invitrogen

Invitrogen™ Collibri™ PCR-free PS DNA Library Prep Kit for Illumina™

USER GUIDE

- For use with Illumina™ next-generation sequencing (NGS) platforms
- For physically sheared DNA

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B.0	11 November 2019	Updated the user guide to include Cat. No. A38615196.
A.0	03 May 2019	New user guide.

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1. Product information

Product description

Invitrogen™ Collibri™ PCR-free PS DNA Library Prep Kits for Illumina™ are designed for the construction of high-efficiency DNA fragment libraries for whole-genome sequencing on Illumina™ next-generation sequencing (NGS) platforms. The kits support library preparation from a wide range of DNA samples and inputs (250 ng–1 µg) starting from appropriately sheared or fragmented double-stranded DNA.

The entire Collibri™ PCR-free PS DNA library prep workflow is integrated into one vial, two-step protocol that takes less than 2 hours. The protocol does not require intermediate sample cleanup between End conversion and Adaptor ligation steps, which minimizes handling errors and saves time and valuable sample.

For convenience, the kits provide color-coded components for visual tracking of library preparation progress. Inert dyes in the reagents do not interfere with enzymatic reactions and do not compromise library prep and sequencing results.

The Collibri™ PCR-free PS DNA Library Prep Kits contain all the necessary reagents that are required for the preparation of up to 96 uniquely indexed DNA libraries, including enzyme mixes, dual-barcoded plate-format adaptors, and cleanup beads.

Note: For an overview of the technology used in the Invitrogen™ Collibri™ PCR-free PS DNA Library Prep Kits, see "Technology overview", page 6.

Product specifications

Assay time	~110 minutes on average
Hands-on time	~30 minutes on average
Sample type	• Low complexity dsDNA (bacteria/phage DNA) • High complexity dsDNA (Mammalian, Mouse, Human, Rat, Plant), including challenging DNA samples (FFPE)
Sample input amount	250 ng– 1 µg of sheared or fragmented DNA with the cleanup protocol 500 ng– 1 µg of sheared or fragmented DNA with the double-sided size selection protocol
Sample input quality	Double-stranded DNA with A_{260}/A_{280} ratio of 1.7–2.0
Fragment size range	150 bp– 1000 bp
Multiplexing	24 Combinatorial Dual (CD) indexes 96 Combinatorial Dual (CD) indexes 4 sets of 24 Unique Dual (UD) indexes (Set A, Set B, Set C, Set D)
System compatibility	iSeq™, HiSeq™ 1000, HiSeq™ 1500, HiSeq™ 2000, HiSeq™ 2500, HiSeq™ 3000, HiSeq™ 4000, HiSeq™ X, MiSeq™, MiniSeq™, NextSeq™ 500, NextSeq™ 550, NovaSeq™ 6000
Sequencing application	Whole-genome sequencing (WGS)

Kit contents and storage

Kit configurations

The Collibri™ PCR-free PS DNA Library Prep Kits for Illumina™ are available in two sizes, providing sufficient reagents to prepare DNA fragment libraries for 24 or 96 samples. The 24 prep sizes are available with Collibri™ DNA CD (Combinatorial Dual) or UD (Unique Dual) Indexes.

Kit configuration	Kit size	DNA index type[1]	Catalog No.
Collibri™ PCR-free PS DNA Library Prep Kit	24 preps	CD	A38608024
		UDI Set A (1–24)	A38609024
		UDI Set B (25–48)	A43608024
		UDI Set C (49–72)	A43609024
		UDI Set D (73–96)	A43610024
	96 preps	CD	A38610096
		UDI Set A–D (1–96)	A38615196

[1] CD: Combinatorial Dual, UDI: Unique Dual Indexes.

Note: Kits containing PCR amplification module that support library preparation from 1 ng of input DNA (Cat. Nos. A38612024, A38614096, A38613024, A43611024, A43612024, and A43613024) are available from Thermo Fisher Scientific. For more information, go to **thermofisher.com**.

Kit components and storage

Upon receipt, immediately store the Collibri™ PCR-free PS DNA Library Prep Kit and the Collibri™ DNA CD or UD Indexes at –20°C. Store the Collibri™ DNA Library Cleanup Kit at 2°C to 8°C.

