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SWIFT PROTOCOL

Accel-NGS® 2S DNA Library Kits

Low Input Library Prep with Enhanced Repair for Fragmented dsDNA For Indexing by Ligation & Indexing by PCR

Protocol for Cat. Nos.:
Accel-NGS® 2S PCR-Free DNA Library Kits (20024, 20096, 200384)
Accel-NGS® 2S Plus DNA Library Kits (21024, 21096, 21384)
Accel-NGS® 2S Hyb DNA Library Kits (23024, 23096, 23384)

DNA Library Kits Compatible with:

Accel-NGS® 2S Indexed Adapters (Indexing by Ligation)
2S Single Indexed Adapters (12-plex to 96-plex)
2S Single Indexed Adapters with MIDs (12-plex to 96-plex)
2S Combinatorial Dual Indexed Adapters (96-plex to 768-plex)

Swift Indexing Primers (Indexing by PCR)
Swift Single Indexing Primers (12-plex)
Swift Combinatorial Dual Indexing Primers (96-plex to 768-plex)
Swift Normalase Combinatorial Dual Indexing Primers (96-plex)
Swift Unique Dual Indexing Primers (96-plex)
Swift Normalase Unique Dual Indexing Primers (96-plex to 384-plex)
Note: All indexing primers require Accel-NGS® 2S Truncated Adapters for library preparation

Swift Hybridization Capture Panels
Swift Exome Hyb Panel (83216)
Swift Pan-Cancer Hyb Panel (83316)
Swift Inherited Diseases Hyb Panel (83416)

Swift Normalase® Kits
Swift Normalase® Kit (66096, 660384)

Agilent SureSelect^{XT}:
2S SureSelect^{XT} Compatibility Module (26424, 26496)

See Appendix Section E for details on Product Ordering Information.

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Swift BIOSCIENCES™

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Support

For additional support please contact Swift at TechSupport@swiftbio.com, or by phone: 734.330.2568 (9:00 am – 5:00 pm ET, Monday through Friday).

Product Information

This guide provides instructions for the preparation of high complexity NGS libraries from fragmented double-stranded DNA (dsDNA) using Accel-NGS 2S DNA Library Kits. These kits offer a unique solution to improve NGS library preparation from low input and damaged samples for sequencing on Illumina® Platforms, supporting a broad range of input quantities from 1 µg down to 10 pg. Numerous indexing options are available for the two Accel-NGS 2S workflows (indexing by ligation and indexing by PCR), see below and Appendix Section D for details.

Indexing by Ligation:

- PCR-free libraries can be prepared using full-length indexed adapters and a minimum of 100 ng to 1 µg input DNA.
- Optional library amplification can be performed using the primers and High-Fidelity Polymerase provided in the 2S Plus Kit.
- Full-length indexed adapters are compatible with the MID workflow where indexed i7 adapters are used with an i5 adapter comprising a 9N molecular identifier sequence (for more information, see the Swift Tech Note on the 2S product page: Increase Specificity of Detecting Low Frequency Alleles with Molecular Identifiers).

Indexing by PCR:

- When indexing by PCR, library amplification is required for completing library preparation as truncated, non-indexed adapters are attached during the ligation steps (truncated adapters TruY2 and TruB2 required for this workflow. Refer to Appendix Section E for Product Order Information.
- For direct sequencing applications, Swift High-Fidelity Polymerase is supplied in the 2S Plus Kit for library amplification.

Hybridization Capture:

- Whether indexing by ligation or by PCR, use the Polymerase recommended by the hybridization capture panel vendor for pre- and post-hyb PCR steps.
- 2S is compatible with:
    - Swift Hybridization Capture Panels
    - IDT xGen® Lockdown Panels
    - Twist Bioscience Panels
    - Arbor Biosciences Panels
    - Agilent SureSelect^{XT} Panels (for compatibility, use the XT Module for ligation steps)

Normalase

- Swift Normalase is compatible with Accel-NGS 2S Plus where Normalase primers are required during library amplification to increase yield and condition libraries for Normalase chemistry, see Appendix Section C.
    - When indexing by ligation, a Normalase kit is required which includes Normalase PCR primers.
    - When indexing by PCR, a Normalase kit and Normalase Indexing primers are required to complete the workflow. Available indexing includes CDI (96-plex) and UDI (96-plex to 384-plex).
- Swift Normalase is not compatible with Accel-NGS 2S PCR-Free as PCR is required. For Accel-NGS 2S Hyb, Normalase is compatible with post-hyb PCR but not for pre-hyb PCR.

Automation

This protocol is readily automatable. A 10% overage volume of reagents is supplied in the 24, 96, and 384 reaction kits to accommodate automation. Swift Biosciences does not supply automated liquid handling instruments or consumables but collaborates with automation solution providers and customers to develop optimized scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Please contact your instrument vendor or Automation@swiftbio.com if you plan to use this kit with your automated liquid handling system.

