SWIFT PROTOCOL

ACCEL-NGS® METHYL-SEQ DNA LIBRARY KIT

Single, Combinatorial Dual and Unique Dual Indexing

Protocol for Cat. Nos. 30024, 30096, 330384, 302304 to be used with:

- Methyl-Seq Set A Indexing Kit (12-plex), Cat. No. 36024
- Methyl-Seq Combinatorial Dual Indexing Kit (96-plex), Cat. No. 38096
- Swift Combinatorial Dual Indexing Primer Kits Set S1-S4 (192- to 768-plex)
  - Cat. Nos. X85192, X86192, X87192, X88192, X89768
- Methyl-Seq Unique Dual Indexing Kit (24-plex), Cat. No. 39096
- Methyl-Seq Unique Dual Indexing Kit (96 plex), Cat.No. 390384

- Normalase Combinatorial Dual Indexing Kit (96-plex), Cat. No. 68096
  - Requires Swift Normalase Kit for complete workflow, Cat.No. 66096

- Swift Unique Dual Indexing Primer Plate (96-plex, 96 reactions), Cat. No. X9096-PLATE
- Swift Unique Dual Indexing Primer Plate (96-plex, 4x96 reactions bundle), Cat. No. X90384-PLATE
- Swift Unique Dual Indexing Primer Plate (96-plex, 24x96 reactions bundle), Cat. No. X92304-PLATE

Visit swiftbiosci.com/protocols for updates.

Tech Notes:

- Accel-NGS 1S Plus and Methyl-Seq Tail Trimming

Swift BIOSCIENCES™

Version 3

Table of Contents

Revision History

Document #	Revision	Date	Description of Change
PRT-019	Version 1.0	10/24/2019	Initial Release.
PRT-019	Version 2.0	12/18/19	Added Normalase compatibility.
PRT-019	Version 3.0	2/14/20	Removed tube transfer step for EtOH washes. Added new kit bundles and UDI Primer Plate Cat Nos.

Product Overview

he` Accel-NGS Methyl-Seq technology utilizes Illumina-compatible adapter sequences and has been validated for whole genome bisulfite sequencing (WGBS) and targeted sequencing through hybridization capture. his` kit has been alidated` with genomic DNA, cfDNA, as well as FFPE.

Libraries can be made from as little as 100 pg of high quality starting input DNA. The Adaptase 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 while maintaining superior library complexity.

Applications

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

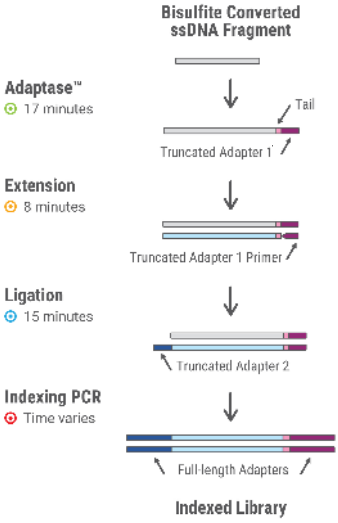
- Whole Genome Bisulfite Sequencing (WGBS)
- Reduced Representation Bisulfite Sequencing (RRBS)*
- Targeted Sequencing by Hybridization capture‡*
- Bisulfite-converted DNA enriched by ChIP or other methods*
- Sequencing ancient DNA samples when retention of fragments containing uracil nucleotides as a result of damage is desired**

**Enrichment methods generally require higher input quantities. Please contact techsupport@swiftbio.com for current hybridization capture and RRBS recommendations.*

‡ 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).

***For samples containing small DNA fragments, like ancient DNA samples, modified bead ratios can be used to retain fragments as small as 40 bases with inputs greater than 10ng (see Appendix, Section A).*

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 performed to generate a uracil-free library molecule.

*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.

*Normalase Indexing Primers can also be used for compatibility with the Normalase workflow (see Appendix Section D and the Swift Normalase Kit protocol for instructions **before starting the Indexing PCR setup**).*

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 Indexing primers), U001-U096 (Unique Dual Indexing primers), or D501N/D7XXN (Normalase Combinatorial Dual Indexing primers), are provided separately in one of the compatible Indexing Kits (see Appendix, Section C).

