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DNBSEQ High-throughput Sequencing Primer Kit

(App-D) Instructions for Use

For Research Use Only.
Not for use in diagnostic procedures.

Complete Genomics, Inc.

About the Instructions for Use

CG intends to provide this product solely for research use.

This Instructions for Use is applicable to DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End) and DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End). The Instructions for Use version is 1.0 and the kit version is 1.0.

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Revision history

	Date	Version
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01

Primer kit overview

This chapter describes the intended use, working principle, sample requirements, primer kit information and basic safety information about the kit. Carefully read and understand the information before use to ensure correct operations, best performance, and personnel safety. Keep this instructions for use at hand for reference at any time.

Intended use

WARNING **This kit is intended only for scientific research and should not be used for clinical diagnosis.**

The primer kit can be used with High-throughput Sequencing Set to perform high-throughput sequencing on the sequencer of DNBSEQ-G400RS or DNBSEQ-T7RS and obtain sample sequence information.

Sample requirements

This primer kit is compatible with the converted libraries from third parties by the CG Library Conversion Kit (App libraries, including TruSeq and Nextera adapter) and the App libraries pooled with CG libraries prepared by CG Library Prep Kits. If third party library preparation kits are used, please contact CG Technical Support for conversion options.

Working principle

The High-throughput Sequencing Primer Kit (App-D) contains sequencing primers and barcode primers for App adapter libraries and CG adapter libraries, so that this primer kit can be used with High-throughput Sequencing Set for sequencing both App and CG libraries.

Available primer kit list

- DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End) is suitable for SE single barcode and SE dual barcode sequencing.
- DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) is suitable for PE single barcode and PE dual barcode sequencing.

Table 1 Available primer kit list

Catalog No.	Product name	Version
940-000916-00	DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End)	V1.0
940-000917-00	DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End)	V1.0

Supported platform and read length

Table 2 Supported platform and read length

Supported platform	Flow cell type	Supported read length
Genetic Sequencer (DNBSEQ-G400RS)	FCL	SE50
		SE100
		PE100
		PE150
	FCS	SE100
		PE100
		PE150
		PE300
Genetic Sequencer (DNBSEQ-T7RS)	T7 FCL	PE100
		PE150

Biological safety

- Reagents and waste chemicals may cause personal injury through skin, eye, or mucosal contact. Follow the safety standards of your laboratory and wear protective equipment (such as a laboratory coat, protective glasses, a mask, gloves, and shoe covers) when using the kit.
- If you accidentally splash reagents or waste liquids on your skin or into your eyes, immediately flush the affected area with large amounts of water and seek medical aid immediately.

- When disposing of expired reagents, waste liquids, waste samples, and consumables, comply with local regulations.
- Use and store the reagents according to the instructions for use. Failure to do so may negatively impact performance.
- Check the expiration date of all reagents before use. Using expired reagents may cause inaccurate results.

User-supplied equipment and consumables

Before using the kit, prepare the following equipment:

Table 3 User-supplied equipment list

Equipment	Recommended brand
Freezer, -25 °C to -15 °C	General lab supplier
Refrigerator, 2 °C to 8 °C	General lab supplier
Ice bucket	General lab supplier
Pipette, 20 µL	Eppendorf or equivalent
Pipette, 200 µL	Eppendorf or equivalent
Pipette, 1000 µL	Eppendorf or equivalent
Electronic pipette	Intergra or equivalent
Vortex mixer	General lab supplier
Qubit Fluorometer	Thermo Fisher
Thermal cycler	Bio-Rad or equivalent
Mini spinner	General lab supplier

It is recommended to use the following reagents/consumables:

Tips are disposable consumables. Do not reuse them.

Table 4 Recommended reagent/consumable list

Reagent/Consumable	Recommended brand	Purpose
Sterile pipette tip (various types)	General lab supplier	Pipetting for diluting and loading wash and loading reagents
Sterile 200 µL wide-bore, non-filtered pipette tip	AXYGEN, Cat.No.: T-205-WB-C	For mixing DNBs

Reagent/Consumable	Recommended brand	Purpose
Qubit ssDNA Assay Kit	General lab supplier	Library and DNB QC
Qubit Assay Tubes	Thermo Fisher	Library and DNB QC
Sterile PCR 8-strip tube, 0.2 mL	Thermo Fisher	Making DNB reaction mixture
Sterile microcentrifuge tube, 1.5 mL	VWR, Cat.No.: 20170-038, or equivalent	Combining volumes when diluting library
Disposable gloves, powder-free	General lab supplier	General purpose

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02

Making DNBs

This chapter describes the recommended library insert size, DNA library amount, making DNBs and quantifying DNBs procedures. Read and follow the instructions to ensure correct operations.

Recommended library insert size

The sequencing kit is applicable to single-stranded circular DNA (ssDNA). For the best sequencing quality, the recommended size distribution of inserts ranges between 50 bp and 700 bp, with the main insert size fragment centered within ±100 bp.

If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.

Table 5 Recommended library insert size

Model	Recommended library insert distribution (bp)
FCL SE50	50 to 230
FCL SE100	200 to 400
FCL PE100	200 to 400
FCL PE150	300 to 500
FCS SE100	200 to 400
FCS PE100	200 to 400
FCS PE150	300 to 500
FCS PE300	400 to 700

DNA library concentration and amount requirement

The concentration of ssDNA library should be no less than 3 fmol/μL.

- If the library concentration is unknown, it is recommended to perform ssDNA library quantitation (ng/μL) by using Qubit ssDNA Assay Kit and Qubit Fluorometer. Use the equation below to convert the concentration of the ssDNA library from ng/μL to fmol/μL:

  C (fmol/μL)=3030×C (ng/μL)/N

  N represents the number of nucleotides (average library length including the adapter) as determined by fragment size analysis. Typically, fragment size analysis is determined during library preparation.

- If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.

Making DNBs

- App Make DNB Buffer can be used to make DNBs for both CG and App libraries.
- Mixed use of reagent components from different batches is not recommended.
- Avoid making and loading DNBs by the filtered pipette tips. It is necessary to use the pipettes and tips with recommended brands and catalog numbers.

Four DNB making protocols are listed in sections as below, please select the appropriate one according to the sequencing model.

