Complete
GENOMICS™
Powered by MGI

DNBSEQ Fast RNA Library Prep Set User Manual

- **Cat. No.: 940-001518-00 (16 RXN)**
  940-001522-00 (B, 96 RXN)
  940-001836-00 (C, 96 RXN)
  940-001515-00 (192 RXN)
- **Set Version: V1.0**

For Research Use Only

About the user manual

CG does not make any promise of this user manual, including (but not limited to) any special commercial purpose and any reasonable implied guarantee. CG has taken measures to guarantee the correctness of this user manual. However, CG is not responsible for any missing parts in the manual and reserves the right to revise the manual and the reagent to improve the reliability, performance, or design.

All the pictures in this user manual are schematic diagrams and are for reference only. The content of the pictures may be slightly different from the actual product or the actual layout.

DNBSEQ™, Agilent®, Agilent Technologies®, ALPAQUA®, Ambion®, Axygen®, Advanced Analytical®, Covaris®, DynaMag™, Invitrogen®, PerkinElmer®, Qubit®, Thermo Fisher®, or any other company, product names, and trademarks are the property of their respective owners.

Manufacturer information

Manufacturer	Complete Genomics, Inc.
Address	2904 Orchard Parkway, San Jose, CA 95134
Customer service telephone	+1 (888) 811-9644
Customer service Email	US-CustomerService@CompleteGenomics.com
Technical support Email	US-TechSupport@CompleteGenomics.com
Website	www.CompleteGenomics.com

Revision history

Manual Rev	Set version	Date	Description
1.0	1.0	Mar. 2024	Initial release

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

Contents

1 Product overview

1.1 Introduction

The DNBSEQ Fast RNA Library Prep Set is designed to prepare libraries for DNBSEQ high-throughput sequencing platforms.

The RNA prep set is optimized to convert 10 ng to 1 μg of total RNA into a DNA library for gene expression profiling, transcriptome analysis, or detection of pathogenic microorganisms in high-throughput RNA sequencing by using MGI devices. This kit is optimized with high-quality enzyme and buffer system to combine cDNA second strand synthesis and end repair steps, which greatly shorten the time of library construction. The second strand synthesis is equipped with two kinds of buffers that can be used to prepare a common or directional library according to your needs . All reagents provided in this set have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

ℹ DNBSEQ Fast RNA Library Prep Set is paired with DNBSEQ UDB Primers Adapter Kit for library construction. The constructed dsDNA library can be combined with the DNBSEQ Dual Barcode Circularization Kit (Cat. No.: 940-001310-00) for circularization and DNB preparation "Circularization and DNB preparation" on page 31.

1.2 Intended use

This library prep set is applicable to the preparation of libraries for samples from common animals, plants, fungus, bacteria, including human, mouse, rice, *Arabidopsis*, yeast, *E. coli*, and so on. Stable performance across all such sample types is expected.

In addition, this library prep set is also used for whole blood, saliva, fresh tissue, and other type of human samples for the detection of pathogenic microorganisms.

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

Sequencing platform	Sequencing type
DNBSEQ-G400RS	SE50/PE100/PE150
DNBSEQ-G99RS	SE50/PE100/PE150
DNBSEQ-T7RS	SE50/PE100/PE150

- When the library insert is 200 bp, it is recommended that you use a sequencing read length of PE100 or SE50.
- When the library insert is 270 bp, it is recommended that you use a sequencing read length of PE150.

1.4 Components

This library prep set comes in four specifications: 16 RXN, 96 RXN (set B), 96 RXN (set C), and 192 RXN. Three separate boxes are included for each specification. For component details, refer to the following table.

Table 2 DNBSEQ Fast RNA Library Prep Set (16 RXN) (Cat. No.: 940-001518-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ Fast RNA Library Prep Kit Cat. No.: 940-001521-00 Specification: 16 RXN	Fragmentation Buffer	🟢 Green	80 μL/tube × 1
	RT Buffer	🟤 Brown	64 μL/tube × 1
	RT Enzyme Mix	🟢 Green	16 μL/tube × 1
	Second Strand Buffer (with dNTP)	🟡 Yellow	405 μL/tube × 1
	Directional Second Strand Buffer (with dUTP)	🟠 Orange	405 μL/tube × 1
	Second Strand Enzyme Mix	🟡 Yellow	76 μL/tube × 1
	Ligation Buffer	🔴 Red	375 μL/tube × 1
	DNA Ligase	🔴 Red	26 μL/tube × 1
	PCR Enzyme Mix	🔵 Blue	400 μL/tube × 1

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ UDB Primers Adapter Kit Cat. No.: 940-001516-00 Specification: 16 RXN	UDB Adapter (10 μM)	○ White	80 μL/tube × 1
	UDB PCR Primer Mix 57-64, 89-96 (20 μM)	● Blue	10 μL/tube × 16
DNBSEQ DNA Clean Beads Cat. No.: 940-001284-00 Specification: 3.2 mL	DNA Clean Beads	○ White	3.2 mL/tube × 1
	TE Buffer	○ White	3.2 mL/tube × 1

Table 3 DNBSEQ Fast RNA Library Prep Set (96 RXN) (Cat. No.: 940-001522-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ Fast RNA Library Prep Kit Cat. No.: 940-001519-00 Specification: 96 RXN	Fragmentation Buffer	● Green	480 μL/tube × 1
	RT Buffer	● Brown	384 μL/tube × 1
	RT Enzyme Mix	● Green	96 μL/tube × 1
	Second Strand Buffer (with dNTP)	● Yellow	1215 μL/tube × 2
	Directional Second Strand Buffer (with dUTP)	● Orange	1215 μL/tube × 2
	Second Strand Enzyme Mix	● Yellow	452 μL/tube × 1
	Ligation Buffer	● Red	1124 μL/tube × 2
	DNA Ligase	● Red	154 μL/tube × 1
	PCR Enzyme Mix	● Blue	1200 μL/tube × 2
DNBSEQ UDB Primers Adapter Kit B Cat. No.: 940-001517-00 Specification: 96 RXN	UDB Adapter (10 μM)	○ White	480 μL/tube × 1
	UDB PCR Primer Mix 97- 192 (20 μM)	◌ Colorless	10 μL/well × 96
DNBSEQ DNA Clean Beads Cat. No.: 940-001283-00 Specification: 15 mL	DNA Clean Beads	○ White	15 mL/tube × 1
	TE Buffer	○ White	17 mL/tube × 1

Table 4 DNBSEQ Fast RNA Library Prep Set C (96 RXN) (Cat. No.: 940-001836-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ Fast RNA Library Prep Kit Cat. No.: 940-001519-00 Specification: 96 RXN	Fragmentation Buffer	● Green	480 μL/tube × 1
	RT Buffer	● Brown	384 μL/tube × 1
	RT Enzyme Mix	● Green	96 μL/tube × 1
	Second Strand Buffer (with dNTP)	● Yellow	1215 μL/tube × 2
	Directional Second Strand Buffer (with dUTP)	● Orange	1215 μL/tube × 2
	Second Strand Enzyme Mix	● Yellow	452 μL/tube × 1
	Ligation Buffer	● Red	1124 μL/tube × 2
	DNA Ligase	● Red	154 μL/tube × 1
	PCR Enzyme Mix	● Blue	1200 μL/tube × 2
DNBSEQ UDB Primers Adapter Kit C Cat. No.: 940-001862-00 Specification: 96 RXN	UDB Adapter (10 μM)	○ White	480 μL/tube × 1
	UDB PCR Primer Mix 193 - 288 (20 μM)	○ Colorless	10 μL/well × 96
DNBSEQ DNA Clean Beads Cat. No.: 940-001283-00 Specification: 15 mL	DNA Clean Beads	○ White	15 mL/tube × 1
	TE Buffer	○ White	17 mL/tube × 1