Sample Types

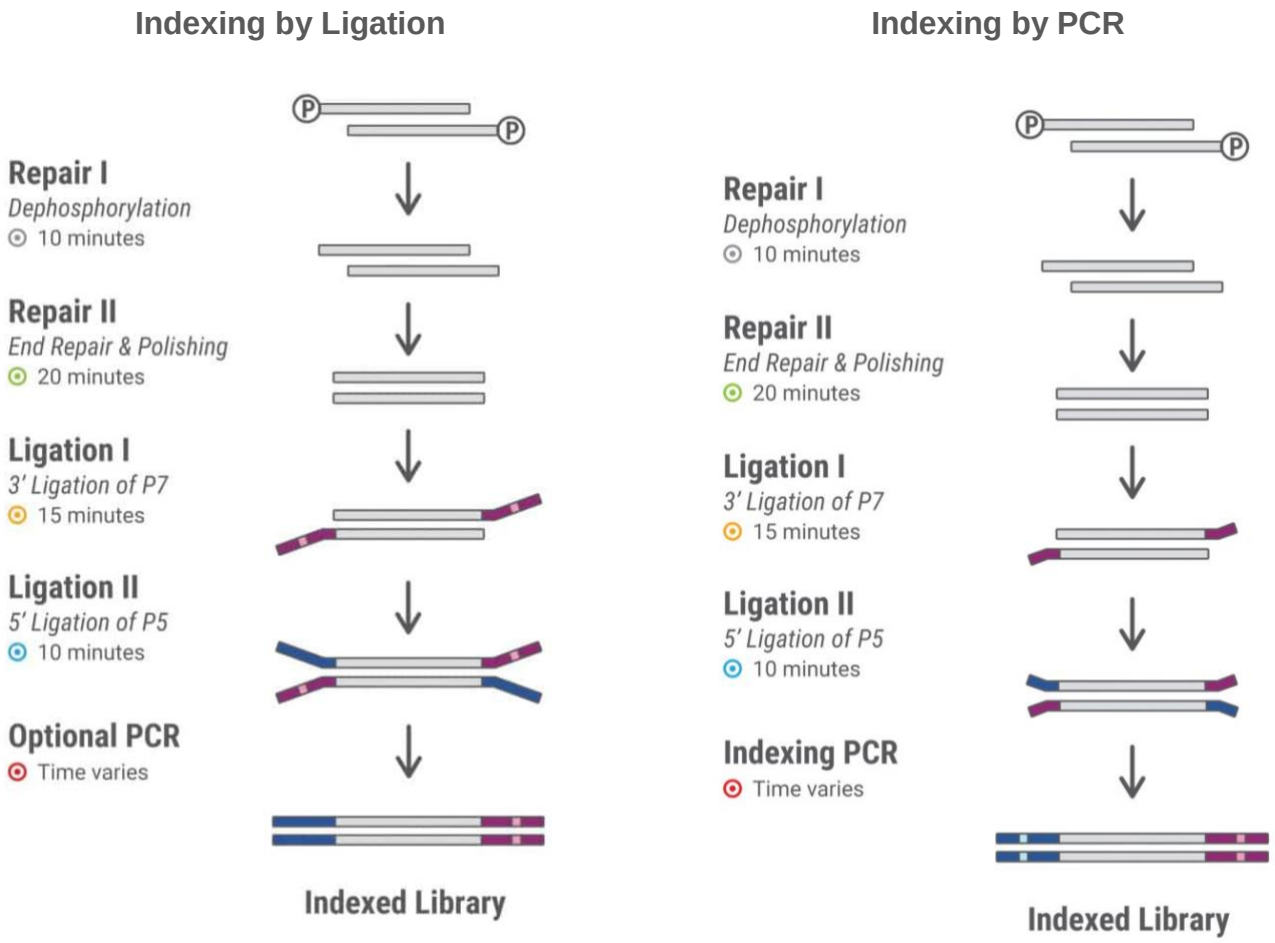
The Accel-NGS 2S DNA Library Kits are suitable for the following sample types and support a broad range of input quantities from 1 µg down to 10 pg.

- Damaged DNA samples including FFPE
- Low cell number ChIP and Hi-C samples
- Low input Cell-free DNA (Liquid biopsy)
- Low input metagenome samples

Damaged samples are supported due to the unique end repair capabilities where both damaged 5’ and 3’ termini can be excised and replaced to improve ligation efficiency.

Low inputs are supported by performing sequential ligation that overcomes the requirement for adapter titration, thereby maintaining efficient ligation at low nanogram and picogram input quantities.

Accel-NGS® 2S Workflows



Repair I: 5’ and 3’ ends are dephosphorylated to prevent chimera formation and improve adapter ligation to 3’ ends.

Repair II: additional 3’ end repair and polishing of 3’ and 5’ overhangs is performed.

Ligation I: the full-length i7 or truncated adapter is ligated to the 3’ ends of the dsDNA substrate.

Ligation II: 5’ end repair and ligation of the full-length i5 adapter or truncated adapter to 5’ ends of the dsDNA substrate.

PCR increases library yield and incorporates indexes if using truncated adapters.

Bead-based purifications are used to remove unused adapters and change buffer composition between steps.

