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DNBSEQ Fast PCR-FREE FS Library Prep Set User Manual

- Cat. No.: 940-001314-00 (16 RXN)
  940-001315-00 (96 RXN)
  940-001316-00 (384 RXN)
- Set Version: V2.0

For Research Use Only

About the user manual

Manufacturer information

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Revision history

Manual Rev	Set version	Date	Description
2.0	V2.0	Jun. 2024	Update the set version
1.0	V1.0	Nov. 2023	Initial release

Use the latest version of the manual and use it with the corresponding kit.

Contents

1 Product overview

1.1 Introduction

The DNBSEQ Fast PCR-FREE FS (Fragmentation Shearing) Library Prep Set is designed to prepare WGS PCR-Free libraries for DNBSEQ high-throughput sequencing platforms. This library prep set is optimized to convert 50 ng - 900 ng genomic DNA (gDNA) into a customized library using a high-quality fast fragmentase to simplify the preparation process, thereby significantly shortening the duration of the DNA library preparation process. All reagents provided in this set have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

The DNBSEQ Fast PCR-FREE FS Library Prep Set uses dual barcode adapters for library preparation. It is recommended that you prepare 8 or more samples with this set due to the adapter design. For fewer than 8 samples, please contact CG Technical Support. The constructed libraries (adapter-ligated dsDNA) can be used with the DNBSEQ Dual Barcode Circularization Kit (not included in this set. Cat. No.: 940-001310-00) to create single-strand circular (ssCir) DNA libraries for subsequent DNB preparation. After DNB preparation, it can be directly sequenced on DNBSEQ high-throughput sequencing platforms.

1.2 Intended use

This library prep set is applicable to samples from human (blood, saliva, oral swabs, and so on), animals (rat, mouse, and so on), plants (*A. thaliana*, *O. sativa*, and so on), bacteria (*E. coli*, and so on), fungi (yeast, and so on), and microorganisms (fungi, bacteria, environmental samples for metagenomics). In addition, the library prep set is also used for long amplicon DNA fragments.

1.3 Applicable sequencing platforms

Select the appropriate sequencing platform and sequencing type based on the recommended application sample.

Table 1 Sequencing platform and sequencing type recommendation

Recommended application sample	Sequencing platform	Sequencing type
Humans (blood, saliva, oral swabs), animals, plants, and microorganisms	DNBSEQ-G400RS	PE100/PE150
	DNBSEQ-T7RS	PE100/PE150
	DNBSEQ-T10x4RS	PE100/PE150
	DNBSEQ-T20x2RS	PE100
Long amplicon DNA, microorganisms	DNBSEQ-G99RS	PE100/PE150

1.4 Components

This library prep set comes in three specifications: 16 RXN, 96 RXN and 384 RXN. For component details, refer to Table 2, Table 3 and Table 4.

Table 2 DNBSEQ Fast PCR-FREE FS Library Prep Set (16 RXN) (Cat. No.: 940-001314-00)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
DNBSEQ Fast PCR-FREE FS Library Prep Module Cat. No.: 940-001300-00	Fast FS Buffer II	Green	215 µL/tube × 1
	Fast FS Enzyme II	Green	105 µL/tube × 1
	Fast Ligation Buffer	Red	450 µL/tube × 1
	Ad Ligase	Red	100 µL/tube × 1
	Ligation Enhancer	Brown	55 µL/tube × 1
	20x Elute Enhancer	Black	7 µL/tube × 1
DNBSEQ UDB PF Adapter Kit Cat. No.: 940-001298-00	UDB Adapters	Blue	5 µL/tube × 16
DNBSEQ DNA Clean Beads Cat. No.: 940-001284-00	DNA Clean Beads	White	3.2 mL/tube × 1
	TE Buffer	White	3.2 mL/tube × 1

Table 3 DNBSEQ Fast PCR-FREE FS Library Prep Set (96 RXN) (Cat. No.: 940-001315-00)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
DNBSEQ Fast PCR-FREE FS Library Prep Module Cat. No.: 940-001295-00	Fast FS Buffer II	Green	1440 µL/tube × 1
	Fast FS Enzyme II	Green	660 µL/tube × 1
	Fast Ligation Buffer	Red	1440 µL/tube × 3
	Ad Ligase	Red	600 µL/tube × 1
	Ligation Enhancer	Brown	360 µL/tube × 1
	20x Elute Enhancer	Black	25 µL/tube × 1
	TE Buffer	White	4 mL/tube × 2
DNBSEQ UDB PF Adapter Kit A Cat. No.: 940-001301-00	UDB Adapters A	-	5 µL/well × 96
DNBSEQ DNA Clean Beads Cat. No.: 940-001283-00	DNA Clean Beads	White	15 mL/tube × 1
	TE Buffer	White	17 mL/tube × 1

Table 4 DNBSEQ Fast PCR-FREE FS Library Prep Set (384 RXN) (Cat. No.: 940-001316-00)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
DNBSEQ Fast PCR-FREE FS Library Prep Module ×4 kits Cat. No.: 940-001295-00	Fast FS Buffer II	Green	1440 µL/tube × 1
	Fast FS Enzyme II	Green	660 µL/tube × 1
	Fast Ligation Buffer	Red	1440 µL/tube × 3
	Ad Ligase	Red	600 µL/tube × 1
	Ligation Enhancer	Brown	360 µL/tube × 1
	20x Elute Enhancer	Black	25 µL/tube × 1
	TE Buffer	White	4 mL/tube × 2
DNBSEQ UDB PF Adapter Kit A Cat. No.: 940-001301-00	UDB Adapters A	-	5 µL/well × 96
DNBSEQ UDB PF Adapter Kit B Cat. No.: 940-001299-00	UDB Adapters B	-	5 µL/well × 96
DNBSEQ UDB PF Adapter Kit C Cat. No.: 940-001296-00	UDB Adapters C	-	5 µL/well × 96
DNBSEQ UDB PF Adapter Kit D Cat. No.: 940-001297-00	UDB Adapters D	-	5 µL/well × 96
DNBSEQ DNA Clean Beads Cat. No.: 940-001281-00	DNA Clean Beads	White	50 mL/tube × 1
	TE Buffer	White	25 mL/tube × 1

1.5 Storage and transportation

Table 5 Kit storage and transportation

Modules	Storage temperature	Transport temperature
DNBSEQ Fast PCR-FREE FS Library Prep Module	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
DNBSEQ UDB PF Adapter Kit		
DNBSEQ UDB PF Adapter Kit A		
DNBSEQ UDB PF Adapter Kit B		
DNBSEQ UDB PF Adapter Kit C		
DNBSEQ UDB PF Adapter Kit D		
DNBSEQ DNA Clean Beads	2 ℃ to 8 ℃	2 ℃ to 8 ℃

- Production date and expiration date: refer to the label.
- For ice packs or dry ice shipments, ensure that there is enough ice or dry ice remaining after transportation.
- With proper transport, storage, and use, all components can maintain complete activity within their designated shelf life.
- In DNBSEQ Fast PCR-FREE FS Library Prep Module, TE Buffer should be stored at 2 ℃ - 8 ℃, and 20 × Elute Enhancer and Ligation Enhancer should be stored at room temperature. Avoid repeated freeze-thawing. The Ligation Enhancer should be stored away from light.

