 µl (ratio: 1.2)	20 µl

18. Add 5 µl of the appropriate indexed adapter primer(s) directly to each sample.

Reagents	Volume Added to Each Sample (Using Cat. No. 36024)
Index X	5 µl

IMPORTANT!
The indexed adapter primers are provided separately as part of the Indexed Adapter Kit.

19. Add 25 µl of the already pre-mixed Indexing PCR Reaction Mix (listed in the table below) to each PCR tube containing 25 µl of sample, using reagents in the order listed below. **KAPA HiFi HotStart Uracil+ ReadyMix should be added to the master mix just before use.**

Assembly Order	Reagents	Volume per Sample
Add just before use	KAPA HiFi HotStart Uracil+ ReadyMix (2x)	25 µl
	Total Volume	**50 µl**

20. Mix by pipetting or gentle vortexing until homogenous. Spin down.
21. Place the samples in the thermocycler and run the program, with lid heating ON.

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 gDNA	11-13	95 °C for 30 seconds PCR Cycles: 98 °C for 10 seconds 60 °C for 30 seconds 72 °C for 60 seconds Hold at 4 °C
10 ng gDNA	14-16	
1 ng gDNA	17-19	

22. Transfer each sample to a 1.5 mL tube and clean up the Indexing PCR Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Insert Size	Sample Volume	Bead Volume	Elution Volume
200 bp (SeqCap Epi)	50 µl	60.0 µl (ratio: 1.2)	20 µl

Perform two SPRI clean-ups instead of one if sequencing on patterned flow cells, according to the following specifications.

Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
1st clean-up	50 µl	60.0 µl (ratio: 1.2)	50 µl
2nd clean-up	50 µl	60.0 µl (ratio: 1.2)	20 µl

Section B: Small Fragment (≥ 40 bp) Retention

Modifications to the standard Accel-NGS Methyl-Seq protocol are necessary when constructing libraries from samples with small fragments. Please use these bead volumes to retain DNA fragments as small as 40 bp. This may result in increased adapter dimer presence in the final library.

1-12. For steps 1-12, follow the instructions outlined in the standard protocol.

13. Transfer each sample to a 1.5 mL tube and clean up the Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Sample Volume	Bead Volume	Elution Volume
84 µl	151 µl (ratio: 1.8)	15 µl

14-16. For steps 14-16, follow the instructions outlined in the standard protocol.

17. Transfer each sample to a 1.5 mL tube and clean up the Ligation Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Sample Volume	Bead Volume	Elution Volume
30 µl	48 µl (ratio: 1.6)	20 µl

18-21. For steps 18-21, follow the instructions outlined in the standard protocol.

22. Transfer each sample to a 1.5 mL tube and clean up the Indexing PCR Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section C for instructions. Follow the clean-up instructions below.

Sample Volume	Bead Volume	Elution Volume
50 µl	80.0 µl (ratio: 1.6)	20 µl

Perform two SPRI clean-ups instead of one if sequencing on patterned flow cells, according to the following specifications.

Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
1st clean-up	50 µl	80.0 µl (ratio: 1.6)	50 µl
2nd clean-up	50 µl	80.0 µl (ratio: 1.6)	20 µl

Section C: Size Selection/Clean-up Protocol

Please use the following protocol for each clean-up step, substituting the correct **Sample Volume**, **Bead 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. Transfer each Sample Volume to a 1.5 mL tube.
3. Add the specified Bead Volume to each sample. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
4. Incubate the samples for 5 minutes at room temperature (off the magnet).
5. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
6. Remove and discard the supernatant without disturbing the pellet (less than 5 µl may be left behind). Leave the tubes on the magnet.
7. Add 500 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.
8. Repeat step 7 once more for a second wash with the 80% ethanol solution.
9. 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.
10. Add the specified Elution Volume of Low EDTA TE buffer and re-suspend the pellet. Mix well by pipetting up and down until homogenous. Incubate at room temperature for 2 minutes off the magnet, then place the tube on the magnet.
11. Transfer the entire eluate to a new 0.2 mL PCR tube. Ensure that eluate does not contain magnetic beads (indicated by brown coloration in the eluate). If magnetic beads are present, pipette eluate into a new tube, place on magnet, and transfer eluate again.

