PROTOCOL

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ACCEL-NGS® 2S DNA LIBRARY KITS

Dual Indexing up to 768-Plex

Protocol for:

- Accel-NGS 2S PCR-Free (Cat. Nos. 20024, 20096)
- Accel-NGS 2S Plus (Cat Nos. 21024, 21096)
- Accel-NGS 2S Hyb (Cat. Nos. 23024, 23096)

To be used with dual indexed adapters:

- 2S Set S1 (Cat. No. 28596)
- 2S Set S2 (Cat. No. 28696)
- 2S Set S3 (Cat. No. 28796)
- 2S Set S4 (Cat. No. 28896)
- 2S Set S1-S4 (Cat. No. 289384)

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About This Guide

This guide provides instructions for the preparation of high complexity NGS libraries from double-stranded DNA (dsDNA) using Accel-NGS 2S DNA Library Kits. These kits are suitable for NGS library prep with broad input range and 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. Read the Protocol thoroughly to ensure that you absorb all important information needed for success.

Product Information

The Accel-NGS 2S DNA Library Kits offer a unique solution to improve next-generation sequencing (NGS) sample preparation of dsDNA for sequencing on Illumina® platforms. The 2S technology utilizes Illumina-compatible adapter sequences and has been validated on the MiniSeq®, MiSeq®, NextSeq®, and HiSeq® platforms.

Note: PCR is NOT required for inputs at 100 ng of high quality genomic DNA or 10 ng of circulating, cell-free DNA (cfDNA). PCR is also NOT required for completing the library preparation as full-length adapters are attached during the ligation steps of the protocol. PCR reagents are included to amplify libraries that are made from inputs down to 10 pg and for lower quality samples to produce enough library to satisfy the loading requirements of the Illumina sequencers. This kit can be used for sequencing applications requiring PCR for which you would like to use a polymerase of choice.

Applications

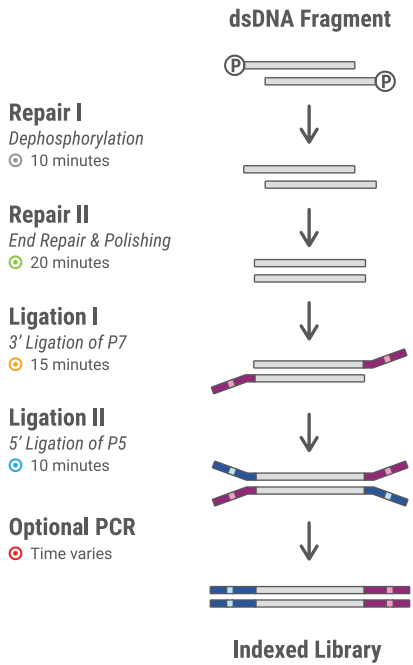
The Accel-NGS 2S DNA Library Kits are suitable for the following applications using ultra-high-throughput, multiplexed sequencing:

- Accel-NGS 2S PCR-Free and 2S Plus Kits
  - Whole genome sequencing for low pass copy number variation
  - Sequencing of chromatin DNA prepared using immunoprecipitation (ChIP)
  - Single-cell sequencing
- Accel-NGS 2S Hyb Kit
  - Targeted enrichment of relevant genomic regions (i.e., the exome) or transcripts of interest
  - Somatic variant detection of SNVs and Indels
  - Germline detection of inherited diseases

Accel-NGS 2S DNA Library Kit Workflow

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

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



For PCR-free applications, the resulting functional library is ready for library quantification and sequencing on the Illumina platform. Alternatively, an optional PCR step may be used to increase yield of indexed libraries, which then may be quantified and sequenced.

The Accel-NGS 2S Dual Indexed Adapters are full-length Swift indexed TruSeq® LT P7 and TruSeq HT P5 adapters. This workflow contains two indexing steps. During the Ligation I step, a unique, full-length TruSeq LT P7 adapter (Reagent Y2) is used to label each library. During the Ligation II step, a full-length TruSeq HT P5 adapter (Reagent B2) is used to provide unique dual indexed combinations.

Kit Contents

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

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

** Provided separately with an indexing kit.*

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

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

Material and Equipment Not Included

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

Storage and Usage Warning

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

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

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

IMPORTANT!

- Assemble all reagent master mixes ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes, use our **Accel-NGS 2S Set S1-S4 Indexed Adapter Master Mix Volume Calculator** and prepare them in advance to ensure the magnetic beads do not over-dry during size selection steps.
- Always add reagents to the master mix *in the specified order* as stated throughout the Protocol. Reagent Y2 and B2 (indexed adapters) are the only reagents that are added individually to each sample for Ligation I and Ligation II steps, respectively.