IMPORTANT! Do **not** freeze the DNA Cleanup Beads.

Component	Cap/reagent color[1]	24 preps	96 preps
Collibri™ PCR-free PS DNA Library Prep Kit (Store at –20°C)			
2X End Conversion Master Mix	Blue	600 µL	2 × 1.2 mL
7X Ligation Master Mix for PS	Red	250 µL	1 mL
Collibri™ DNA Library Cleanup Kit (Store at 2°C to 8°C. **IMPORTANT!** Do **not** freeze.)			
DNA Cleanup Beads	Orange	10 mL	30 mL
Wash Buffer (Concentrated)	Blue	4.5 mL	18 mL
Elution Buffer	White	5 mL	20 mL
Collibri™ DNA CD[2] or UD[3] Indexes[4] (Store at –20°C)			
Dual Index Adaptors (7 µM)	—	10 µL/well (24 wells)	10 µL/well (96 wells)

[1] In the Collibri™ PCR-free PS DNA Library Prep Kit, the cap colors match the color of the reagent in the vial. However, this is not the case for caps and reagents in the Collibri™ DNA Library Cleanup Kit.
[2] Combinatorial Dual-Indexed Adaptors (CD) are available with Catalog Nos. A38608024, A38610096.
[3] Unique Dual-Indexed Adaptors (UD) are available with Catalog Nos. A38609024, A43608024, A43609024, A43610024.
[4] For the index sequences and plate layouts, see "Appendix B: Adaptor index sequences and plate layouts" (page 33).

Required materials not supplied

For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Thermal cycler with heated lid, such as: • Veriti™ 96-well Thermal Cycler • ProFlex™ 96-well PCR System • ProFlex™ 3 × 32-well PCR System • QuantStudio™ 3 Real-Time PCR System • QuantStudio™ 5 Real-Time PCR System • QuantStudio™ 6 Flex Real-Time PCR System • QuantStudio™ 6 Pro Real-Time PCR System • QuantStudio™ 7 Flex Real-Time PCR System • QuantStudio™ 7 Pro Real-Time PCR System • StepOnePlus™ Real-Time PCR System • Applied Biosystems™ 7500 Fast Real-Time PCR System	 • 4375786 • 4484075 • 4484073 • thermofisher.com • thermofisher.com • thermofisher.com • thermofisher.com • thermofisher.com • thermofisher.com • thermofisher.com • thermofisher.com
Agilent™ 2100 Bioanalyzer™ instrument[1]	Agilent, G2938A
Agilent™ High Sensitivity DNA Kit[1]	Agilent, 5067-4626
Tools for physical DNA shearing, such as: • Covaris™ S2 Focused-ultrasonicator™[2] • Covaris™ M220 Focused-ultrasonicator™	 • Discontinued[2] • 4482277
Magnetic rack, such as: • Invitrogen™ DynaMag™-2 Magnet (for 1.5-mL tubes) • Invitrogen™ DynaMag™-96 Side Magnet (for PCR strips or 96-well 0.2-mL plates)	 • 12321D • 12331D
Benchtop microcentrifuge	MLS
Vortex mixer	MLS
Heating block and/or thermomixer	MLS
Nuclease-free 1.5-mL tubes, such as Eppendorf™ DNA LoBind™ Tubes	Eppendorf, 022431021
0.2-mL thin-wall PCR tubes or plates	MLS
Cooling rack for 0.2-mL PCR tubes/plates	MLS
Calibrated single-channel or multi-channel pipettes (1 µL– 1,000 µL)	MLS
Nuclease-free pipette tips	MLS
Disposable gloves	MLS

[1] You can also use comparable method to evaluate the quality of prepared library.

[2] Discontinued, but supported. Contact manufacturer for details.

Item	Source
One of the following Tris buffers: • 10 mM Tris-HCl buffer, pH 7.5–8.5 • TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) • Low TE buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA)	MLS
Ethanol 96–100%, molecular biology grade	MLS
(Optional) Qubit™ 4 Fluorometer[3]	Q33226
(Optional) Qubit™ DNA HS Assay Kit[3]	Q32854
Invitrogen™ Collibri™ Library Quantification Kit	A38524100, A38524500

[3] You can also use the Qubit™ 3.0 Fluorometer, the NanoDrop™ instrument, or a comparable method. The Qubit™ 2.0 Fluorometer is supported, but it is no longer available for purchase.