Reagents	Quantity (µl) 24 rxn	Quantity (µl) 96 rxn	Storage (°C)
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

NOTE: 4x96 and 24x96 kits and UDI Primer Plates are provided in bundles of 96 reaction kits for high-throughput use.

IMPORTANT! Place the enzymes on ice for at least 10 minutes prior to pipetting.

Reagents	Quantity	Storage (°C)
Low EDTA TE	20 mL	Room Temp
Alu 115 and Alu 247 Primers	209 µl of each	-20

Indexing Reagents Provided Separately

Reagents	Swift Cat. No.	Multiplexing	Storage
Single Index Reagent R1 (IX)	36024	12	-20
Combinatorial Dual Index (D50X/D7XX)	38096	96	-20
Unique Dual Index (U001-U024)	39096	24	-20
Unique Dual Index (U001-U096)	390384	96	-20
Normalase Combinatorial Dual Index (D50XN/D7XXN)	68096	96	-20
Swift Unique Dual Indexing Primer Plate (U001-U096)	X9096-PLATE X90384-PLATE X92304-PLATE	96	-20

Material and Equipment Not Included

- A compatible Accel-NGS Methyl-Seq Indexing Kit (see above table and Appendix Section C).
- Swift Normalase Kit (Cat. No. 66096), if using Normalase Indexing Primer kit.
- Bisulfite conversion kit that 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., Permagen® 0.2 mL PCR strip magnet or Agencourt® SPRIPlate™
- qPCR Library quantification kit with primers that target Illumina TruSeq-based adapters
- 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 shearing or MspI digestion
- Microfuge
- Programmable thermocycler
- 0.2 mL PCR tubes
- 1.5 mL microfuge tubes
- Serological pipettes (5 mL - 25 mL)
- 50 mL conical 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 Notes

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 pipetting accuracy of enzyme reagents when ready to use, remove enzyme tubes from -20˚C storage and place on ice for at least 10 minutes prior to pipetting. Attempting to pipette enzymes at -20˚C may result in higher volumes being added to the mastermix, leading to reagent loss.

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.

Although a 10% volume overage is provided, plan to prepare a minimum of 6 reactions for a 24-reaction kit or 24 reactions for a 96-reaction kit to avoid excessive reagent loss from preparing >4 master mixes with 5% overage each.

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.

Always add reagents to the master mix in the specified order as stated throughout the Protocol. Indexing Primers 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 bisulfite conversion, 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 A 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.

Recommended PCR Cycles

Below are examples of recommended PCR cycles for high quality genomic DNA for direct sequencing. Yields are approximate and will vary across sample types. If not using Normalase, please follow the recommended PCR cycles for ≥4nM. If using catalog number 68096 (Normalase Combinatorial Dual Indexing Primers) please follow our recommended PCR cycles for yields of ≥1 2nM. When using reduced quality samples, additional cycles may be required to achieve the minimum threshold. **The (-) in the table indicates inputs not tested.** For targeted sequencing by hyb capture please contact techsupport@swiftbio.com.

Input Material	Input Quantity (ng)	PCR Cycles (≥4nM)	PCR Cycles (≥12nM)
gDNA	100	4-6	8
	10	7-9	11
	1	11-13	-
	0.1	14-16	17
cfDNA	5	7-9	-

Input Material Considerations

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 higher cfDNA input quantities.

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 be observed.

If using more than 100 ng of input material please substitute our Swift high-fidelity polymerase with a high-fidelity uracil tolerant polymerase for library amplification. Please contact techsupport@swiftbio.com for more information.

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 FFPE or other 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 *Optional*
ChIP DNA	Quantify with Qubit or similar fluorometric method
cfDNA	Quantify by qPCR with Alu primer pairs or by electrophoretic methods (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 of genomic 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 in order to assess for bisulfite conversion efficiency. 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 0.1-0.5% (w/w), as specified in the Standards and Guidelines for Whole Genome Shotgun Bisulfite Sequencing (roadmapepigenomics.org/protocols).