Tips and Techniques

Avoiding Cross-Contamination

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

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

DNA Fragmentation

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

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

Size Selection During Clean-Up Steps

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

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

Recommended PCR Cycles for Direct Sequencing

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

Input Material (ng)	PCR Cycles	Average Yield (nM)
100	0	≥ 4
10	6	≥ 4
1	9	≥ 4
0.1	12	≥ 4
0.01	15	≥ 4

IMPORTANT!
For hybridization (hyb) capture workflows, follow the PCR recommendations provided by your capture enrichment of choice.

Input Material Considerations

The Accel-NGS 2S DNA Library Kits enable the preparation of high complexity NGS libraries from dsDNA.

PCR-free libraries may be generated from as low as 100 ng of high quality genomic DNA or 10 ng of cfDNA, while PCR reagents are included for inputs below 100 ng and down to 10 pg and for lower quality samples.

If performing WGA prior to library construction, please use a WGA kit that is compatible with downstream NGS library preparation, as some WGA kits incorporate synthetic adapter or primer sequences on WGA product termini that make them incompatible with Illumina sequencing instrument cluster registration (all reads initiate with an identical synthetic sequence). For example, if using a Sigma WGA kit, please choose SeqPlex™ Enhanced DNA Amplification Kit for WGA that has a 'primer digestion' step to remove such synthetic sequences, as the Sigma GenomePlex® WGA kits do not include such a step.

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, so it is recommended to use the highest input available, up to 250 ng, for best results.

Prepare the DNA Sample

Input DNA Quantification

For high quality samples, it is recommended to determine dsDNA concentration using Qubit, or a similar fluorometric method, as it will accurately represent the double-stranded, adaptable DNA content of your sample. For cfDNA or low quality DNA samples, we recommend quantification by qPCR using Alu primer pairs provided in this kit 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 10 pg–250 ng input DNA per library preparation.
- Input DNA should be re-suspended in 40 µl of Low EDTA TE buffer. Contact TechSupport@swiftbiosci.com if you would like to work with larger volumes.
- PCR-free library preparation: minimum input of 100 ng gDNA or 10 ng cfDNA
- Library amplification by PCR: minimum input of 10 pg DNA

	Supported Input	
Input Material	**PCR-Free**	**With PCR**
High Quality gDNA	≥ 100 ng	10 pg–250 ng
ChIP DNA	—	≥ 10 pg
cfDNA	≥ 10 ng	≥ 1 ng
FFPE DNA	—	≥ 1 ng

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

DNA Fragmentation

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

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

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

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

Prepare the Reagent Master Mixes and Ethanol

1. To create the master mix, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes based on the number of reactions of choice, use our Accel-NGS 2S Set S1-S4 Indexed Adapter Master Mix Volume Calculator.This tool automatically incorporates 5% excess volume to compensate for pipetting loss.

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

IMPORTANT!

Prepare the reagents in advance to ensure the magnetic beads do not dry out during size selection steps. Always add reagents in specified order. This applies to all reagents except for Reagent Y2 and B2, unique indexed adapters provided separately in the indexed adapter kit that should be added individually to uniquely index each library for Ligation I and Ligation II steps, respectively.

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

Prepare the DNA Libraries

Repair I

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

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

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

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

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

* Alternatively, the thermocycler lid may be left open.

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

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	–
Less than 10 ng gDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	–
10 ng–250 ng gDNA	200 bp	60 µl	60 µl (ratio: 1.0)	–
	350 bp	60 µl	54 µl (ratio: 0.9)	–
	450 bp	60 µl	42 µl (ratio: 0.7)	–
DNA for Hyb Capture	All Sizes	60 µl	108 µl (ratio: 1.8)	–

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

Repair II

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

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

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

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

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
Less than 10 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
10 ng–250 ng gDNA	200 bp	50 µl	—	42.5 µl (ratio: 0.85)
	350 bp	50 µl	—	37.5 µl (ratio: 0.75)
	450 bp	50 µl	—	27.5 µl (ratio: 0.55)
DNA for Hyb Capture	All Sizes	50 µl	—	82.5 µl (ratio: 1.65)