- *Making DNBs for DNBSEQ-G400RS (FCL SE50, FCL SE100, FCL PE100, FCL PE150, FCS SE100, FCS PE100 and FCS PE150) on Page 9.*
- *Making DNBs for DNBSEQ-G400RS FCS PE300 on Page 13.*
- *Making DNBs for DNBSEQ-T7RS FCL PE100 on Page 16.*
- *Making DNBs for DNBSEQ-T7RS FCL PE150 on Page 20.*

Making DNBs for DNBSEQ-G400RS (FCL SE50, FCL SE100, FCL PE100, FCL PE150, FCS SE100, FCS PE100 and FCS PE150)

Preparing reagents for making DNBs

Perform the following steps:

1. Place the libraries on ice until use.
2. Remove App Make DNB Buffer from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End) or DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) and thaw the reagent at room temperature.
3. Remove Low TE Buffer and Stop DNB Reaction Buffer from DNBSEQ-G400RS High-throughput Sequencing Kit (FCL) or DNBSEQ-G400RS High-throughput Rapid Sequencing Kit (FCS) and thaw the reagents at room temperature.
4. Remove Make DNB Enzyme Mix I from DNBSEQ-G400RS High-throughput Sequencing Kit (FCL) or DNBSEQ-G400RS High-throughput Rapid Sequencing Kit (FCS) and thaw the reagent for approximately 30 minutes on ice.
5. After thawing, vortex each reagent tube for 5 seconds to mix reagent thoroughly. Centrifuge briefly by using a mini spinner and place on ice until use.

Calculating the number of DNB reactions

- Using the sequencer to load DNBs

  All lanes in the flow cell must be loaded with same DNBs.
- Using DNBSEQ-G400RS mini loader DL-200H to load DNBs

Different DNBs can be loaded into different lanes.

Table 6 Required number of make DNB reactions for each flow cell

Flow Cell type	Loading system	DNB volume (µL) / lane	Make DNB reaction (µL)	Required number of make DNB reactions / flow cell
FCL	Sequencer	50	100	2
	DL-200H	25	50	2 to 4
FCS	Sequencer	50	100	1
	DL-200H	25	50	1 to 2

Calculating the required amount of ssDNA libraries

The required volume of ssDNA libraries is determined by the required library amount (fmol) and library concentration quantified in section *DNA library concentration and amount requirement on Page 8*.

- If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.
- *C* mentioned in the following table represents the concentration of libraries (fmol/µL).
- Calculate the required ssDNA libraries for each Make DNB reaction and fill it in *Table 8 on Page 11* as V.

Table 7 Volume of ssDNA libraries

Library type	Required ssDNA volume of 100 µL DNB reaction (µL)	Required ssDNA volume of 50 µL DNB reaction (µL)
App libraries	V = 60 fmol / C	V = 30 fmol / C
CG libraries	V=40 fmoll / C	V=20 fmoll / C

Making DNBs

Perform the following steps:

1. Take out a 0.2 mL PCR tube. Prepare Make DNB Reaction Mixture 1 according to the table below:

   The following table only illustrates the volume used for one make DNB reaction. The amount of required number of make DNB reactions are determined by the actual application as described in *Calculating the number of DNB reactions on Page 9*.

Table 8 Make DNB Reaction Mixture 1

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Low TE Buffer	20 - V	10 - V
App Make DNB Buffer	20	10
ssDNA libraries	V	V
Total volume	40	20

2. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds by using a mini spinner and place it on ice until use.
3. Place the mixture into a thermal cycler and start the primer hybridization reaction. Thermal cycler settings are shown in the table below:

Table 9 Primer hybridization reaction conditions

Temperature	Time
105 °C (heated lid)	On
95 °C	1 min
65 °C	1 min
40 °C	1 min
4 °C	Hold

4. Remove Make DNB Enzyme Mix II (LC) from DNBSEQ-G400RS High-throughput Sequencing Kit (FCL) or DNBSEQ-G400RS High-throughput Rapid Sequencing Kit (FCS) and place it on ice. Centrifuge briefly for 5 seconds by using a mini spinner and place on ice.

   - Do not keep Make DNB Enzyme Mix II (LC) at room temperature.
   - Avoid holding the tube for a prolonged time.

5. Take out the hybridization reaction tube out of the thermal cycler when the temperature reaches 4 °C.
6. Centrifuge briefly for 5 seconds, and then place the tube on ice, and prepare the Make DNB Reaction Mixture 2 according to the table below:

   Do not discard the Make DNB Enzyme Mix II (LC) after you finish this step, it will be used in DNB loading operations.

Table 10 Make DNB Reaction Mixture 2

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Make DNB Enzyme Mix I	40	20
Make DNB Enzyme Mix II (LC)	4	2
Total volume	44	22

7. Add all the Make DNB Reaction Mixture 2 into the Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds by using a mini spinner.
8. Place the tubes into the thermal cycler for the Rolling Circle Replication (RCR) reaction. The conditions are shown in the table below:

   - When a reaction protocol is ran, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled to operating temperature. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature during the DNB reactions.
   - It is recommended to set the temperature of the heated lid to 35 °C or as close as possible to 35 °C.

Table 11 RCR conditions

Temperature	Time
35 °C (heated lid)	On
30 °C	25 min
4 °C	Hold

9. Immediately add Stop DNB Reaction Buffer into the RCR reaction tube once the temperature reaches 4 °C. The volume of Stop DNB Reaction Buffer is shown in the table below. Mix gently by pipetting 8 times by using a wide-bore, non-filtered pipette tip.

   - It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip.
   - Do not centrifuge, vortex, or shake the tube.
   - Store the DNBs at 4 °C and perform sequencing within 48 hours.

Table 12 Volume of Stop DNB Reaction Buffer

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Stop DNB Reaction Buffer	20	10

Making DNBs for DNBSEQ-G400RS FCS PE300

Preparing reagents for making DNBs

Perform the following steps:

1. Place the libraries on ice until use.
2. Remove App Make DNB Buffer from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) and thaw the reagent at room temperature.
3. Remove Low TE Buffer and Stop DNB Reaction Buffer from DNBSEQ-G400RS High-throughput Rapid Sequencing Kit and thaw reagents at room temperature.
4. Remove Make DNB rapid Enzyme Mix II from DNBSEQ-G400RS High-throughput Rapid Sequencing Kit and thaw the reagent for approximately 30 minutes on ice.
5. After thawing, vortex each reagent tube for 5 seconds to mix reagent thoroughly. Centrifuge briefly by using a mini spinner and place on ice until use.

Calculating the number of DNB reactions

Each FCS contains 2 lanes. DNBs can be loaded into the flow cell by using the sequencer or DL-200H.

- Using the sequencer to load DNBs

  All lanes in the flow cell must be loaded with the same DNBs.

- Using DL-200H to load DNBs

  Different DNBs can be loaded into different lanes.