DNA Fragmentation

Input Material	Fragmentation Required
High Quality gDNA	Yes
ChIP DNA	No
cfDNA	No
FFPE DNA	Yes

When working with high molecular weight genomic DNA, the DNA must be fragmented prior to bisulfite conversion. Fragmentation may be performed via mechanical shearing, such as sonication to produce 350 bp DNA fragments. After shearing, samples will be further fragmented to an average of 170bp - 200bp during bisulfite conversion; however, no further fragmentation is observed during bisulfite conversion when working with cfDNA samples (Please see page 13 of this protocol for more information). If using other fragment sizes please contact techsupport@swiftbio.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 digest the gDNA followed by DNA purification and isolation of small fragments (100-220 bp) therefore fragmentation by Covaris® is not necessary.

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 a majority of library molecules that are too large for efficient cluster generation on the flow cell resulting in reduced cluster efficiency. Therefore, we do not recommend omitting the fragmentation step.

Note: We recommend analysis of the sheared DNA samples prior to library preparation using electrophoretic methods.

Optional Concentration Step

If 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 NanoDrop on the RNA setting as the DNA will be single-stranded following bisulfite conversion. Low input quantities may not be detectable on the Nanodrop.

IMPORTANT!

This 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 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 Adaptase step for recipe), and place on ice.
2. Pre-heat the thermocycler to 95 °C.
3. Transfer the sample to a 0.2 mL PCR tube and adjust the volume 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 Adaptase program, with **lid heating ON (105 °C**).

Adaptase 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.

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

10. Add 44 µl of the pre-assembled 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.
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 (105 °C)**.

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

13. Clean up the Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section B 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

Safe Stopping Point: Store eluate at 4 ºC until ready to proceed.

Ligation

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

14. Add 15 µl of the pre-assembled Ligation Reaction Mix (listed in the table above) 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**.

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 OFF,** followed by a 4 °C hold

17. Clean up the Ligation Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section B 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

Safe Stopping Point: Store eluate at 4 ºC until ready to proceed or -20 ºC for long term storage.

Indexing PCR

18. Add the appropriate volume of indexing primer listed below 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 and 390384)
Index X	5 µl	–	–
Index D50X	–	2.5 µl	–
Index D7XX	–	2.5 µl	–
Index U001-U096	–	–	5 µl

If using Normalase Indexing Primers (Cat. No. 68096), see Appendix Section D and the Normalase Kit protocol for specific instructions.

IMPORTANT!

The indexing primers are provided separately as part of the Indexing Kit.

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**

**Please contact techsupport@swiftbio.com for compatibility with hyb capture.*

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.**

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.

For inputs greater than 100ng the Swift library amplification reagents provided in the kit must be substituted with a high-fidelity uracil tolerant polymerase mastermix to enable efficient library amplification. The Swift library amplification polymerase is not uracil tolerant and will be inhibited in the presence of a high background of uracil containing DNA in the sample.

Input	Recommended PCR Cycles (>4nM)	Indexing PCR 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. Clean up the Indexing PCR Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section B 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

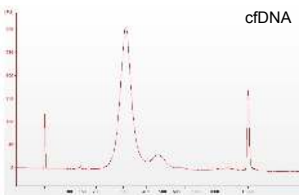
If concerned about index hopping, perform two SPRI clean-ups instead of one if sequencing on patterned flow cells. Reduction in indexing PCR primer carryover into the ExAmp clustering reaction reduces index hopping overall. This modification is not required if sequencing on non-patterend flow cells, if using UDIs, or if you are incorporating the Normalase workflow.

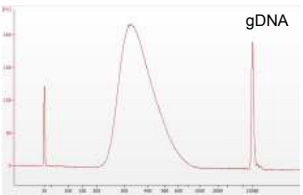
Input	Number of Clean-ups	Sample Volume	Bead Volume	Elution Volume
cfDNA	1st clean-up	50 µl	40.0 µl (ratio: 0.8)	50 µl
	2nd clean-up	50 µl	40.0 µl (ratio: 0.8)	20 µl
gDNA	1st clean-up	50 µl	42.5 µl (ratio: 0.85)	50 µl
	2nd clean-up	50 µl	42.5 µl (ratio: 0.85)	20 µl

Expected Results

Library Quantification

Accurate library quantification is essential to properly load the sequencing instrument. Libraries can be quantified using electrophoretic, fluorometric or qPCR-based methods. Electophoretic-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 4nM for use with our standard indexing primers and 12 nM for use with Normalase Indexing Primers.