Table 5 DNBSEQ Fast RNA Library Prep Set (192 RXN) (Cat. No.: 940-001515-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ Fast RNA Library Prep Kit Cat. No.: 940-001519-00 Specification: 96 RXN **x 2 kits**	Fragmentation Buffer	● Green	480 μL/tube × 1
	RT Buffer	● Brown	384 μL/tube × 1
	RT Enzyme Mix	● Green	96 μL/tube × 1
	Second Strand Buffer (with dNTP)	● Yellow	1215 μL/tube × 2

Item & Cat. No.	Component	Cap color	Spec & Quantity
	Directional Second Strand Buffer (with dUTP)	● Orange	1215 μL/tube × 2
	Second Strand Enzyme Mix	● Yellow	452 μL/tube × 1
	Ligation Buffer	● Red	1124 μL/tube × 2
	DNA Ligase	● Red	154 μL/tube × 1
	PCR Enzyme Mix	● Blue	1200 μL/tube × 2
DNBSEQ UDB Primers Adapter Kit A Cat. No.: 940-001520-00 Specification: 96 RXN	UDB Adapter (10 μM)	○ White	480 μL/tube × 1
	UDB PCR Primer Mix 01-08 100009-100016, 17- 96 (20 μM)	◌ Colorless	10 μL/well × 96
DNBSEQ UDB Primers Adapter Kit B Cat. No.: 940-001517-00 Specification: 96 RXN	UDB Adapter (10 μM)	○ White	480 μL/tube × 1
	UDB PCR Primer Mix 97- 192 (20 μM)	◌ Colorless	10 μL/well × 96
DNBSEQ DNA Clean Beads Cat. No.: 940-001283-00 Specification: 15 mL **x 2 kits**	DNA Clean Beads	○ White	15 mL/tube × 1
	TE Buffer	○ White	17 mL/tube × 1

1.5 Storage and transportation

Table 6 Kit storage and transportation temperatures

Item	Storage temperature	Transportation temperature
DNBSEQ Fast RNA Library Prep Kit	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
DNBSEQ UDB Primers Adapter Kit		
DNBSEQ UDB Primers Adapter Kit A		
DNBSEQ UDB Primers Adapter Kit B		
DNBSEQ UDB Primers Adapter Kit C		
DNBSEQ DNA Clean Beads	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 shelf life.

1.6 User-supplied materials

Table 7 Order information for CG products

Catalog number	Model	Name
940-001812-00	16 RXN	DNBDEQ rRNA Depletion Kit
940-001811-00	96 RXN	DNBDEQ rRNA Depletion Kit
940-001810-00	16 RXN	DNBSEQ rRNA & Globin Depletion Kit
940-001813-00	96 RXN	DNBSEQ rRNA & Globin Depletion Kit
940-001310-00	16 RXN	DNBSEQ Dual Barcode Circularization Kit

Table 8 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
Magnetic rack DynaMag -2, or equivalent	Thermo Fisher Scientific, Cat. No. 12321D
Qubit Fluoromete, or equivalent	Thermo Fisher, Cat. No. Q33216
Agilent 2100 Bioanalyzer, or equivalent	Agilent Technologies , Cat. No. G2939AA

Table 9 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Dynabeads mRNA Purification Kit	Invitrogen, Cat. No. 61006
Library Preparation VAHTS mRNA Capture Beads	Vazyme, Cat. No. N403-02
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)	General lab supplier

Reagent/consumable	Recommended brand
Qubit ssDNA Assay Kit	Invitrogen, Cat. No. Q10212, or equivalent
Qubit dsDNA HS Assay Kit	Invitrogen, Cat. No. Q32854), or equivalent
Agilent High Sensitivity DNA Kit	Agilent, Cat. No. 5067-4626, or equivalent
Agilent DNA 1000 Kit	Agilent, Cat. No. 5067-1504, or equivalent
Pipette tips	General lab supplier
1.5 mL tube	General lab supplier
0.2 mL PCR tube or 96-well plate	General lab supplier
Qubit Assay Tubes or 0.5mL Thin Wall PCR Tubes	Invitrogen or Axygen or equivalent

1.7 Precautions and warnings

- This product is for research use only, not for in vitro diagnosis. Please 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 for pipetting 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.
- Aerosol contamination may cause inaccurate results. It is recommended that you prepare separate working areas in the laboratory for PCR reaction preparation, PCR reaction, and PCR product cleanup. Use designated equipment for each area and clean the area regularly to ensure a sterile working environment (use 0.5% Sodium Hypochlorite or 10% bleach to clean the working area).
- Avoid skin and eyes contact with samples and reagents. Do not eat or drink the 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.**

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	RNA enrichment	1 hr - 2 hr 20 min	20 - 50 min
3.2	RNA fragmentation	11 min	5 min
3.3	Reverse transcription	50 - 55 min	10 - 15 min
3.4	Second strand synthesis and End repair	55 - 60 min	10 - 15 min
3.5	Adapter ligation	25 min	10 min
3.6	Cleanup of adapter-ligated product ⏸	30 - 50 min	20 - 40 min
3.7	PCR ⏸	50 min	10 min
3.8	Cleanup of PCR product ⏸	30 - 40 min	20 - 30 min
3.9	QC of PCR product ⏸	30 - 40 min	10 - 20 min

ℹ
- Total time: The theoretical use time of 8 reactions. The time will be extended if the number of reactions increases.
- Hands-on time: The total required hands-on time in the process.
- ⏸ Stop point.

2 Sample preparation

2.1 Total RNA input requirement

- It is strongly recommended that you use a total RNA input of 10 ng to 1 μg.
- For low-abundance mRNA species such as plants, a total RNA input of 1 μg - 2.5 μg is recommended.
- For pathogenic microorganisms, human whole blood samples and intestinal samples need to be treated with DNBSEQ rRNA Depletion Kit , and the input total RNA amount of the samples used is 200 ng.

2.2 QC of sample

- Use an Agilent 2100 Bioanalyzer to perform quality control of extracted total RNA samples. RIN value should be ≥ 7. If RIN < 7, use an RNA input of no more than 2.5 μg and appropriately increase the number of PCR cycles in the library construction.
- RNA integrity: $OD_{260/280} = 1.8 - 2.0$, $OD_{260/230} \geq 2$.
- For library construction of FFPE RNA samples, refer to appendix 'Library preparation from low quality FFPE sample'.
- For library construction of Pathogenic microorganism RNA samples, refer to appendix 'Library preparation for RNA pathogen sample'.
- If DNA contamination is visible in the RNA sample, perform a DNase I digestion to remove DNA before starting the procedure below.

3 Library preparation protocol

3.1 RNA enrichment

Select one of the following two RNA Enrichment methods based on your needs.

- "rRNA Depletion Kit" on page 10.
- "Dynabeads mRNA Purification Kit " on page 10.

3.1.1 rRNA Depletion Kit

For human blood Total RNA samples:

- If you want to deplete the rRNA and Globin mRNA at the same time, follow the instructions provided in the DNBSEQ rRNA & Globin Depletion Kit.
- If you only want to deplete the rRNA, follow the instructions provided in the DNBSEQ rRNA Depletion Kit.

After depletion, directly proceed to the "RNA fragmentation" on page 12.