Table 13 Required number of make DNB reactions for each flow cell

Loading system	DNB loading volume (µL)/lane	Make DNB reaction (µL)	Required number of make DNB reactions / flow cell
Sequencer	45	90	1
DL-200H	22.5	90	1 to 2

Calculating the required amount of ssDNA libraries

The required volume of ssDNA libraries is determined by the required library amount (fmol) and library concentration quantified in the section *DNA library concentration and amount requirement on Page 8*.

- If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.
- *C* mentioned in the following table represents the concentration of libraries (fmol/μL).
- Calculate the required ssDNA libraries for each Make DNB reaction and fill it in *Table 15 on Page 14* as V.

Table 14 Volume of ssDNA libraries for FCS PE300 of DNBSEQ-G400RS

Library type	Required ssDNA volume of 90 μL DNB reaction (μL)	Required ssDNA volume of 45 μL DNB reaction (μL)
App libraries	V = 60 fmol / C	V = 30 fmol / C
CG libraries	V = 40 fmol / C	V = 20 fmol / C

Making DNBs

Perform the following steps:

1. Take out a 0.2 mL PCR tube. Prepare Make DNB Reaction Mixture 1 according to the table below:

   - Do not discard the Low TE Buffer after you finish this step, it will be used in DNB dilution operations.
   - The following table only illustrates one make DNB reaction. The amount of required number of make DNB reactions are determined by the actual application as described in *Calculating the number of DNB reactions on Page 13*.

   Table 15 Make DNB Reaction Mixture 1 for FCS PE300 of DNBSEQ-G400RS

Component	Volume of 90 μL DNB reaction (μL)	Volume of 45 μL DNB reaction (μL)
Low TE Buffer	20 - V	10 - V
App Make DNB Buffer	20	10
ssDNA libraries	V	V
Total volume	40	20

2. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds and place it on ice until use.

3. Place the mixture into a thermal cycler and start the primer hybridization reaction. Thermal cycler settings are shown in the table below:

Table 16 Primer hybridization reaction conditions for FCS PE300 of DNBSEQ-G400RS

Temperature	Time
105 °C (heated lid)	On
95 °C	1 min
65 °C	1 min
40 °C	1 min
4 °C	Hold

4. Remove Make DNB Enzyme Mix II (LC) from DNBSEQ-G400RS High-throughput Rapid Sequencing Kit and place on ice. Centrifuge briefly for 5 seconds by using a mini spinner and hold on ice.

   - Do not keep Make DNB Enzyme Mix II (LC) at room temperature.
   - Avoid holding the tube for a prolonged time.

5. Take out the hybridization reaction tube out of the thermal cycler when the temperature reaches 4 °C.
6. Centrifuge briefly for 5 seconds by using a mini spinner, place the tube on ice, and prepare the Make DNB Reaction Mixture 2 according to the table below:

Table 17 Make DNB Reaction Mixture 2 for FCS PE300 of DNBSEQ-G400RS

Component	Volume of 90 µL DNB reaction (µL)	Volume of 45 µL DNB reaction (µL)
Make DNB rapid Enzyme Mix II	40	20
Make DNB Enzyme Mix II (LC)	1.6	0.8
Total volume	41.6	20.8

7. Add all the Make DNB Reaction Mixture 2 into the Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds by using a mini spinner and place it on ice until use.

8. Place the tube into the thermal cycler for the next reaction. The conditions are shown in the table below:

   - When a reaction protocol is ran, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled to operating temperature. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature during the DNB reactions.
   - It is recommended to set the temperature of the heated lid to 35 °C or as close as possible to 35 °C.

   Table 18 RCR conditions for FCS PE300 of DNBSEQ-G400RS

Temperature	Time
35 °C (heated lid)	On
30 °C	15 min
4 °C	Hold

9. Immediately add Stop DNB Reaction Buffer once the temperature reaches 4 °C. The volume of Stop DNB Reaction Buffer is shown in the table below. Mix gently by pipetting 8 times using a wide-bore, non-filtered pipette tip.

   - Keep DNBs on ice during the entire operation to prevent DNBs from performing secondary replication.
   - It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip.
   - Do not centrifuge, vortex, or shake the tube.
   - Do not stop here, immediately go to the next step: *Quantifying DNBs on Page 24*.

   Table 19 Volume of Stop DNB Reaction Buffer for FCS PE300 of DNBSEQ-G400RS

Component	Volume of 90 µL DNB reaction (µL)	Volume of 45 µL DNB reaction (µL)
Stop DNB Reaction Buffer	10	5

Making DNBs for DNBSEQ-T7RS FCL PE100

Preparing reagents for making DNBs

Perform the following steps:

1. Place the libraries on ice until use.

2. Remove App Make DNB Buffer from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) and thaw the reagent at room temperature.
3. Remove Low TE Buffer and Stop DNB Reaction Buffer from DNBSEQ-T7RS DNB Make Reagent Kit and thaw reagents at room temperature.
4. Remove Make DNB Enzyme Mix I from DNBSEQ-T7RS DNB Make Reagent Kit and thaw the reagent for approximately 30 minutes on ice.
5. After thawing, vortex each reagent tube for 5 seconds to mix reagent thoroughly. Centrifuge briefly by using a mini spinner and place on ice until use.

Calculating the number of DNB reactions and the required amount of ssDNA libraries

- 270 µL of DNBs is required to load one flow cell for FCL PE100.
- One Make DNB reaction can make either 100 µL or 50 µL of DNBs. The volume of the Make DNB reaction depends on the amount of data required for sequencing per sample and the types of DNA libraries.
- The required ssDNA library volume to make either 100 µL or 50 µL of DNBs are shown in the table below.

  - If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.
  - *C* mentioned in the following table represents the concentration of libraries (fmol/µL).
  - Calculate the required ssDNA libraries for each Make DNB reaction and fill it in *Table 21 on Page 18* as *V*.

Table 20 Volume of ssDNA libraries for FCL PE100 of T7

Library type	Required ssDNA volume of 100 µL DNB reaction (µL)	Required ssDNA volume of 50 µL DNB reaction (µL)
App libraries	V = 60 fmol / C	V = 30 fmol / C
CG libraries	V = 60 fmol / C	V = 30 fmol / C

- For a given sample A, if it requires “a” million base data output and the total theoretical expected data output for this flow cell is “b” million bases, then the required DNB volume (V) in the pooling for sample A is as follows:

$$V = a/b \times 270\ (\mu L)$$

- If the total sample number pooled is less than 6, it is recommended that you select the volume of 100 µL for each DNB reaction. The number of 100 µL Make DNB reaction is equal to (V/100)+1 rounded down to the nearest whole number.
- For example:
  - If V=80, it requires one 100 µL Make DNB reaction.