Representative Bioanalyzer traces of libraries produced by Accel-NGS Methyl-Seq are shown above. We recommend using the High Sensitivity Chip or similar. A library made from cfDNA results is an average library size of 300bp. A library made from gDNA sheared to 350bp results in a average library size of 330bp.

Considerations for Sequencing

Bisulfite converted DNA libraries are relatively low in base composition complexity due to C depletion. Therefore, it is important to assess the need for PhiX or other balanced high complexity library spike in. Please see Illumina's recommendations based on each of their sequencing instruments. These precautions consistently lead to highly successful sequencing runs to maintain data quality and mapping rates. These recommendations are subject to change, depending on the version of sequencing software. Please contact Illumina for the most up to date PhiX recommendations.

IMPORTANT!

To ensure optimal mapping efficiency and precise methylation information, bioinformatic trimming of the low complexity Adaptase tail from these libraries is required. Please refer to page 14 of this document for more details.

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 "Tail Trimming for Better Data: Accel-NGS Methyl-Seq, Adaptase Module and 1S Plus DNA Library Kits".

The Accel-NGS Methyl-Seq Kit adds bases to 3' termini during the Adaptase tailing step, including unmethylated cytosines. This tail adds a synthetic sequence, adding 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 and bisulfite conversion efficiency.

Many informatics pipelines already include trimming of up to 10 bases from the beginning of both R1 and R2 to eliminate any synthetic 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.

Appendix

Section A: 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 consider that you will need an input of greater than 10ng in order for this to be successful. 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. Clean up the Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section B 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 Extension Reaction using beads and freshly prepared 80% ethanol. See Appendix, Section B 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 B 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 (please see page 12 for more detials).

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 B: 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. Prepare a fresh solution of 80% EtOH. It is important that the solution is fresh as evaporation of EtOH can occur with prolonged storage, resulting in DNA being eluted off the beads during wash steps.
2. Ensure the magnetic beads are at room temperature and vortex beads to homogenize the suspension before use.
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 200 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 (see page 12 for more details).

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 C: Indexing Kits (Cat. No. 36024, 38096, 39096, 390384, and 68096)

During the Indexing PCR step, you must use a unique indexing primer Index X, D50X/D7XX, D50XN/D7XXN 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 pooled for cluster generation and co-sequenced on the same Illumina flow cell. Please note that the reverse complement of the i5 sequence must be entered into the sample sheet for the following sequencers: iSeq 100, MiniSeq, NextSeq, HiSeq X, HiSeq 4000, and HiSeq 3000.

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.

Set A Indexing Primers (Cat.No. 36024)

TruSeq Universal Adapter:

5′ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

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

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

TruSeq Index 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
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

Combinatorial Dual Indexing Primers (Cat. No. 38096)

TruSeq Index 1 (i7) Adapter (D501-D508):

5′ AATGATACGGCGACCACCGAGATCTACAC<u>XXXXXXXX</u>ACACTCTTTCCCTACACGACGCTCTTCCGATCT

TruSeq Index 2 (i5) Adapter (D701-D712):

5′ GATCGGAAGAGCACACGTCTGAACTCCAGTCAC<u>XXXXXXXX</u>ATCTCGTATGCCGTCTTCTGCTTG

i5 Adapters	i5 Index Seqeunce for NovaSeq, MiSeq, HiSeq2500	i5 Index Seqeunce for HiSeq4000, NextSeq, MiniSeq
Index D501	TATAGCCT	AGGCTATA
Index D502	ATAGAGGC	GCCTCTAT
Index D503	CCTATCCT	AGGATAGG
Index D504	GGCTCTGA	TCAGAGCC
Index D505	AGGCGAAG	CTTCGCCT
Index D506	TAATCTTA	TAAGATTA
Index D507	CAGGACGT	ACGTCCTG
Index D508	GTACTGAC	GTCAGTAC

i7 Adapters	Sequence
Index D701	ATTACTCG
Index D702	TCCGGAGA
Index D703	CGCTCATT
Index D704	GAGATTCC
Index D705	ATTCAGAA
Index D706	GAATTCGT
Index D707	CTGAAGCT
Index D708	TAATGCGC
Index D709	CGGCTATG
Index D710	TCCGCGAA
Index D711	TCTCGCGC
Index D712	AGCGATAG