3.1.2 Dynabeads mRNA Purification Kit

⚠ **CAUTION** Use non-stick tubes for the mRNA enrichment. Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

3.1.2.1 Preparation

Table 10 Preparing the reagents

Reagent	Requirement
Beads	User-supplied. Take out 30 min in advance to equilibrate to room temperature (RT) and mix thoroughly by vortexing before each use.
Binding Buffer	User-supplied. Mix by vortexing, centrifuge briefly, and place at RT.

Reagent	Requirement
Washing Buffer	
10 mM Tris-HCl	
NF Water	

3.1.2.2 Resuspend the beads

According to the desired reaction number, resuspend the beads as follows. The preparation volume of reagents listed blow is enough for 1 sample.

1. Mix the magnetic beads by vortexing for 1 min. Transfer 50 μL of beads to a new 1.5 mL non-stick tube. Place the tube on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
2. Remove the non-stick tube from the magnetic rack and add 50 μL of Binding Buffer to the tube. Pipette at least 10 times until all beads are suspended. Place the tube on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
3. Repeat step 2.
4. Add 25 μL of Binding Buffer to the tube, and pipette 10 times to mix thoroughly.

3.1.2.3 mRNA purification

1. Preheat the Thermomixer to 65 ℃. Add 200 ng (determined by the species and concentration of sample) of total RNA sample to a 1.5 mL non-stick tube and add NF Water to make a total volume of 25 μL.
2. Place the sample tube(s) on the Thermomixer for denaturation at 65 ℃ for 5 min. Take out the tube(s) and immediately add 25 μL of resuspended beads to the sample. **Pipette** 10 times to mix it well.
3. Incubate at room temperature for 5 min. Set the Thermomixer to 80 ℃ .
4. Place the tube(s) on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
5. Remove the non-stick tube(s) from the magnetic rack and add 50 μL of Washing Buffer to each sample. Gently **pipette** at least 10 times to mix it well. Place the tube(s) on the magnetic rack for 2 min. Carefully remove and discard the supernatant.
6. Repeat step 5.
7. Add 25 μL of 10 mM Tris-HCl to each sample tube and mix it well by gently **pipetting**. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min to elute mRNA from magnetic beads.
8. Take out the tube(s) and immediately add 25 μL of Binding Buffer to each sample. **Pipette** 10 times to mix it well and incubate at room temperature for 5 min. Place the tube(s) on the magnetic rack for 2 min. Carefully remove and discard the supernatant.
9. Repeat step 5 two more times.

10. Add 12 μL of 10 mM Tris-HCl to each sample tube and mix it well by gently **pipetting**. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min.
11. Immediately place the tube(s) on the magnetic rack for 1 to 2 min until the liquid is clear. Carefully transfer 10 μL of supernatant to a new 0.2 mL PCR tube.

3.2 RNA fragmentation

⚠ CAUTION

- Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.
- RNA fragmentation, Reverse transcription, Second strand synthesis and End repair are recommended to be performed without interruption.

3.2.1 Preparation

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

Table 11 Preparing the reagent

Reagent	Requirement
Fragmentation Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.

3.2.2 RNA fragmentation

1. Add 5 μL of Fragmentation Buffer to each sample tube (from section 3.1). Gently **pipette** the solution at least 5 times, centrifuge briefly, and place on ice.

   ⚠ CAUTION Do not vortex. When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

2. Place the PCR tube(s) into the thermocycler when the temperature reaches the reaction temperature. Incubate according to the recommended fragmentation protocol for your target insert size (105 ℃ heated lid).

   ⚠ CAUTION Preheat the thermocycler to the reaction temperature in advance.

Table 12 Recommended conditions for RNA fragmentation (Volume: 15 μL)

Insert size	RNA fragmentation temperature	RNA fragmentation time
200 bp	94 ℃	6 min
270 bp	87 ℃	6 min

3. When the program is completed, immediately place the PCR tube(s) on ice for 2 min. Centrifuge the tube(s) for 10 sec and immediately proceed to the reverse transcription step.

3.3 Reverse transcription

⚠ CAUTION Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

3.3.1 Preparation

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

Table 13 Preparing the reagents

Reagent	Requirement
RT Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
RT Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

3.3.2 Reverse transcription

1. According to the desired reaction number, prepare the reverse transcription mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 14 Reverse transcription mixture

Reagent	Volume per reaction
RT Buffer	4 μL
RT Enzyme Mix	1 μL
Total	5 μL

2. Add 5 μL of reverse transcription mixture to each sample tube (from step 3 in section 3.2.2). Gently **pipette** at least 5 times to mix it well. Centrifuge briefly and place on ice.

⚠ CAUTION Do not vortex. When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

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

Table 15 Reverse transcription reaction conditions (Volume: 20 μL)

Temperature	Time
75 ℃ Heated lid	On
25 ℃	10 min
42 ℃	15 min
70 ℃	15 min
4 ℃	Hold

ℹ Prepare the "Second strand synthesis and End repair" on page 14 in advance during Step 3.

4. When the program is completed, centrifuge the tube(s) for 5 sec and place on ice.

3.4 Second strand synthesis and End repair

⚠ CAUTION Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

3.4.1 Preparation

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

Table 16 Preparing the reagents

Reagent	Requirement
Second Strand Buffer (with dNTP) or Directional Second Strand Buffer (with dUTP)	Thaw at RT, mix well, centrifuge briefly and place on ice.
Second Strand Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

⚠ CAUTION The Second Strand Buffer can be selected according to actual needs: If constructing a common RNA library, use the Second Strand Buffer (withdNTP). If constructing a directional RNA library, use the Directional Second Strand Buffer (with dUTP).

3.4.2 Second strand synthesis and End repair

1. According to the desired reaction number, prepare the reverse transcription mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each). Centrifuge briefly and place on ice.

Table 17 Second strand synthesis and End repair mixture

Reagent	Volume per reaction
Second Strand Buffer (with dNTP) or Directional Second Strand Buffer (with dUTP)	25.3 μL
Second Strand Enzyme Mix	4.7 μL
Total	30 μL

2. Add 30 μL of Second strand synthesis and End repair mixture to each sample tube (from step 4 in section 3.3.2). Gently **pipette** at least 10 times to mix well. Centrifuge briefly and place on ice.

⚠ CAUTION

- Do not vortex. Set the pipette to 40 μL for mixing. To prevent bubbles from forming, do not pour out all of the liquid each time.
- When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

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

Table 18 Second strand synthesis and End repair reaction conditions (Volume: 50 μL)

Temperature	Time
70 ℃ Heated lid	On
16 ℃	30 min
65 ℃	15 min
4 ℃	Hold

ℹ Prepare the "Adapter ligation" on page 16 in advance during Step 3.

4. When the program is completed, centrifuge the tube(s) for 10 sec and place on ice.

3.5 Adapter ligation

- The amount of adapter used in Adapter Ligation depends on the amount of RNA input. Read "Barcode pooling guide" on page 28 carefully before operation.
- Adapter quality and quantity directly affect the efficiency and quality of the library construction. Refer to the table below and the actual amount of total RNA to determine the corresponding times of adapter dilution.

3.5.1 Preparation

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

Table 19 Preparing the reagents

Reagent	Requirement
TE Buffer	User-supplied. Place at RT.
Adapters	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
Ligation Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
DNA Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

CAUTION

- Mix the adapter well before use. Adapter should not be mixed directly with the adapter ligation mixture.
- The Ligation Buffer is highly viscous. Mix it well by vortexing 6 times (3 sec each) and centrifuge briefly for 10 sec. If white precipitation is visible inside the tube cap, cover the tube cap and turn it upside down and then shake and mix well to dissolve the white precipitate.