- If V=120, it requires two 100 µL Make DNB reactions.
- If the total sample number pooled is no less than 6, it is suggested that you select the volume of 50 µL for each DNB reaction, and the number of 50 µL Make DNB reactions is equal to (V/50)+1 rounded down to the nearest whole number.

Making DNBs

Perform the following steps:

1. Take out a 0.2 mL PCR tube. Prepare Make DNB Reaction Mixture 1 according to the table below:

   The following table only illustrates one make DNB reaction. The amount of required number of make DNB reactions are determined by the actual application as described in *Calculating the number of DNB reactions and the required amount of ssDNA libraries on Page 17*.

Table 21 Make DNB Reaction Mixture 1

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Low TE Buffer	20 - V	10 - V
App Make DNB Buffer	20	10
ssDNA libraries	V	V
Total volume	40	20

2. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds by using a mini spinner and place it on ice until use.
3. Place the mixture into a thermal cycler and start the primer hybridization reaction. Thermal cycler settings are shown in the table below:

Table 22 Primer hybridization reaction conditions

Temperature	Time
105 °C (heated lid)	On
95 °C	1 min
65 °C	1 min
40 °C	1 min
4 °C	Hold

4. Remove Make DNB Enzyme Mix II (LC) from DNBSEQ-T7RS DNB Make Reagent Kit and place it on ice. Centrifuge briefly for 5 seconds by using a mini spinner and hold on ice.

   - Do not keep Make DNB Enzyme Mix II (LC) at room temperature.
   - Avoid holding the tube for a prolonged time.

5. Take out the hybridization reaction tube out of the thermal cycler when the temperature reaches 4 °C.
6. Centrifuge briefly for 5 seconds, and then place the tube on ice, and prepare the Make DNB Reaction Mixture 2 according to the table below:

   Do not discard the Make DNB Enzyme Mix II (LC) after you finish this step, it will be used in DNB loading operations.

Table 23 Make DNB Reaction Mixture 2

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Make DNB Enzyme Mix I	40	20
Make DNB Enzyme Mix II (LC)	4	2
Total volume	44	22

7. Add all the Make DNB Reaction Mixture 2 into the Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer, centrifuge for 5 seconds by using a mini spinner.
8. Place the tubes into the thermal cycler for the Rolling Circle Replication (RCR) reaction. The conditions are shown in the table below:

   - When a reaction protocol is ran, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled to operating temperature. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature during the DNB reactions.
   - It is recommended to set the temperature of the heated lid to 35 °C or as close as possible to 35 °C.

Table 24 RCR conditions

Temperature	Time
35 °C (heated lid)	On
30 °C	25 min
4 °C	Hold

9. Immediately add Stop DNB Reaction Buffer into the RCR reaction tube once the temperature reaches 4 °C. The volume of Stop DNB Reaction Buffer is shown in the table below. Mix gently by pipetting 8 times by using a wide-bore, non-filtered pipette tip.

   - It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip.
   - Do not centrifuge, vortex, or shake the tube.
   - Store the DNBs at 4 °C and perform sequencing within 48 hours.

Table 25 Volume of Stop DNB Reaction Buffer

Component	Volume of 100 µL DNB reaction (µL)	Volume of 50 µL DNB reaction (µL)
Stop DNB Reaction Buffer	20	10

Making DNBs for DNBSEQ-T7RS FCL PE150

Preparing reagents for making DNBs

Perform the following steps:

1. Place the libraries on ice until use.
2. Remove App Make DNB Buffer from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) and thaw the reagent at room temperature.
3. Remove Low TE Buffer and Stop DNB Reaction Buffer from DNBSEQ-T7RS DNB Make Reagent Kit and thaw the reagents at room temperature.
4. Remove Make DNB Rapid Enzyme Mix II from DNBSEQ-T7RS DNB Make Reagent Kit and thaw it on ice for approximately 30 minutes.
5. After thawing, vortex each reagent tube for 5 seconds to mix reagent thoroughly. Centrifuge briefly by using a mini spinner and place on ice until use.

Calculating the number of DNB reactions and the required amount of ssDNA libraries

- 300 µL of DNBs is required to load one flow cell for the FCL PE150. One Make DNB reaction can make 90 µL of DNB. The volume of the Make DNB reaction system depends on the amount of data required for sequencing per sample and the types of DNA libraries.
- The required ssDNA library volume to make 90 µL of DNBs (one DNB reaction) are shown in the table below.

- If there are any special requirements or specifications for the CG library preparation kit, then the requirements of the kit should be followed.
- *C* mentioned in the following table represents the concentration of libraries (fmol/μL).
- Calculate the required ssDNA libraries for each Make DNB reaction and fill it in *Table 27 on Page 21* as V.

Table 26 Volume of ssDNA libraries for FCL PE150 of DNBSEQ-T7RS

Library type	Required ssDNA volume of 100 μL DNB reaction (μL)	Required ssDNA volume of 50 μL DNB reaction (μL)
App libraries	V = 60 fmol / C	V = 30 fmol / C
CG libraries	V = 60 fmol / C	V = 30 fmol / C

- For a given sample A, if it requires "a" million base data output and the total theoretical expected data output for this flow cell is "b" million bases, then the required DNB volume (V) in the pooling for sample A is as follows:

$$V = a/b \times 300\ (\mu L)$$

- The number of the 90 μL Make DNB reactions is equal to (V/90)+1 rounded down to the nearest whole number.

Making DNBs

Perform the following steps:

1. Take out a 0.2 mL PCR tube. Prepare Make DNB Reaction Mixture 1 according to the table below:

   - Do not discard the Low TE Buffer after you finish this step, it will be used in DNB dilution operations.
   - The following table only illustrates one make DNB reaction. The amount of required number of make DNB reactions are determined by the actual application as described in *Calculating the number of DNB reactions and the required amount of ssDNA libraries on Page 20*.

   Table 27 Make DNB Reaction Mixture 1 for FCL PE150 of DNBSEQ-T7RS

Component	Volume of 90 μL DNB reaction (μL)
Low TE Buffer	20 - V
App Make DNB Buffer	20
ssDNA libraries	V
Total volume	40

2. Mix Make DNB Reaction Mixture 1 thoroughly by using a vortex mixer. Centrifuge it for 5 seconds by using a mini spinner and place it on ice until use.
3. Place the mixture into a thermal cycler and start the primer hybridization reaction. Thermal cycler settings are shown in the table below:

Table 28 Primer hybridization reaction conditions for FCL PE150 of DNBSEQ-T7RS

Temperature	Time
105 °C (heated lid)	On
95 °C	1 min
65 °C	1 min
40 °C	1 min
4 °C	Hold

4. Remove Make DNB Enzyme Mix II (LC) from DNBSEQ-T7RS DNB Make Reagent Kit and place it on ice. Centrifuge briefly for 5 seconds by using a mini spinner and hold on ice.