NOTE: Normalase primers can be used during PCR for compatibility with the Normalase workflow (see Appendix Section C and the Normalase protocol for instructions).

Kit Contents

- The Accel-NGS 2S Kits contain sufficient reagents for the preparation of 24 or 96 libraries (10% excess volume provided). The 384 reaction kits are a bundle of 4x96 reactions.
- Indexed adapters or indexing primers are sold separately (see Appendix Section D) for indexing information. **IMPORTANT:** If using indexing primers, the truncated adapters (TruY2, TruB2) Cat. No. 28196 are required for adapter ligation steps. The truncated adapters can be purchased separately or together with the indexing primers. See Appendix Section E for Product Ordering Information.

Protocol stage	Component	Volume (µl)		Storage
		24 rxns	96 rxns	
Repair I	• Buffer W1	158	634	-20°C**
	• Enzyme W2	28	106	
Repair II	• Buffer G1	132	528	
	• Reagent G2	344	1374	
	• Enzyme G3	28	106	
	• Enzyme G4	28	106	
Ligation I	• Buffer Y1	80	316	
	• Indexed i7 Adapter Y2 or Reagent TruY2*	-	-	
	• Enzyme Y3	53	212	
Ligation II	• Buffer B1	132	528	
	• Indexed i5 Adapter B2 or Reagent TruB2*	-	-	
	• Reagent B3	238	950	
	• Enzyme B4	28	106	
	• Enzyme B5	53	212	
	• Enzyme B6	28	106	
Library Amplification	• Reagent R1 Primers or Indexing Primers*	-	-	
	• Reagent R2	106	424	
	• Buffer R3	264	1056	
	• Enzyme R4	53	212	
Additional Reagents	Low EDTA TE	20 mL	20 mL	Room Temp
	PEG NaCl Solution	20 mL	20 mL	

***** Adapters and primers are sold separately, see Appendix Section E for Product Ordering Information.
If the SureSelect^{XT} Module is used, use Reagent Y-XT, B2, and R-XT for Ligation I, Ligation II and Library Amplification step.
****** Temperature range -15° C to -25° C.

Storage and Usage Recommendations

- Upon receipt, store the Accel-NGS 2S DNA Library Kit products at -20ºC 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 -20ºC storage and place on ice for 10 minutes prior to pipetting. Attempting to pipette enzymes at -20ºC may result in shortage of enzyme reagents.
- After thawing reagents on ice, briefly vortex (except enzymes) to mix well, then pulse spin to collect contents before proceeding.

Materials and Equipment Not Included

- Compatible Indexed Adapters if indexing by ligation (see Appendix Sections D and E)
- Compatible Indexing Primers if indexing by PCR (see above and Appendix Sections D and E).
  Note: if indexing by PCR, truncated adapters (TruY2, TruB2) Cat. No. 28196 are also required. These are sold in combination with indexing primers or separately.
- Swift Normalase Kit (Cat. No. 66096, 660384), if using the Normalase workflow.
  Note: Normalase Indexing Primers are available, if using the Normalase workflow and indexing by PCR (see Appendix Section C)
- SPRIselect beads (Beckman Coulter, Cat. Nos. B23317/B23318/B23319) or Agencourt AMPure XP beads (Beckman Coulter, Cat. Nos. A63880/A63881/A63882)
- Permagen Magnetic Separator (Cat. No. MSR812), Agencourt SPRIPlate or similar magnetic rack for magnetic bead clean-ups
- Qubit® or other fluorometric-based assay for determining dsDNA concentration
- If assessing damaged human DNA samples, the Input DNA Quantification Assay can be used to determine DNA quantity and integrity (Input DNA Quantification Primers Cat. No. 90396 are sold separately).
- Method for fragmentation of input DNA (validated with Covaris® shearing)
- Agilent Bioanalyzer or other electrophoretic instrument to determine fragment and library size
- qPCR-, electrophoretic-, or fluorometric-based library quantification assay for Illumina libraries
- Microcentrifuge
- Vortex
- Programmable thermocycler
- 0.2 mL PCR tubes or 96-well plate
- Aerosol-resistant tips and pipettes ranging from 1-1000 µl
- 200-proof/absolute ethanol and nuclease-free water (both molecular biology grade) for preparation of fresh 80% ethanol

Tips and Techniques

Avoid 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.

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). See Appendix Section B for instructions. If other beads are used, solutions and conditions for DNA binding may differ. Consider the information below for performing efficient size selection:

- Post shearing, confirm the size distribution of each sample by electrophoretic methods to determine the median fragment size of your dsDNA samples.
- The size selection steps in this protocol perform a Left Side Size Selection and are designed to remove unused oligonucleotides and small DNA fragments to select the specified insert size.

- To customize size selection, please use Beckman Coulter's SPRIselect User Guide for desired conditions not included in this protocol.

Prepare DNA Samples

Input DNA Quantification

- Determine the dsDNA concentration using Qubit, or a similar fluorometric method, as it will accurately represent the double-stranded, adaptable DNA content of your sample.
- If working with damaged human samples, the Input DNA Quantification Assay can be used to determine DNA quantity and integrity (Input DNA Quantification Primers Cat. No. 90396 are sold separately), See Appendix, Section A for details.
- This kit has been validated for use with a wide range of DNA inputs; 10 pg to 1 µg.