1.6 User-supplied materials

Table 6 Order information for CG products

Catalog number	Model	Name
940-001310-00	16 RXN	DNBSEQ Dual Barcode Circularization Kit
940-001309-00	96 RXN	DNBSEQ Dual Barcode Circularization Module (Customized)
940-001281-00	50 mL	DNBSEQ DNA Clean Beads

Prepare reagent kits based on application requirements. The circularization kit and DNA clean beads can be prepared along with the library preparation set to complete the library making and get a circularized ssDNA from the adapter ligated library.

Table 7 User-supplied equipment list

Equipment	Recommended brand
Vortex mixer	General lab supplier
Desktop centrifuge	General lab supplier
Pipettes	General lab supplier
Thermocycler	General lab supplier
96M Magnum Plate	ALPAQUA, Part A000400 (Recommended)
Qubit® Fluorometer 2.0 or higher	Thermo Fisher (Cat. No.: Q33216), or equivalent
Agilent 2100 Bioanalyzer or Tape station	Agilent Technologies (Cat. No.: G2939AA), Tape station, or equivalent

Table 8 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Nuclease Free (NF) water	Ambion (Cat. No.: AM9937), or equivalent
TE Buffer, pH 8.0	Ambion (Cat. No.: AM9858), or equivalent
100% Ethanol (Analytical Grade)	Fisher BioReagents™ (Cat. No.: BP2818500), or equivalent
Qubit ssDNA Assay Kit	Invitrogen (Cat. No.: Q10212), or equivalent
Qubit dsDNA HS Assay Kit/Quant-iT	Invitrogen (Cat. No.: Q32854), or equivalent
Agilent High Sensitivity DNA Kit or Tape station High sensitive DNA kit	Agilent (Cat. No.: 5067-4626), or equivalent
Pipette tips	Axygen, or equivalent
1.5 mL tube	Axygen, or equivalent
0.2 mL PCR tube or 96-well plate	Axygen (Cat. No.: PCR-02-C or PCR-96M2-HS-C), or equivalent
Qubit Assay Tubes or 0.5 mL Thin Wall PCR Tubes	Invitrogen (Cat. No.: Q32856) or Axygen (Cat. No.: PCR-05-C), or equivalent

1.7 Precautions

1.7.1 Using the UDB PF Adapters

This kit is designed for the construction of dual barcode libraries only, and the barcode sequences are designed to be located on the adapter sequences. When multiple samples are mixed together for sequencing, barcodes can be used to accurately attribute the sequencing results to the appropriate samples. To meet the requirements for batch processing of library construction and multiplex sequencing, the best adapter combinations were selected based on the principle of balanced base composition.

Based on the principles of balanced base composition, adapters must be used in specific groups. Follow the instructions below to use the adapters in the proper combinations. There are three specifications of UDB PF Adapter Reagent Kits, depending on the number of reactions.

Table 9 The UDB PF Adapters of the DNBSEQ Fast PCR-FREE FS Library Prep Set

Model	Reagent Kits	Note
16 RXN	DNBSEQ UDB PF Adapter Kit	2 groups, each with 8 barcodes
96 RXN	DNBSEQ UDB PF Adapter Kit A	12 groups, each with 8 barcodes
384 RXN	DNBSEQ UDB PF Adapter Kit A	12 groups, each with 8 barcodes
	DNBSEQ UDB PF Adapter Kit B	12 groups, each with 8 barcodes
	DNBSEQ UDB PF Adapter Kit C	1 group, each with 96 barcodes
	DNBSEQ UDB PF Adapter Kit D	1 group, each with 96 barcodes

“UDB PF Adapter Kit's barcode number and sequence information” on page 34

1.7.1.1 Note for UDB PF Adapters

- Pay attention to the adapter number of different specifications of DNBSEQ UDB PF Adapter Kit. Among the adapters from the sets, adapters with the same ID number share the same sequence and thus cannot be sequenced in the same lane.
- All adapters are double stranded. To prevent structural changes that might affect performance, such as denaturation, do not place the adapters above 30 ℃.
- To prevent cross-contamination, change tips when pipetting different adapters.
- Before use, centrifuge the adapters to collect liquid at the bottom of tubes or plates. Gently remove the cap/sealing film to prevent spilling and cross-contamination. Mix adapters with a pipette before use. Remember to reseal the adapters immediately after use.
- For Adapters-96 (Plate), if the seal film is contaminated, discard the contaminated seal film and use a new one to reseal the 96-well plate.

- Adapters from other CG Library Prep Kits are designed differently and cannot be mixed with the adapters described here. Otherwise, errors will occur during barcode demultiplexing procedures on DNBSEQ platforms.

1.7.1.2 UDB PF Adapters pooling guide

It is recommended that you optimize the base balance by planning UDB PF Adapter with diverse sequences when pooling libraries across DNBSEQ systems. Pooling combines at least eight libraries to sequence in one lane.

The following three application scenarios are predefined for the recommended method of selecting UDB PF Adapter.

1. The sequencing data output requirement is the same for all samples in one lane. Choose the UDB PF Adapter combinations in Table 10.

   i Here X means positive integer. For example: 8X = 8 multiplied by X , which means there are 8X samples.

Table 10 UDB PF Adapter pooling guide

Sample/lane	Instruction (Example)
8X	Add 1 UDB PF Adapter per sample, from X set of 8 UDB PF Adapters (X column total). For example: X is equal to 1. The selected UDB PF adapter is 393-400. Add adapter 393 to sample 1, adapter 394 to sample 2, ... adapter 400 to sample 8.
8X+1	Add X set of 8 UDB PF Adapters + 1 random well of UDB PF Adapter
8X+2	Add X set of 8 UDB PF Adapters + 2 random wells of UDB PF Adapters
8X+3	Add X set of 8 UDB PF Adapters + 3 random wells of UDB PF Adapters
8X+4	Add X set of 8 UDB PF Adapters + 4 random wells of UDB PF Adapters
8X+5	Add X set of 8 UDB PF Adapters + 5 random wells of UDB PF Adapters
8X+6	Add X set of 8 UDB PF Adapters + 6 random wells of UDB PF Adapters
8X+7	Add X set of 8 UDB PF Adapters + 7 random wells of UDB PF Adapters