- Do not keep Make DNB Enzyme Mix II (LC) at room temperature.
- Avoid holding the tube for a prolonged time.

5. Take out the hybridization reaction tube out of the thermal cycler when the temperature reaches 4 °C.
6. Centrifuge briefly for 5 seconds by using a mini spinner, place the tube on ice, and prepare Make DNB Reaction Mixture 2 according to the table below:

Table 29 Make DNB Reaction Mixture 2 for FCL PE150 of DNBSEQ-T7RS

Component	Volume of 90 µL DNB reaction (µL)
Make DNB Rapid Enzyme Mix II	40
Make DNB Enzyme Mix II (LC)	1.6
Total volume	41.6

7. Add all the Make DNB Reaction Mixture 2 into Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer. Centrifuge it for 5 seconds by using a mini spinner .
8. Place the tube into a thermal cycler for the next reaction. The condition is shown in the table below.

- When a reaction protocol is ran, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled to operating temperature. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature during the DNB reactions.
- It is recommended that you set the temperature of the heated lid to 35 °C or as close as possible to 35 °C.

Table 30 RCR conditions for FCL PE150 of DNBSEQ-T7RS

Temperature	Time
35 °C (heated lid)	On
30 °C	10 min
4 °C	Hold

9. Immediately add 10 µL of Stop DNB Reaction Buffer once the temperature reaches 4 °C. Mix gently by pipetting 8 times by using a wide-bore, non-filtered pipette tip.

   - Keep DNBs on ice during the entire operation to prevent DNBs from performing secondary replication.
   - It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip.
   - Do not centrifuge, vortex, or shake the tube.
   - Do not stop here, immediately go to the next step: *Quantifying DNBs on Page 24*.

Quantifying DNBs and pooling

Quantifying DNBs

Perform the following steps:

1. When DNB making is completed, take 2 µL of DNBs, and use Qubit ssDNA Assay Kit and Qubit Fluorometer to quantify the DNBs. For details, refer to *Instructions for using Qubit to quantify the DNBs on Page 37.*

Table 31 DNB concentration standard

Sequencer	Model	DNB concentration
DNBSEQ-G400RS	FCL SE50, FCL SE100, FCL PE100, FCL PE150, FCS SE100, FCS PE100, FCS PE150	≥8 ng/µL
	FCS PE300	≥8 ng/µL
DNBSEQ-T7RS	FCL PE100	≥8 ng/µL
	FCL PE150	≥5 ng/µL

- If the concentration of libraries prepared by customers is lower than that specified in the table above, refer to *Chapter 07 FAQs* of *DNBSEQ-G400RS System Guide* or *Chapter 08 FAQs* of *DNBSEQ-T7RS System Guide* for details.
- If there are too many samples in a single test, it is recommended to quantify in batches to avoid inaccurate DNB quantification due to fluorescence quenching.

2. If the concentration exceeds 40 ng/µL, the DNBs should be diluted to 20 ng/µL according to the table below:

- For DNBSEQ-G400RS FCS PE300 and DNBSEQ-T7RS FCL PE150, do not use DNB Loading Buffer I to dilute the DNBs, use Low TE Buffer instead.
- To ensure sequencing quality, it is recommended that you pool and load DNBs as soon as possible. If sequencing for four flow cells is performed simultaneously, you can make the DNBs together.

Table 32 DNB dilution scheme

Sequencer	Model	Dilution reagent	Storage conditions	Storage time
DNBSEQ-G400RS	FCL SE50, FCL SE100, FCL PE100, FCL PE150, FCS SE100, FCS PE100, FCS PE150	DNB Load Buffer I	4 °C	≤48 h
	FCS PE300	Low TE Buffer	4 °C	≤4 h
DNBSEQ-T7RS	FCL PE100	DNB Load Buffer I	4 °C	≤48 h
	FCL PE150	Low TE Buffer	4 °C	≤8 h

DNB pooling for DNBSEQ-T7RS sequencing

When the App libraries need to be pooled with CG libraries, some libraries with similar barcode sequence in the adapter should avoid being pooled together for sequencing. For details, refer to *Conflicting adapter list on Page 39.*

Refer to *"DNB Pooling"* in *Chapter 04 Sequencing* of *DNBSEQ-T7RS System Guide* for details.

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03

Loading DNBs

This chapter describes the loading DNBs procedure. Read and follow the instructions to ensure correct operations.

Loading DNBs for DNBSEQ-G400RS sequencing

Refer to *"Preparing the flow cell"* to *"Loading DNBs"* in *Chapter 04 Sequencing* of *DNBSEQ-G400RS System Guide* for details to load DNBs.

Loading DNBs for DNBSEQ-T7RS sequencing

Perform the following steps:

1. Remove App-D Insert primer 1 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End) or DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End). Thaw the reagent at room temperature for approximately 30 minutes.
2. Refer to *"Preparing the flow cell"* to *"Loading DNBs"* in *Chapter 04 Sequencing* of *DNBSEQ-T7RS System Guide* for details to prepare the flow cell, DNB Load Plate and buffers.
3. After DNB Load Plate is thoroughly thawed, use a pipette to completely remove all the reagent in well No. 1 of DNB Load Plate, then add 2 mL of App-D Insert primer 1.

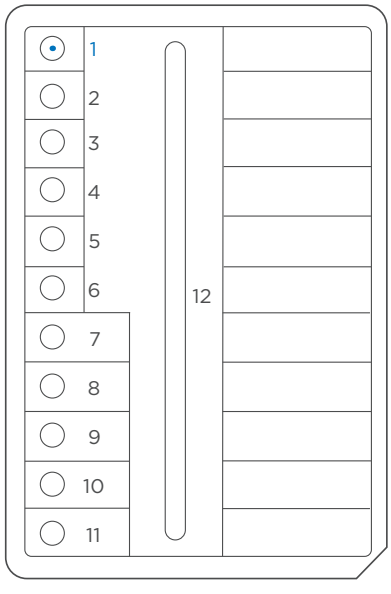

Figure 1 Well No.1 of DNB Load Plate

4. Refer to *"Loading DNBs"* in *Chapter 04 Sequencing* of *DNBSEQ-T7RS System Guide* for details to perform DNB Loading.

04

Sequencing

This chapter describes the sequencing procedure, device maintenance and FAQs. Read and follow the instructions to ensure correct operations.

Preparing the sequencing reagent cartridge

The primer kit can be used with High-throughput Sequencing Set to perform high-throughput sequencing. After the basic preparations of sequencing cartridge, App-D primer replacement operation is required.

