PROTOCOL

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ACCEL-NGS® 2S PLUS DNA LIBRARY KITS

Dual Indexing

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

- 2S Dual Indexing (Cat. No. 28096)

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About This Guide

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

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 Plus DNA Library Kit offers 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 required for completing the library preparation as truncated adapters are attached during the ligation steps of the protocol. PCR reagents are included to support indexing, inputs down to 10 pg, and lower quality samples. For sequencing applications requiring PCR for which you would like to use a polymerase of choice, please use the Accel-NGS 2S PCR-Free DNA Library Kit (Cat. No. 20024 or 20096).

Applications

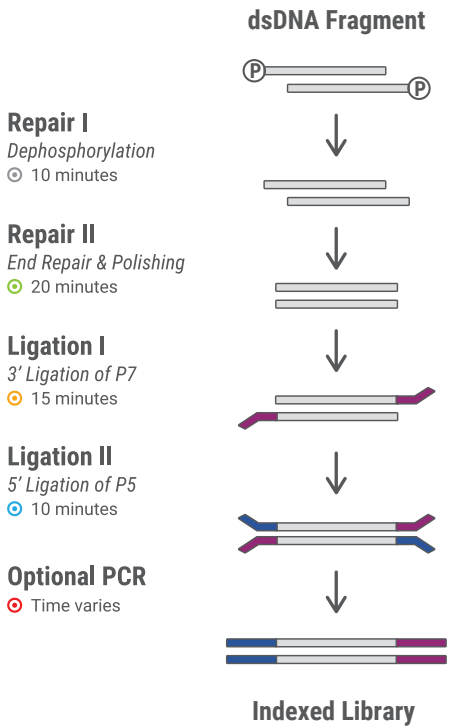
The Accel-NGS 2S Plus DNA Library Kit is suitable for the following applications:

- Whole genome sequencing [including whole genome amplification (WGA) samples]
- Cell-free DNA sequencing
- FFPE DNA sequencing
- Long range PCR amplicons
- Metagenomic sequencing

Accel-NGS 2S Plus DNA Library Kit Workflow

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

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



The Indexing PCR step is used to increase yield and add the indexed adapter sequences. Please refer to the table on Page 6 for the recommended library sizes and input requirements.

Kit Contents

The Accel-NGS 2S Plus DNA Library Kit is available in two sizes with reagents (10% excess volume) for the preparation of either 24 or 96 libraries. Reagent Y2 (truncated adapter), Reagent B2 (truncated adapter), and D50X/D7XX are provided separately in one of the available Accel-NGS 2S Indexing Kits (see Appendix, Section C).

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
Index D50X	*	*	-20
Index D7XX	*	*	-20
Reagent R2	106	424	-20
Buffer R3	264	1056	-20
Enzyme R4	28	106	-20

** Provided separately with an Indexing Kit*

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

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

Material and Equipment Not Included

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

Storage and Usage Warning

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

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

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

IMPORTANT!

- Assemble all reagent master mixes ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes, use our Accel-NGS 2S Master Mixing Volume Calculator (located on our Accel-NGS 2S product web pages), 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.

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, this step is omitted.

Input Material	Fragmentation	Supported Fragment Size (bp)
High Quality gDNA	✓	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

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

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

Input Material Considerations

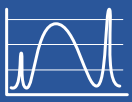
The Accel-NGS 2S Plus DNA Library Kit designed for Illumina platforms enables the preparation of high complexity NGS libraries from dsDNA.

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.

From Sample to Sequencing



Prepare the DNA Sample

- Quantify your DNA sample
- Fragment the DNA sample and analyze the results
- (Optional) Store your DNA at -20 °C until ready to use

Prepare the Reagent Master Mixes and Ethanol

- Place the reagents on ice for 10 minutes
- Calculate volumes using the Accel-NGS 2S Master Mixing Volume Calculator
- Briefly vortex non-enzyme reagents and spin all reagents
- Assemble master mixes and leave on ice
- Prepare 80% ethanol solution

Prepare the DNA Libraries

- Ensure DNA sample is in 40 µl of Low EDTA TE buffer
- Follow the Accel-NGS 2S Plus workflow on page 2
- Library quantification

Sequence the DNA Libraries

Compatible with Illumina instruments:

- MiniSeq
- MiSeq
- HiSeq
- NextSeq

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 (Appendix, Section A) to accurately assess the usable amount of DNA in the samples and their integrity.

High Quality gDNA	Quantify with Qubit or similar fluorometric method
cfDNA	Quantify by qPCR with Alu primer pairs (see Appendix, Section A)
FFPE DNA	Quantify by qPCR with Alu primer pairs (see Appendix, Section A)

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

Input Material	Supported Input with PCR
High Quality gDNA	10 pg–250 ng
cfDNA	≥ 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 200–450 bp. If using larger fragments, please contact TechSupport@swiftbiosci.com. This kit has been specifically validated on Covaris®-fragmented DNA.