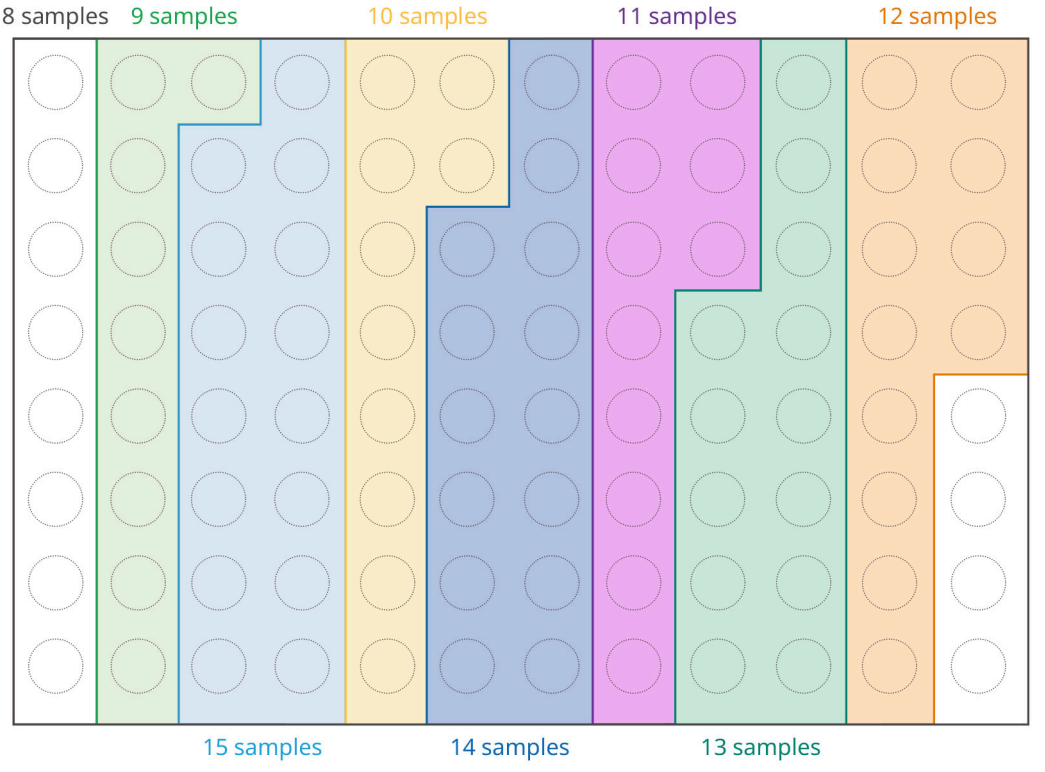

Figure 1 Dual barcode 8 to 15 samples pooling example

2. Under exceptional circumstances (for example, insufficient reagents for a well), when the requirement of at least one balanced UDB PF Adapter combination for standard pooling cannot be met, or if the required data amount of each library pooled is not equal, make sure to determine the pooling strategy by calculating the content of each base in each sequencing cycle. It is necessary to ensure that each base content is not less than 12.5% and is not greater than 62.5% in single sequencing position in the same lane.

Table 11 Balanced 8 UDB PF Adapter Pooling strategy (8 UDB PF Adapter from one entire column)

	Position of base in adapter sequence									
	Base 1	Base 2	Base 3	Base 4	Base 5	Base 6	Base 7	Base 8	Base 9	Base 10
Adapter 1	A	G	G	A	C	G	T	A	G	A
Adapter 2	C	T	G	A	A	C	C	G	A	A
Adapter 3	G	A	A	C	G	T	G	T	C	G
Adapter 4	T	C	C	G	T	G	A	C	T	C
Adapter 5	A	A	T	T	C	A	C	T	G	T
Adapter 6	C	C	T	G	A	A	G	G	A	T
Adapter 7	T	T	C	C	T	T	A	C	T	G
Adapter 8	G	G	A	T	G	C	T	A	C	C
Signal % per base	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0

Table 12 Unbalanced 9 UDB PF Adapter Pooling strategy (UDB PF Adapters from different columns)

	Position of base in adapter sequence									
	Base 1	Base 2	Base 3	Base 4	Base 5	Base 6	Base 7	Base 8	Base 9	Base 10
Adapter 1	A	G	G	A	C	G	T	A	G	T
Adapter 2	A	C	G	A	A	G	G	T	C	C
Adapter 3	G	A	A	C	G	T	G	T	C	G
Adapter 4	T	C	C	G	T	G	A	C	T	C
Adapter 5	A	A	T	T	C	A	C	T	G	T
Adapter 6	G	C	T	G	A	A	G	G	A	T
Adapter 7	T	G	C	C	T	T	A	C	T	G
Adapter 8	G	G	A	T	G	A	T	A	C	C
Adapter 9	G	A	C	G	G	T	C	G	A	G
A signal %	33.3	33.3	22.2	22.2	22.2	33.3	22.2	22.2	22.2	0
T signal %	22.2	0	22.2	22.2	22.2	33.3	22.2	33.3	22.2	33.3
C signal %	0	33.3	33.3	22.2	22.2	0	22.2	22.2	33.3	33.3
G signal %	44.4	33.3	22.2	33.3	33.3	33.3	33.3	22.2	22.2	33.3

1.7.2 General precautions and warnings

- This product is for research use only, not for clinical diagnosis. Read this manual carefully before use.
- Familiarize yourself with the precautions and operation methods of various instruments before performing the experiment.
- This manual aims to provide a standard protocol. Changes can be made for different applications, but changes must be tested prior to starting the protocol.
- It is recommended that you use pipette tips with filters to prevent cross-contamination. Use a new tip each time you pipette different solutions or samples.
- It is recommended that you use the thermocyclers with heated lids for reactions. Preheat the thermocyclers to reaction temperature before use. If the thermocycler does not allow for lid temperature adjustments, the preset lid temperature of 105 ℃ is sufficient.
- To prevent yield loss, try to avoid transferring reaction product to a new tube for bead purification.
- Avoid skin and eye contact with samples and reagents. In case of contact with skin and eyes, rinse immediately with plenty of water and seek medical advice.
- Conform to the law and regulations when disposing of all samples and reagents.
- If you have questions, contact Technical Support: **US-TechSupport@CompleteGenomics.com**.

2 Sample preparation

2.1 Sample type

This library preparation set is applicable to samples from human (blood, saliva, oral swabs , and so on), animals (rat, mouse , and so on), plants (*A. thaliana*, *O. sativa*, and so on), bacteria (*E. coli*, and so on), fungi (yeast, and so on) and microorganisms (fungi, bacteria, environmental samples for metagenomics). In addition, it is also used for long amplicon DNA fragments.

Different types of samples should be performed in a fragmentation demo test before library preparation to achieve the best results. For the relevant experimental manipulations, see " Fragment demo test (optional)" on page 17.

2.2 Sample purity and requirements

It is strongly recommended that you use high-quality genomic DNA ($1.8 \leq OD_{260/280} \leq 2.0$, $OD_{260/230} \geq 1.7$) for fragmentation. If the sample purity does not satisfy the recommended standards, or if enzyme inhibitors are present, there is a likelihood of low library yield.

Fast FS Enzyme II is sensitive to the pH and component of the DNA storage buffer. It is recommended that you use TE Buffer (pH 8.0) for DNA dissolution.