Preparing for DNBSEQ-G400RS SE sequencing

Perform the following steps:

1. Refer to *"Preparing the sequencing reagent cartridge"* in *Chapter 04 Sequencing of DNBSEQ-G400RS System Guide* for detailed basic SE sequencing preparations.
2. Thaw primers at room temperature. Perform the steps according to the appropriate model:
   - For single barcode App-D SE sequencing:

     Remove App-D Insert Primer 1, App-D Barcode Primer 1 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End).
   - For dual barcode App-D SE sequencing:

     Remove App-D Insert Primer 1, App-D Barcode Primer 1, App-D Barcode Primer 4 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End).
3. After thawing, gently tap these tubes on the bench to bring the liquid to the bottom. Place them on ice until use.
4. Pierce the foil seals. Perform the steps according to the appropriate model:
   - For single barcode App-D SE sequencing:

     Pierce the foil seals of well No.3 and No. 5 with clean pipettes.
   - For dual barcode App-D SE sequencing:

     Pierce the foil seals of well No.3, No. 4 and No. 5 with clean pipettes.
5. Discard the reagents in each tubes by using a pipette.
6. Adding the primers using appropriate pipettes according to the table below:

   - App-D barcode primer 4 is only for dual barcode App-D SE sequencing.
   - When adding the mixture, ensure that there are no bubbles at the bottom of the tube.

Table 33 Primer loading correspondence for DNBSEQ-G400RS SE sequencing

Reagent name	Well	Volume (mL)
App-D Insert Primer 1	No. 3	2.20
App-D Barcode Primer 4	No. 4	2.90
App-D Barcode Primer 1	No. 5	2.90

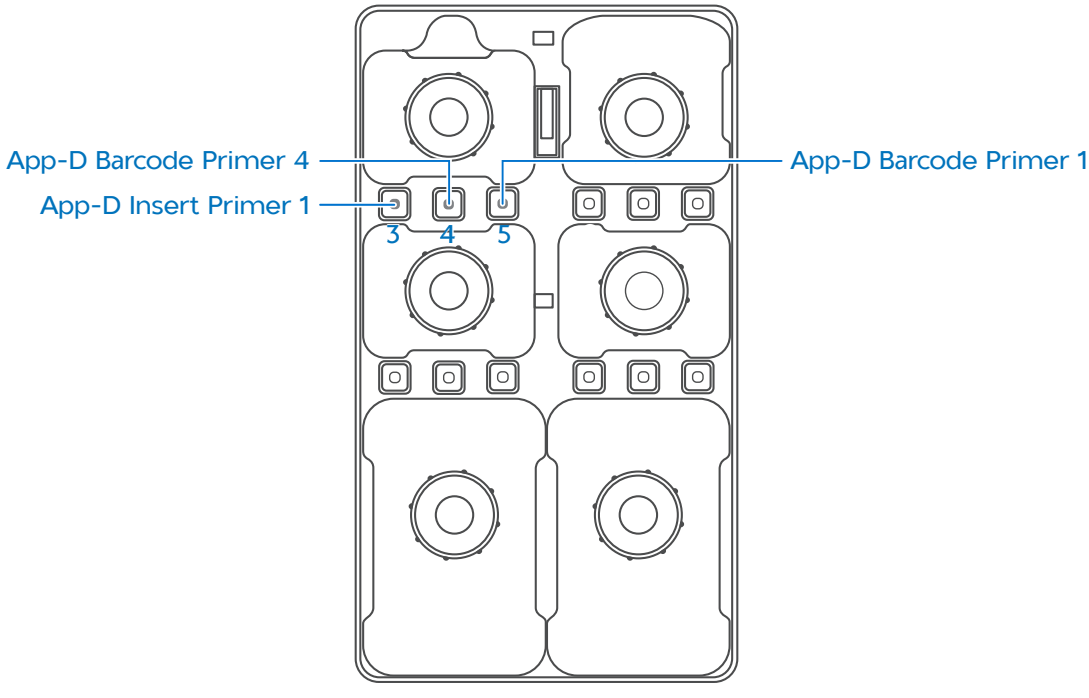

Figure 2 DNBSEQ-G400RS SE sequencing primer adding diagram

Preparing for DNBSEQ-G400RS PE sequencing

Perform the following steps:

1. Refer to *"Preparing the sequencing reagent cartridge"* in *Chapter 04 Sequencing of DNBSEQ-G400RS System Guide* for detailed basic PE sequencing preparations.
2. Thaw primers at room temperature. Perform the steps according to the appropriate model:
   - For single barcode App-D PE sequencing:

     Remove App-D Insert Primer 1, App-D Insert Primer 2, App-D MDA primer and App-D Barcode Primer 2 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End).
   - For dual barcode App-D PE sequencing:

     Remove App-D Insert Primer 1, App-D Insert Primer 2, App-D MDA primer, App-D Barcode Primer 2 and App-D Barcode Primer 3 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End).
3. After thawing, gently tap these tubes on the bench to bring the liquid to the bottom. Place them on ice until use.
4. Pierce the foil seals. Perform the steps according to the appropriate model:
   - For single barcode App-D PE sequencing:

     Pierce the foil seals of well No.3, No. 6, No. 7 and No. 8 with clean pipettes.
   - For dual barcode App-D PE sequencing:

     Pierce the foil seals of well No.3, No. 4, No. 6, No. 7 and No. 8 with clean pipettes.
5. Discard the reagents in each tubes by using a pipette.
6. Adding the primers using appropriate pipettes according to the table below:

   - App-D barcode primer 3 is only for dual barcode App-D PE sequencing.
   - When adding the mixture, ensure that there are no bubbles at the bottom of the tube.