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

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

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

Prepare the Reagent Master Mixes and Ethanol

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

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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 B for instructions. Follow the clean-up instructions below.

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

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

Repair II

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

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

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

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

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

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

Ligation I

10. Add 30 µl of pre-mixed Ligation I Master Mix (listed in the table below) to the beads for each sample. **Note:** Reagent Y2, a truncated adapter, is provided separately in the Dual Indexing Adapter Kit.

Reagents	Volume per Sample
Low EDTA TE	20 µl
Reagent Y2	5 µl
Buffer Y1	3 µl
Enzyme Y3	2 µl
Total Volume	**30 µl**

11. 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.

12. Clean up the Ligation I reaction using a magnetic rack, PEG NaCl solution, and freshly prepared 80% ethanol. See Appendix, Section B 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)

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

Ligation II

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

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

Note: Reagent B2 (non-indexed adapter) is provided separately in the Indexed Adapter Kit.

15. 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.

16. Clean up the Ligation II Reaction using a magnetic rack, PEG NaCl solution, and freshly prepared 80% ethanol. See Appendix, Section B 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

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

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

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

PCR-Library Amplification

20. Add indexing reagent(s) directly to the entire eluted library (20 µl). Mix by pipeting. **Note:** Index D50X/D7XX, the index primers, are provided as part of the Dual Indexing Adapter Kit.

Reagents	Volume per Sample
Index D50X	2.5 µl
Index D7XX	2.5 µl
Sample	20.0 µl
Library + Primer Mix	**25.0 µl**

21. Add 25 µl of the pre-mixed Indexing PCR Master Mix (listed in the table below) to the entire eluted library (20 µl). Mix by pipetting.

Reagents	Volume per Sample
Low EDTA TE	10 µl
Reagent R2	4 µl
Buffer R3	10 µl
Enzyme R4	1 µl
Total Volume	**25 µl**

22. Place the sample tubes in the thermocycler and run the Indexing PCR Thermocycler Program in the order listed below.

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

Input	Recommended PCR Cycles	Thermocycler Program
100 ng	3	98 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
10 ng	6	
1 ng	9	
100 pg	12	
10 pg	15	
10 ng cfDNA	0–2	
1 ng cfDNA	5–6	

23. Clean up the PCR Reaction using magnetic beads and freshly prepared 80% ethanol. See Appendix, Section B 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

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

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

26. 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 ºC (or long term at -20 ºC. The library is now ready for quantification. Analysis of libraries by fluorometric methods may be performed to assess size distribution. Please note the sensitivity limits specified by the fluorometric assay of choice, and consult the application note released by Covaris titled “Analysis of DNA Fragments Using the Agilent 2100 Bioanalyzer” to ensure proper analysis of library size.

Appendix

Section A: Input DNA Quantification

Fluorometric-based (Qubit) quantification will provide accurate DNA concentrations for samples with high quality DNA but is not recommended for cfDNA and FFPE samples. If working with cfDNA or FFPE samples, we recommend quantification by qPCR using supplied Alu primers for both short and long amplicons to accurately determine the usable concentration and quality of the sample DNA.

Alu sequences are highly abundant in the human genome and can be used for the sensitive quantification of human genomic DNA. Included in this kit are qPCR primers that can be used to amplify two differently sized amplicons: short (115 bp; Alu115) and long (247 bp; Alu247) amplicons from genomic Alu repeats. Following input analysis, the appropriate amount of sample DNA can be used as input for NGS library preparation.

The following assay describes the quantification of low quality dsDNA samples using the Alu repeat primers for qPCR quantification. For additional information, please refer to the Protocol: Input DNA Quantification Assay. For further information, refer to Hao, et al, Br J Cancer 2014 Oct 14; 111(8); 1482-9.

Alu Assay

1. Prepare a standard curve using serial dilutions of human genomic DNA of known quantities (11 ng, 1.1 ng, 0.11 ng, 0.011 ng, 0.0011 ng) for each Alu primer pair in duplicate.

2. Prepare to run each sample and a no template control in duplicate for sample quantification. Determine the volume of sample DNA to load so as to increase the likelihood it will fall within the standards and, therefore, the dynamic range of the assay. For limiting samples, a minimum of 1 µl is required. If your DNA is more concentrated than the highest standard, dilute it to fall between the standards.

3. Prepare the qPCR reaction in a 1.5 mL tube by adding reagents in the order listed below. We suggest the use of iTaq™ Universal SYBR Green Supermix (Bio-Rad, 172-5120).