Technology overview

The Collibri™ PCR-free PS DNA Library Prep Kit provides a fast and efficient NGS library construction method by combining the DNA fragment End conversion and Adaptor ligation steps into a convenient one-tube protocol (Figure 1).

End conversion and Adaptor ligation

First, fragmented DNA is end-repaired (5′- and 3′-overhangs are blunted, 5′-ends are phosphorylated) and a single dA-overhang is added at the 3′-end of each strand. In the following step, Illumina™-compatible NGS adaptors with 3′-dTMP overhangs are added to each end of the 3′-dA-tailed DNA molecules.

Indexing

Illumina™-compatible NGS adaptors contain sequences required for binding of DNA fragments to a flow cell and PCR amplification of adaptor-ligated library fragments, and sequences complementary to the Illumina™ sequencing primers. Collibri™ PCR-free PS DNA Library Prep Kits include dual-barcoded adaptors in a 24-well or 96-well plate format. Each well in the Dual Index Adaptor plate contains a single-use adaptor that consists of a unique combination of two 8-nucleotides identification indexes (see page 33 for Adaptor index sequences). Combination of one D5 barcode with one D7 barcode in each ready-to-use adaptor allows you to pool up to 24 or 96 different samples for the sequencing run.

Library purification

Unligated adaptors and adaptor dimer molecules are efficiently removed from the library in a single cleanup or size selection step using the DNA Cleanup beads magnetic particles (included in the kit) while preserving high library yields.

Library quantification

For best results, we recommend qPCR-based quantification of libraries using the Invitrogen™ Collibri™ Library Quantification Kit (Cat. No. A38524100, A38524500) before sequencing.

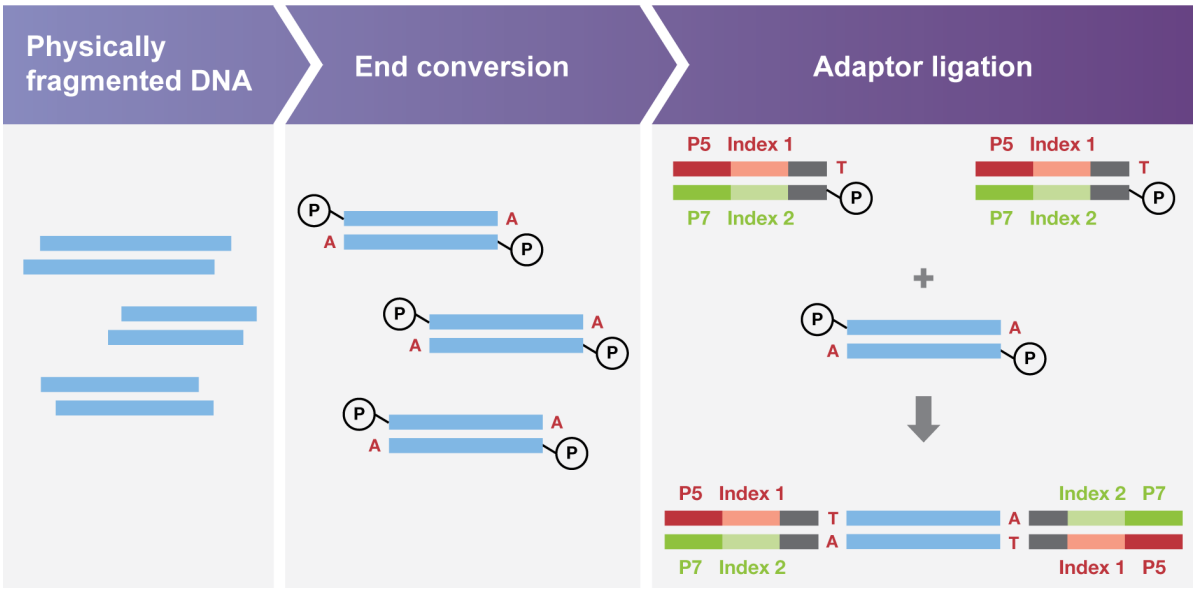


Figure 1 Simplified schematic representation of technology used in the Collibri™ PCR-free PS DNA Library Prep Kit.

