Swift BIOSCIENCES™

Protocol

Accel-NGS® DNA Library Kit for the Ion Torrent™ Platform

PCR-Free NGS Library Preparation

Cat. No. 11010/11050

Contents

Introduction

The Accel-NGS DNA Library Kit for Ion Torrent is designed to enable you to make high complexity, PCR-free next-generation sequencing (NGS) libraries from single- or double-stranded DNA with input ranging from 5 ng to 5 µg. The technology underlying Accel-NGS does not require intact double-stranded DNA (dsDNA), making it compatible with single-stranded, denatured, or nicked samples. The unique, sequential adaptation process also reduces adapter dimer formation to further maximize sequencing output. The kit has been validated for use on the Ion PGM™ and Ion Proton™ instruments.

The Accel-NGS DNA Library Kit for Ion Torrent is suitable for the following sample types and applications:

Sample Types
Single-stranded samples
Double-stranded samples (heat-denatured)
Nicked DNA samples (inquire for recommended changes to bead-based clean-ups)
Samples with a mixture of single-stranded and double-stranded DNA
First strand cDNA

Applications
Whole Genome Sequencing (genomic DNA and Whole Genome Amplification samples)
Amplicon Sequencing (long range PCR fragments and multiplex PCR)
ChIP-Seq

Our Technical Support team may be reached at TechnicalSupport@swiftbiosci.com or by calling 734.330.2568 and pressing 2 when prompted.

Before You Start

- Upon receipt, store the kit at -20 ºC.
- Please read this manual carefully before starting.

Kit Contents

Kits contain enough reagents for the preparation of either 10 or 50 libraries (10% excess volume provided).

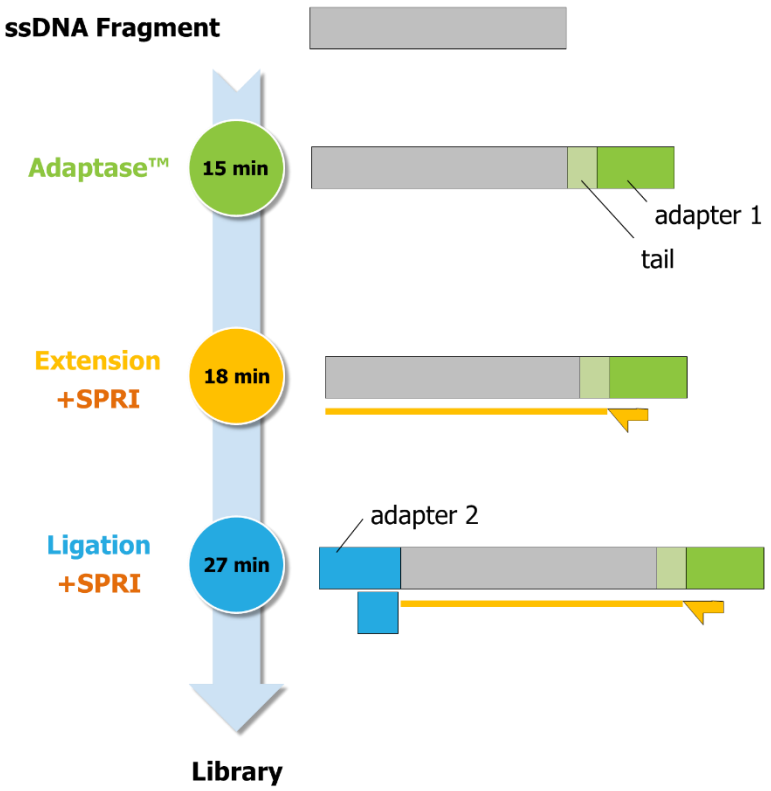
Kit	Reagents	10 Reactions	50 Reactions	Kit	Reagents	10 Reactions	50 Reactions
Adaptase Reagents	Reagent G1	44.0 µl	220.0 µl	Extension Reagents	Reagent Y1	33 µl	165.0 µl
	Reagent G2	88.0 µl	440.0 µl		Enzyme Y2	460 µl	2.2 ml
	Reagent G3	27.5 µl	137.5 µl	Ligation Reagents	Buffer B1	44 µl	220.0 µl
	Enzyme G4	33.0 µl	165.0 µl		Non-barcoded Reagent B2*	110 µl	550.0 µl
Buffer	Low EDTA	2.0 ml	5.0 ml		Enzyme B3	33 µl	165.0 µl

* A barcoded adapter may be used in place of non-barcoded Reagent B2 when multiplexing. This requires the Accel-NGS Barcoding Kit for Ion Torrent (Cat. No. 11010/11050) (see Appendix).