Post-Extension Clean-Up

Input	Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
cfDNA	Single clean-up	84 µl	101 µl (ratio: 1.2)	15 µl
≥ 10 ng gDNA				
< 10 ng gDNA	1st clean-up	84 µl	101 µl (ratio: 1.2)	50 µl
	2nd clean-up	50 µl	60 µl (ratio: 1.2)	15 µl

Post-Ligation Clean-Up

Input	Sample Volume	Bead Volume	Elution Volume
cfDNA	30 µl	36 µl (ratio: 1.2)	20 µl
gDNA	30 µl	30 µl (ratio: 1.0)	20 µl

Post-Indexing PCR Clean-Up

Input	Sample Volume	Bead Volume	Elution Volume
cfDNA	50 µl	40 µl (ratio: 0.8)	20 µl
gDNA	50 µl	42.5 µl (ratio: 0.85)	20 µl

Perform two SPRI clean ups instead of one if sequencing on patterned flow cells, according to the following specifications.

Input	Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
cfDNA	1st clean-up	50 µl	40.0 µl (ratio: 0.8)	50 µl
	2nd clean-up	50 µl	40.0 µl (ratio: 0.8)	20 µl
gDNA	1st clean-up	50 µl	42.5 µl (ratio: 0.85)	50 µl
	2nd clean-up	50 µl	42.5 µl (ratio: 0.85)	20 µl

Section D: Indexing Kits (Cat. No. 36024, 38096, 39096, and 390384)

During the Indexing PCR step, you must use a unique indexed adapter primer Index X, D50X/D7XX, or U001-U096 to label each library. If no multiplex sequencing is being performed, all libraries may be labeled with a single index 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 of the single and combinatorial dual indices, where the underlined text is replaced by the indexed adapter sequences in the tables below are as follows.

P5 TruSeq® LT Adapter:

5′ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

P7 TruSeq LT Adapter (I2, I4, I5, I6, I7, I12):

5′ GATCGGAAGAGCACACGTCTGAACTCCAGTCAC<u>XXXXXX(XX)</u>CTCGTATGCCGTCTTCTGCTTG

P7 TruSeq LT Adapter (I13, I14, I15, I16, I18, I19):

5′ GATCGGAAGAGCACACGTCTGAACTCCAGTCAC<u>XXXXXX(XX)</u>ATCTCGTATGCCGTCTTCTGCTTG

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 6 bp index sequences, but can be used for 8 bp index reads.

Set A Adapters	Sequence	Cat. No. 36024
Index (I2)	CGATGT(AT)	11 µl
Index (I4)	TGACCA(AT)	11 µl
Index (I5)	ACAGTG(AT)	11 µl

Set A Adapters	Sequence	Cat. No. 36024
Index (I6)	GCCAAT(AT)	11 µl
Index (I7)	CAGATC(AT)	11 µl
Index (I12)	CTTGTA(AT)	11 µl
Index (I13)	AGTCAA(CA)	11 µl
Index (I14)	AGTTCC(GT)	11 µl
Index (I15)	ATGTCA(GA)	11 µl
Index (I16)	CCGTCC(CG)	11 µl
Index (I18)	GTCCGC(AC)	11 µl
Index (I19)	GTGAAA(CG)	11 µl

P5 TruSeq HT Adapter (D501-D508):

5' AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT

P7 TruSeq LT Adapter (D701-D712):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

Dual Index Adapters	Sequence	Cat. No. 38096
Index D501	TATAGCCT	33 µl
Index D502	ATAGAGGC	33 µl
Index D503	CCTATCCT	33 µl
Index D504	GGCTCTGA	33 µl
Index D505	AGGCGAAG	33 µl
Index D506	TAATCTTA	33 µl
Index D507	CAGGACGT	33 µl
Index D508	GTACTGAC	33 µl
Index D701	ATTACTCG	22 µl
Index D702	TCCGGAGA	22 µl
Index D703	CGCTCATT	22 µl
Index D704	GAGATTCC	22 µl
Index D705	ATTCAGAA	22 µl
Index D706	GAATTCGT	22 µl
Index D707	CTGAAGCT	22 µl
Index D708	TAATGCGC	22 µl
Index D709	CGGCTATG	22 µl
Index D710	TCCGCGAA	22 µl
Index D711	TCTCGCGC	22 µl
Index D712	AGCGATAG	22 µl