2. Methods

Workflow

Figure 2 (below) and Figure 3 (page 8) illustrate the Collibri™ PCR-free PS DNA Library Prep Kit workflow to construct sequencing-ready DNA fragment libraries for whole-genome DNA sequencing.

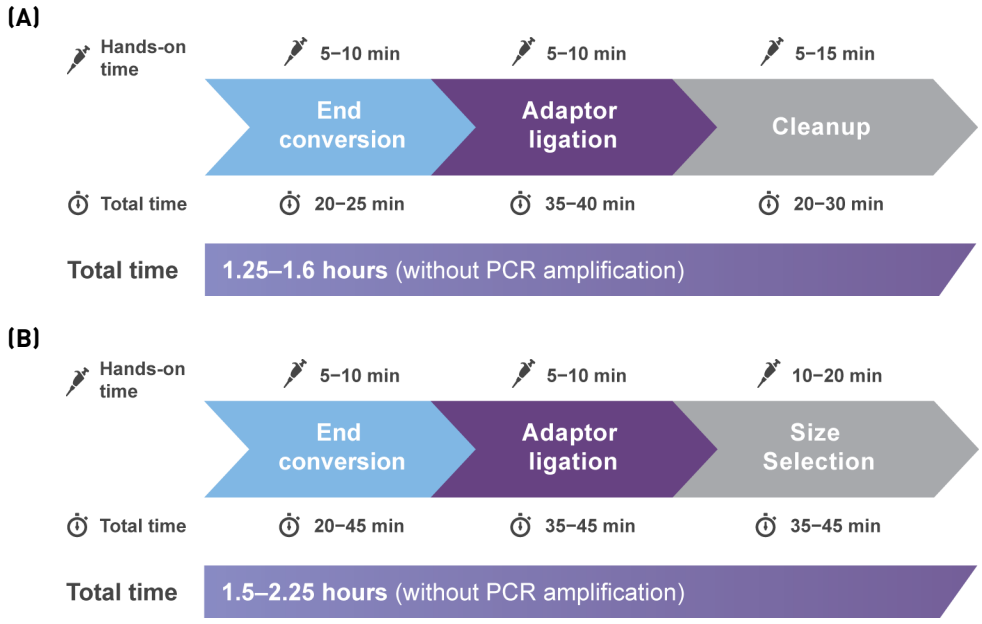


Figure 2 Collibri™ PCR-free PS DNA Library Prep Kit workflow to construct sequencing-ready DNA fragment libraries for whole-genome DNA sequencing with **(A)** library cleanup and **(B)** double-sided size selection protocols.