- If DNA is dissolved in other buffers, such as 10 mM Tris (pH 6.8-8.0), AE Buffer (pH 8.5), 0.1x TE (pH 8.0) or other special buffers, perform a demo fragmentation test by adjusting the incubation time of 30 ℃ in "Table 22 Fragmentation reaction conditions (Volume: 60 µL)" on page 18. The incubation time can be titrated from 7.5 to 15 min.
- If the sample contains many impurities and inhibitors, it is recommended to re-purify the sample DNA with 1.8x magnetic beads and elute it with TE Buffer (pH 8.0). After repurification, carry out the fragmentation test.

2.3 Sample input

50 ng - 900 ng gDNA can be used for library preparation. If the amount of gDNA is sufficient, it is recommended that a high input of genomic DNA be used for library preparation. Qubit is recommended for quantification of sample concentrations.

It is strongly recommended that you consider the sample input in conjunction with size selection method.Refer to Table 13 for scheme selection.

Table 13 Recommended sample input range

Input range (ng)	Recommended input (ng)	Size selection method
50 - 500	All input	Single size selection
500 - 900	500	Single size selection
≥ 900	900	Double size selection

2.3.1 Choosing a size selection method

A narrow size distribution of fragmented DNA is preferable. Better sequencing quality can be obtained with a narrow size distribution, while a wide distribution results in lower quality. The single size selection library has a wider insert size distribution than the double size selection library, which results in lower sequencing quality. It is recommended that you use double size selection method to construct the library. Do not pool double size selection library with single size selection library together for sequencing.

- The recommended peak size of single size selection library is 600 bp - 850 bp.
- The recommended peak size of double size selection library is 400 bp - 600 bp.
- For experimental steps related to size selection, see “Cleanup of fragmentation product ” on page 21.

2.3.2 The Make DNB protocol during sequencing

From gDNA to DNB, it is recommended that you use sufficient genomic DNA to construct the library.

- If gDNA input is less than 200 ng, the yield of ssCir is usually insufficient for sequencing once. In this case, library pooling with other PCR-free libraries may be necessary.
- If gDNA input is less than 900 ng, there is an increased possibility of low library yield through double size selection purification.
- For the relevant experimental manipulations, see “Circularization and DNB preparation” on page 29.

3 Library preparation protocol

3.1 Workflow

Table 14 Time consumption of workflow

Workflow	Hands-on time (one reaction)	Actual time taken (one reaction)
Fragmentation	2 min	27 min
Cleanup of fragmentation product	1 - 2 min	7 - 13 min
Adapter ligation	2 min	12 min
Cleanup of adapter-ligated product ⏸	5 - 10 min	23 - 36 min
QC of adapter-ligated product ⏸	2 min	4 min
Total	12 -18 min	73 - 92 min

- Hands-on time: The total required hands-on time in the process.
- Actual time taken: When sample input is more than 200 ng, the theoretical use time of one reaction. The time will be extended if the number of reactions increases.
- ⏸ **Stop point**.

The protocol in this chapter is only for dual barcode library preparation.

50 ng - 900 ng gDNA sample can be quickly converted to adapter-ligated libraries by fragmentation, end repair, simple purification, adapter ligation, and purification. The adapter-ligated product (dsDNA) can be converted to DNB. Use the DNBSEQ Dual Barcode Circularization Kit/Module to convert dsDNA library to ssCir and prepare DNB by using the High-throughput Sequencing kit. Refer to "Circularization and DNB preparation" on page 29.

3.2 Reagent preparation

3.2.1 Preparation

Table 15 Preparing the reagents

Reagent	Requirement
Nuclease-Free Water	User-supplied; place at room temperature (RT); mix thoroughly
TE Buffer	
20x Elute Enhancer	Place at RT; mix thoroughly
DNA Clean Beads	

3.2.2 Operation

CAUTION The preparation volume of reagents listed below is enough for 8 samples. Increase the preparation reagent volumes proportionally if there are more samples.

1. Prepare the 1x Elute Enhancer according to Table 16. Mix it by vortexing, and centrifuge briefly. Store at room temperature before using. The shelf life of the 1x Elute Enhancer is 7 days.

Table 16 1x Elute Enhancer

Reagent	Volume
20x Elute Enhancer	1 µL
Nuclease-Free Water	19 µL
Total	20 µL

2. Prepare the En-TE according to Table 17. Mix it by vortexing, and centrifuge briefly. Store at 4 ℃ before using. The shelf life of the En-TE is 60 days.

Table 17 En-TE

Reagent	Volume
1x Elute Enhancer	3 µL
TE Buffer	1497 µL
Total	1500 µL

3. Prepare the En-Beads according to Table 18. Mix it by vortexing, and centrifuge briefly. Store at 4 ℃ before using. The shelf life of the En-Beads is 60 days.

Table 18 En-Beads

Reagent	Volume
1x Elute Enhancer	10 μL
DNA Clean Beads	990 μL
Total	1000 μL

3.3 Fragmentation

The extent of fragmentation (size distribution of DNA fragments) is controlled by time and temperature. Make sure the time and temperature during the fragmentation reaction are consistent with Table 22 or Table 25. Samples and enzyme mix should always be kept on ice during preparation.

3.3.1 Preparation

Mix the reagents before using and store back the remaining reagents immediately after use.

Table 19 Preparing the reagents

Reagent	Requirement
TE Buffer (pH 8.0)	User-supplied; place at RT
Fast FS Buffer II	Thaw at RT; vortex; centrifuge briefly; place on ice
Fast FS Enzyme II	Keep on ice
80% ethanol	User-supplied; freshly prepared
En-TE	Refer to Table 17; place at RT
En-Beads	Refer to Table 18; allow 30 min to equilibrate to RT before use; mix thoroughly by vortexing before each use

3.3.2 Fragment demo test (optional)

Different types of samples should be performed in a fragmentation demo test before library preparation to achieve the best results. The following steps are recommended for Fragment demo testing. This section can be skipped if optimal conditions have been determined.

1. Normalize gDNA referring to Table 20. Based on the sample concentration, transfer the appropriate gDNA (recommended 50 ng - 900 ng) to a new 0.2 mL PCR tube. Add TE Buffer (pH 8.0) to make a total volume of 45 μL. Place the normalized gDNA on ice.

Table 20 Normalization of gDNA dissolved in TE (pH 8.0)

Components	Volume
TE Buffer (pH 8.0)	45-X μL
gDNA (50 ng - 900 ng)	X μL
Total	45 μL

This Fast FS Enzyme II is pH-sensitive. The normalization buffer should be the same buffer as the DNA elution buffer.

2. Set and run the program (refer to Table 22). The thermocycler will perform the first step reaction described in Table 22 and be kept at 4 ℃.
3. Mix Fast FS Enzyme II by inverting 10 times and flicking the bottom of the tube gently. Ensure that no residual reagent is left at the bottom each time. Centrifuge briefly and place it on ice until use.

CAUTION DO NOT vortex the Fast FS Enzyme II. Strictly follow the manual instructions. Insufficient mixing of prepared mixtures will affect the fragmentation process. Samples and enzyme mix should always be kept on ice during preparation.