DNA Shearing

This step is required for intact DNA samples but not samples such as ChIP or cell-free DNA. This protocol was validated with Covaris shearing and supports 200, 350 and 450 bp sheared DNA in low EDTA TE (10 mM Tris, 0.1 mM EDTA). If the DNA volume is less than 40 µl, add Swift Low EDTA TE to a final volume of 40 µl. Other shear sizes are compatible, please contact Swift at TechSupport@swiftbio.com.

Prepare Reaction Mixes and Ethanol

This is a "with bead" protocol, meaning beads containing the crowding agent, 20% polyethylene glycol (PEG), 2.5 M NaCl, added to clean up the Repair I reaction will be retained and re-used throughout subsequent enzymatic reactions, and clean-ups where additional PEG NaCl is added to the re-cycled beads to cause the negatively-charged DNA to bind with the carboxyl groups on the bead surface. As the immobilization is dependent on the concentration of PEG and salt in the reaction, the volumetric ratio of beads to DNA is critical**.** After each cleanup step, the beads are resuspended in the next reaction mix.

IMPORTANT! Prepare the reaction mixes in advance to ensure that each mix is added without delay to prevent drying of the beads following each cleanup step.

- Assemble all Reaction Mixes (see instructions on pages 7-10) for Repair I, Repair II, Ligation I and Ligation II steps **ON ICE** and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. For convenience, please use our Accel-NGS 2S Master Mix Volume Calculator (located on our Accel-NGS® 2S product webpage protocols and tools tab).
- After thawing reagents, briefly vortex (except enzymes) to mix well, and pulse spin to collect contents prior to opening.
- Always add reagents in the specified order, thoroughly mix each Reaction Mix, pulse spin to collect contents and store **ON ICE** prior to using.
- Prepare fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Approximately 2 mL of 80% ethanol will be used per sample.

Prepare DNA Libraries

Repair I

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

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

Reagents	Volume per Sample
Low EDTA TE	13 µl
• Buffer W1	6 µl
• Enzyme W2	1 µl
Repair I Reaction Mix	**20 µl**
Sample	40 µl
Total Volume	**60 µl**

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

Sample Type	Thermocycler Program
cfDNA	37 °C, 5 min, lid heating ON
	65 °C, 2 min, lid heating ON
	37 °C, 5 min, lid heating ON
All Other Inputs	37 °C, 10 min, lid heating OFF

4. Clean up the Repair I reaction using SPRISelect beads, a magnetic rack, and freshly prepared 80% ethanol. See Appendix, Section B for instructions. Follow the clean-up instructions below:

   For Direct Sequencing:

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)	-

   For Hyb Capture:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
All Inputs	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 the pre-mixed Repair II Reaction 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
Repair II Reaction Mix	**50 µl**
Sample	Beads
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 PEG NaCl solution, a magnetic rack, and freshly prepared 80% ethanol. See Appendix, Section B for instructions. Follow the clean-up instructions below:

For Direct Sequencing:

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)

For Hyb Capture:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
All Inputs	All Sizes	50 µl	-	82.5 µl (ratio: 1.65)

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

Ligation I

10. Refer to the tables below for preparing the Ligation I Reaction Mix when using either an indexed i7 adapter (left table, Reagent Y2) or truncated adapter (right table, Reagent TruY2).

11. Add 25 µl (for indexing by ligation, left) or 30 µl (for indexing by PCR, right) pre-mixed Ligation I Reaction Mix to the beads for each sample and re-suspend by pipetting, then add the individual indexed i7 adapter to each sample *ONLY* if indexing by ligation (left).

Indexing by Ligation	
Reagents	**Volume per Sample**
Low EDTA TE	20 µl
• Buffer Y1	3 µl
• Enzyme Y3	2 µl
Reaction Mix	**25 µl**
Sample	Beads
• Reagent Y2*	5 µl (uniquely added to each sample)
Total Volume	**30 µl**

Indexing by PCR	
Reagents	**Volume per Sample**
Low EDTA TE	20 µl
• Buffer Y1	3 µl
• Enzyme Y3	2 µl
• Reagent TruY2**	5 µl
Reaction Mix	**30 µl**
Sample	Beads
Total Volume	**30 µl**

*** For indexing by ligation**, Reagent Y2 is the indexed i7 adapter and should be added individually to each sample.
**** For indexing by PCR**, Reagent TruY2 is the truncated adapter and should be added directly to the reaction mix. For Agilent SureSelect^{XT}, use 5 µl Y-XT adapter supplied in the XT Module.

12. The final reaction volume for each sample is 30 µl (listed in the table below). Place samples in the thermocycler and run the Ligation I Thermocycler Program at 25 °C for 15 minutes with lid heating OFF.