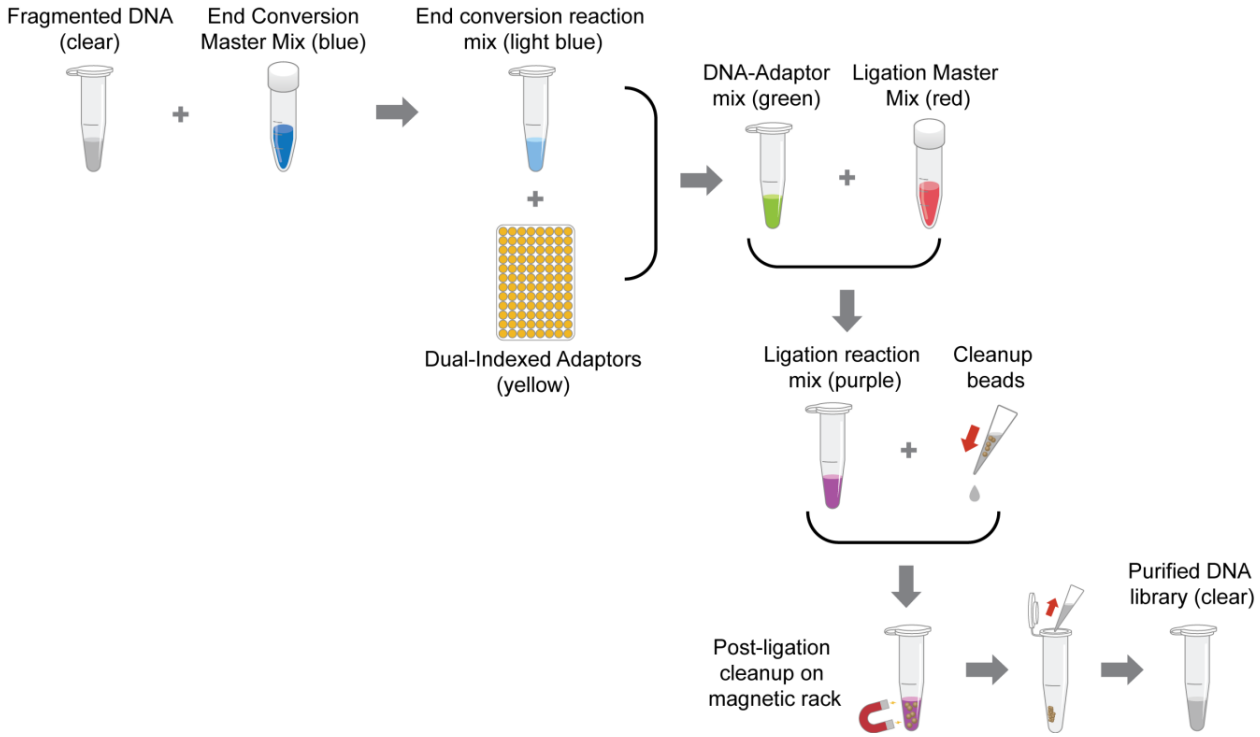


Figure 3 Collibri™ PCR-free PS DNA Library Prep Kit components are colored with inert dyes to provide a visual control of the proper workflow progress – reaction mix changes color in every step to ensure that the correct component is added.

Important procedural guidelines

Input DNA requirements

"Input" typically refers to the amount of DNA used in the End conversion reaction. If DNA was quantified before fragmentation and the fragmented DNA was subjected to cleanup or size selection, the actual input into library construction can be significantly lower. Losses during cleanup or size selection should be taken into account when evaluating the process efficiency. DNA input recommendations for PCR-free library construction workflow are listed in Table 1.

Table 1 Input DNA requirements

NGS library read length	300 bp or 2 ×150 bp	500 bp or 2 ×250 bp
Target insert size	~350 bp	~550 bp
Recommended DNA input	250 ng–1 µg[1]	1 µg

[1] 500 ng input DNA is sufficient to prepare size-selected PCR-free libraries of ≥4 nM concentration. For higher concentration PCR-free libraries, use 500 ng–1 µg input DNA.

Guidelines for DNA quality

- The success of DNA library preparation and reliable DNA sequencing results strongly depend on the quality and quantity of input DNA used. Proper sample handling, appropriate DNA isolation method, and accurate measurement of DNA concentration are essential for successful sequencing.
- Residual traces of contaminating proteins, organic solvents, and salts can degrade the DNA or decrease the activity of enzymes that are necessary for efficient DNA library preparation. Ensure that your input DNA is free of such contaminants.
- Single-stranded DNA, RNA, or free nucleotides can interfere with accurate quantification of purified DNA, especially when UV spectrometry-based methods are used for measurement. For best results, we recommend using fluorometric-based methods for input DNA quantification, such as the Invitrogen™ Qubit™ dsDNA HS Assay Kit with the Qubit™ 4 Fluorometer (or a similar instrument) (page 4).
- For high-quality gDNA purification from various sources, use specialized commercial kits.