The following are the full-length adapter sequences of the unique dual indices (UDI)

Index 1 (i7) Adapters:

GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

Index 2 (i5) Adapters:

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT

UDI #	i7 Index Sequence	i5 Index Sequence
U001	CAACACAG	CTTCACAT
U002	ACACCTCA	CACCCAAA
U003	ACCATAGG	TCGAAGTG
U004	CAGGTAAG	TTGACTCT
U005	AACGCACA	TCTCGGTT
U006	TAGTCTCG	ATCACGTT
U007	CAGTCACA	AGCCAACT
U008	CCAACACT	CCACATTG
U009	ACATGCCA	TTGAGCTC
U010	ATTCCGCT	TGACCGTT
U011	CAAGGTAC	GCAACCAT
U012	CCATGAAC	CGCCTTAT
U013	TCAGCCTT	CTTGCTTC
U014	CAGTGCTT	CCGACAAG
U015	CTCGAACA	CACTCGAG
U016	ACAGTTCG	CTGTACGG
U017	ATCCTTCC	CATGAATG
U018	CGAAGTCA	CTATCATG
U019	CTCTATCG	CCACCGAT
U020	ACTCTCCA	CCTAGTAT
U021	TCCTCATG	CAAGACCT
U022	AACAACCG	CGCTTCCT
U023	CTCGTTCT	CCGTATCT
U024	TCAGTAGG	AGTCTGTA
U025	GCTTCACA	CACGTCGT
U026	CGATGTTT	CAATTGAT
U027	TTAGGCAT	ATATTGTA
U028	ACAGTGGT	ATAGCACG
U029	GCCAATGT	ACACATGT
U030	CAGATCTG	ATGTAATG
U031	ACTTGATG	AGTATCTG
U032	TAGCTTGT	ATGCTTGA
U033	TGGTTGTT	AACTGTAT
U034	TGTACCTT	CAGGCATT
U035	TCTGCTGT	AAGGCGAT
U036	TTGGAGGT	GCGTCGAA
U037	TCGAGCGT	GAACGACA
U038	TGATACGT	GGCAAGCA
U039	TGCATAGT	GTAACCGA
U040	TGCGATCT	GCTATGGA
U041	TTCCTGCT	GACACTTA
U042	TACAGGAT	GGTTGGAC
U043	TGTGGTTG	TCAGATTC
U044	TTCCATTG	TATGCCAG
U045	TAACGCTG	TGGCTCAG
U046	TTGGTATG	TCATTGAG
U047	TGAACTGG	TGTATGCG
U048	TACTTCGG	TCCAGTCG

UDI #	i7 Index Sequence	i5 Index Sequence
U049	TCCAGTCG	TACTTCGG
U050	TGTATGCG	TGAACTGG
U051	TCATTGAG	TTGGTATG
U052	TGGCTCAG	TAACGCTG
U053	TATGCCAG	TTCCATTG
U054	TCAGATTC	TGTGGTTG
U055	GGTTGGAC	TACAGGAT
U056	GACACTTA	TTCCTGCT
U057	GCTATGGA	TGCGATCT
U058	GTAACCGA	TGCATAGT
U059	GGCAAGCA	TGATACGT
U060	GAACGACA	TCGAGCGT
U061	GCGTCGAA	TTGGAGGT
U062	AAGGCGAT	TCTGCTGT
U063	CAGGCATT	TGTACCTT
U064	AACTGTAT	TGGTTGTT
U065	ATGCTTGA	TAGCTTGT
U066	AGTATCTG	ACTTGATG
U067	ATGTAATG	CAGATCTG
U068	ACACATGT	GCCAATGT
U069	ATAGCACG	ACAGTGGT
U070	ATATTGTA	TTAGGCAT
U071	CAATTGAT	CGATGTTT
U072	CACGTCGT	GCTTCACA
U073	AGTCTGTA	TCAGTAGG
U074	CCGTATCT	CTCGTTCT
U075	CGCTTCCT	AACAACCG
U076	CAAGACCT	TCCTCATG
U077	CCTAGTAT	ACTCTCCA
U078	CCACCGAT	CTCTATCG
U079	CTATCATG	CGAAGTCA
U080	CATGAATG	ATCCTTCC
U081	CTGTACGG	ACAGTTCG
U082	CACTCGAG	CTCGAACA
U083	CCGACAAG	CAGTGCTT
U084	CTTGCTTC	TCAGCCTT
U085	CGCCTTAT	CCATGAAC
U086	GCAACCAT	CAAGGTAC
U087	TGACCGTT	ATTCCGCT
U088	TTGAGCTC	ACATGCCA
U089	CCACATTG	CCAACACT
U090	AGCCAACT	CAGTCACA
U091	ATCACGTT	TAGTCTCG
U092	TCTCGGTT	AACGCACA
U093	TTGACTCT	CAGGTAAG
U094	TCGAAGTG	ACCATAGG
U095	CACCCAAA	ACACCTCA
U096	CTTCACAT	CAACACAG