Table 34 Primer loading correspondence for DNBSEQ-G400RS PE sequencing

Reagent name	Well	Volume (mL)
App-D Insert Primer 1	No. 3	2.20
App-D Barcode Primer 3	No. 4	2.90
App-D Barcode Primer 2	No. 6	2.90
App-D MDA Primer	No. 7	3.10
App-D Insert Primer 2	No. 8	3.30

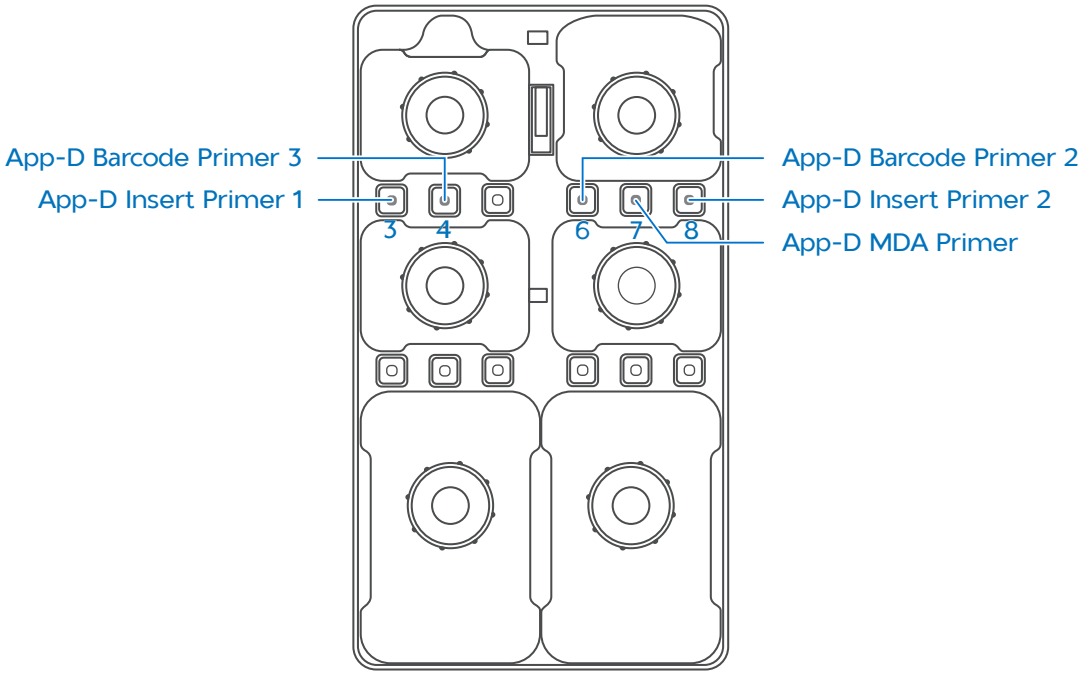

Figure 3 DNBSEQ-G400RS PE sequencing primer adding diagram

Preparing for DNBSEQ-T7RS PE sequencing

Perform the following steps:

1. Refer to *"Preparation before sequencing"* in *Chapter 04 Sequencing* of *DNBSEQ-T7RS System Guide* for detailed basic PE sequencing preparations.
2. Thaw primers at room temperature. Perform the steps according to the appropriate model:
   - For single barcode App-D PE sequencing:

     Remove App-D Insert Primer 2, App-D MDA primer and App-D Barcode Primer 2 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End).
   - For dual barcode App-D PE sequencing:

     Remove App-D Insert Primer 2, App-D MDA primer, App-D Barcode Primer 2 and App-D Barcode Primer 3 from DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End).
3. After thawing, gently tap these tubes on the bench to bring the liquid to the bottom. Place them on ice until use.
4. Pierce the foil seals. Perform the steps according to the appropriate model:
   - For single barcode App-D PE sequencing:

Pierce the foil seals of well No.4, No.6 and No.13 with clean pipettes.

- For dual barcode App-D PE sequencing:

  Pierce the foil seals of well No.3, No.4, No.6 and No.13 with clean pipettes.

5. Adding the primers by using the appropriate pipette according to the table below:

   - App-D barcode primer 3 is just for dual barcode App-D PE sequencing.
   - When adding the mixture, ensure that there are no bubbles at the bottom of the tube.

Table 35 Primer loading correspondence for DNBSEQ-T7RS PE sequencing

Reagent name	Well	Volume (mL)
App-D Barcode Primer 3	No.3	3.5
App-D Barcode Primer 2	No.4	3.5
App-D MDA Primer	No.6	4.2
App-D Insert Primer 2	No.13	4.2

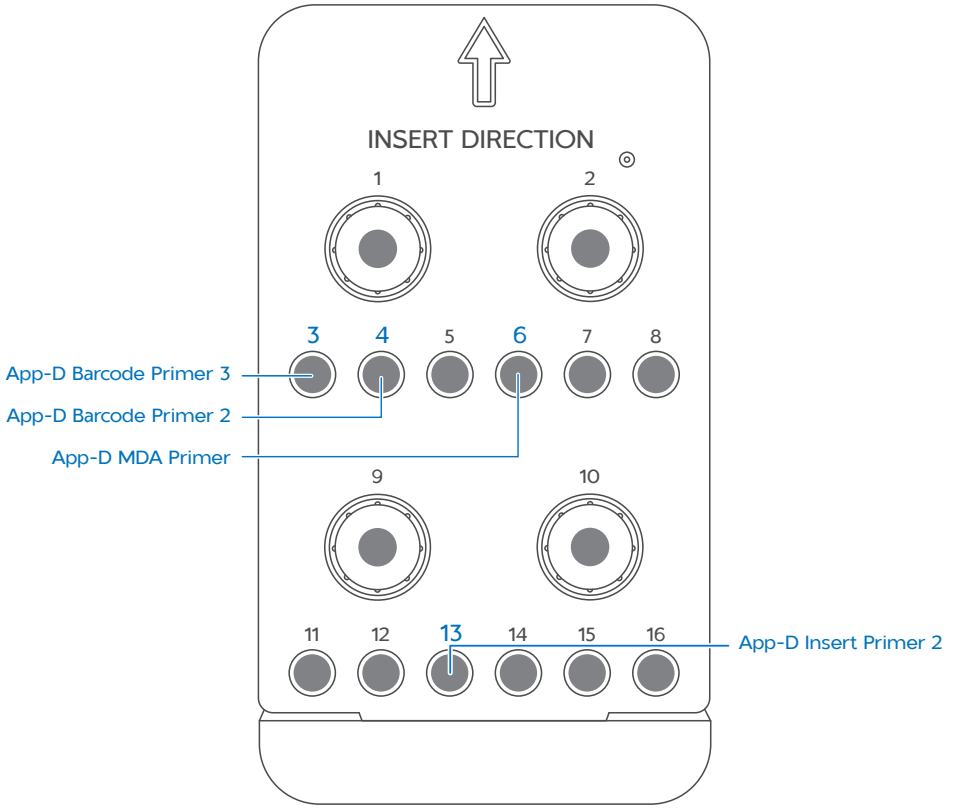

Figure 4 DNBSEQ-T7RS PE sequencing primer adding diagram

Performing a sequencing run

Performing a sequencing run as below:

Sequencer	Reference
DNBSEQ-G400RS	Refer to *Chapter 04 Sequencing* of *DNBSEQ-G400RS System Guide* for details.
DNBSEQ-T7RS	Refer to *Chapter 04 Sequencing* of *DNBSEQ-T7RS System Guide* for details.

