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DNBSEQ Universal Library Conversion Kit User Manual

- Cat. No.: 940-000963-00 (16 RXN)
- Kit Version: 1.0

For Research Use Only

About the user manual

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@mgi-tech.com
Technical support Email	US-TechSupport@mgi-tech.com
Website	www.CompleteGenomics.com

Revision history

Manual Rev	Kit version	Date	Description
1.0	V1.0	Jun. 2023	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 Universal Library Conversion Kit is designed for linear dsDNA library conversion and is optimized for DNBSEQ sequencing platforms. The linear DNA library can be converted to a single stranded circular (ssCir) DNA library and sequenced on DNBSEQ sequencing platforms with the High-throughput Sequencing Set (App-D). All reagents provided in this kit have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

1.2 Intended use

The DNBSEQ Universal Library Conversion Kit is used to convert dsDNA linear libraries prepared with Next Generation Sequencing Library Preparation Kits other than CG to libraries suitable for DNBSEQ sequencing platforms.

For information on suitable library types, refer to the *App Series Products Ordering Guide* or contact your local sales representative.

1.3 Applicable sequencing platform

The prepared libraries are applicable to the High-throughput Sequencing Set (App-D) and the corresponding DNBSEQ sequencing platforms.

1.4 Components

For component details, refer to the following table.

Table 1 DNBSEQ Universal Library Conversion Kit (16 RXN) (Cat. No.: 940-000963-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
DNBSEQ Universal Library Conversion Kit Cat. No.: 940-000963-00	AC-PCR Primer	Blue	48 µL/tube × 1
	AC-PCR Amplification Master Mix	Blue	400 µL/tube × 1
	Conversion Splint Buffer	Red	186 µL/tube × 1
	Ligation Enzyme	Red	8 µL/tube × 1
	Digestion Buffer	White	23 µL/tube × 1
	Digestion Enzyme	White	42 µL/tube × 1
	Digestion Stop Buffer	White	120 µL/tube × 1

1.5 Storage and transportation

DNBSEQ Universal Library Conversion Kit

- Storage temperature: -25 ℃ to -15 ℃
- Transportation temperature: -25 ℃ to -15 ℃

- Production date and expiration date: refer to the label.
- With proper transport, storage, and use, all components can maintain complete activity within their shelf life.

1.6 User-supplied materials

Table 2 Order information for CG products

Catalog number	Model	Name
940-001281-00	50 mL	DNBSEQ DNA Clean Beads

Table 3 User-supplied equipment list

Equipment	Recommended brand
Vortex mixer	/
Desktop centrifuge	/
Pipettes	/
Thermocycler	/
Magnetic rack DynaMag -2, or equivalent	Thermo Fisher Scientific, Cat. No. 12321D
Qubit Fluorometer, or equivalent	Thermo Fisher, Cat. No. Q33216
Agilent 2100 Bioanalyzer, or equivalent	Agilent Technologies, Cat. No. G2939AA

Table 4 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Nuclease Free (NF) water	Ambion, Cat. No. AM9937, or equivalent
TE Buffer, pH 8.0	Ambion, Cat. No. AM9858, or equivalent
100% Ethanol (Analytical Grade)	/
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	/
1.5 mL tube	/
0.2 mL PCR tube, or 96-well plate	/
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 use 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 decrease the accuracy of 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, please contact Technical Support: **US-TechSupport@mgi-tech.com.**

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	Adapter conversion PCR amplification ⏸	40 - 45 min	10 min
3.2	Cleanup of PCR product ⏸	40 min	10 - 15 min
3.3	QC of PCR product ⏸	15 - 60 min	10 - 20 min
4.1	Denaturation, single strand circularization	45 - 50 min	15 min
4.2	Digestion	35 - 40 min	10 min
4.3	Cleanup of digestion product ⏸	50 min	10 - 15 min
4.4	QC of digestion product ⏸	15 - 20 min	10 - 15 min

- 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.
- ⏸ : The stop point.

2 Sample preparation

2.1 Sample requirements

Sample types: Linear dsDNA library.

Linear dsDNA library size distribution: Between 100 to 500 bp, and the peak size should be at approximately 200 bp (center of the distribution) ±100 bp on either side.