Required Materials Not Supplied

- If multiplexing is desired, a compatible Accel-NGS Barcoding Kit for Ion Torrent (Reagent B2) (see Appendix)
  - Barcoding Kit for Ion Torrent (Barcodes 1-10, Set A), Cat. No. 16110
  - Barcoding Kit for Ion Torrent (Barcodes 11-20, Set B), Cat. No. 16210
  - Barcoding Kit for Ion Torrent (Barcodes 1-10, Set A), Cat. No. 16150
  - Barcoding Kit for Ion Torrent (Barcodes 11-20, Set B), Cat. No. 16250
- SPRIselect™ (Beckman Coulter, Cat. No. B23317/B23318/B23319)
- Invitrogen DynaMag™, Agencourt® SPRIPlate™, or similar magnetic rack for bead clean-ups
- qPCR-based library quantification kit, such as:
  - KAPA Biosystems, Cat. No. KK4827
  - Life Technologies, Cat. No. 4468802
- NanoDrop®, Qubit® or other device for determining DNA concentration
- Fragmentation device and associated reagents for DNA shearing (e.g., Covaris®)
- Microcentrifuge
- Programmable thermocycler
- 0.2 ml PCR tubes
- 1.5 ml microfuge tubes
- Aerosol-resistant tips and P2 through P1000 range pipettes
- 200-proof/absolute ethanol (molecular biology grade)
- Nuclease-free water (molecular biology grade)

Protocol Overview



The protocol uses a unique, sequential adaptation process to attach adapters to the ends of single-stranded DNA fragments.

- The Adaptase step is a highly efficient, proprietary reaction that simultaneously performs end repair, tailing of 3' ends, and ligation of the first adapter to 3' ends.
- The Extension step is used to facilitate ligation of the second adapter. The synthesized strand lacks full-length adapters and does not get sequenced.
- Bead-based SPRI clean-ups are used to remove oligonucleotides and small fragments, and to change enzymatic buffer composition.
- The Ligation reaction is used to add the second adapter to the 5' ends.
- Libraries can be prepared from as low as 5 ng input material. The method is ideal for samples containing single-stranded or nicked DNA, as well as first strand cDNA.

Notes on Starting Input Material

- 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.
- To reduce the risk of DNA and library contamination, particularly at low input:
  1. Clean lab areas using 0.5% Sodium Hypochlorite (10% Bleach).
  2. Use specialty barrier pipette tips that seal when exposed to potential contaminants.

Prepare the Library

For best results, please follow these suggestions:

- To maximize efficient use of enzyme reagents, remove enzyme tubes from -20 °C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow reagents 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, invert or briefly vortex (except enzymes) to mix them well. Spin down tubes before opening.
- If you have a limiting amount of starting input material, fragment your sample to the smallest acceptable size in order to maximize yield, as indicated in the table below. This is due to the impact of fragmentation and size selection on yield.
- For heavily damaged samples, it is important to use a quality control metric to analyze DNA integrity and purity. If you have questions related to sample quality, please contact us.
- If preparing multiple libraries at once, assemble reagent master mixes for each step and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Multiplexing samples for sequencing on the same chip requires the Accel-NGS Barcoding Kit for Ion Torrent (Cat. No. 11010/11050, sold separately).
- Always add enzymes last to master mixes, immediately before adding to samples.
- Before starting, prepare a fresh 80% ethanol solution. Approximately 720 µl will be used per sample; formula is for proper volumetric addition and excess.
- All loading calculations for PCR-free libraries must be based on quantification by qPCR, not Bioanalyzer, in order to accurately load the OneTouch 2. For these reasons, we recommend using the KAPA Library Quantification Kit – Illumina/Universal, Cat. No. KK4824 to quantify your libraries.
- Pre-program a thermocycler with the following programs to expedite the workflow:

Denaturation Thermocycler Program
95 °C for 2 minutes
4 °C hold

Extension Thermocycler Program
98 °C for 1 minute
65 °C for 5 minutes
72 °C for 1 minute
4 °C hold

Adaptase Thermocycler Program
37 °C for 10 minutes
95 °C for 2 minutes for enzyme inactivation
4 °C hold

Ligation Thermocycler Program
25 °C for 15 minutes
4 °C hold

DNA Fragmentation

1. Determine DNA concentration and purity using a NanoDrop with A260/280 ratio, Qubit, or similar method. Accurate determination of DNA input amount and purity is critical for Accel-NGS performance. However, it is not necessary to specifically quantify dsDNA, as Accel-NGS is compatible with both ssDNA and dsDNA.
2. Fragment the DNA. Multiple fragmentation methods are available; this kit was validated on Covaris-fragmented DNA in the appropriate size range. Do not denature double-stranded samples prior to fragmentation. When possible, fragment double-stranded DNA as yield of desired insert size will be more consistent. Fragmentation of single-stranded DNA may result in shorter fragments and lower library yield. Depending on the method used, optimization may be required. Please note that single-stranded DNA cannot be visualized by Bioanalyzer. Either other means are required, or finished libraries can be assessed by Bioanalyzer.