Unique Dual Indexing Primers (Cat. No. 39096, 390384, X9096-PLATE, X90384-PLATE and X-92304-PLATE)

The following are the full-length adapter sequences of the unique dual indices (UDI) TruSeq Index 1 (i7) Adapters:

GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

TruSeq Index 2 (i5) Adapters:

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT

UDI #	i7 Index Sequence	i5 Index Sequence for NovaSeq, MiSeq, HiSeq2500	i5 Index Sequence for HiSeq4000, NextSeq, MiniSeq
U001	CAACACAG	CTTCACAT	ATGTGAAG
U002	ACACCTCA	CACCCAAA	TTTGGGTG
U003	ACCATAGG	TCGAAGTG	CACTTCGA
U004	CAGGTAAG	TTGACTCT	AGAGTCAA
U005	AACGCACA	TCTCGGTT	AACCGAGA
U006	TAGTCTCG	ATCACGTT	AACGTGAT
U007	CAGTCACA	AGCCAACT	AGTTGGCT
U008	CCAACACT	CCACATTG	CAATGTGG
U009	ACATGCCA	TTGAGCTC	GAGCTCAA
U010	ATTCCGCT	TGACCGTT	AACGGTCA
U011	CAAGGTAC	GCAACCAT	ATGGTTGC
U012	CCATGAAC	CGCCTTAT	ATAAGGCG
U013	TCAGCCTT	CTTGCTTC	GAAGCAAG
U014	CAGTGCTT	CCGACAAG	CTTGTCGG
U015	CTCGAACA	CACTCGAG	CTCGAGTG
U016	ACAGTTCG	CTGTACGG	CCGTACAG
U017	ATCCTTCC	CATGAATG	CATTCATG
U018	CGAAGTCA	CTATCATG	CATGATAG
U019	CTCTATCG	CCACCGAT	ATCGGTGG
U020	ACTCTCCA	CCTAGTAT	ATACTAGG
U021	TCCTCATG	CAAGACCT	AGGTCTTG
U022	AACAACCG	CGCTTCCT	AGGAAGCG
U023	CTCGTTCT	CCGTATCT	AGATACGG
U024	TCAGTAGG	AGTCTGTA	TACAGACT
U025	GCTTCACA	CACGTCGT	ACGACGTG
U026	CGATGTTT	CAATTGAT	ATCAATTG
U027	TTAGGCAT	ATATTGTA	TACAATAT
U028	ACAGTGGT	ATAGCACG	CGTGCTAT
U029	GCCAATGT	ACACATGT	ACATGTGT
U030	CAGATCTG	ATGTAATG	CATTACAT
U031	ACTTGATG	AGTATCTG	CAGATACT
U032	TAGCTTGT	ATGCTTGA	TCAAGCAT
U033	TGGTTGTT	AACTGTAT	ATACAGTT
U034	TGTACCTT	CAGGCATT	AATGCCTG
U035	TCTGCTGT	AAGGCGAT	ATCGCCTT
U036	TTGGAGGT	GCGTCGAA	TTCGACGC
U037	TCGAGCGT	GAACGACA	TGTCGTTC
U038	TGATACGT	GGCAAGCA	TGCTTGCC
U039	TGCATAGT	GTAACCGA	TCGGTTAC
U040	TGCGATCT	GCTATGGA	TCCATAGC
U041	TTCCTGCT	GACACTTA	TAAGTGTC