4. According to the desired reaction number, prepare the fragmentation mixture in a 1.5 mL centrifuge tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 21 Fragmentation mixture

Reagent	Volume per reaction
Fast FS Buffer II	10 μL
Fast FS Enzyme II	5 μL
Total	15 μL

5. Add 15 µL of fragmentation mixture to each sample tube from step 1 (volume: 45 µL). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
6. Place the PCR tube(s) into the thermocycler and skip the first step (4 ℃ Hold) to start the reaction.

Table 22 Fragmentation reaction conditions (Volume: 60 µL)

Temperature	Time
70 ℃ Heated lid	On
4 ℃	Hold
30 ℃	X min
65 ℃	15 min
4 ℃	Hold

Incubation time for X here, 7.5 - 15 minutes at 30 °C is recommended. The time can be shortened or extended depending on the situation.

The extent of fragmentation (size distribution of DNA fragments) is controlled by time and 30 ℃ temperature. Make sure the time and temperature are correct during the fragmentation reaction. When the reaction is finished and in the 4°C hold state, do not stop there. Take out the tube in time for subsequent operations.

7. Add 0.8x magnetic beads (48 µL of En-Beads) to each sample tube. Mix with a vortexer until all beads are suspended.
8. Incubate the tube(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard the supernatant.
10. While keeping the PCR tube(s) on the magnetic rack, add 160 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
11. Repeat step 10. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
12. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.
13. Remove the tube(s) from the magnetic rack and add 47 µL of En-TE to elute the DNA. Mix with a vortexer until all beads are suspended.

If gDNA input is less than 100 ng, add 15 µL of En-TE for eluting in step 13.

14. Incubate the tube(s) at room temperature for 5 min.
15. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 45 µL of supernatant to a new 0.2 mL PCR tube.

If gDNA input is less than 100 ng, transfer 13 µL of supernatant to a new 0.2 mL PCR tube.

16. Use 2 µL of supernatant to quantify the concentration with Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit. The concentration is more than 1.5 ng/µL, it is a qualified ligation product.

CAUTION Different instruments may show some differences in test results. Figure 2 shows the results in Agilent 2100. Use 1 µL of elute product for Agilent 2100 High Sensitivity test and ensure that the smear size is 100 bp - 3000 bp with the peak size between 600 bp - 850 bp. For example: 200 ng gDNA dissolved in TE Buffer (pH 8.0), when fragmentation time is 8.5 min, the size distribution of fragments is shown in Figure 2.

If the peak size is too large or too small, it is recommended that you re-adjust the incubation time at step 6 in section 3.3.2 (incubation time of 7.5 min - 15 min at 30 ℃ is recommended).

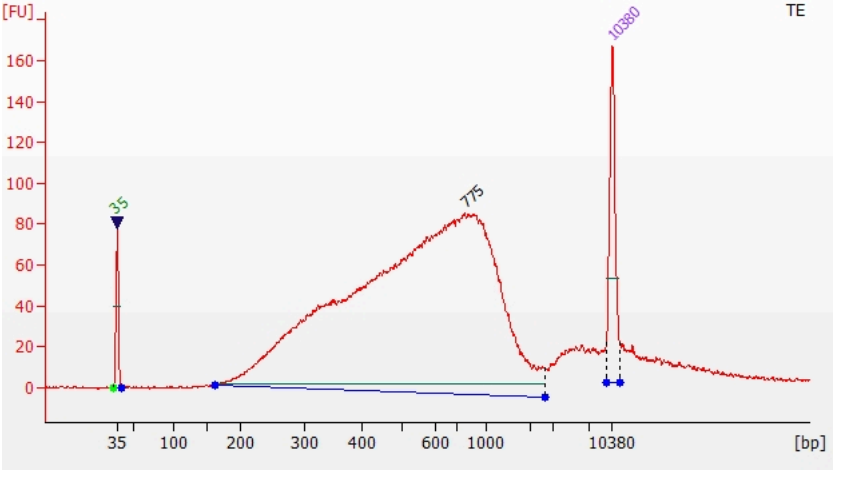

Figure 2 Agilent 2100 Bioanalyzer results of 0.8x beads purification fragmentation product

3.3.3 Fragmentation

If the optimal fragmentation time by the fragment demo test have been determined, or if you have no plan to perform the demo test, the following steps can be performed to construct libraries, and the incubation time is X or the recommended 8.5 min to fragment.

1. Normalize gDNA referring to Table 23. Based on the sample concentration, transfer the appropriate gDNA (recommended 50 ng - 900 ng) to a new 0.2 mL PCR tube. Add TE Buffer (pH 8.0) to make a total volume of 45 μL. Place the normalized gDNA on ice.

Table 23 Normalization of gDNA dissolved in TE (pH 8.0)

Components	Volume
TE Buffer (pH 8.0)	45-X μL
gDNA (50 ng - 900 ng)	X μL
Total	45 μL

This Fast FS Enzyme II is pH-sensitive. The normalization buffer should be the same buffer as the DNA elution buffer.

2. Set and run the program (refer to Table 25). The thermocycler will perform the first step reaction described in Table 25 and be kept at 4 ℃.
3. Mix Fast FS Enzyme II by inverting 10 times and flicking the bottom of the tube gently. Ensure that no residual reagent is left at the bottom each time. Centrifuge briefly and place it on ice until use.

CAUTION DO NOT vortex the Fast FS Enzyme II. Strictly follow the manual instructions. Insufficient mixing of prepared mixtures will affect the fragmentation process. Samples and enzyme mix should always be kept on ice during preparation.

4. According to the desired reaction number, prepare the fragmentation mixture in a 1.5 mL centrifuge tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 24 Fragmentation mixture

Reagent	Volume per reaction
Fast FS Buffer II	10 μL
Fast FS Enzyme II	5 μL
Total	15 μL

5. Add 15 µL of fragmentation mixture to each sample tube from step 1 (volume: 45 µL). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
6. Place the PCR tube(s) into the thermocycler and skip the first step (4 ℃ Hold) to start the reaction.

Table 25 Fragmentation reaction conditions (Volume: 60 µL)

Temperature	Time
70 ℃ Heated lid	On
4 ℃	Hold
30 ℃	X or 8.5 min
65 ℃	15 min
4 ℃	Hold

The value of X here comes from "Table 22 Fragmentation reaction conditions (Volume: 60 µL)" on page 18.

The extent of fragmentation (size distribution of DNA fragments) is controlled by time and 30 ℃ temperature. Make sure the time and temperature are correct during the fragmentation reaction. When the reaction is finished and in the 4°C hold state, do not stop there. Take out the tube in time for subsequent operations.

7. After the reaction, centrifuge the tube(s) briefly and immediately proceed to the next step.

CAUTION DO NOT STOP AT THIS STEP. Proceed to section 3.4.