Reagents	1 rxn
iTaq Universal SYBR Green Supermix	10 µl
Alu115 or Alu247 primers	2 µl
DNA	X µl
Low EDTA TE	up to 20 µl
Total Volume	**20 µl**

4. Place in the thermocycler and run the Alu Primer PCR Quantification program as described below.

Alu Primer qPCR Quantification Thermocycler Program	95 °C for 3 minutes, lid heating ON	
	95 °C for 5 seconds, lid heating ON 62 °C for 30 seconds, lid heating ON	(x35 cycles)

5. Plot Ct values (y-axis) vs. DNA quantity of the serial dilutions (x-axis) on a log scale to produce the standard curve. Identify the slope and the y-intercept. Solve using the following formula to determine the sample DNA concentration.

ng/µl = 10 ^ (Ct – y-intercept) / slope

6. The concentration for the Alu115 amplicon can be used to determine the total quantity of usable DNA in ng/µl. Verify that the calculated concentration of your sample is between the DNA standards of the assay.

7. Use a ratio of the Alu247 and Alu115 amplicons to calculate a DNA integrity score. High quality DNA is expected to have a DNA integrity score of 1.0, while lower quality DNA will have a score between 0.1 and 1.0 due to either damage or high molecular weight DNA contamination in FFPE or cfDNA samples, respectively. The DNA integrity score is intended to be used as an indicator of probability of successful library construction. Due to the diversity of sample types and protocols, specific recommendations concerning library construction and sequencing metrics are difficult to define in terms of the DNA integrity score. Use your best judgement.

DNA integrity score = (ng/µl of Alu247) / (ng/µl of Alu115)

Additional Notes:

DNA extracted from FFPE samples can exhibit varying degrees of DNA damage and the consequence of this damage will be a more pronounced amplification of the longer (Alu247) amplicon. Therefore, with FFPE samples, the Alu115 qPCR results accurately detect the total quantity of usable DNA (ng/µl).

As cfDNA exhibits a narrow size distribution around 165 bp, the Alu115 qPCR result will accurately detect the total quantity of cfDNA and high molecular weight genomic DNA. Alu247 qPCR results indicate only the presence of high molecular weight genomic DNA. Subtract the DNA mass in Alu247 value from the DNA mass in the Alu115 value to obtain the quantity of cfDNA in the sample.

Alu115	Forward: 5′ -CCTGAGGTCAGGAGTTCGAG- 3′ Reverse: 5′ -CCCGAGTAGCTGGGATTACA- 3′
Alu247	Forward: 5′ -GTGGCTCACGCCTGTAATC- 3′ Reverse: 5′ -CAGGCTGGAGTGCAGTGG- 3′

Section B: Size Selection/Clean-Up Protocol

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

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

Post-Repair I Clean-Up

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

Post-Repair II Clean-Up

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

Post-Ligation I Clean-Up

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

Post-Ligation II Clean-Up

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

Post-PCR Clean-Up

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

Section C: Indexing Kits (Cat. No. 28096)

During the Indexing PCR step, you must use a unique combination of index primers to label each library. Libraries made with uniquely indexed adapters may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell. The full-length adapter sequences where X is replaced by the indexed adapter sequences in the tables below are as follows:

P5 TruSeq® HT Adapter:

5′ AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′

P7 TruSeq HT Adapter:

5′ GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

The number on the product tube label indicates which indexed adapter is provided in the tube. Unique indexed adapters in quantity ordered (see table below), which should be used where this manual calls for 2.5 µl of each Index Primer in the Indexing PCR step:

D5 Adapters	Sequence	28096
D501	TATAGCCT	33 µl
D502	ATAGAGGC	33 µl
D503	CCTATCCT	33 µl
D504	GGCTCTGA	33 µl
D505	AGGCGAAG	33 µl
D506	TAATCTTA	33 µl
D507	CAGGACGT	33 µl
D508	GTACTGAC	33 µl

D7 Adapters	Sequence	28096
D701	ATTACTCG	22 µl
D702	TCCGGAGA	22 µl
D703	CGCTCATT	22 µl
D704	GAGATTCC	22 µl
D705	ATTCAGAA	22 µl
D706	GAATTCGT	22 µl
D707	CTGAAGCT	22 µl
D708	TAATGCGC	22 µl
D709	CGGCTATG	22 µl
D710	TCCGCGAA	22 µl
D711	TCTCGCGC	22 µl
D712	AGCGATAG	22 µl

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

Reagents	28096
Reagent B2	212 µl
Reagent Y2	528 µl

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

Starting Material Supported input	Insert Size	Sequencing Recommended Read Length
10 pg-250 ng	350 bp	2 X 100
	200 bp	2 X 75
1 ng-250 ng cfDNA	165 bp	2 X 75

Section D: Helpful Information and Troubleshooting

Problem	Possible Cause	Suggested Remedy
Library migrates unexpectedly on Bioanalyzer.	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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Swift Biosciences, Inc.
674 S. Wagner Road, Suite 100 • Ann Arbor, MI 48103 • 734.330.2568 • www.swiftbiosci.com