UDI #	i7 Index Sequence	i5 Index Sequence for NovaSeq, MiSeq, HiSeq2500	i5 Index Sequence for HiSeq4000, NextSeq, MiniSeq
U049	TCCAGTCG	TACTTCGG	CCGAAGTA
U050	TGTATGCG	TGAACTGG	CCAGTTCA
U051	TCATTGAG	TTGGTATG	CATACCAA
U052	TGGCTCAG	TAACGCTG	CAGCGTTA
U053	TATGCCAG	TTCCATTG	CAATGGAA
U054	TCAGATTC	TGTGGTTG	CAACCACA
U055	GGTTGGAC	TACAGGAT	ATCCTGTA
U056	GACACTTA	TTCCTGCT	AGCAGGAA
U057	GCTATGGA	TGCGATCT	AGATCGCA
U058	GTAACCGA	TGCATAGT	ACTATGCA
U059	GGCAAGCA	TGATACGT	ACGTATCA
U060	GAACGACA	TCGAGCGT	ACGCTCGA
U061	GCGTCGAA	TTGGAGGT	ACCTCCAA
U062	AAGGCGAT	TCTGCTGT	ACAGCAGA
U063	CAGGCATT	TGTACCTT	AAGGTACA
U064	AACTGTAT	TGGTTGTT	AACAACCA
U065	ATGCTTGA	TAGCTTGT	ACAAGCTA
U066	AGTATCTG	ACTTGATG	CATCAAGT
U067	ATGTAATG	CAGATCTG	CAGATCTG
U068	ACACATGT	GCCAATGT	ACATTGGC
U069	ATAGCACG	ACAGTGGT	ACCACTGT
U070	ATATTGTA	TTAGGCAT	ATGCCTAA
U071	CAATTGAT	CGATGTTT	AAACATCG
U072	CACGTCGT	GCTTCACA	TGTGAAGC
U073	AGTCTGTA	TCAGTAGG	CCTACTGA
U074	CCGTATCT	CTCGTTCT	AGAACGAG
U075	CGCTTCCT	AACAACCG	CGGTTGTT
U076	CAAGACCT	TCCTCATG	CATGAGGA
U077	CCTAGTAT	ACTCTCCA	TGGAGAGT
U078	CCACCGAT	CTCTATCG	CGATAGAG
U079	CTATCATG	CGAAGTCA	TGACTTCG
U080	CATGAATG	ATCCTTCC	GGAAGGAT
U081	CTGTACGG	ACAGTTCG	CGAACTGT
U082	CACTCGAG	CTCGAACA	TGTTCGAG
U083	CCGACAAG	CAGTGCTT	AAGCACTG
U084	CTTGCTTC	TCAGCCTT	AAGGCTGA
U085	CGCCTTAT	CCATGAAC	GTTCATGG
U086	GCAACCAT	CAAGGTAC	GTACCTTG
U087	TGACCGTT	ATTCCGCT	AGCGGAAT
U088	TTGAGCTC	ACATGCCA	TGGCATGT
U089	CCACATTG	CCAACACT	AGTGTTGG

U039	TGCATAGT	GTAACCGA	TCGGTTAC
U040	TGCGATC.	GCTATGGA	TCCATAGC
U041	TTCCTGCT	GACACTTA	TAAGTGTC
U042	TACAGGAT	GGTTGGAC	GTCCAACC
U043	TGTGGTTG	TCAGATTC	GAATCTGA
U044	TTCCATTG	TATGCCAG	CTGGCATA
U045	TAACGCTG	TGGCTCAG	CTGAGCCA
U046	TTGGTATG	TCATTGAG	CTCAATGA
U047	TGAACTGG	TGTATGCG	CGCATACA
U048	TACTTCGG	TCCAGTCG	CGACTGGA

U087	TGACCGTT	ATTCCGCT	AGCGGAAT
U088	TTGAGCTC	ACATGCCA	TGGCATGT
U089	CCACATTG	CCAACACT	AGTGTTGG
U090	AGCCAACT	CAGTCACA	TGTGACTG
U091	ATCACGTT	TAGTCTCG	CGAGACTA
U092	TCTCGGTT	AACGCACA	TGTGCGTT
U093	TTGACTCT	CAGGTAAG	CTTACCTG
U094	TCGAAGTG	ACCATAGG	CCTATGGT
U095	CACCCAAA	ACACCTCA	TGAGGTGT
U096	CTTCACAT	CAACACAG	CTGTGTTG

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

Swift Normalase™ Combinatorial Dual Indexing PrimerKit (Cat. No. 68096)

TruSeq Index 1 (i5) Adapter (D501N-D508N):