3.5.2 Adapter ligation

1. Dilute the adapter with TE Buffer according the table below and mix well by vortexing 3 times (3 sec each). Centrifuge the diluted adapter(s) briefly and place on ice.

Table 20 Recommended adapter input according to the amount of total RNA

Total RNA (ng)	UDB Adapter	
-	Adapter dilution ratio	Adapter input after dilution (μL)
201-2500	No dilution	5
51-200	5	5
10-50	10	5

ℹ The table above lists the corresponding dilution factor of the UDB Adapter when high-quality total RNA samples of varying quality are input. For samples with different degrees of degradation, refer to "Recommended amount of FFPE sample input" on page 37 .

2. **Add 5 μL of diluted adapter to the corresponding sample tube** (from step 4 in section 3.4.2). Vortex 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 0.2 mL PCR tube on ice. Mix it well by vortexing 6 times (3 sec each). Centrifuge briefly and place on ice.

Table 21 Adapter ligation mixture

Reagent	Volume per reaction
Ligation Buffer	23.4 μL
DNA Ligase	1.6 μL
Total	25 μL

4. Slowly pipette 25 μL of adapter ligation mixture to each sample tube and vortex 6 times (3 sec each). Centrifuge briefly to collect the liquid at the bottom of the tube and place on ice.

   ℹ The adapter ligation mixture is highly viscous. Be careful to aspirate and not extend the tip too far below the liquid surface. This prevent it from sticking to the tip wall; When adding liquid, add the liquid slowly: there should be no liquid left in the tip.

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

Table 22 Adapter ligation reaction conditions (Volume: 80 μL)

Temperature	**Time**
30 ℃ Heated lid	On
23 ℃	15 min
4 ℃	Hold

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 next reaction.

- If you need to stop here, store the product(s) at -20 ℃ for no longer than 16 hr. There is a risk of reduced yield.

3.6 Cleanup of adapter-ligated product

- For a 200 bp insert size, refer to section 3.6.2. For a 270 bp insert size, refer to section 3.6.3.
- 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 23 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.6.2 Cleanup (200 bp insert size)

1. Add 40 μL of TE Buffer into each sample tube (from step 6 in section 3.5.2) to 120 μL.

   CAUTION
   - Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.
   - When adding TE Buffer and DNA Clean Beads, the liquid should be added slowly with a single tip to ensure accurate pipetting. Otherwise, the final product yield and fragment size may be affected.

2. Add 30 μL of DNA Clean Beads to each sample tube. Mix with a vortexer until all beads are suspended. Centrifuge briefly, and pay attention to maintain uniform dispersion of magnetic beads.
3. Incubate the sample(s) at room temperature for 5 min.

4. Centrifuge the sample 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.

   ⚠ CAUTION If the liquid is difficult to clarify, gently rotate the sample tube on the magnetic rack to make the magnetic beads attract more concentratedly.

5. While keeping the centrifuge tube(s) on the magnetic rack, add 200 μL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
6. Repeat step 5. 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.
7. Keep the centrifuge 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.

8. Remove the centrifuge tube(s) from the magnetic rack and add 23 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
9. Incubate the sample(s) at room temperature for 5 min.
10. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 21 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 ℃.

3.6.3 Cleanup (270 bp insert size)

1. Add 60 μL of TE Buffer into each sample tube (from step 6 in 3.5.2) to 140 μL.

   ⚠ CAUTION
   - Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.
   - When adding TE Buffer and DNA Clean Beads, the liquid should be added slowly with single tip to ensure accurate pipetting. Otherwise the final product yield and fragment size may be affected.

2. Add 23 μL of DNA Clean Beads to each sample tube. Mix with a vortexer until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing.
3. Incubate the sample(s) at room temperature for 5 min.

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

4. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 153 μL of supernatant to a new PCR tube or centrifuge tube.

5. Add 15 μL of DNA Clean Beads to each sample tube (containing 153 μL supernatant). Mix with a vortexer until all beads are suspended. Centrifuge briefly and pay attention to maintain uniform dispersion of magnetic beads.

   ⚠ **CAUTION** When adding DNA Clean Beads, ensure that you are pipetting accurately. Otherwise, the final product yield and fragment size may be affected.

6. Incubate the sample(s) at room temperature for 5 min.
7. Centrifuge the sample 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.
8. While keeping the centrifuge tube(s) on the magnetic rack, add 200 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 centrifuge 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.

11. Remove the centrifuge tube(s) from the magnetic rack and add 23 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
12. Incubate the sample(s) at room temperature for 5 min.
13. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 21 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 PCR

3.7.1 Preparation

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

Table 24 Preparing the reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
UDB PCR Primer Mix	

3.7.2 PCR

1. Add 25 μL PCR Enzyme Mix to each sample tube (from step 10 in section 3.6.2 or step 13 in section 3.6.3).
2. Add 4 μL of the corresponding UDB PCR Primer Mix according to "Notes for UDB PCR Primer Mix" on page 25. Vortex 3 times (3 sec each) and centrifuge briefly to collect the solution at the bottom of the tube.

Table 25 PCR mixture

Reagent	Volume per reaction
Adapter-ligated product (from step 10 in section 3.6.2 or step 13 in section 3.6.3)	21 μL
PCR Enzyme Mix	25 μL
Corresponding UDB PCR Primer Mix	4 μL
Total	50 μL

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

Table 26 PCR reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	On	-
95 ℃	3 min	1
95 ℃	15 s	X (see Table 27)
60 ℃	30 s	
72 ℃	30 s	
72 ℃	5 min	1
4 ℃	Hold	-

Table 27 The recommended PCR cycles for 420 ng PCR yield

Total RNA (ng)	Directional PCR cycles	Common PCR cycles
10	17-19	16-18
50	16-17	15-16
200	14-15	13-14
1000	12-13	11-12

The table above lists the number of PCR cycles required when inputting 10-1000 ng of high-quality sample total RNA (for the requirements of high-quality total RNA, refer to "QC of sample" on page 9). For samples of different species and different degrees of degradation, the number of PCR cycles should be adjusted according to the actual situation.

4. When the program is completed, centrifuge the tube(s) briefly.

   Stop point PCR product(s) can be stored at -20 ℃.

3.8 Cleanup of PCR 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.

3.8.1 Preparation

Table 28 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.8.2 Cleanup of PCR product

Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the DNA Clean Beads thoroughly. Add 40 μL of DNA Clean Beads to each sample tube (from step 4 in 3.7.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 5 min.
3. Centrifuge the sample 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 centrifuge tube(s) on the magnetic rack, add 200 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 centrifuge 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.

7. Remove the tube(s) from the magnetic rack and add 32 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 30 μL of supernatant to a new 0.2 mL PCR tube.

   ‖ Stop point After cleanup, PCR product can be stored at -20 ℃.

3.9 QC of PCR product

- **dsDNA fluorescence quantification method**: Quantify purified PCR products with dsDNA fluorescence assay kits and instructions.
- **Electrophoresis method**: Validate the size range of purified PCR products with electrophoresis-based equipment and instructions.

Table 29 Different QC methods and standards for library

Method	Equipment/Reagent	Standard
dsDNA fluorescence quantification method	Qubit dsDNA HS Assay Kit, Quant-iT PicoGreen dsDNA Assay Kit.	Yield for PCR products: ≥ 1.5 pmol
Electrophoresis method	Tapestation (Agilent Technologies), Bioanalyzer, LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical)	200 bp insert size: Main size: 280 bp - 370 bp
		270 bp insert size: Main size: 380 bp - 470 bp

Formula 1 Conversion between N pmol and mass in ng of PCR product

N pmol PCR product (ng) = N × PCR product peak size (bp) × 0.66

Set N = 1.5 in Formula 1 when calculating the mass of 1.5 pmol PCR product.