STARTING MATERIAL		SEQUENCING
Supported Input	**Insert Size**	**Sequencing Type**
5 ng-5 µg	100 bases	100-base-read
10 ng-5 µg	200 bases	200-base-read

Other insert sizes are possible by adjusting SPRISelect bead ratios.

Optional Concentration Step

If you have performed enzymatic reactions, including enzymatic fragmentation, OR your fragmented DNA concentration is too low to provide sufficient quantity in the 10 µl DNA starting volume specified in the Adaptase step, concentrate with Zymo Research's DNA Clean & Concentrator™, or other method, and elute in 10 µl of the Low EDTA TE buffer supplied.

Otherwise, skip to the **Denaturation Step**.

Denaturation

1. Use the **Denaturation Thermocycler Program** on the thermocycler and pause it at the first step to pre-heat to 95 °C until all samples are ready to be loaded.
2. Add 10 µl of fragmented DNA to a 0.2 ml PCR tube.
3. Place each sample in the thermocycler and run the program to denature the DNA. Place on ice **immediately** for 2 minutes. Proceed immediately to the **Adaptase** step.

Adaptase

1. Use the **Adaptase Thermocycler Program** on the thermocycler and pause it at the first step to pre-heat to 37 °C until all samples are mixed and loaded.
2. On ice, make the **Adaptase Reaction Mix** with the following amounts of each reagent. Add the reagents in the specified order.

Reagent	Volume (1 Reaction)
Low EDTA TE	12.5 µl
Reagent G1	4.0 µl
Reagent G2	8.0 µl
Reagent G3	2.5 µl
Enzyme G4	3.0 µl

3. Mix the **Adaptase Reaction Mix** well, and then add 30 µl to each PCR tube containing 10 µl of denatured DNA. Place each sample in the thermocycler and run the program.

Extension

1. Load the **Extension Thermocycler Program** on the thermocycler and pause it at the first step (98 °C) until all samples are mixed and loaded.
2. On ice, make an **Extension Reaction Mix** with the following amounts of each reagent. Add the reagents in the specified order.

Reagent	Volume (1 Reaction)
Reagent Y1	3 µl
Enzyme Y2	43 µl

3. Mix the **Extension Reaction Mix** well, and then add 46 µl to each PCR tube containing a 40 µl **Adaptase Reaction**. Mix by pipetting. Place in the thermocycler and run the program.

SPRI Step 1

Clean up the **Extension Reaction** using SPRIselect beads (for protocol, see Appendix) and freshly prepared 80% ethanol.

Insert Size	Sample Volume	SPRI Volume	Elution Volume
100 bp	86 µl	120 µl (ratio: 1.4)	20 µl
200 bp	86 µl	69 µl (ratio: 0.8)	20 µl

Store eluate at 4 °C until ready to proceed.

Ligation

1. Load the **Ligation Thermocycler Program** on the thermocycler and pause it at the first step (25 °C) until all samples are mixed and loaded.
2. Make a **Ligation Reaction Mix** with the following amounts of each reagent. Keep on ice.

   When barcoding multiple samples, use a barcoded Reagent B2 (Accel-NGS Barcoding Kit for Ion Torrent; see Appendix).

   If multiplexing: Make the Ligation Reaction Mix below with all components except non-barcoded Reagent B2. Add 10 µl of the appropriate barcoded Reagent B2 (Cat. No. 11010/11050) directly to each 20 µl eluate, and then add 10 µl of pre-mixed Ligation Reaction Mix to each sample. The final reaction volume for each sample is 40 µl.

   If not multiplexing: Make the Ligation Reaction Mix below, including 10 µl per reaction of non-barcoded Reagent B2. Add 20 µl of the Ligase Reaction Mix to each PCR tube containing a 20 µl eluate. The final reaction volume for each sample is 40 µl. Add the reagents in the specified order.