5' AATGATACGGCGACCACCGAGATCTACAC<u>XXXXXXXX</u>ACACTCTTTCCCTACACGACGCTCTTCCGATCT

TruSeq Index 2 (i7) Adapter (D701N-D712N):

5' GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

i5 Adapters	i5 Index Seqeunce for NovaSeq, MiSeq, HiSeq2500	i5 Index Seqeunce for HiSeq4000, NextSeq, MiniSeq
Index D501N	TATAGCCT	AGGCTATA
Index D502N	ATAGAGGC	GCCTCTAT
Index D503N	CCTATCCT	AGGATAGG
Index D504N	GGCTCTGA	TCAGAGCC
Index D505N	AGGCGAAG	CTTCGCCT
Index D506N	TAATCTTA	TAAGATTA
Index D507N	CAGGACGT	ACGTCCTG
Index D508N	GTACTGAC	GTCAGTAC

i7 Adapters	Sequence
Index D701N	ATTACTCG
Index D702N	TCCGGAGA
Index D703N	CGCTCATT
Index D704N	GAGATTCC
Index D705N	ATTCAGAA
Index D706N	GAATTCGT
Index D707N	CTGAAGCT
Index D708N	TAATGCGC
Index D709N	CGGCTATG
Index D710N	TCCGCGAA
Index D711N	TCTCGCGC
Index D712N	AGCGATAG

Section D: Normalase Instructions

Please review this section and the Swift Normalase™ Kit protocol before setting up your Indexing PCR reaction. In order to achieve the expected results, amplify each library using Normalase Indexing Primers with the appropriate number of cycles and thermocycling conditions below to obtain a library yield of 12nM or greater in a 20 µl eluate.

1. Add 2 µl of each Normalase Combinatorial Dual Index to each sample (24 µl total volume).

Normalase Reagent	Volume added to each sample
D50XN	2 µl
D7XXN	2 µl

**See Appendix Section C for the sequences corresponding to each index.*

2. Assemble the PCR master mix on ice. Mix thoroughly and pulse spin to collect contents. Add 26 µl of the mix to each sample tube, mix thoroughly and pulse spin to collect contents (50 µl total reaction volume).

Components	Volume per Sample
PCR Master Mix	25 µl
Reagent R6	1 µl
Total Volume	**26 µl**

3. Run the following thermocycler program, adjusting the number of cycles depending on the input amount and sample quality (see table below):

Thermocycler Program	
98 °C, 2 min	
98 °C, 20 sec	**Perform X number of cycles***
60 °C, 30 sec	
72 °C 30 sec	
72 °C, 5 minute	
4 °C hold	

**The recommended minimum number of cycles for each input in order to provide ≥12nM yields suitable for the Normalase workflow is as follows:*

Input	Recommended PCR Cycles (≥12nM)
5 ng cfDNA	-
100 ng gDNA	8
10 ng gDNA	11
1 ng gDNA	-
100 pg gDNA	17

4. Proceed to SPRI clean-up, Normalase I, Pooling and Normalase II in the Swift Normalase Kit protocol.

Section E: 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 your gDNA with Qubit prior to bisulfite conversion. For cfDNA and FFPE, refer to section "Input DNA Quantification" for detailed information.
	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 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 beads and reaction are at room temperature prior to starting SPRI clean-up steps. • 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 hybridization capture, ensure that you are using the amplification polymerase provided in your hybridization capture kit. Please contact techsupport@swiftbio.com for more information
Low mapping rates/low bisulfite conversion rates	Failing to bioinformatically trim Adaptase tails	• Please refer to the Accel-NGS 1S and Methyl-Seq Tail Trimming technical note

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

Section F: High Throughput Use

The protocol is readily automatable. A 10% overage volume of reagents is supplied in both the 24 and 96 reaction kits to accommodate automation. Please contact us at Automation@swiftbio.com if you require additional reagent overage volume or would like to learn about our custom packaging options. While Swift Biosciences does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop and qualify optimized automated scripts for use of our kits, with liquid handling platforms routinely used in NGS library preparation. Please contact us at Automation@swiftbio.com to discuss automating your Accel-NGS Methyl-Seq kit with your automated liquid handling system.

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