2.2 Sample preparation

- Quantitate the linear dsDNA library with a dsDNA Fluorescence Assay Kit such as Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit.
- The input amount of linear dsDNA library is based on the available linear dsDNA library (see the table below). For example, if the amount of the linear dsDNA library available is 20 ng, the linear dsDNA library input should be 10 ng, and the linear dsDNA library concentration should be at least 0.5 ng/μL.

Table 5 The relationship between linear dsDNA library input and total amount of dsDNA library

Library input (ng)	Library amount (ng)	Library concentration (ng/μL)
10	Amount ≤ 25	≥ 0.5
25	25 < Amount ≤ 50	≥ 1.2
50	Amount > 50	≥ 2.3

i Because the PCR cycles affect downstream data analysis (e.g. InDel analysis), it is preferred that the linear dsDNA library input be increased and the number of PCR cycles corresponds to library input. Please refer to Table 9 for details.

The linear dsDNA library volume can be calculated according to the following formula.

Formula 1 Calculation of linear dsDNA library volume

$$\text{Volume of linear dsDNA library (μL)} = \frac{\text{Linear dsDNA library input (ng)}}{\text{Linear dsDNA library concentration (ng/μL)}}$$

3 Adapter conversion protocol

3.1 Adapter conversion PCR amplification

3.1.1 Preparation

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

Table 6 Preparing the reagents

Reagent	Requirement
Nuclease Free (NF) water	User-supplied; place at room temperature (RT).
AC-PCR Amplification Master Mix	Mix by vortexing, centrifuge briefly, and place on ice.
AC-PCR Primer	

3.1.2 Adapter conversion PCR

1. Transfer the linear dsDNA library into a new 0.2 mL PCR tube and add **NF water** to make a total volume of 22 μL.
2. According to the desired reaction number, prepare the AC-PCR amplification 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 7 AC-PCR amplification mixture

Reagent	Volume per reaction
AC-PCR Amplification Master Mix	25 μL
AC-PCR Primer	3 μL
Total	28 μL

3. Add **28 μL of AC-PCR amplification mixture** to each sample tube (from step 1). Vortex 3 times (3 sec each) and centrifuge briefly.

4. Place the PCR tube(s) into the thermocycler and run the program with the following conditions. The PCR cycles for different library input amounts are listed in table 9.

Table 8 AC-PCR amplification reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated Lid	On	/
98 ℃	3 min	1 cycle
98 ℃	30 sec	N cycles (see table 9)
62 ℃	15 sec	
72 ℃	30 sec	
72 ℃	5 min	1 cycle
4 ℃	Hold	/

Table 9 The PCR cycles for different linear dsDNA library input

Linear dsDNA library input (ng)	PCR cycles
10	10
25	8
50	5

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

Stop point AC-PCR product can be stored at -20 ℃.

3.2 Cleanup of PCR product

- For use with DNBSEQ DNA Clean Beads. If you use the magnetic beads from other brands, optimize the cleanup conditions before getting started.
- Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

3.2.1 Preparation

Table 10 Preparing the reagents

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

3.2.2 Cleanup of PCR product

i 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 **60 µL of DNA Clean Beads** to each sample tube (from step 5 in 3.1.2). 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 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 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 1.5 mL centrifuge tube or PCR tube.

   ⏸ Stop point After cleanup, AC-PCR product(s) can be stored at -20 ℃.

3.3 QC of PCR product

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

Table 11 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 pmol
Electrophoresis method	Tapestation (Agilent Technologies), Bioanalyzer, LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical)	/

Refer to Formula 2 in "Pooling (option)" on page 9 or the table below to calculate the mass (in ng) that corresponds to 1 pmol of dsDNA sample with different fragment sizes.

Table 12 The corresponding yield in 1 pmol for PCR products with different fragment sizes

AC-PCR product peak size (bp)	Corresponding yield in 1 pmol (ng)	AC-PCR product peak size (bp)	Corresponding yield in 1 pmol (ng)
150	99	350	231
200	132	400	264
250	165	450	297
300	198	500	330

- For 1 sample sequencing in 1 lane, please proceed to "Circularization and digestion" on page 13.
- For multiple samples pooled sequencing, refer to "Pooling (option)" on page 9.

3.4 Pooling (option)

⚠ CAUTION Do not pool AC-PCR products with different insert size distributions in the same lane.

There are three stages for multiple samples pooling: purified PCR products (dsDNA libraries) pooling, ssCir libraries (ssDNA, purified digestion products) pooling, and DNB pooling. Unless

otherwise required, you can choose one of the three stages for samples pooling based on your needs.