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

For Direct Sequencing:

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)

For Hyb Capture:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
All Inputs	All Sizes	30 µl	-	49.5 µl (ratio: 1.65)

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

Ligation II

Add 48 µl (for indexing by ligation) or 50 µl (for indexing by PCR) of pre-mixed Ligation II Reaction Mix (listed in the table below) to the beads for each sample and re-suspend by pipetting, then add the individual indexed i5 adapter to each sample *ONLY* if indexing by ligation (left).

Indexing by Ligation	
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
Reaction Mix	**48 µl**
Sample	Beads
• Reagent B2*	2 µl
Total Volume	**50 µl**

Indexing by PCR	
Reagents	**Volume per Sample**
Low EDTA TE	30 µl
• Buffer B1	5 µl
• Reagent TruB2**	2 µl
• Reagent B3	9 µl
• Enzyme B4	1 µl
• Enzyme B5	2 µl
• Enzyme B6	1 µl
Reaction Mix	**50 µl**
Sample	Beads
Total Volume	**50 µl**

* **For indexing by ligation**, Reagent B2 is the indexed i5 adapter and should be added individually to each sample.
** **For indexing by PCR**, Reagent TruB2 is the truncated adapter and should be added directly to the reaction mix. For Agilent SureSelect^{XT}, use 2 µl Reagent B2 adapter supplied in the XT Module.

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.

For Direct Sequencing:

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	-	45.5 µl (ratio: 0.85)	20 µl
>10 ng – 250 ng gDNA	All Sizes	50 µl	-	60.0 µl (ratio: 1.2)	20 µl

For Hyb Capture:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
All Inputs	All Sizes	50 µl	-	82.5 µl (ratio: 1.65)	20 µl

17. At the end of the clean-up step, 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.

---- SAFE STOPPING POINT ----
Store freshly prepared libraries at 4 °C (or long term at -20 °C).

Library Amplification

Library amplification is optional if using at least 100 ng input and full length, indexed adapters. Indexing PCR is required if using truncated adapters in order to complete the adapter sequences and index each library.

Use step 19-A if using full length, indexed Y2 and B2 adapters and Reagent R1 or the XT Module.

Use step 19-B if using truncated TruY2 and TruB2 adapters and Indexing Primers.

19-A For optional library amplification of fully indexed libraries, add 30 µl of the PCR Reaction Mix (listed in the table below) to the entire eluted library (20 µl). Be sure to include Reagent R1 (library amplification primers) in the reaction mix. Mix by moderate vortexing for 5 seconds and spin down in a centrifuge.

NOTE: If using Normalase Primers (Reagent R5), see Appendix Section C and the Normalase Kit Protocol for specific instructions.

Indexing by Ligation	
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
PCR Reaction Mix	**30 µl**
Eluted Sample	20 µl
Total Volume	**50 µl**

* For SureSelect^{XT} use 5 µl R-XT primer pair from the XT Module.

19-B For Indexing PCR, add 25 µl PCR Reaction Mix to each sample and then add a unique combination of indexing primers directly to each sample following the table below as a guideline. Mix by moderate vortexing for 5 seconds and spin down in a centrifuge.

IMPORTANT: If using the Normalase workflow see Appendix Section C and the Normalase Kit Protocol for specific instructions for Normalase PCR.

Indexing Options	Reagents	Volume per Sample
Single Indexing	Pre-mixed primer pair	5 µl
Unique Dual Indexing (UDI)	Pre-mixed primer pair	5 µl
Combinatorial Dual Indexing (CDI)	i5 primer i7 primer	2.5 µl 2.5 µl
Normalase Unique Dual Indexing* (UDI)	Pre-mixed primer pair Reagent R7	4 µl 1 µl

* The Normalase workflow is not required when using these indexing primers, add Reagent R7 to the PCR Reaction Mix, see Appendix Section C.

Indexing by PCR	
Reagents	**Volume per Sample**
Low EDTA TE	10 µl
• Reagent R2	4 µl
• Buffer R3	10 µl
• Enzyme R4	1 µl
PCR Reaction Mix	**25 µl**
Sample	20 µl
Indexing Primers	5 ul
Total Volume	**50 µl**

20. Place the sample tubes in the thermocycler and run the Indexing PCR Thermocycler Program below.

Input	Recommended PCR Cycles for Direct Sequencing*	Thermocycler Program
100 ng	3	98 °C for 30 seconds
10 ng	6	**PCR Cycles:**
1 ng	9	98 °C for 10 seconds
100 pg	12	60 °C for 30 seconds
10 pg	15	68 °C for 60 seconds
10 ng cfDNA	0 - 2	Hold at 4 °C – Proceed
1 ng cfDNA	5 - 6	immediately to clean-up step

IMPORTANT: The number of cycles require to produce 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.

*Please refer to vendor specific thermocycling instructions to achieve the desired library yield for pre-hybridization capture pooling, being sure to also use the recommended polymerase.

21. 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.

21-A For Direct Sequencing:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	All Sizes	50 µl	42.5 µl (ratio: 0.85)	-	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	70.0 µl (ratio: 1.4)	-	20 µl

IMPORTANT: If sequencing on patterned flow cells, the **optional second bead purification** below will reduce indexing primer carryover and lower index hopping artefacts. This purification is not required if sequencing on non-patterned flow cells, performing Hyb Capture or if using UDIs.