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

Ligation I

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

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

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

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

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

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

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	165 bp	30 µl	—	31.5 µl (ratio: 1.05)
Less than 10 ng gDNA	All Sizes	30 µl	—	25.5 µl (ratio: 0.85)
10 ng–250 ng gDNA	All Sizes	30 µl	—	36.0 µl (ratio: 1.2)
DNA for Hyb Capture	All Sizes	30 µl	—	49.5 µl (ratio: 1.65)

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

⊙ Ligation II

15. Add 48 µl of pre-mixed Ligation II Master Mix (listed in the table below) to the beads for each sample.

Reagents	Volume per Sample
Low EDTA TE	30 µl
⊙ Buffer B1	5 µl
⊙ Reagent B3	9 µl
⊙ Enzyme B4	1 µl
⊙ Enzyme B5	2 µl
⊙ Enzyme B6	1 µl
Total Volume	**48 µl**

16. Add 2 µl of the appropriate indexed Reagent B2 to each sample and re-suspend by pipetting.

Reagents	Volume per Sample
Master Mix	48 µl
Sample	Beads
⊙ Reagent B2	2 µl
Total Volume	**50 µl**

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

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

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	20 µl
DNA for Hyb Capture	All Sizes	50 µl	—	82.5 µl (ratio: 1.65)	20 µl

IMPORTANT!
If sequencing PCR-free libraries on patterned flow cells, follow steps 19-20. It is important to ensure optimal removal of the indexed adapter carryover by performing a second clean-up using PEG NaCl solution and freshly prepared 80% ethanol. Otherwise, proceed to step 21.

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

20. To ensure optimal removal of indexed adapters, perform a second clean-up using PEG NaCl solution and freshly prepared 80% ethanol.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	20 µl
DNA for Hyb Capture	All Sizes	50 µl	—	82.5 µl (ratio: 1.65)	20 µl

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

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

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

Safe Stopping Point

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

Optional PCR-Library Amplification

Direct Sequencing for Accel-NGS 2S Plus Library Kit

24a. Add 30 µl of the pre-mixed Optional PCR Master Mix (listed in the table below) to the entire eluted library (20 µl). Mix by pipetting. If analysis of the PCR-free library by qPCR is desired before PCR amplification, set aside 3 µl of the final eluate for this analysis and instead add 17 µl of eluate plus 3 µl of Low EDTA TE buffer to the 30 µl of Optional PCR Master Mix.

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

Note: Reagent R1 (PCR primer mix) is provided separately as part of the Dual Indexed Adapter Kit.

25a. Place the sample tubes in the thermocycler and run the Optional PCR Thermocycler Program in the order listed below.

IMPORTANT!

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

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

26a. Clean up the PCR Reaction using magnetic beads and freshly prepared 80% ethanol. See Appendix, Section A for instructions. Follow the size selection instructions below.

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

27a. At the end of the clean-up, resuspend the beads in 20 µl of Low EDTA TE buffer.

28a. Place the sample tubes on a magnetic rack and wait 2 minutes.

29a. Carefully transfer the supernatant containing the final library to a clean tube without carrying any beads.

Pre-Hybridization PCR – Library Amplification for Accel-NGS 2S Hyb Library Kit

24b. Final libraries should be amplified to obtain adequate input for the hybridization capture. Use the PCR primers provided separately as part of the indexing kit with the polymerase recommended with the hybridization capture reagents.

For xGen® Lockdown® Only:

Accel-NGS 2S Dual Indexed Adapter Kits contain the following PCR primers:

Primer 1:

5′ AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGAC 3′

Primer 2:

5′ CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTG 3′

Where **XXXXXXXX** indicates the 8 bp index sequence. See Appendix, Section B for index sequences. Note that index sequences for P7 adapter Set S1-S4 will be used as a reverse complement in place of the 8 bp X sequences in Primer 2.

The PCR primers are at a concentration of 6 µM. In place of the primers supplied with the hybridization capture of choice, users constructing dual indexed libraries for xGen Lockdown hybridization capture should use these primers at 600 nM (final concentration in the PCR reaction) with the polymerase supplied with xGen Lockdown reagents.

25b. Place the samples in the thermocycler programmed according to the thermocycler conditions recommended by your hybridization capture of choice.

26b. Add 90 µl (ratio: 1.8) of magnetic bead volume to each sample.

27b. Clean up the pre-Hybridization PCR reaction using a magnetic rack and freshly prepared 80% ethanol. See Appendix, Section A for instructions.

28b. At the end of the clean-up, re-suspend the beads in 25 µl of nuclease water. It is important to elute in water to prevent residual EDTA from interfering with downstream applications, such as hybridization capture.