Guidelines for DNA fragmentation

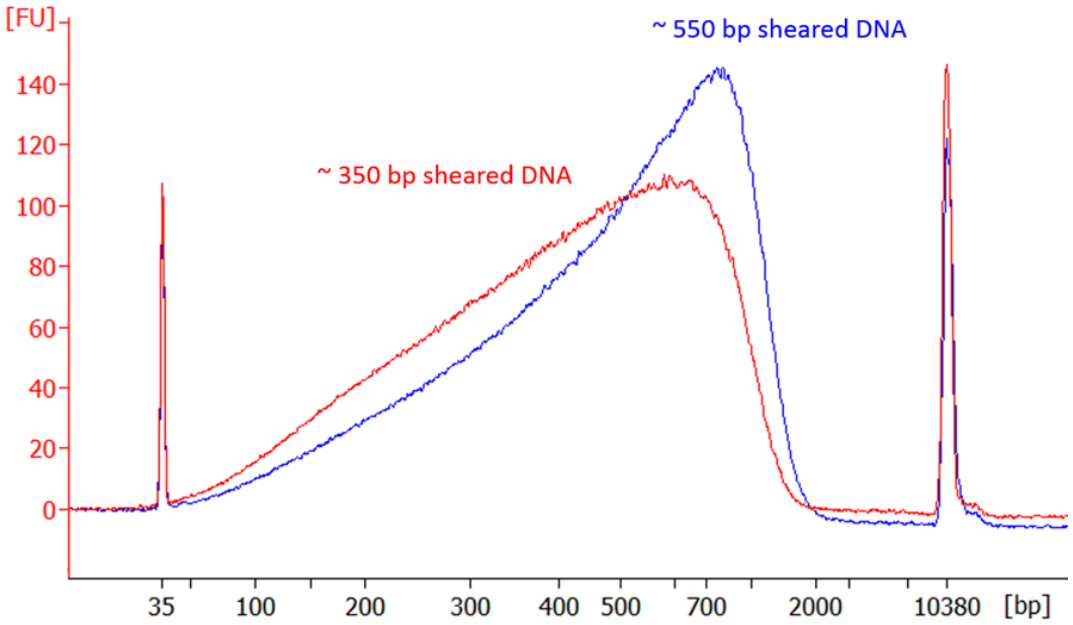
- The DNA library construction workflow requires high-quality fragmented DNA obtained using enzymatic or physical (such sonication or nebulization) fragmentation methods that are commonly used in NGS library construction workflows. Follow manufacturer's recommendations to obtain fragmented DNA of desired fragment length and concentration.
- Dissolve the fragmented double-stranded DNA in 10 mM Tris (pH 7.5–8.5) buffer, TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA), or Low TE buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA).

  IMPORTANT! Do **not** use nuclease-free water.

- Evaluate the quality of fragmented DNA by agarose gel electrophoresis or using the Agilent™ 2100 Bioanalyzer™ (or a similar instrument) (Figure 4, page 10).

Note: You can modify the instrument settings for physical shearing of dsDNA to meet the specific requirements of your experiments and achieve the desired fragment size distribution.

(A)



(B)

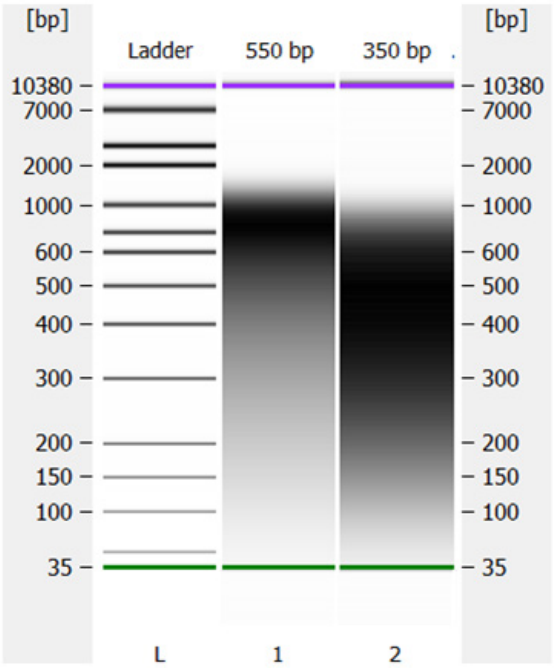


Figure 4 Double-stranded DNA was physically sheared up to ~350 bp and ~550 bp using the Covaris™ E220 Evolution instrument and analyzed with the Agilent™ High Sensitivity DNA Kit and the Agilent™ 2100 Bioanalyzer instrument. **(A)** Trace view of the analysis results Peaks at 35 bp and 10380 bp represent low and high-molecular weight markers. **(B)** The results of the same analysis are shown in gel view.