Optional Second Bead Purification:

At the end of the first 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 elute.

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
cfDNA	All Sizes	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

21-B For Hyb Capture:

dsDNA Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
All Inputs	All Sizes	50 µl	90 µl (ratio: 1.8)	-	20 µl of H_2O*

* It is important elute in water to prevent residual EDTA from interfering with downstream applications.

22. At the end of the clean-up, resuspend the beads in 20 µl of Low EDTA TE buffer for direct sequencing or 20 µl H_2O for Hyb Capture.

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

24. 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).

Quantification

PCR-free libraries must be quantified by qPCR as fluorometric methods such as Qubit® are unable to distinguish fully from partially adapted dsDNA. PCR-free libraries also migrate abnormally on electrophoretic chips such as Bioanalzyer due to the partially single stranded adapters so this method is not recommended.

PCR amplified library yields can be assessed by fluorometric, electrophoretic or qPCR-based methods.

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.

To quantify low-quality dsDNA samples using Input DNA Quantification Primers Cat. No. 90396. Please refer to the Protocol: Input DNA Quantification Assay for instructions.

Section B: Size Selection/Clean-Up Protocol

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

1. 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.
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 microcentrifuge.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the samples 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).
7. 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.
8. Repeat step 6 once more for a second wash with the 80% ethanol solution.
9. Quick spin the samples in a microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube.
10. Remove the samples from the magnetic rack.
11. Add the specified elution volume of Low EDTA TE buffer and re-suspend the pellet. Mix well by pipetting up and down until homogenous.
12. Place the samples back on the magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
13. Transfer the specified eluate volume 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, place on magnet for 2 minutes and transfer eluate again to a new 0.2 mL PCR tube.

Section C: Normalase PCR Instructions

Please review this section and the Normalase Kit protocol (https://swiftbiosci.com/protocols/) before setting up your Normalase PCR for Accel-NGS 2S Plus or post-hyb PCR. In order to achieve expected results, amplify each library using Normalase primers with the appropriate number of cycles and thermocycling conditions shown below to obtain a library yield of 12 nM or greater in a 20 µl eluate.

Normalase PCR Setups:

For Indexing by Ligation, use Normalase Primers (Reagent R5)

1. If you typically obtain the required minimum threshold (i.e. ≥ 12 nM following library amplification), simply utilize Normalase primers (Reagent R5 included in the Normalase Kit) and add one additional PCR cycle to your program.

2. If prior to amplification your library yields are ≥ 12 nM, a minimum of 3 cycles is still required to condition the libraries for downstream Normalase enzymology.

3. Assemble your PCR Master Mix using standard PCR reagents as shown in the table below, except substitute standard primers (Reagent R1 included with indexed adapters) with 5 µl of Reagent R5. Thoroughly mix by moderate vortexing, pulse spin to collect contents and place in the thermocycler.

Swift Hot Start High Fidelity Polymerase	
PCR RXN	**Master Mix**
Sample: 20 µl Master Mix: 30 µl **Final Volume: 50 µl**	Low EDTA TE: 10 µl Reagent R2: 4 µl Buffer R3: 10 µl Enzyme R4: 1 µl Reagent R5: 5 µl **Final Volume: 30 µl**

4. Run the following thermocycler program, adjusting the number of cycles depending on the input amount and sample quality. Lid heating set to 105 °C.

Temperature	Time
98 °C	30 sec
98 °C	10 sec
60 °C	30 sec
68 °C	60 sec
Perform X cycles*	
68 °C	5 min
4 °C	hold

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

DNA Input (ng)	Minimum number of cycles for ≥ 12 nM
≥ 100	4
10	8
1	11
0.1	14
0.01	17

5. Proceed to SPRI clean-up step 21-24 on page 12-13 of this protocol.

6. Proceed to Normalase I, Pooling, and Normalase II in the Normalase Kit Protocol.

For Indexing by PCR, use Normalase Indexing Primers

Normalase indexing primers complete the adapter sequences, amplify and condition libraries for downstream Normalase steps. Assemble using standard PCR reagents as shown below, except substitute standard indexing primers with Normalase Indexing Primers and Reagent R6 or R7.

1. To each sample library (20 µl eluate), add 2 µl of each Normalase Combinatorial Dual Indexing Primer or 4 µl of Normalase Unique Dual Indexing Primers for a total volume of 24 µl. See Appendix Section D for Index information.

Indexing Options	Reagents	Volume per Sample
Normalase CDI	i5 primer i7 primer	2.0 µl 2.0 µl
Normalase UDI	Pre-mixed primer pair	4.0 µl

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 final PCR volume) and place in the thermocycler.