3.4 Cleanup of fragmentation product

- Before starting the experiment, carefully read "Sample preparation" on page 12. Select the appropriate size selection method.
- Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.4.1 Single size selection (option 1)

3.4.1.1 Preparation

Table 26 Preparing the reagents

Reagent	Requirement
En-TE	Refer to Table 17; place at RT.
En-Beads	Refer to Table 18; allow 30 min to equilibrate to RT before use; mix thoroughly by vortexing before each use

3.4.1.2 Size selection

1. Check the volume of the fragmentation product (from step 7 in section 3.3.3). If the volume is less than 60 µL, add EN-TE to make a total volume of 60 µL.
2. Mix the En-Beads thoroughly. Add 48 µL of En-Beads to each sample tube. Mix with a vortexer until all beads are suspended.
3. Incubate at room temperature for 5 min.
4. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard all of the supernatant. If some liquid remains on the tube wall, centrifuge the tube(s) briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
5. Remove the tube(s) from the magnetic rack and add 47 µL of En-TE to elute the DNA. Mix with a vortexer until all beads are suspended, and centrifuge briefly.

3.4.1.3 QC of single size product (optional)

When single size selection is selected, the peak size of the single size product is approximately 600 bp - 850 bp. The size distribution of the fragments is similar to Figure 3 on page 24.

CAUTION DO NOT STOP AT THIS STEP. Proceed to section 3.5. Take the beads to the next reaction.

3.4.2 Double size selection (option 2)

3.4.2.1 Preparation

Table 27 Preparing the reagents

Reagent	Requirement
En-TE	Refer to Table 17; place at RT
En-Beads	Refer to Table 18; allow 30 min to equilibrate to RT before use; mix thoroughly by vortexing before each use

3.4.2.2 Size selection

1. Check the volume of the fragmentation product (from step 7 in section 3.3.3). If the volume is less than 60 µL, add EN-TE to make total volume of 60 µL.
2. Mix the En-Beads thoroughly. Add 32 µL of En-Beads to each sample tube. Mix with a vortexer until all beads are suspended.
3. Incubate at room temperature for 5 min.
4. Centrifuge the tube(s) briefly and place it on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully transfer 92 µL of supernatant to a new 0.2 mL PCR tube.

i In this step, keep the supernatant and discard the beads.

5. Add 16 µL of En-Beads to each sample tube (from step 4, volume: 92 µL). Mix with a vortexer until all beads are suspended.
6. Incubate at room temperature for 5 min.
7. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard all of the supernatant. If some liquid remains on the tube wall, centrifuge the tube(s) briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
8. Remove the tube(s) from the magnetic rack and add 47 µL of En-TE to elute the DNA. Mix with a vortexer until all beads are suspended, and centrifuge briefly.

⚠ **CAUTION** DO NOT STOP AT THIS STEP. Proceed to section 3.5. Take the beads to the next reaction.

3.4.2.3 QC of double size product (optional)

When double selection is selected for the first time, the following steps can be used to detect the size distribution of double size product.

1. Transfer 10 µL of double size product (from step 6 in section 3.4.2.2) into a new 0.2 mL PCR tube.
2. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard the supernatant.
3. While keeping the PCR tube(s) on the magnetic rack, add 160 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
4. Repeat step 3. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
5. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.
6. Remove the tube(s) from the magnetic rack and add 5 µL of En-TE to elute the DNA. Mix with a vortexer until all beads are suspended.
7. Incubate the tube(s) at room temperature for 5 min.
8. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully transfer 4 µL of supernatant to a new 0.2 mL PCR tube.

Different instruments may have some differences in test results. Figure 3 shows the results in Agilent 2100. Take 1 µL of elute product for Agilent 2100 High Sensitivity test. The peak size of the selected fragments is approximately 400 bp - 600 bp.

For example: 900 ng gDNA dissolved in TE Buffer (pH 8.0), when fragmentation time is 8.5 min, the size distribution of the fragments is shown in Figure 3.

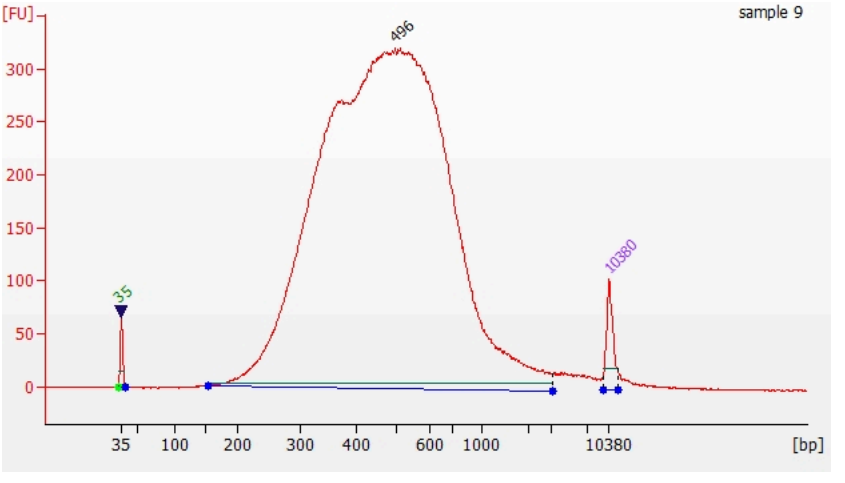

Figure 3 Agilent 2100 Bioanalyzer results of fragmentation product by double-sided size purification

3.5 Adapter ligation

The barcode is in the adapter. Before operation, carefully read "Using the UDB PF Adapters " on page 7 and "UDB PF Adapter Kit's barcode number and sequence information" on page 34.

3.5.1 Preparation

Mix the reagents before using, and store back the remaining reagents immediately after use.

Table 28 Preparing the reagents

Reagent	Requirement
UDB PF Adapter Kits Series	Thaw at RT; mix thoroughly; centrifuge briefly; place on ice
Fast Ligation Buffer	
Ad Ligase	Flick and/or invert the tube gently; centrifuge briefly; place on ice
Ligation Enhancer	Mix thoroughly; centrifuge briefly; place at RT

- Mix the adapter thoroughly before use. Adapters should not be mixed directly with the adapter ligation mixture.
- The Fast Ligation Buffer is highly viscous. Mix it thoroughly by vortexing 6 times (3 sec each), and centrifuge briefly.
- Mix Ad Ligase by inverting the tube 10 times and flicking the bottom gently. Ensure that no residual reagent is left at the bottom. Centrifuge briefly and place it on ice until use.
- After Ligation Enhancer is used for the first time, store it at 10 ℃ - 30 ℃ away from light.

3.5.2 Adapter ligation

1. Dilute the UDB PF Adapter with TE Buffer based on gDNA input.

Table 29 Relationship between UDB PF Adapter dilution ratio and gDNA input

gDNA input (*N* ng)	Dilution ratio of UDB PF Adapter	Volume after dilution
500< N ≤ 900	No Dilution	3
200 ≤ N ≤ 500	1.5	3
100 ≤ N < 200	3	3
50 ≤ N< 100	10	3

2. Add 3 µL of UDB PF Adapter or diluted adapters to the corresponding sample tube (from step 5 in section 3.4.1.2 or from step 8 in section 3.4.2.2, volume: 47 µL). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. According to the desired reaction number, prepare the adapter ligation mixture in a 1.5 mL centrifuge tube on ice. Vortex it 6 times (3 sec each), centrifuge briefly, and place on ice.