Guidelines for adaptor ligation

- Indexed adaptors are used to uniquely label sequencing libraries that are generated from individual biological samples. This allows pooling of indexed libraries before cluster generation and enables multiplexed sequencing, which simplifies sample preparation and reduces sequencing costs.
- Pooling applications on Illumina™ sequencing platforms require the use of specific index combinations. For optimal results, we recommend that you follow Illumina™ multiplexing guidelines.
- Depending on the Collibri™ PCR-free PS DNA Library Prep Kit, the Collibri™ Dual-Indexed Adaptor plate contains a set of 24 or 96 Adaptors, each carrying two 8-nucleotide indexes (barcodes). For the names and sequences of the indexes and the adaptor plate layouts for 24- and 96-prep kits, go to Appendix B (page 33).
- Collibri™ Dual-Indexed Adaptors are supplied in fully skirted PCR plates, which are sealed with non-pierceable, non-porous, Easy-Peal™ seals to minimize cross-contamination during handling. Adaptors are provided at a concentration of 7 µM, and each well of the plate contains 10 µL of adaptor required for one library prep (plus a generous excess volume required for automated preps).
- Collibri™ Dual-Indexed Adaptors are duplexed oligonucleotides. Do **not** expose the adaptors to temperatures above room temperature to prevent denaturation.
- Use appropriate laboratory practices to avoid cross-contamination of indexed adaptors. Wipe the seal surface with 70% ethanol before each use, and use new, sterile pipette tips for every well of the adaptor plate.
- To ensure equal read distribution when multiplexing libraries, carefully quantify individual libraries and normalize before pooling. We recommend using the Collibri™ Library Quantification Kit (Cat. No. A38524100, A38524500) as the preferred qPCR-based method to accurately and reproducibly quantify sequenceable molecules.

Guidelines for post-ligation library cleanup

- Post-ligation library cleanup or size selection is required to remove unligated adaptors and/or adaptor-dimer molecules from the library before the library amplification or cluster generation steps.
- The Collibri™ DNA Library Cleanup Kit (included in the Collibri™ PCR-free PS DNA Library Prep Kit) eliminates unused adaptors and adaptor dimers efficiently. Therefore, the library prep workflow requires only a single post-ligation cleanup step, which saves time and results in higher library yields.
- Equilibrate the DNA Cleanup Beads to room temperature before use and carry out all library cleanup steps at room temperature. This is essential for achieving the specified library size distribution and yields.
- DNA Cleanup Beads tend to gradually settle at the bottom of the tube. Before each use, thoroughly resuspend the cleanup beads by pipetting up and down several times or by vortexing. When properly resuspended, the bead solution has a uniform color with no visible clumping on the walls or at the bottom of the tube.
- To ensure optimal DNA recovery, it is critical that you mix the DNA and the cleanup beads thoroughly by vortexing or extensive pipetting.
- The beads are superparamagnetic and are collected by placing the reaction plate or tube in a magnetic stand. The time required for complete separation varies depending on the strength of your magnet, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet. Optimize the bead capture times accordingly.
- To ensure the best DNA yields, do not lose any magnetic beads during the cleanup procedure. Always verify that you do not discard or transfer any beads when removing or transferring the supernatant.
- Supplement the Wash Buffer with the appropriate volume of 96% ethanol, as noted on the bottle.
- You can adjust the volume of Wash Buffer used to accommodate various reaction vessels, but it is important that cleanup beads are entirely submerged during the wash steps.
- Remove all traces of ethanol before proceeding with subsequent reactions. However, over-drying the beads can make them difficult to resuspend, which can result in considerable DNA loss.
- The volume of Elution Buffer used to elute the library DNA depends on the downstream workflow. Generally, we recommend using 25 µL of Elution Buffer, which results in 22–23 µL of eluted DNA. This leaves sufficient volume of DNA library (2–3 µL) required for quality control purposes.
- You can store the purified DNA in elution buffer at 2°C to 8°C for 1–2 weeks, or at –20°C for long-term storage.