The following methods are suitable for the purified PCR products pooling. For pooling methods in other stages, please refer to the instructions of the library preparation kits, High-throughput sequencing kits, or DNBSEQ sequencing platforms.

- Purified PCR products pooling

CAUTION Before pooling, carefully read Appendix 1 on page 18.

Quantify the purified PCR products before pooling. The total yield after pooling should be 1 pmol, with a total volume ≤ 48 µL.

Calculate the percentage of the required amount of sequencing data for samples on the same lane. Refer to Formula 2 and 3 to calculate the required mass of each sample. Formula 4 shows the calculation of sample volume.

Formula 2 Conversion between 1 pmol of dsDNA sample and mass in ng

$$\text{Mass (ng) corresponding to 1 pmol PCR products} = \frac{\text{PCR product peak size (bp)}}{\text{1000 bp}} \times 660 \text{ ng}$$

Formula 3 Calculation of each sample mass before pooling

Sample mass (ng)= Mass corresponding to 1 pmol PCR product (ng) × Ratio of sample data (%)

Formula 4 Calculation of sample volume

$$\text{Sample volume (µL)} = \frac{\text{Sample mass (ng)}}{\text{Sample concentration (ng/µL)}}$$

For example: For 4 samples (belong to 300 bp insert size libraries) pooled sequencing. The PCR products should have a total mass of 198 ng and be equal to a total yield of 1 pmol.

1. Calculation the mass of each sample.

- The expected amount of sequencing data for each sample is the same. The ratio of each sample sequencing data is 25%. Referring to Formula 3, the required mass of each PCR sample is 198 ng × 25% = 49.5 ng.
- The expected amount of sequencing data for each sample is different. The ratios of sequencing data for samples 1-4 are 20%, 20%, 30%, and 30%. Referring to Formula 3, the required mass of sample 1 is 39.6 ng. Calculate the mass of samples 2 to 4 in the same way.

2. The concentration of sample 1 is 10 ng/µL. Refer to Formula 4 and the required volume is "A µL". Calculate the volume of samples 2 to 4 in the same way.
3. Transfer A µL of sample 1 into a new 0.2 mL PCR tube.
4. Add other samples into the same PCR tube.

5. Add TE Buffer to make a total volume of 48 μL.

Table 13 Multiple samples pooling (each sample volume should be at least 1 μL)

Name	Volume
Sample 1	A μL
Sample 2	B μL
Sample 3	C μL
Sample 4	D μL
TE Buffer	48 - (A+B+C+D) μL
Total	48 μL

The volumes of A, B, C, and D should each be ≥ 1 μL.

Follow one of the two methods below to pool the samples when the required volume of a sample is less than 1 μL. Method 1 is recommended.

Method 1: Increase the volume of all samples by Z (Z > 1) times. After mixing the samples, take 1/Z of the total volume W μL. Add TE Buffer to make a total volume of 48 μL.

Table 14 Samples mixture: All samples volume increases by Z times

Name	Volume
Sample 1	A × Z μL
Sample 2	B × Z μL
Sample 3	C × Z μL
Sample 4	D × Z μL
Total	W μL

Table 15 Method 1: Multiple samples pooling

Name	Volume
Samples mixture	(W ÷ Z) μL
TE Buffer	48 - (W ÷ Z) μL
Total	48 μL

If necessary, quantify the samples mixture and calculate a new volume X μL (1 pmol). Replace "(W ÷ Z) μL" with "X μL".

Method 2: Dilutes a high concentration sample by Y (Y > 1) times if the required volume is less than 1 μL. Quantify the diluted sample and calculate a new volume. Pool the diluted sample with other samples.

For example: The required volume of sample 3 is < 1 μL. It needs to be diluted by Y times.

Table 16 Diluted sample: Dilute the high concentration sample by Y times

Name	Volume
Sample 3	5 μL*
TE Buffer	5Y - 5 μL
Total	5Y μL

*: The volume of high concentration sample is recommended to be more than 5 μL.

Quantify the diluted sample. Refer to Formula 4 to calculate a new volume "E μL". Pool the diluted sample with other samples. Add TE Buffer to make a total volume of 48 μL.