Table 30 Adapter ligation mixture

Reagent	Volume per reaction
Fast Ligation Buffer	23 µL
Ad Ligase	5 µL
Ligation Enhancer	2 µL
Total	30 µL

Use the adapter ligation mixture within 30 min.

4. Slowly pipette 30 µL of adapter ligation mixture to each sample tube. Vortex it 6 times (3 sec each), centrifuge briefly, and place on ice.

The adapter ligation mixture is highly viscous. Slowly aspirate and dispense to ensure that the volume is accurate.

5. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 31 Adapter ligation reaction conditions (Volume: 80 µL)

Temperature	Time
30 ℃ Heated lid	On
25 ℃	10 min
4 ℃	Hold

If the library yield is low, the reaction time at 25 °C can be extended to 30 minutes.

6. When the program is completed, centrifuge the PCR tube(s) briefly and place on ice.

CAUTION DO NOT STOP AT THIS STEP. Proceed to section 3.6.

3.6 Cleanup of adapter-ligated product

Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.6.1 Preparation

Table 32 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied; freshly prepared
En-TE	Refer to Table 17; place at RT
En-Beads	Refer to Table 18; allow 30 min to equilibrate to RT before use; mix thoroughly by vortexing before each use

3.6.2 Cleanup of adapter-ligated product

1. Add 20 µL of En-TE to each sample tube (from step 6 in section 3.5.2, volume: 80 µL).
2. Mix the En-Beads thoroughly. Add 20 µL of En-Beads to each sample tube. Mix with a vortexer until all beads are suspended.
3. Incubate the sample tube(s) at room temperature for 5 min.
4. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard all the supernatant. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
5. Remove the tube(s) from the magnetic rack. Add 30 µL of En-TE and 20 µL of En-Beads to each sample tube. Mix with a vortexer until all beads are suspended.
6. Incubate the tube(s) at room temperature for 5 min.
7. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully remove and discard the supernatant.
8. While keeping the PCR tube(s) on the magnetic rack, add 160 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
9. Repeat step 8. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.

10. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

i Over-drying the beads will result in reduced yield.

11. Remove the tube(s) from the magnetic rack and add 51 μL of En-TE to elute the DNA. Mix with a vortexer until all beads are suspended.

i If gDNA input is less than 100 ng, add 15 μL of En-TE for eluting in step 11.

12. Incubate the tube(s) at room temperature for 5 min.
13. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min - 5 min until the liquid is clear. Carefully transfer 49 μL of supernatant to a new 0.2 mL PCR tube.

i If gDNA input is less than 100 ng, transfer 13 μL of supernatant to a new 0.2 mL PCR tube.

Stop point After cleanup, the adapter-ligated product(s) can be stored at -20 ℃.

3.7 QC of ligation product

Use 1 μL of supernatant to quantify the concentration with Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit.

- If the concentration is more than 1 ng/μL, it is a qualified ligation product.
- If the concentration is between 0.8 ng/μL and 1 ng/μL, library preparation can still be attempted, but with a greater risk of failure.
- If the concentration is less than 0.8 ng/μL, do not continue with this sample.

CAUTION To avoid adapter contamination and attain better uniformity of sequencing data, it is recommended that you pool samples at DNB. However, if the gDNA input is less than 200 ng, library pooling with other PCR-free libraries may be required.

4 Circularization and DNB preparation

DNBSEQ Dual Barcode Circularization Kit/ Module (user-supplied, 940-001309-00, 940-001310-00) and DNBSEQ DNA Clean Beads (user-supplied, 940-001281-00) are required for ssCir preparation.

CAUTION Check the name and Cat. No. of the kit carefully before use.

4.1 Denaturation and single-strand circularization

4.1.1 Preparation

Mix the reagents before using and store back the remaining reagents immediately after use.

Table 33 Preparing the reagents

Reagent	Requirement
Dual Barcode Splint Buffer	Thaw at RT; mix thoroughly; centrifuge briefly; place on ice
DNA Rapid Ligase	Flick and/or invert the tube gently; centrifuge briefly; place on ice
TE Buffer, pH 8.0	User-supplied; place at RT

4.1.2 Denaturation

1. Pipette 100 ng - 300 ng adapter-ligated product (from step 13 in section 3.6.2) into a new 0.2 mL PCR tube. If the volume is less than 48 µL, add TE Buffer to make a total volume of 48 µL.

i In the range of 100 ng -300 ng, more yield of ssCir will be attained with more adapter-ligated product.

2. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 34 Denaturation reaction conditions (Volume: 48 μL)

Temperature	Time
100 ℃ Heated lid	On
95 ℃	3 min
4 ℃	10 min

3. After the reaction, centrifuge the tube briefly and place on ice.

4.1.3 Single-strand circularization

1. According to the desired reaction number, prepare the circularization reaction mixture in a new 0.2 mL PCR tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 35 Circularization reaction mixture

Reagent	Volume per reaction
Dual Barcode Splint Buffer	11.5 μL
DNA Rapid Ligase	0.5 μL
Total	12 μL

2. Add 12 μL of circularization reaction mixture to each sample tube (from step 3 in section 4.1.2, volume: 48 μL). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 36 Single strand DNA circularization reaction conditions (Volume: 60 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	10 min
4 ℃	Hold

i Prepare the "Table 38 Digestion mixture" on page 31 in advance of this step.

4. When the program is completed, place the PCR tube(s) on ice, centrifuge briefly, and immediately proceed to the next step.

4.2 Digestion

4.2.1 Preparation

Table 37 Preparing the reagents

Reagent	Requirement
Digestion Buffer	Thaw at RT; mix thoroughly; centrifuge briefly; place on ice.
Digestion Enzyme	Flick and/or invert the tube gently; centrifuge briefly; place on ice.
Digestion Stop Buffer	Thaw at RT; mix thoroughly; centrifuge briefly; place at RT.

4.2.2 Digestion

1. According to the desired reaction number, prepare the digestion mixture in a 0.2 mL PCR tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 38 Digestion mixture

Reagent	Volume per reaction
Digestion Buffer	1.4 μL
Digestion Enzyme	2.6 μL
Total	4.0 μL

2. Add 4 μL of digestion mixture to each sample tube (from step 4 in section 4.1.3, volume: 60 μL). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 39 Digestion reaction conditions (Volume: 64 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	10 min
4 ℃	Hold

4. When the program is completed, centrifuge the tube briefly and immediately **add 7.5 μL of Digestion Stop Buffer** to each sample tube. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

i DO NOT STOP AT THIS STEP. Proceed to next reaction.

4.3 Cleanup of digestion product

Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

4.3.1 Preparation

Table 40 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied; freshly prepared.
TE Buffer, pH 8.0	User-supplied; place at RT.
DNA Clean Beads	User-supplied; allow 30 min to equilibrate to RT before use; mix thoroughly by vortexing before each use.