Swift Hot Start High Fidelity Polymerase		
PCR RXN	**CDI Master Mix per Sample**	**UDI Master Mix per Sample**
Sample + Primers: 24 µl Master Mix: 26 µl **Final PCR Volume: 50 µl**	Low EDTA TE: 10 µl Reagent R2: 4 µl Buffer R3: 10 µl Enzyme R4: 1 µl Reagent R6: 1 µl **Master Mix: 26 µl**	Low EDTA TE: 10 µl Reagent R2: 4 µl Buffer R3: 10 µl Enzyme R4: 1 µl Reagent R7: 1 µl **Master Mix: 26 µl**

3. Repeat steps 4 - 6 of the above protocol for indexing by ligation.

Section D: Indexed Adapter Sequences

Below is a summary of indexing options for Accel-NGS 2S:

Indexing Kit	Reagent	Reagent Contents	Volume per Sample
Single Indexed Adapters (12 to 96-plex)	Set A+B (I1-I27) Set S1-S4 (S701-S796)	Indexed i7 Adapter	5 µl
	Reagent B2	Universal P5 Adapter	2 µl
Single Indexed Adapters + MID (12 to 96-plex)	Set A+B (I1-I27) Set S1-S4 (S701-S796)	Indexed i7 Adapter	5 µl
	Reagent B2-MID	MID i5 Adapter	2 µl
Combinatorial Dual Indexed Adapters (192 to 768-plex)	Set S1-S4 (S701-S796)	i7 Adapter	5 µl
	D501-D508	i5 Adapter	2 µl
Combinatorial Dual Indexing Primers (96-plex)	D501-D508	i5 primer	2.5 µl
	D701-D712	i7 primer	2.5 µl
Normalase Combinatorial Dual Indexing Primers (96-plex)	D501N-D508N	i5 primer	2 µl
	D701N-D712N	i7 primer	2 µl
Unique Dual Indexing Primers (24 to 96-plex)	U001-U096	Pre-mixed i5 and i7 primers	5 µl
Normalase Unique Dual Indexing Primers (96 to 384-plex)	SU001-SU384	Pre-mixed i5 and i7 primers	4 µl

To access the index sequences, please see the Swift Index Sequences Master List on the Protocols page at https://swiftbiosci.com/protocols/.

The full-length adapter sequences of the single, combinatorial dual, and unique dual indexes are below. The underlined text indicates the location of the index sequences. All Swift adapter sequences are Illumina TruSeq based (TS).

Swift Single Indexed Adapter Sequences (Set A+B, Set S1-S4, Universal and MID)

TS Universal Adapter:
5’ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

TS Index (i5) Adapter (MID):
5’ AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNACACTCTTTCCCTACACGACGCTCTTCCGATCT

TS Index (i7) Adapter (I1-I12):
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXX(AT)CTCGTATGCCGTCTTCTGCTTG

TS Index (i7) Adapter (I13-I27):
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXX(XX)ATCTCGTATGCCGTCTTCTGCTTG

TS Index (i7) Adapter (S701-S796):
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

The number on the product tube label indicates which index is provided in the tube. For Set A+B, the bases in parentheses are not considered part of the 6-base index sequences but can be used for 8-base index reads. For Set S1-S4, the index sequences are 8-base and the MID is a 9-base index read.

Each tube i5 adapters contains 22 µl and each i7 adapters contains 22 µl of the appropriate indexed adapters.

Swift Combinatorial Dual Indexed Adapter Sequences

TS Index (i7) Adapter (D701-D712 for 96-plex; S701-S796 for 192 to 768-plex):
5’ GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG

TS Index (i5) Adapter (D501-D508):
5’ AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT

For 96-plex Combinatorial Dual Indexing Primers, each D50X tube contains 33 µl and each D7XX tube contains 22 µl.

For 96-plex Normalase Combinatorial Dual Indexing Primers, each D50XN tube contains 27 µl and each D7XXN tube contains 18 µl. The Reagent R6 tube contains 106 µl.

For 192-plex Combinatorial Dual Indexed Adapters, each D50X tube contains 53 µl and each S7XX tube contains 44 µl.

For 768-plex Combinatorial Dual Indexed Adapters, each D50X tube contains 53 µl and each S7XX tube contains 44 µl.

Swift Unique Dual Indexed Adapter Sequences

The UDI 96-plex Set are 8-base index sequences, whereas the UDI 96 to 384-plex set is 10-base index sequences as depicted by the two additional (XX) bases.

TS Index (i7) Adapters:
5’ – GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX(XX)ATCTCGTATGCCGTCTTCTGCTTG – 3’

TS Index (i5) Adapters:
5’ – AATGATACGGCGACCACCGAGATCTACACXXXXXXXX(XX)ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3’

Each U0XX tube contains 22 µl of the appropriate indexing primer set. Each U0XX/SUXXX plate well contains 12 µl.