Table 17 Method 2: Multiple samples pooling

Name	Volume
Sample 1	A μL
Sample 2	B μL
Sample 4	D μL
Diluted sample 3	E μL
TE Buffer	48 - (A+B+D) - E μL
Total	48 μL

4 Circularization and digestion

4.1 Denaturation, single strand circularization

Calculate the required purified PCR product volume based on the main fragment size of purified PCR product, concentration of sample, and Formula 2.

4.1.1 Preparation

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

Table 18 Preparing the reagents

Reagent	Requirement
TE Buffer	User-supplied; place at RT.
Conversion Splint Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Ligase Enzyme	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

4.1.2 Denaturation

1. Add **1 pmol of PCR product** into a new 0.2 mL PCR tube and add **TE Buffer** to make a total volume of 48 µL.
2. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 19 Denaturation reaction conditions (Volume: 48 µL)

Temperature	Time
105 ℃ Heated lid	On
95 ℃	3 min

3. When the program is completed, immediately place the PCR tube(s) on ice for 2 min. Centrifuge briefly and place on ice.

4.1.3 Single strand circularization

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

Table 20 Single strand circularization mixture

Reagent	Volume per reaction
Conversion Splint Buffer	11.6 μL
Ligase Enzyme	0.5 μL
Total	12.1 μL

2. Add **12.1 μL of single strand circularization mixture** to each sample tube(from step 3 in 4.1.2). Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 21 Single strand circularization reaction conditions (Volume: 60.1 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	30 min
4 ℃	Hold

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

4.2 Digestion

4.2.1 Preparation

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

Table 22 Preparing the reagents

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

4.2.2 Digestion

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

Table 23 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 4.1.3). Vortex 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 24 Digestion reaction conditions (Volume: 64.1 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	30 min
4 ℃	Hold

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

4.3 Cleanup of digestion product

- For use with DNBSEQ DNA Clean Beads. If you use the magnetic beads from other brands, optimize the cleanup conditions before getting started.
- Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

4.3.1 Preparation

Table 25 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied; freshly prepared.
TE Buffer	User-supplied; place at RT.
DNA Clean Beads	User-supplied; take out 30 min in advance to equilibrate to RT. Mix thoroughly by vortexing before each use.

4.3.2 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 4.2.2). 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.

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

7. Remove the tube(s) from the magnetic rack and add **27 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 **25 µL** of supernatant to a new 1.5 mL centrifuge tube or PCR tube.

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

4.4 QC of digestion product

Quantify the purified digestion product (ssDNA library) by following the instructions of the Qubit ssDNA Assay Kit.

- The final yield should be at least 60 fmol and the cyclization efficiency should be at least 5%.

Refer to the formula or table below to calculate the mass of 60 fmol ssCir.

Formula 5 Conversion between circular ssDNA fmol and mass in ng

$$60\ \text{fmol ssDNA (ng)} = 0.06\times \frac{\text{PCR product peak size (bp)}}{1000\ \text{bp}} \times 330\ \text{ng}$$

Table 26 The conversion table between ng and PCR product size for 60 fmol of ssDNA

PCR product size (bp)	60 fmol ssDNA (ng)	PCR product size (bp)	60 fmol ssDNA (ng)
150	3	350	7
200	4	400	8
250	5	450	9
300	6	500	10

Appendix 1 Sample barcode pooling strategies

- For pooled sequencing, sample barcode pooling should follow the principle of base balance.
- Using an 8 bp barcode as an example, the ratio of ATGC at 1-8 bp bases should be 25%, as shown in the table below.

Table 27 Example of sample barcode pooling strategies

Barcode	Sequence	1	2	3	4	5	6	7	8
Example 1	TAGGTCCG	T	A	G	G	T	C	C	G
Example 2	GGACGGAA	G	G	A	C	G	G	A	A
Example 3	CTTACTGC	C	T	T	A	C	T	G	C
Example 4	ACCTAATT	A	C	C	T	A	A	T	T
Barcode 1-8 bp A%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp T%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp G%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp C%		25%	25%	25%	25%	25%	25%	25%	25%

- If the proportion cannot reach 25%, then ATGC should appear in each cycle. The minimum base proportion should not be less than 12.5% and the maximum base proportion should not be greater than 62.5%.
- If the proportion is not between 12.5% and 62.5%, then sequencing quality could be reduced. In this case, it is possible that the sample barcodes might not be properly split.

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