29b. Place the sample tubes on a magnetic rack and wait 2 minutes. Carefully transfer the supernatant containing the final library to a clean tube without carrying any beads.

Appendix

Section A: Size Selection/Clean-Up Protocol

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

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

Post-Repair I Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
Less than 10 ng gDNA	All Sizes	60 µl	84 µl (ratio: 1.4)	—
10 ng–250 ng gDNA	200 bp	60 µl	60 µl (ratio: 1.0)	—
	350 bp	60 µl	54 µl (ratio: 0.9)	—
	450 bp	60 µl	42 µl (ratio: 0.7)	—
DNA for Hyb Capture	All Sizes	60 µl	108 µl (ratio: 1.8)	—

Post-Repair II Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
Less than 10 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)
10 ng–250 ng	200 bp	50 µl	—	42.5 µl (ratio: 0.85)
	350 bp	50 µl	—	37.5 µl (ratio: 0.75)
	450 bp	50 µl	—	27.5 µl (ratio: 0.55)
DNA for Hyb Capture	All Sizes	50 µl	—	82.5 µl (ratio: 1.65)

Post-Ligation I Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
cfDNA	165 bp	30 µl	—	31.5 µl (ratio: 1.05)
Less than 10 ng gDNA	All Sizes	30 µl	—	25.5 µl (ratio: 0.85)
10 ng–250 ng gDNA	All Sizes	30 µl	—	36.0 µl (ratio: 1.2)
DNA for Hyb Capture	All Sizes	30 µl	—	49.5 µl (ratio: 1.65)

Post-Ligation II Clean-Up

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	165 bp	50 µl	—	52.5 µl (ratio: 1.05)	20 µl
Less than 10 ng gDNA	All Sizes	50 µl	—	42.5 µl (ratio: 0.85)	20 µl
10 ng–250 ng gDNA	All Sizes	50 µl	—	60.0 µl (ratio: 1.2)	20 µl
DNA for Hyb Capture	All Sizes	50 µl	—	82.5 µl (ratio: 1.65)	20 µl

Post-PCR Clean-Up for Direct Sequencing

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

If performing hybridization capture, add 90 µl (ratio: 1.8) of magnetic bead volume to each sample.

Section B: Indexed Adapters (Cat. Nos. 28596, 28696, 28796, 28896, and 289384)

During the Ligation I and II steps, you must use a unique indexed adapter (Reagent Y2 and B2) to label each library respectively. Libraries made with dual indexed adapters may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell. The full-length adapter sequences where X is replaced by the index sequences in the tables below are as follows:

P5 TruSeq HT Indexed Adapter

5´ AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3´

P7 TruSeq LT Indexed Adapter:

5´GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**XXXXXXXX**ATCTCGTATGCCGTCTTCTGCTTG 3´

The number on the product tube label indicates which indexed adapter is provided in the tube.

Sample Sheet Creation:

- In Illumina Experiment Manager software, select your sequencer of choice.
- On the Application Selection page, select other – FASTQ Only.
- Choose TruSeq HT. By selecting HT, you can alter the index sequences read in the sample sheet.
- On the next step, select the place holder for index sequences until sample sheet status is valid.

- Click finish to generate the .CSV file.
- Alter your sample sheet (by editing the .CSV file in Excel). To specify the 96 index sequences and i5 index, download our Index Sequences for Accel-NGS 2S Indexed Adapter Kits. Depending on the sequencer of choice, determine whether you use the reverse complement of the i5 index sequences.
- Be sure to re-save your edits as a .CSV file.

D5 Adapters	Sequence	28596	28696	28796	28896	289384
Index D501	TATAGCCT	26 µl	26 µl	26 µl	26 µl	104 µl
Index D502	ATAGAGGC	26 µl	26 µl	26 µl	26 µl	104 µl
Index D503	CCTATCCT	26 µl	26 µl	26 µl	26 µl	104 µl
Index D504	GGCTCTGA	26 µl	26 µl	26 µl	26 µl	104 µl
Index D505	AGGCGAAG	26 µl	26 µl	26 µl	26 µl	104 µl
Index D506	TAATCTTA	26 µl	26 µl	26 µl	26 µl	104 µl
Index D507	CAGGACGT	26 µl	26 µl	26 µl	26 µl	104 µl
Index D508	GTACTGAC	26 µl	26 µl	26 µl	26 µl	104 µl