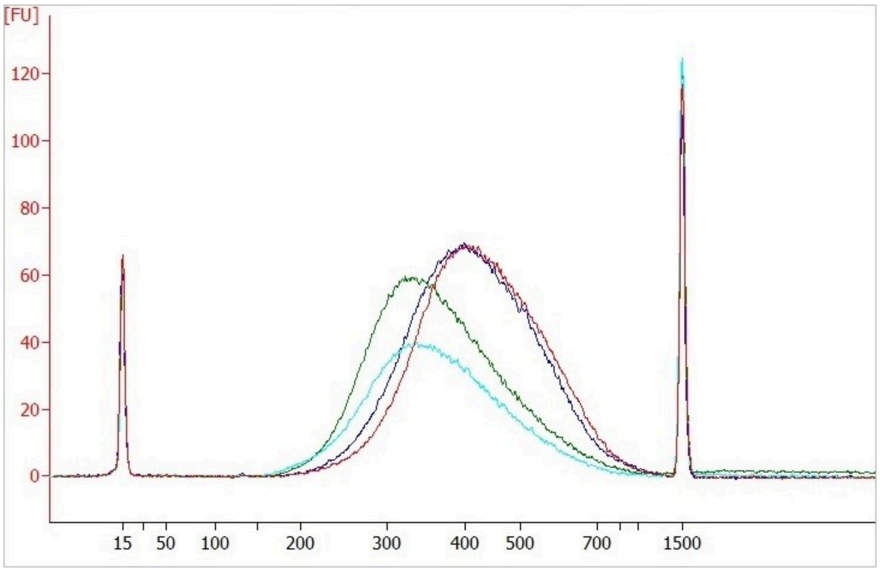


Figure 1 The Agilent 2100 Bioanalyzer results of purified PCR product

- Stop here if the library will be delivered to a service lab for sequencing.
- Proceed to "Circularization and DNB preparation" on page 31 if the library will be sequenced in your lab.

4 Appendix

4.1 Instructions for DNBSEQ UDB Primers Adapter Kit

DNBSEQ UDB Primers Adapter Kit (16 RXN) provides primers in tubes, 96 RXN provides primers in plates, and 192 RXN provides two plates of primers. This kit was developed to meet requirements for batch processing of library construction and multiplex sequencing. The maximum 192-well of UDB PCR Primer Mix and one tube of UDB Adapter Mix are provided, which provides 192 samples of multiplex sequencing.

4.1.1 Notes for UDB Adapter

- UDB Adapter is double stranded. To prevent structural changes, such as denaturation, which might affect performance, do not place the adapters in an area that exceeds 30 ℃.
- Before use, centrifuge UDB Adapter to collect liquid at the bottom of the tubes. Mix UDB Adapter thoroughly before use. Close the cap immediately after use.
- The length of UDB Adapter is 132 bp.

4.1.2 Notes for UDB PCR Primer Mix

For detailed sequence information of each barcode, please reach out to our Technical Support team.

- The dual-barcode library can be used for both single-barcode sequencing and dual-barcode sequencing. Please refer to the corresponding single-barcode/dual-barcode sequencing user manual.
- Before use, centrifuge to collect liquid at the bottom of the tubes or plates.
- Change tips when pipetting different solutions to prevent cross-contamination.
- For tubes, gently remove the cap to prevent liquid from spilling and cross-contamination. Cover the tube immediately after use.
- For the 96-well plate, use 75% alcohol and wipe the surface of the aluminum film of the plate with absorbent paper. The aluminum film is penetrable. Do not touch the surface of the

aluminum film with sharp objects. Pierce the aluminum film to pipette the solutions for first-time use. After use, separately transfer the remaining reagents to 1.5 mL centrifuge tube(s) or 0.2 mL PCR tube(s), label the tubes clearly, and store them at -20 ℃.

- Based on the principles of balanced base composition, UDB PCR Primer Mix must be used in specific groups. Follow the instructions below to use UDB PCR Primer Mix in proper combination.

1. There are 16 tubes of UDB PCR Primer Mix in the 16 RXN kit, one UDB PCR Primer Mix per tube, 8 tubes as a group: UDB PCR Primer Mix 57 to 64 and UDB PCR Primer Mix 89 to 96.

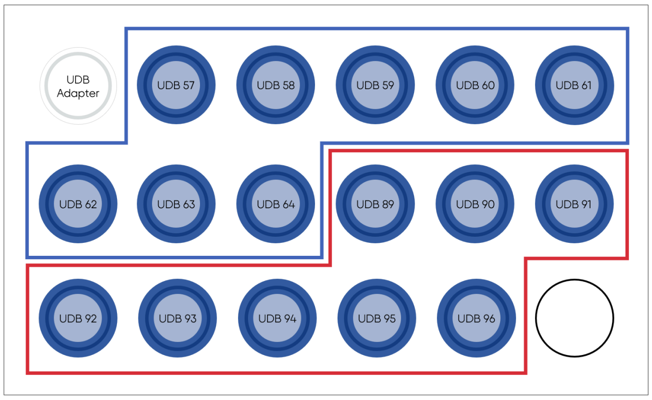


Figure 2 UDB PCR Primer Mix (16 tube) layout

2. There is 1 plate of UDB PCR Primer Mix in Set A, Set B, and Set C. Each plate contains 96 UDB PCR Primer Mix, and 8 wells of each column are preset as a balanced dual barcode combination. The detailed layouts are as follows.

ℹ The 8- and 12-column Barcode numbers of Set A overlap with those of the 16 RXN kit. The base sequences are the same and cannot be sequenced in the same lane.

Table 30 Set A UDB PCR Primer Mix layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB1	UDB100009	UDB17	UDB25	UDB33	UDB41	UDB49	**UDB57**	UDB65	UDB73	UDB81	**UDB89**
B	UDB2	UDB100010	UDB18	UDB26	UDB34	UDB42	UDB50	**UDB58**	UDB66	UDB74	UDB82	**UDB90**
C	UDB3	UDB100011	UDB19	UDB27	UDB35	UDB43	UDB51	**UDB59**	UDB67	UDB75	UDB83	**UDB91**
D	UDB4	UDB100012	UDB20	UDB28	UDB36	UDB44	UDB52	**UDB60**	UDB68	UDB76	UDB84	**UDB92**
E	UDB5	UDB100013	UDB21	UDB29	UDB37	UDB45	UDB53	**UDB61**	UDB69	UDB77	UDB85	**UDB93**
F	UDB6	UDB100014	UDB22	UDB30	UDB38	UDB46	UDB54	**UDB62**	UDB70	UDB78	UDB86	**UDB94**
G	UDB7	UDB100015	UDB23	UDB31	UDB39	UDB47	UDB55	**UDB63**	UDB71	UDB79	UDB87	**UDB95**
H	UDB8	UDB100016	UDB24	UDB32	UDB40	UDB48	UDB56	**UDB64**	UDB72	UDB80	UDB88	**UDB96**