Reagent	Volume (1 Reaction)
Buffer B1	4 µl
Reagent B2	10 µl
Enzyme B3	3 µl
Low EDTA TE	3 µl

3. Mix the reaction well. Place each sample in the thermocycler and run the program.

After incubation, add 10 µl of Low EDTA TE buffer to the completed 40 µl reaction and proceed to SPRI Step 2.

SPRI Step 2

Clean up the **Ligation Reaction** using SPRIselect beads (for protocol, see Appendix) and freshly prepared 80% ethanol.

Insert Size	Sample Volume	SPRI Volume	Elution Volume
100 bp	50 µl	70 µl (ratio: 1.4)	20 µl
200 bp	50 µl	40 µl (ratio: 0.8)	20 µl

Store freshly prepared libraries at 4 °C. Your library is now ready for quantification, which should be performed by qPCR.

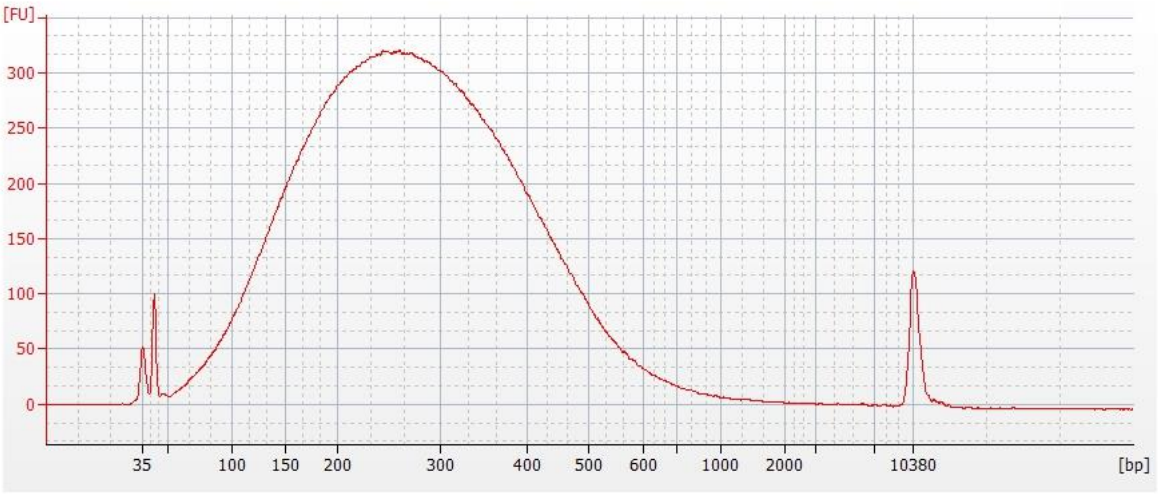
Note: When preparing PCR-free libraries, it is important to specifically quantify functionally-adapted molecules prior to using the OneTouch 2. Since PCR-free libraries are unenriched for functional library molecules, a Bioanalyzer trace will also reflect unused adapters and partially adapted library molecules. Hence, quantifying by this method inaccurately estimates the number of library molecules. Using a qPCR-based method, such as the Ion Library Quantitation Kit (Life Technologies Cat. No. 4468802) or KAPA's Library Quantification Kit for Ion Torrent (Cat. No. KK4827), is recommended since it will lead to more accurate loading of the correct number of functional library molecules into the emulsion PCR.

Expected Results

Expected dilution factors for input quantities, regardless of insert size:

Input Quantity	Expected Dilution Factor
5 ng	> 1
10 ng	> 1
100 ng	> 20

Example library size distribution by Agilent Bioanalyzer for a 150 bp insert size library prepared from *E. coli* DNA



With lower input quantities, library concentration may be too low to visualize by Bioanalyzer without PCR, even when using the High Sensitivity Chip.

Appendix

SPRIselect Clean-Up Protocol

Please use the following protocol for each SPRI Step, substituting in the correct **Sample Volume**, **SPRI Volume,** and **Elution Volume** as indicated in the table for each step:

1. Invert or briefly vortex beads to homogenize the suspension before use.
2. For samples with total volumes (**Sample Volume** + **SPRI Volume**) greater than 180 µl, transfer each **Sample Volume** sample to a 1.5 ml tube. Add **SPRI Volume** beads to each sample. Mix by pipetting 10 times or until homogenous. Ensure no bead-sample suspension droplets are left on the sides of the tube.
3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microfuge. Place the sample tubes 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.
6. Add 180 µl of freshly prepared ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, and then carefully remove the ethanol solution.
7. Repeat step 6 once for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microfuge, place back onto the magnet and remove any residual ethanol solution from the bottom of the tube.
9. Air-dry the pellet, watching the pellet to avoid cracking or over-drying.
10. Add **Elution Volume** of Low EDTA TE to resuspend the pellet, mixing well by pipetting up and down until homogenous. If droplets of the resuspension are on the side of the tube, pulse-spin the tube in a microfuge to collect contents. After at least 2 minutes, place the tube on the magnet. 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 eluate). If magnetic beads are present, pipette eluate into a new tube, place on magnet, and transfer eluate again.