Set S1 Adapters	Sequence	28596
Reagent Y2 (Index S701)	CAACACAG	22 µl
Reagent Y2 (Index S702)	ACACCTCA	22 µl
Reagent Y2 (Index S703)	ACCATAGG	22 µl
Reagent Y2 (Index S704)	CAGGTAAG	22 µl
Reagent Y2 (Index S705)	AACGCACA	22 µl
Reagent Y2 (Index S706)	TAGTCTCG	22 µl
Reagent Y2 (Index S707)	CAGTCACA	22 µl
Reagent Y2 (Index S708)	CCAACACT	22 µl
Reagent Y2 (Index S709)	ACATGCCA	22 µl
Reagent Y2 (Index S710)	ATTCCGCT	22 µl
Reagent Y2 (Index S711)	CAAGGTAC	22 µl
Reagent Y2 (Index S712)	CCATGAAC	22 µl
Reagent Y2 (Index S713)	TCAGCCTT	22 µl
Reagent Y2 (Index S714)	CAGTGCTT	22 µl
Reagent Y2 (Index S715)	CTCGAACA	22 µl
Reagent Y2 (Index S716)	ACAGTTCG	22 µl
Reagent Y2 (Index S717)	ATCCTTCC	22 µl
Reagent Y2 (Index S718)	CGAAGTCA	22 µl
Reagent Y2 (Index S719)	CTCTATCG	22 µl
Reagent Y2 (Index S720)	ACTCTCCA	22 µl
Reagent Y2 (Index S721)	TCCTCATG	22 µl
Reagent Y2 (Index S722)	AACAACCG	22 µl
Reagent Y2 (Index S723)	CTCGTTCT	22 µl
Reagent Y2 (Index S724)	TCAGTAGG	22 µl

Set S2 Adapters	Sequence	28696
Reagent Y2 (Index S725)	GCTTCACA	22 µl
Reagent Y2 (Index S726)	CGATGTTT	22 µl
Reagent Y2 (Index S727)	TTAGGCAT	22 µl
Reagent Y2 (Index S728)	ACAGTGGT	22 µl
Reagent Y2 (Index S729)	GCCAATGT	22 µl
Reagent Y2 (Index S730)	CAGATCTG	22 µl
Reagent Y2 (Index S731)	ACTTGATG	22 µl
Reagent Y2 (Index S732)	TAGCTTGT	22 µl
Reagent Y2 (Index S733)	TGGTTGTT	22 µl
Reagent Y2 (Index S734)	TGTACCTT	22 µl
Reagent Y2 (Index S735)	TCTGCTGT	22 µl
Reagent Y2 (Index S736)	TTGGAGGT	22 µl
Reagent Y2 (Index S737)	TCGAGCGT	22 µl
Reagent Y2 (Index S738)	TGATACGT	22 µl
Reagent Y2 (Index S739)	TGCATAGT	22 µl
Reagent Y2 (Index S740)	TGCGATCT	22 µl
Reagent Y2 (Index S741)	TTCCTGCT	22 µl
Reagent Y2 (Index S742)	TACAGGAT	22 µl
Reagent Y2 (Index S743)	TGTGGTTG	22 µl
Reagent Y2 (Index S744)	TTCCATTG	22 µl
Reagent Y2 (Index S745)	TAACGCTG	22 µl
Reagent Y2 (Index S746)	TTGGTATG	22 µl
Reagent Y2 (Index S747)	TGAACTGG	22 µl
Reagent Y2 (Index S748)	TACTTCGG	22 µl

Set S3 Adapters	Sequence	28796
Reagent Y2 (Index S749)	TCCAGTCG	22 µl
Reagent Y2 (Index S750)	TGTATGCG	22 µl
Reagent Y2 (Index S751)	TCATTGAG	22 µl
Reagent Y2 (Index S752)	TGGCTCAG	22 µl
Reagent Y2 (Index S753)	TATGCCAG	22 µl
Reagent Y2 (Index S754)	TCAGATTC	22 µl
Reagent Y2 (Index S755)	GGTTGGAC	22 µl
Reagent Y2 (Index S756)	GACACTTA	22 µl
Reagent Y2 (Index S757)	GCTATGGA	22 µl
Reagent Y2 (Index S758)	GTAACCGA	22 µl
Reagent Y2 (Index S759)	GGCAAGCA	22 µl
Reagent Y2 (Index S760)	GAACGACA	22 µl
Reagent Y2 (Index S761)	GCGTCGAA	22 µl
Reagent Y2 (Index S762)	AAGGCGAT	22 µl
Reagent Y2 (Index S763)	CAGGCATT	24 µl
Reagent Y2 (Index S764)	AACTGTAT	24 µl
Reagent Y2 (Index S765)	ATGCTTGA	22 µl