Table 31 Set B UDB PCR Primer Mix layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB97	UDB105	UDB113	UDB121	UDB129	UDB137	UDB145	UDB153	UDB161	UDB169	UDB177	UDB185
B	UDB98	UDB106	UDB114	UDB122	UDB130	UDB138	UDB146	UDB154	UDB162	UDB170	UDB178	UDB186
C	UDB99	UDB107	UDB115	UDB123	UDB131	UDB139	UDB147	UDB155	UDB163	UDB171	UDB179	UDB187
D	UDB100	UDB108	UDB116	UDB124	UDB132	UDB140	UDB148	UDB156	UDB164	UDB172	UDB180	UDB188
E	UDB101	UDB109	UDB117	UDB125	UDB133	UDB141	UDB149	UDB157	UDB165	UDB173	UDB181	UDB189
F	UDB102	UDB110	UDB118	UDB126	UDB134	UDB142	UDB150	UDB158	UDB166	UDB174	UDB182	UDB190
G	UDB103	UDB111	UDB119	UDB127	UDB135	UDB143	UDB151	UDB159	UDB167	UDB175	UDB183	UDB191
H	UDB104	UDB112	UDB120	UDB128	UDB136	UDB144	UDB152	UDB160	UDB168	UDB176	UDB184	UDB192

Table 32 Set C UDB PCR Primer Mix layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB193	UDB201	UDB209	UDB217	UDB225	UDB233	UDB241	UDB249	UDB257	UDB265	UDB273	UDB281
B	UDB194	UDB202	UDB210	UDB218	UDB226	UDB234	UDB242	UDB250	UDB258	UDB266	UDB274	UDB282
C	UDB195	UDB203	UDB211	UDB219	UDB227	UDB235	UDB243	UDB251	UDB259	UDB267	UDB275	UDB283
D	UDB196	UDB204	UDB212	UDB220	UDB228	UDB236	UDB244	UDB252	UDB260	UDB268	UDB276	UDB284
E	UDB197	UDB205	UDB213	UDB221	UDB229	UDB237	UDB245	UDB253	UDB261	UDB269	UDB277	UDB285
F	UDB198	UDB206	UDB214	UDB222	UDB230	UDB238	UDB246	UDB254	UDB262	UDB270	UDB278	UDB286
G	UDB199	UDB207	UDB215	UDB223	UDB231	UDB239	UDB247	UDB255	UDB263	UDB271	UDB279	UDB287
H	UDB200	UDB208	UDB216	UDB224	UDB232	UDB240	UDB248	UDB256	UDB264	UDB272	UDB280	UDB288

4.1.3 Barcode pooling guide

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

The following three application scenarios are predefined for the recommended method of selecting UDB PCR Primer Mix.

1. The sequencing data output requirement is the same for all samples in one lane. Choose the UDB PCR Primer Mix combinations in the table below.

   - Add only one UDB PCR Primer Mix to each sample.
   - Here X means positive integer. For example: 8X = 8 multiplied by X , which means there are 8X samples.

Table 33 UDB PCR Primer Mix pooling guide

Sample/lane	Instruction
4	Only UDB PCR Primer Mix 193-196 or UDB PCR Primer Mix 197-200 can be used.
5	Use the method for (4 samples/lane) above + 1 random UDB PCR Primer Mix.
6	Use the method for (4 samples/lane) above + 2 random UDB PCR Primer Mixes.
7	Use the method for (4 samples/lane) above + 3 random UDB PCR Primer Mixes.
8X	From X set of 8 UDB PCR Primer Mixes (X column total).
8X+1	Add X set of 8 UDB PCR Primer Mixes + 1 random UDB PCR Primer Mix.
8X+2	Add X set of 8 UDB PCR Primer Mixes + 2 random UDB PCR Primer Mixes.
8X+3	Add X set of 8 UDB PCR Primer Mixes + 3 random UDB PCR Primer Mixes.
8X+4	Add X set of 8 UDB PCR Primer Mixes + 4 random UDB PCR Primer Mixes.
8X+5	Add X set of 8 UDB PCR Primer Mixes + 5 random UDB PCR Primer Mixes.
8X+6	Add X set of 8 UDB PCR Primer Mixes + 6 random UDB PCR Primer Mixes.
8X+7	Add X set of 8 UDB PCR Primer Mixes + 7 random UDB PCR Primer Mixes.

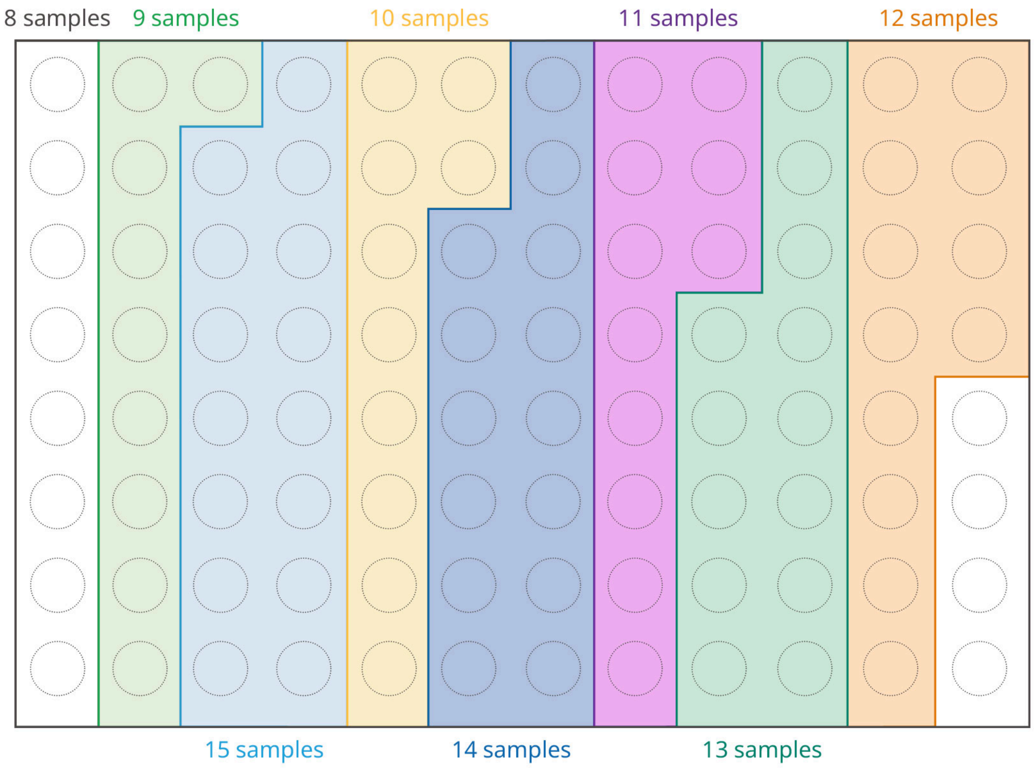


Figure 3 Dual barcode 8 to 15 samples pooling example

2. Under exceptional circumstances (for example, insufficient reagents for a well), when it cannot meet the requirement of at least one balanced UDB Adapter combination for standard pooling 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 **<u>is not less than 12.5% and is not greater than 62.5%</u>** in single sequencing position in the same lane.

Table 34 Balanced 8 UDB Adapter Pooling strategy (8 UDB 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 35 Unbalanced 9 UDB Adapter Pooling strategy (UDB PCR Primer Mix 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

4.2 Circularization and DNB preparation

4.2.1 Denaturation and single strand circularization

- According to the distribution of the main band and concentration of the purified PCR product, refer to Formula 1 in section 3.9 (set N = 1.5) to calculate the required volume of the purified PCR product.
- For multiple samples pooled sequencing, refer to "Barcode pooling guide" on page 28. Quantify the purified PCR products before pooling. The total yield after pooling should be 1.5 pmol, with a total volume of ≤ 48 μL.