Section E: Product Ordering Information

Workflow Component	Product Name	Catalog Number
Library Prep Kits	Accel-NGS® 2S PCR-Free Library Kit (24, 96 or 4x96 rxns Bundle)	20024/20096/200384
	Accel-NGS® 2S Plus DNA Library Kit (24, 96 or 4x96 rxns Bundle)	21024/21096/210384
	Accel-NGS® 2S Hyb DNA Library Kit (24, 96 or 4x96 rxns Bundle)	23024/23096/230384
2S Hyb SureSelect^{XT}	2S SureSelect^{XT} MID Compatibility Module (24 or 96 rxns)	27424/27496
	2S SureSelect^{XT} Compatibility Module (24 or 96 rxns)	26424/26496
Normalase Kits	Swift Normalase® Kit (96 or 4x96 rxns Bundle)	66096/660384
Single Indexed Adapters (with and without MIDs)	2S Set A Single Indexed Adapters (12-plex, 48 rxns)	26148
	2S Set B Single Indexed Adapters (12-plex, 48 rxns)	26248
	2S Set A+B Single Indexed Adapters (24-plex, 96 rxns)	26396
	2S Set A MID Indexed Adapters (12-plex, 48 rxns)	27148
	2S Set B MID Indexed Adapters (12-plex, 48 rxns)	27248
	2S Set A+B MID Indexed Adapters (24-plex, 96 rxns)	27396
	2S Set S1 Single Indexed Adapters (24-plex, 96 rxns)	26596
	2S Set S2 Single Indexed Adapters (24-plex, 96 rxns)	26696
	2S Set S3 Single Indexed Adapters (24-plex, 96 rxns)	26796
	2S Set S4 Single Indexed Adapters (24-plex, 96 rxns)	26896
	2S Set S1-S4 Single Indexed Adapters (96-plex, 384 rxns)	269384
	2S Set S1 MID Indexed Adapters (24-plex, 96 rxns)	27596
	2S Set S2 MID Indexed Adapters (24-plex, 96 rxns)	27696
	2S Set S3 MID Indexed Adapters (24-plex, 96 rxns)	27796
	2S Set S4 MID Indexed Adapters (24-plex, 96 rxns)	27896
	2S Set S1-S4 MID Indexed Adapters (96-plex, 384 rxns)	279384
CDI Adapters	2S Set S1 Combinatorial Dual Indexed Adapters (192-plex, 192 rxns)	28596
	2S Set S2 Combinatorial Dual Indexed Adapters (192-plex, 192 rxns)	28696
	2S Set S3 Combinatorial Dual Indexed Adapters (192-plex, 192 rxns)	28796
	2S Set S4 Combinatorial Dual Indexed Adapters (192-plex, 192 rxns)	28896
	2S Set S1-S4 Combinatorial Dual Indexed Adapters (768-plex, 768 rxns)	289384
UDI Primers (with and without Truncated Adapters	Swift Unique Dual Indexing Primers (24-plex, 96 rxns **+ Truncated Adapters**)	29096
	Swift Unique Dual Indexing Primers (96-plex, 384 rxns **+ Truncated Adapters**)	290384
	Swift Unique Dual Indexing Primers (24-plex, 96 rxns)	X9096
	Swift Unique Dual Indexing Primers (96-plex, 384 rxns)	X90384
	Swift Unique Dual Indexing Primer Plate (96-plex, 96 rxns)	X9096-PLATE
	Swift Unique Dual Indexing Primer Plates (96-plex, 4x96 rxns Bundle)	X90384-PLATE
	Swift Normalase® Unique Dual Indexing Primer Plates (384-plex, 4x96 rxns Bundle)	X91384-PLATES
	Swift Normalase® Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU001-SU096)	X91096-1-PLATE
	Swift Normalase® Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU097-SU192)	X91096-2-PLATE
	Swift Normalase® Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU193-SU288)	X91096-3-PLATE
	Swift Normalase® Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU289-SU384)	X91096-4-PLATE
CDI Primers (with and without Truncated Adapters)	Swift Combinatorial Dual Indexing Primers (96-plex, 96 rxns **+ Truncated Adapters**)	28096
	Swift Combinatorial Dual Indexing Primers (96-plex, 96 rxns)	X8096
	Swift Normalase® Combinatorial Dual Indexing Primers (96-plex, 96 rxns **+ Truncated Adapters**)	69096
	Swift Normalase® Combinatorial Dual Indexing Primers (96-plex, 96 rxns)	68096
Truncated Adapters ONLY	Accel-NGS® 2S Truncated Adapters (96 rxns)	28196

Section F: 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. PCR-Free libraries migrate abnormally due to partially single stranded adapters.	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.
Incomplete resuspension of beads after ethanol wash during purification steps.	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension. To avoid over-drying, re-suspend beads immediately after the removal of residual ethanol. Make sure to prepare master mixes in advance so bead resuspension can be performed without delay, following removal of residual ethanol.
Shortage of enzyme reagents.	Pipetting enzymes at -20 °C.	Place enzyme reagents on ice for 10 minutes prior to pipetting.
Retention of liquid in pipette tip	Viscous reagents (i.e., enzymes) may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to ensure all liquid is released from the pipette tip.
Unexpected increase in adapter dimers	Improper bead purification Lower than expected input DNA quantity	Use the specified bead volume particularly for the post-PCR purification Carefully quantify input to ensure appropriate cleanup steps are performed

Support

For additional support please contact Swift at TechSupport@swiftbio.com, or by phone: 734.330.2568 (9:00 am – 5:00 pm ET, Monday through Friday).

Revision History

Document #	Revision	Date	Description of Change
PRT-037	Version 1	1/12/2021	Initial release.

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