Set S3 Adapters	Sequence	28796
Reagent Y2 (Index S766)	AGTATCTG	22 µl
Reagent Y2 (Index S767)	ATGTAATG	22 µl
Reagent Y2 (Index S768)	ACACATGT	22 µl
Reagent Y2 (Index S769)	ATAGCACG	22 µl
Reagent Y2 (Index S770)	ATATTGTA	22 µl
Reagent Y2 (Index S771)	CAATTGAT	22 µl
Reagent Y2 (Index S772)	CACGTCGT	22 µl

Set S4 Adapters	Sequence	28896
Reagent Y2 (Index S773)	AGTCTGTA	22 µl
Reagent Y2 (Index S774)	CCGTATCT	22 µl
Reagent Y2 (Index S775)	CGCTTCCT	22 µl
Reagent Y2 (Index S776)	CAAGACCT	22 µl
Reagent Y2 (Index S777)	CCTAGTAT	22 µl
Reagent Y2 (Index S778)	CCACCGAT	22 µl
Reagent Y2 (Index S779)	CTATCATG	22 µl
Reagent Y2 (Index S780)	CATGAATG	22 µl
Reagent Y2 (Index S781)	CTGTACGG	22 µl
Reagent Y2 (Index S782)	CACTCGAG	22 µl
Reagent Y2 (Index S783)	CCGACAAG	22 µl
Reagent Y2 (Index S784)	CTTGCTTC	22 µl
Reagent Y2 (Index S785)	CGCCTTAT	22 µl
Reagent Y2 (Index S786)	GCAACCAT	22 µl
Reagent Y2 (Index S787)	TGACCGTT	22 µl
Reagent Y2 (Index S788)	TTGAGCTC	22 µl
Reagent Y2 (Index S789)	CCACATTG	22 µl
Reagent Y2 (Index S790)	AGCCAACT	22 µl
Reagent Y2 (Index S791)	ATCACGTT	22 µl
Reagent Y2 (Index S792)	TCTCGGTT	22 µl
Reagent Y2 (Index S793)	TTGACTCT	22 µl
Reagent Y2 (Index S794)	TCGAAGTG	22 µl
Reagent Y2 (Index S795)	CACCCAAA	22 µl
Reagent Y2 (Index S796)	CTTCACAT	22 µl

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

Reagents	28596	28696	28796	28896	289384
Reagent B2	211 µl	211 µl	211 µl	211 µl	844 µl
Reagent R1	528 µl	528 µl	528 µl	528 µl	2,112 µl

To minimize the amount of sequencing overlap, refer to the recommended read lengths in the table below:

Starting Material Supported Input	Insert Size	Sequencing Recommended Read Length
10 ng–250 ng gDNA	350 bp	2 x 100
	200 bp	2 x 75
1 ng–250 ng cfDNA	165 bp	2 x 75

Section C: Helpful Information and Troubleshooting

Problem	Possible Cause	Suggested Remedy
Library migrates unexpectedly on Bioanalyzer.	When analyzed on the Agilent High Sensitivity chip, migration behavior overestimates library size of PCR-free libraries made from DNA fragmented to the 200–300 base range (as required in this protocol).	• "200 bp insert" PCR-free libraries should migrate to a ≈500 bp peak and "350 bp insert" PCR-free libraries should migrate to a ≈800 bp peak on the High Sensitivity Chip. • Consult the Expected Results section and the application note released by Covaris titled "Analysis of DNA Fragments Using the Agilent 2100 Bioanalyzer" to ensure proper analysis of library size.
	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally.	• Quantify library by qPCR, as other quantification methods will not accurately detect heteroduplex library molecules. • Perform the minimum number of PCR cycles necessary to avoid over-amplification.
DNA does not fragment properly: broad or lopsided (high molecular weight) sonication profile of fragmented DNA.	Impure DNA or fragmentation device malfunction.	• Isopropanol purification, bead clean-up, column purification, or other method before fragmentation. • Ensure fragmentation device is functioning within manufacturer's parameters.
Incomplete resuspension of beads after ethanol wash during SPRI™ steps.	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
Shortage of enzyme reagents.	Pipetting enzymes at -20 °C instead of 0-4 °C.	Allow enzyme reagents to equilibrate to 0-4 °C for 10 minutes prior to pipetting.
Retention of liquid in pipette tip	Viscous reagents may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to ensure all liquid and/or beads are released from the pipette tip.

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

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