  For example: For 8 libraries with main insert fragment of 200 bp (UDB Adapter is 132 bp, PCR product fragment is 332 bp), add 41.1 ng of the PCR product of each sample into a new 0.2 mL PCR tube. The PCR product should have a total mass of 328.7 ng and be equal to a total yield of 1.5 pmol. Add TE Buffer to make a total volume of 48 μL.

4.2.1.1 Preparation

Reagent: For use with DNBSEQ Dual Barcode Circularization Kit. User-supplied.

Table 36 Preparing the reagents

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

4.2.1.2 Denaturation

1. Pipette 1.5 pmol of PCR purified product or mixed purified products into a new 0.2 mL PCR tube. Add TE Buffer to make a total volume of 48 μL.
2. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 37 Denaturation reaction conditions (Volume: 48 μL)

Temperature	Time
100 ℃ Heated lid	On
95 ℃	3 min

3. After the reaction, immediately place the PCR tube(s) on ice for 2 min. Centrifuge briefly and place on ice.

4.2.1.3 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 38 Circularization reaction mixture

Reagent	Volume per reaction
Dual Barcode Splint Buffer	11.5 μL
DNA Rapid Ligase	0.5 μL
Total	12.0 μL

2. Add 12.0 μL of circularization reaction mixture to each sample tube (from step 2 in section 4.2.1.2). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 39 Single strand DNA circularization reaction conditions (Volume: 60 μL)

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

ℹ Prepare the "Table 41 Digestion mixture" on page 33 in advance during Step 3.

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

4.2.2 Digestion

4.2.2.1 Preparation

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

Table 40 Preparing the reagents

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

4.2.2.2 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 41 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 4.2.1.3). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 42 Digestion reaction conditions (Volume: 64 μL)

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

4. After the reaction, centrifuge the tube briefly and immediately add **7.5 μL of Digestion Stop Buffer** to each sample tube.
5. Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction).

⚠ CAUTION It is not recommended that you stop here; continue to complete the cleanup of digestion product. If it must be stopped, place it in a -20 ℃ refrigerator for no longer than 16 hr, but there is a risk of reduced yield or reduced performance.

4.2.3 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.

4.2.3.1 Preparation

Table 43 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

4.2.3.2 Cleanup of digestion product

1. Mix the DNA Clean Beads thoroughly. Add 170 μL of DNA Clean Beads to each sample tube (from step 5 in section 4.2.2.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 10 min.
3. Centrifuge the sample 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 500 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 22 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 10 min.

9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 20 μL of supernatant to a new 1.5 mL centrifuge tube.

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

4.2.4 QC of digestion product

Quantify the purified digestion product by following the instructions provided in the Qubit ssDNA Assay Kit.

The yield of digestion product should be not less than 120 fmol (enough for two sequencing runs). Refer to Formula 2 for calculations.

Formula 2 Conversion between M pmol and mass in ng of circular ssDNA

M pmol circular ssDNA (ng) = M × PCR product peak size (bp) × 0.33

Set M = 0.12 in Formula 2 to calculate the mass of 120 fmol ssDNA product.

4.2.5 DNB preparation

Refer to MGISEQ-200RS High-throughput (Rapid) Sequencing Set User Manual, DNBSEQ-G400RS High-throughput (Rapid) Sequencing Set User Manual or DNBSEQ-T7RS High-throughput Sequencing Set User Manual to prepare DNB: 60 fmol (according to Formula 2, set N=0.06) ssDNA product was prepared into a DNB library suitable for an MGI sequencer.

If multiple ssDNA libraries need to be pooled, it is recommended that you mix them based on their molar ratio. The molar ratio of pooled ssDNA depends on the expected data volume ratio of the different samples by the customers. However, the UDB corresponding to the pooled sample must comply with "Barcode pooling guide" on page 28.

4.3 Library preparation from low quality FFPE sample

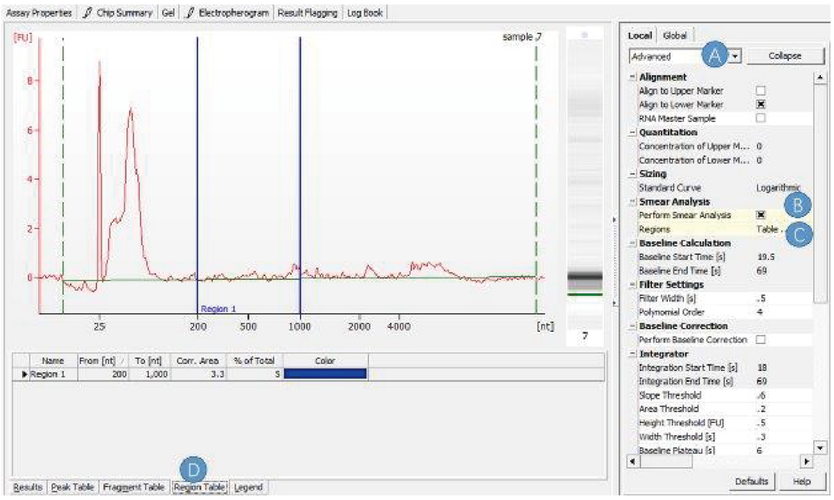
This procedure is used for low-quality total RNA samples such as FFPE. However, as the result of large differences between the quality of different FFPE samples, it is not guaranteed that libraries can be successfully prepared from all FFPE samples. The following instructions take the library construction from the DNBSEQ Fast RNA Library Prep Set as an example and list the problems that you need to address in the library construction to account for different quality FFPE samples.

4.3.1 Quality evaluation of FFPE sample

The RIN value is the most common parameter for the evaluation of RNA quality. However, the RIN value cannot accurately assess the quality of the degraded FFPE samples. In particular, in the NGS library construction, the FFPE samples' RIN value is not always proportional to the overall success rate of library construction. Therefore, DV_{200} is also used for assessing the success rate of library construction from FFPE samples. The DV_{200} indicates the proportion of RNA fragments larger than 200 nucleotides in the sample. For severely degraded FFPE samples, the DV_{200} value is a reliable indicator of the sample quality.

4.3.1.1 The calculation of DV_{200}

Here is an Agilent 2100 Bioanalyzer result as an example for the DV_{200} calculation. The detailed calculation is shown below.



Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
Region 1	200	1,000	3.3	5	▇

Advanced

Alignment	
Align to Upper Marker	☐
Align to Lower Marker	☒
RNA Master Sample	☐
Quantitation	
Concentration of Upper M...	0
Concentration of Lower M...	0
Sizing	
Standard Curve	Logarithmic
Smear Analysis	
Perform Smear Analysis	☒
Regions	Table ...
Baseline Calculation	
Baseline Start Time [s]	19.5
Baseline End Time [s]	69
Filter Settings	
Filter Width [s]	.5
Polynomial Order	4
Baseline Correction	
Perform Baseline Correction	☐
Integrator	
Integration Start Time [s]	18
Integration End Time [s]	69
Slope Threshold	.6
Area Threshold	.2
Height Threshold [FU]	.5
Width Threshold [s]	.3
Baseline Plateau [s]	6

Figure 4 The calculation of DV_{200}

A: In the Agilent 2100 Bioanalyzer result interface, choose **Advanced** under the **Local** tab.

B: Check the Perform Smear Analysis option under **Smear Analysis**.

C: Double-click **Table** and enter the range of fragments to be calculated. The figure shows a range from 200 nt to 1000 nt.

D: Obtain the proportion of selected fragments shown as **% of Total** in the **Region Table**.

If you need to determine the DV_{200} of an FFPE sample, perform the Agilent 2100 Bioanalyzer analysis (using the RNA analysis chip) on the FFPE sample, and calculate DV_{200} according to the method above. For detailed information, refer to *DV_{200} determination for FFPE RNA samples*. (https://www.agilent.com/en/promotions/dv200-determination).