Helpful Information and Troubleshooting

Data Analysis and Informatics Considerations

Swift's Adaptase technology adds a homopolymer tail with an average read length of 6-12 nucleotides to the 3' end of each fragment during the addition of the first adapter molecule. Therefore, it is normal and expected to observe such tails at the end of the read. Alignment to reference genomes does not require trimming of the tails.

Quality control software, such as FastQC (Babraham Bioinformatics), may raise "Per base sequence content" or "Per base GC content" flags. These flags are expected due to the homopolymer tail. At low input, an "overrepresented sequence" flag may be raised due to adapter dimer contamination. Please contact TechnicalSupport@swiftbiosci.com with further questions.

Troubleshooting Common Problems

Problem	Possible Cause	Suggested Remedy
Library shows Bioanalyzer peaks at ≈ 30-45 bp.	PCR-free protocol allows short oligonucleotides through the final SPRI clean-up and into the product.	Quantify the library by qPCR for emPCR; the short oligonucleotides are not dimers and will not affect sequencing.
Input is more dilute than 5 ng of pure DNA in a 10 µl volume.	Input DNA is too dilute.	Concentrate DNA with column purification kit, SpeedVac™, or other method.
DNA does not fragment properly/broad or lop-sided (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.

If you experience problems with your library prep, please contact us at TechnicalSupport@swiftbiosci.com or by calling 734.330.2568.

Indexed Adapter Sequences

When you reach the **Ligation** step in the protocol, you may use a unique barcoded adapter in place of non-barcoded Reagent B2 to label each library. Libraries made with unique barcoded adapters may be multiplexed during emulsion PCR and co-sequenced on the same Ion Torrent sequencing chip.

CONTENTS: Twenty unique barcoded adapters, Barcode 1 (B2) through Barcode 20 (B2), provided at the same concentration as the non-barcoded Reagent B2 (provided in Cat. No. 11010/11050), and which should be used as a direct replacement for non-barcoded Reagent B2 at the 10 µl volume indicated in the **Ligation** step in the library preparation manual.

Barcoded Adapter	Sequence*	16110	16150	16210	16250
Barcode 1 (B2)	CTAAGGTAAC	10 µl	50 µl	---	---
Barcode 2 (B2)	TAAGGAGAAC	10 µl	50 µl	---	---
Barcode 3 (B2)	AAGAGGATTC	10 µl	50 µl	---	---
Barcode 4 (B2)	TACCAAGATC	10 µl	50 µl	---	---
Barcode 5 (B2)	CAGAAGGAAC	10 µl	50 µl	---	---
Barcode 6 (B2)	CTGCAAGTTC	10 µl	50 µl	---	---
Barcode 7 (B2)	TTCGTGATTC	10 µl	50 µl	---	---
Barcode 8 (B2)	TTCCGATAAC	10 µl	50 µl	---	---
Barcode 9 (B2)	TGAGCGGAAC	10 µl	50 µl	---	---
Barcode 10 (B2)	CTGACCGAAC	10 µl	50 µl	---	---
Barcode 11 (B2)	TCCTCGAATC	---	---	10 µl	50 µl
Barcode 12 (B2)	TAGGTGGTTC	---	---	10 µl	50 µl
Barcode 13 (B2)	TCTAACGGAC	---	---	10 µl	50 µl
Barcode 14 (B2)	TTGGAGTGTC	---	---	10 µl	50 µl
Barcode 15 (B2)	TCTAGAGGTC	---	---	10 µl	50 µl
Barcode 16 (B2)	TCTGGATGAC	---	---	10 µl	50 µl
Barcode 17 (B2)	TCTATTCGTC	---	---	10 µl	50 µl
Barcode 18 (B2)	AGGCAATTGC	---	---	10 µl	50 µl
Barcode 19 (B2)	TTAGTCGGAC	---	---	10 µl	50 µl

*These sequences are identical to the IonXpress_001 through IonXpress_020 barcodes.

The number on the product tube label indicates which barcoded adapter is provided in the tube. During library prep, make sure to note which barcoded adapter you are using with your sample and do not use the same barcoded adapter on two different samples you plan to multiplex together.

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