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.

Section E: Data Analysis and Informatics

Swift Biosciences' Adaptase technology, used in the Accel-NGS Methyl-Seq DNA Library Kit, adds a low complexity polynucleotide tail with an average length of 8 bases to the 3' end of each fragment during the addition of the first NGS adapter molecule. If these tails are not trimmed bioinformatically from the sequencing data, it is normal and expected to observe them at the beginning of Read 2 (R2). When read length is close to fragment size, the tail may also be observed toward the end of Read 1 (R1) data.

For specific tail trimming recommendations, please consult our Technical Note titled "Accel-NGS 1S Plus and Methyl-Seq: Tail Trimming for Better Data".

Illumina sequencing chemistry, which uses the initial base of each read to perform cluster registration and establish focus and color balance, is sensitive to low complexity base composition at the start of the read. Therefore, it is important to sequence Accel-NGS Methyl-Seq libraries on the MiSeq using MiSeq Software Updater v2.2.0.2 (containing RTA v.1.17.28 rel. 3/18/2013) or later. If using the HiSeq, adding a high-complexity spike-in is recommended. These precautions consistently lead to highly successful sequencing runs with Q-scores above 90%. Quality control software, such as FastQC (Babraham Bioinformatics), may raise "per base sequence content" or "per base GC content" flags at the beginning of R2. These flags are expected due to the low complexity tail.

The Accel-NGS Methyl-Seq Kit adds bases to 3' termini during the Adaptase tailing step, including unmethylated cytosines. This tail adds both artifactual sequence and methylation information to the dataset. Therefore, trimming is required for Accel-NGS Methyl-Seq libraries to obtain improved mapping efficiency (with tools like Bismark or BSMAP) and precise methylation information.

Many informatics pipelines for our Accel-NGS Methyl-Seq library analysis already include trimming of up to 10 bases from the beginning of both R1 and R2 to eliminate any artifactual cytosine methylation introduced as a result of filling in overhangs during end repair steps of conventional dsDNA library preparation and low quality bases due to bisulfite treatment.

Section F: Helpful Information and Troubleshooting

Problem	Possible Cause	Suggested Remedy
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.
Low library yields.	Inaccurate input quantification	• Quantify DNA with Qubit prior to bisulfite conversion
	Significant loss of library following Covaris shearing	• Perform post-shearing clean-up step with 1.8X-2.0X ratio of SPRI beads to maximize recovery of fragmented DNA.
	Less than 50% recovery from bisulfite conversion.	• Quantify DNA present before and after conversion. Add more DNA into conversion or increase number of PCR cycles if recovering less than 50%
	Low quality sample.	• Use the Alu primers included in the Accel-NGS kit to determine quality of sample and adjust input quantity accordingly.
	Suboptimal performance of SPRI clean-up steps	• Ensure that all residual ethanol is removed by performing a spin down of the beads after the second wash. • Ensure that beads do not over-dry during ethanol wash steps. • Ensure that the reaction warms to room temperature prior to starting SPRI clean-up step. • Ensure adequate time (5 minutes) is allowed for DNA-bead binding while off magnet. • Ensure adequate time (2 minutes) is allowed for resuspension of DNA in Low EDTA TE solution. • Ensure that no eluate is left behind during tube transfer.
	Use of the included polymerase for pre-hybridization PCR amplification	• For libraries going into NimbleGen SeqCap Epi hybridization capture, ensure that you are using the amplification polymerase provided in the SeqCap Epi Accessory Kit (Cat. No. 07145519001/07185707001) for Pre-Hybridization LM-PCR during the Indexing PCR Step of this protocol.

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).

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