4.3.2 Cleanup of digestion product

1. Mix the DNA Clean Beads thoroughly. Add 130 µL of DNA Clean Beads to each sample tube (from step 4 in section 4.2.2, volume: 71.5 µL). Mix with a vortexer until all beads are suspended.
2. Incubate at room temperature for 5 min.
3. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, add 160 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
6. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

Over-drying the beads will result in reduced yield.

7. Remove the tube(s) from the magnetic rack and add 25 µL of TE Buffer to elute the DNA. Mix with a vortexer until all beads are suspended.
8. Incubate at room temperature for 5 min.

9. Centrifuge the tube briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 24 μL of supernatant to a new 1.5 mL centrifuge tube.

Stop point After cleanup, the digestion product(s) (ssCir) can be stored at -20 ℃ for 30 days.

4.4 QC of digestion product

Quantify the ssCir with Qubit ssDNA Assay Kit. The final yields should be more than 50 fmol (about 10 ng). Refer to the formula below to calculate the mass of 50 fmol ssCir:

Formula 1 Conversion between fmol and mass in ng of circular ssDNA

50 fmol ssDNA (ng) = 0.05 × [DNA fragment peak size (bp) + the length of adapter (bp)] × 0.33

The length of dual barcode PCR-Free adapter is 131 bp, and the length of single barcode PCR-Free adapter is 84 bp.

4.5 DNB preparation

Refer to *DNBSEQ-G400RS Highthroughput (Rapid) Sequencing Set User Manual*| or *DNBSEQ-T7RS High-throughput Sequencing Set User Manual*to prepare DNB. 50 fmol (about 10 ng) ssCir is required for DNB preparation.

If multiple ssCir libraries need to be pooled, it is recommended that you mix them based on their molar ratio. The molar ratio of pooled ssCir depends on the expected data volume ratio of the different samples by the customers. However, the barcode corresponding to the pooled sample must comply with "UDB PF Adapters pooling guide" on page 8.

- The insert size and the size range affect sequencing quality and data amount of effective sequencing reads. Therefore, when pooling libraries with different insert sizes or those prepared by different purification methods (such as pooling single size selection purification products with double size selection purification products for sequencing), there might be a decrease in sequencing quality and effective sequencing reads.
- If mixed sequencing is necessary, it is recommended that you pool the PCR-free libraries that have similar insert size and size range.

5 Appendix

5.1 UDB PF Adapter Kit's barcode number and sequence information

For detailed sequence information of each barcode, please reach out to our Technical Support team at: **US-TechSupport@CompleteGenomics.com**.

5.1.1 Instructions for UDB PF Adapter Kit (16 RXN)

This kit contains 16 adapters grouped into 2 sets:

- UDB Adapter-393 to UDB Adapter-400 (see the blue box in Figure 4)
- UDB Adapter-401 to UDB Adapter-408 (see the red box in Figure 4)

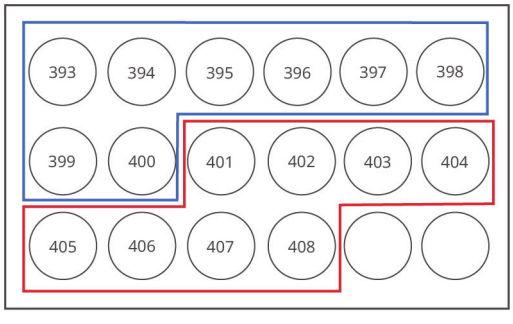

Figure 4 UDB PF Adapters (16 RXN) layout and combination instructions

5.1.2 Instructions for UDB PF Adapter Kit A/B/C/D (96 RXN)

- Adapter Plate: There are four plates of UDB PF Adapters in total. Each set of 8 adapters is balanced in base distribution.
- UDB Adapters A/B: 8 adapters within the same column on the plate are grouped into the same set and are balanced in base distribution. There are 12 columns on each plate and thus there are 12 sets of adapters on each plate.
- UDB Adapters C/D: All 96 adapters are within a single set in terms of balanced base distribution, and this type of plate must be used in its entirety.

Table 41 UDB PF Adapter Kit A (96 RXN) layout

Adapter	1	2	3	4	5	6	7	8	9	10	11	12
A	385	393	401	409	417	425	433	441	449	457	465	473
B	386	394	402	410	418	426	434	442	450	458	466	474
C	387	395	403	411	419	427	435	443	451	459	467	475
D	388	396	404	412	420	428	436	444	452	460	468	476
E	389	397	405	413	421	429	437	445	453	461	469	477
F	390	398	406	414	422	430	438	446	454	462	470	478
G	391	399	407	415	423	431	439	447	455	463	471	479
H	392	400	408	416	424	432	440	448	456	464	472	480

Table 42 UDB PF Adapter Kit B (96 RXN) layout

Adapter	1	2	3	4	5	6	7	8	9	10	11	12
A	481	489	497	505	513	521	529	537	545	553	561	569
B	482	490	498	506	514	522	530	538	546	554	562	570
C	483	491	499	507	515	523	531	539	547	555	563	571
D	484	492	500	508	516	524	532	540	548	556	564	572
E	485	493	501	509	517	525	533	541	549	557	565	573
F	486	494	502	510	518	526	534	542	550	558	566	574
G	487	495	503	511	519	527	535	543	551	559	567	575
H	488	496	504	512	520	528	536	544	552	560	568	576

Table 43 UDB PF Adapter Kit C (96 RXN) layout

Adapter	1	2	3	4	5	6	7	8	9	10	11	12
A	577	585	593	601	609	617	625	633	641	649	657	665
B	578	586	594	602	610	618	626	634	642	650	658	666
C	579	587	595	603	611	619	627	635	643	651	659	667
D	580	588	596	604	612	620	628	636	644	652	660	668
E	581	589	597	605	613	621	629	637	645	653	661	669
F	582	590	598	606	614	622	630	638	646	654	662	670
G	583	591	599	607	615	623	631	639	647	655	663	671
H	584	592	600	608	616	624	632	640	648	656	664	672

Table 44 UDB PF Adapter Kit D (96 RXN) layout

Adapter	1	2	3	4	5	6	7	8	9	10	11	12
A	673	681	689	697	705	713	721	729	737	745	753	761
B	674	682	690	698	706	714	722	730	738	746	754	762
C	675	683	691	699	707	715	723	731	739	747	755	763
D	676	684	692	700	708	716	724	732	740	748	756	764
E	677	685	693	701	709	717	725	733	741	749	757	765
F	678	686	694	702	710	718	726	734	742	750	758	766
G	679	687	695	703	711	719	727	735	743	751	759	767
H	680	688	696	704	712	720	728	736	744	752	760	768

5.2 Acronyms and abbreviations

Table 45 Acronyms and abbreviations

Acronym	Definition
Cat. No.	Catalog Number
DNB	DNA Nanoballs
dsDNA	double-stranded DNA
FS	Fragmentation Shearing
gDNA	genomic DNA
PE	Pair-End
PF	PCR-Free
QC	Quality Control
RT	Room Temperature
RXN	Reaction
ssCir	single-strand Circular

Doc. No.: H-940-001314-00