4.3.2 Recommended amount of FFPE sample input

Use rRNA depleted RNA for NGS library construction.

- In “RNA Fragmentation”section, use different conditions of RNA fragmentation for different samples.
- In “Adapter Ligation” section, pay attention to the amount of UDB Adapter.
- In “PCR”section, note the corresponding different number of PCR cycles for different DNA sample inputs. See the following tables for detailed conditions.

Table 44 Recommended conditions of library construction from FFPE sample

FFPE DV_{200}	Recommended amount of total RNA input	RNA fragmentation	PCR cycles
＞70%	200 ng	94 ℃, 5 min	15
50-70%	200~400 ng	94 ℃, 4 min	16
30-50%	500 ng	No fragmentation	16
＜30%	0.5-1 μg (with a risk of failure of library construction)	No fragmentation	16

Table 45 Recommended adapter of library construction from FFPE sample

FFPE DV_{200}	Recommended amount of total RNA input	MGI adapter dilution ratio	MGI adapter input after dilution (μL)
＞70%	200 ng	10	5
50-70%	200-400 ng	10	5
30-50%	500 ng	20	5
＜30%	0.5-1 μg (with a risk of failure of library construction)	50	5

4.3.3 Library preparation protocol for FFPE sample

4.3.3.1 RNA enrichment

Use the MGI rRNA Depletion Kit. Follow the instructions provided in the rRNA Depletion Kit User Manual to enrich RNA.

4.3.3.2 RNA fragmentation

Refer to "Recommended amount of FFPE sample input" on page 37 to set up different conditions for RNA fragmentation for samples with different levels of degradation.

If fragmentation is not required for the RNA Enrichment product, perform the following steps.

1. According to the desired reaction number, add 5 × (n+1) μL of Fragmentation Buffer to a new 0.2 mL PCR tube.
2. Incubate the RNA enrichment product(s) and the PCR tube (contains Fragmentation Buffer) at 65°C for 5 min. Immediately place the sample and PCR tube on ice for 2 minutes and centrifuge for 10 sec for further use.
3. Add 5 μL Fragmentation Buffer to each sample and immediately proceed to the next reaction.

4.3.3.3 Reverse transcription

Refer to "Reverse transcription " on page 13.

4.3.3.4 Second strand synthesis product and End repair

Refer to "Second strand synthesis and End repair" on page 14.

4.3.3.5 Adapter ligation

Refer to "Adapter ligation" on page 16. Refer to "Recommended amount of FFPE sample input" on page 37. Use a different amount of adapter for different FFPE samples.

4.3.3.6 Cleanup of adapter-ligated product

Refer to "Cleanup of adapter-ligated product" on page 18 (200 bp insert size).

4.3.3.7 PCR

Refer to "PCR" on page 20. Refer to "Recommended amount of FFPE sample input" on page 37. Use different numbers of PCR cycles for different FFPE samples.

4.3.3.8 Cleanup to QC of PCR product

Refer to "Cleanup of PCR product" on page 22 and "QC of PCR product" on page 24.

4.4 Library preparation for RNA pathogen sample

4.4.1 Applicable types of RNA pathogen samples

The kit is suitable for the detection of RNA pathogenic microorganisms from human whole blood and intestinal samples.

For human blood total RNA samples: if you want to deplete the rRNA and Globin mRNA at the same time, follow the instructions provided in the DNBSEQ rRNA & Globin Depletion Kit.

4.4.2 Recommended amount of RNA pathogen sample input

The recommended amount of RNA pathogen sample input is 200 ng. The rRNA of human whole blood or intestinal samples needs to be removed with DNBSEQ rRNA Depletion Kit.

4.4.3 Library preparation from RNA pathogen samples

4.4.3.1 RNA enrichment

Use the rRNA Depletion Kit to remove the rRNA of human whole blood or intestinal samples. Follow the instructions provided by the rRNA Depletion Kit User Manual to enrich RNA.

4.4.3.2 RNA fragmentation

Refer to “RNA fragmentation” on page 12.

The RNA sample is incubated at 94 ℃ for 6 min according to the conditions for 200 bp.

4.4.3.3 Reverse transcription to Cleanup of adapter-ligated product

Refer to all procedures starting with “Reverse transcription ” on page 13 and ending with “Cleanup of adapter-ligated product” on page 18 (200 bp insert size).

4.4.3.4 PCR

Refer to “PCR” on page 20.

For rRNA depleted samples, run 15 cycles of PCR to amplify the sample.

4.4.3.5 Cleanup of PCR product

Refer to "Cleanup of PCR product" on page 22.

4.4.3.6 QC of PCR product

The standards of library quality control are shown in the table below. Libraries that do not meet quality control requirements run the risk of failing in the sequencing run.

Table 46 Standards of library quality control

QC	DNBSEQ RNA Library Prep Set	Standards of library QC
PCR product yield	Quantify using Qubit dsDNA HS	≥ 420 ng
PCR product size	Agilent 2100 chip inspection	Size range: 280-370 bp, Average: 200-700 bp
Adapter residue	Agilent 2100 chip inspection	Visual observation, No obvious peak around 130 bp

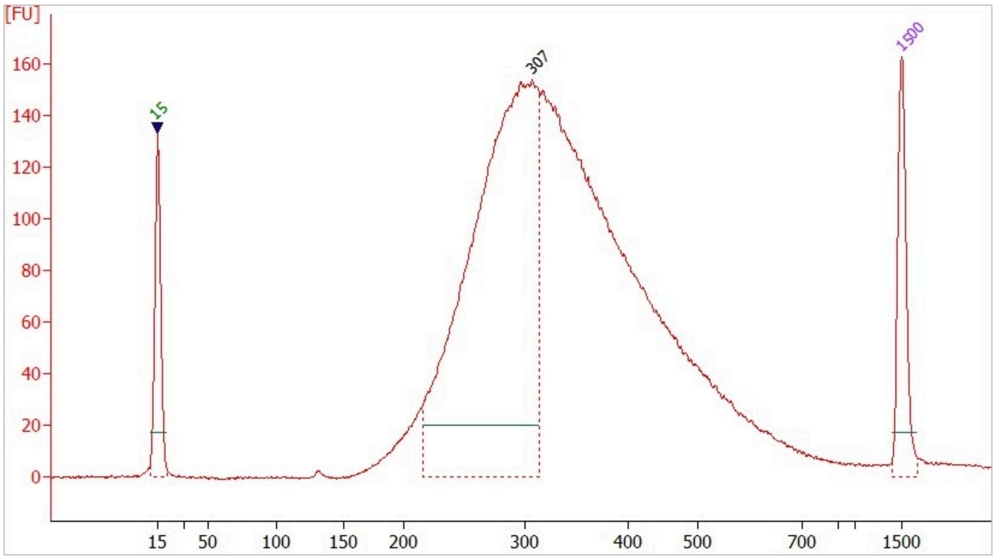


Figure 5 The agilent 2100 Bioanalyzer results of purified PCR product

4.5 Acronyms and abbreviations

Table 47 Acronyms and abbreviations

Acronym	Definition
Cat. No.	Catalog Number
cDNA	complementary DNA
DNB	DNA Nanoballs
dsDNA	double-stranded DNA
PE	Pair-End
QC	Quality Control
RIN	RNA Integrity
RT	Room Temperature
RXN	Reaction
SE	Single-End
ssCir	single-strand Circular
UDB	Unique Dual Barcode

Doc. No.: H-940-001518-00