- For the App library with a barcode length of 8 bp, when sequencing with the CG library at the same time, please customize the Barcode/Dualbarcode read length as 10 bp. In addition, please add a 2 bp fixed sequence "AC" before the original 8 bp Barcode/Dualbarcode sequence in the barcode list of the App library.

  For example, the original 8 bp barcode sequence is "xxxxxxxx", the sequence in the barcode list should be "ACxxxxxxxx".

- If the App libraries need to be pooled with CG libraries, the libraries with similar barcode sequences in the adapter should avoid being pooled together for sequencing. For details, refer to *Conflicting adapter list on Page 39*.

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Instructions for using Qubit to quantify the DNBs

- Working solution should be used within 30 minutes after preparation.
- Avoid touching the wall of tapered detection tubes.
- Avoid introducing bubbles in detection tubes.

Perform the following steps:

1. Prepare the Qubit working solution by diluting the Qubit ssDNA Reagent 1:200 in Qubit ssDNA Buffer. Use a clean plastic tube each time you prepare Qubit working solution. Do not mix the working solution in a glass container.

   The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit working solution, and each sample tube requires 180–199 µL.

   Prepare sufficient Qubit working solution to accommodate all standards and samples.

   For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Qubit reagent plus 1990 µL of Qubit Buffer).
2. Add 190 µL of Qubit working solution to each of the tubes used for standards.
3. Add 10 µL of each Qubit standard to the appropriate tube, and then mix by vortexing 3–5 seconds. Be careful not to create bubbles.
4. Set up the required number of 0.5 mL tubes for standards and samples. The Qubit ssDNA Assay requires 2 standards.

   - Use only thin-wall, clear, 0.5 mL PCR tubes. Acceptable tubes include Qubit assay tubes (Cat. No. Q32856) or Axygen PCR-05-C tubes (Cat. No. 10011-830).
   - Number of Qubit test tubes needed are the number of samples plus 2 standards tubes. For example, if you have 3 samples, you will need 5 tubes.
5. Label the tube lids. Do not label the side of the tube.
6. Prepare the solutions used for standards and sample tests according to the table below:

Table 36 Working solution

	S1 (μL)	S2 (μL)	D1 (μL)	D2 (μL)	D3 (μL)
Working solution	190	190	198	198	198
S1（0 ng/μL）	10	/	/	/	/
S2（20 ng/μL）	/	10	/	/	/
Sample（μL）	/	/	2	2	2
Total volume	200	200	200	200	200

7. Mix the tubes by using a vortex mixer, centrifuge briefly for 5 seconds, and then incubate at room temperature for 2 minutes.
8. Refer to the Qubit user manual for instructions on reading standards and samples. Follow the appropriate procedure for your instrument.

Conflicting adapter list

When the App libraries need to be pooled with CG libraries, the libraries with the following adapters should avoid being pooled together for sequencing.

Table 37 Conflicting adapter list 1

CG barcode ID	Index 1 (i7) name
26	[H/N]716
93	[H/N]704
106	[H/N]710
106	UDI0018
126	UDI0071
506	UDI0067
547	[H/N]726

Table 38 Conflicting adapter list 2

CG barcode ID	index 2 (i5) name
22	UDI0037
22	UDI0055
86	UDI0087
92	UDI0021
101	UDI0024
101	UDI0092
533	[E/H/N/S]517

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List of primer kit components

A primer kit includes App Make DNB Buffer and primers for App-D sequencing.

Table 39 Components of DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End) Cat. No.: 940-000916-00

Component	Cap color	Spec&quantity	Storage temperature	Transportation temperature	Validity period
App-D Insert Primer 1 Cat. No.: 530-002536-00		2.2 mL/tube ×1 tube	-25 °C to -15 °C	-80 °C to -15 °C	12 months
App-D Barcode Primer 1 Cat. No.: 530-002537-00		3.5 mL/tube ×1 tube			
App-D Barcode Primer 4 Cat. No.: 530-002534-00		3.5 mL/tube ×1 tube			
App Make DNB Buffer Cat. No.: 530-002539-00		400 µL/tube ×1 tube			

Table 40 Components of DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End) Cat. No.: 940-000917-00

Component	Cap color	Spec&quantity	Storage temperature	Transportation temperature	Validity period
App-D Insert Primer 1 Cat. No.: 530-002536-00		2.2 mL/tube ×1 tube	-25 °C to -15 °C	-80 °C to -15 °C	12 months
App-D MDA Primer Cat. No.: 530-002540-00		4.2 mL/tube ×1 tube			
App-D Insert Primer 2 Cat. No.: 530-002538-00		4.2 mL/tube ×1 tube			
App-D Barcode Primer 2 Cat. No.: 530-002541-00		3.5 mL/tube ×1 tube			
App-D Barcode Primer 3 Cat. No.: 530-002535-00		3.5 mL/tube ×1 tube			
App Make DNB Buffer Cat. No.: 530-002539-00		400 µL/tube ×1 tube			

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Research use only

Complete Genomics has labeled the product solely for research use only and specified “RS” in the model name which means it should not be used for clinical diagnosis. Please refer to FDA Guidance, *Distribution of In Vitro Diagnostic Products Labeled for Research Use Only or Investigational Use Only* (Nov. 2013) (available at: *https://www.fda.gov/media/87374/download*). If you have any question, please contact Complete Genomics at +1 (888) 811-9644.

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Manufacturer information

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Technical support E-mail	US-TechSupport@mgi-tech.com
Customer service telephone	+1 (888) 811-9644
Website	www.completegenomics.com

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Order information

Catalog number	Name	Version	Recommended brand
940-000916-00	DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Single-End)	V1.0	CG
940-000917-00	DNBSEQ High-throughput Sequencing Primer Kit (App-D) (Paired-End)	V1.0	CG

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Acronyms and abbreviations

Item	Description
App Library	Converted libraries from third parties by the CG Library Conversion Kit (including TruSeq and Nextera adapter)
bp	Base-pair
DL-200H	Portable DNB Loader (For DNBSEQ-G400)
DNA	Deoxyribonucleic Acid
DNB	DNA Nanoball
dsDNA	double-stranded DNA
FAQ	Frequently Asked Questions
FCL	Flow Cell Large, 4 lanes per flow cell in DNBSEQ-G400RS Sequencing FCL Flow Cell, 1 lane per flow cell in DNBSEQ-T7RS Sequencing FCL Flow Cell
FCS	Flow Cell Small, 2 lanes per flow cell in DNBSEQ-G400 Sequencing FCS Flow Cell
PE	Pair-end sequencing
QC	Quality Control
RCR	Rolling Circle Replication
SE	Single-end sequencing
ssDNA	single-stranded DNA

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Part No.: H-020-000666-00