Guidelines for evaluation of successful library construction

- Verify the size distribution of the prepared DNA library by an electrophoretic method, such as performing an analysis with the Agilent™ High Sensitivity DNA Kit on the Agilent™ 2100 Bioanalyzer™ instrument (or similar) (Figure 5).
- **IMPORTANT!** Note that the libraries carrying Y-shape Adaptors in the PCR-free workflows appear to have a longer fragment size distribution than would be predicted or derived from the sequencing data. The apparent larger size is due to the characteristic migration of the fragments on the Bioanalyzer™ chip, which is caused by the structural features of Y-shape Adaptors (Figure 5).

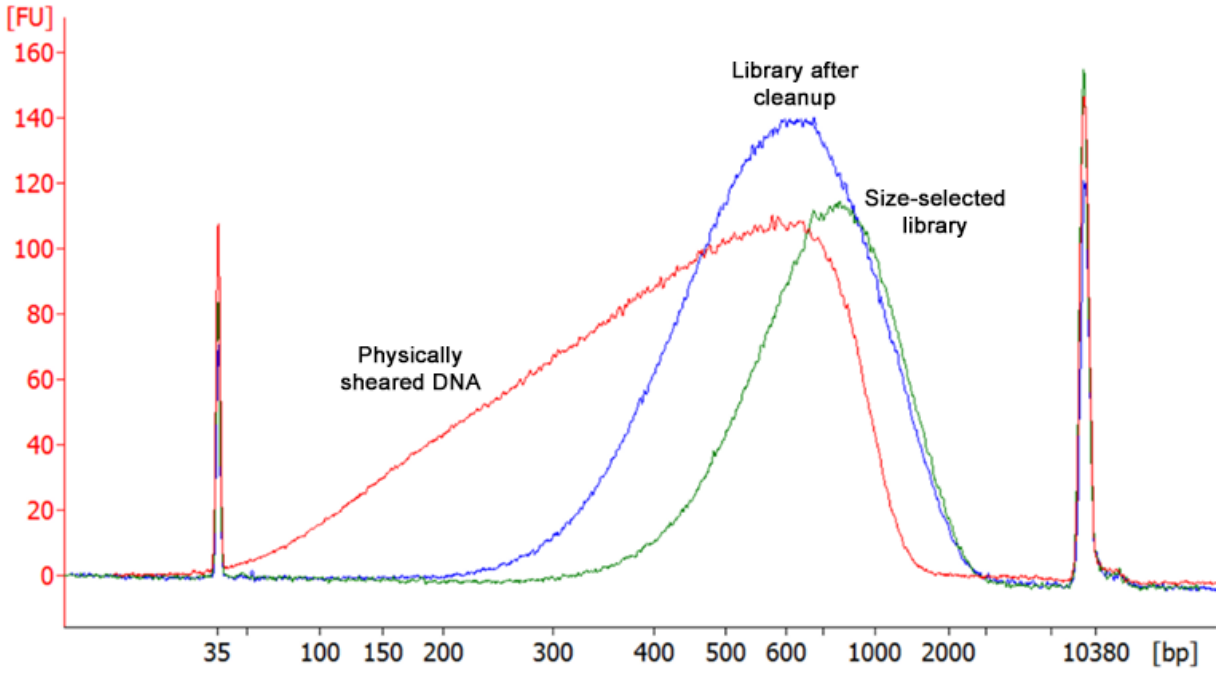


Figure 5 DNA was physically sheared using Covaris™ E220 instrument and libraries were prepared from 500 ng DNA using the Collibri™ PCR-free PS DNA Library Prep Kit. Libraries were purified following post-ligation cleanup or double-sided size selection protocol. Aliquots of the sample were collected, then electrophoregrams were generated on an Agilent™ 2100 Bioanalyzer™ instrument.

Note: Libraries appear longer than expected due to Y-shape adaptors.

- To achieve the highest quality sequencing data, it is essential to create optimal cluster densities across the flow cell. Optimization of cluster densities requires accurate quantification of DNA libraries, and the best quantification methods are based on qPCR.
- We recommend using the Collibri™ Library Quantification Kit for qPCR-based quantification of prepared libraries before sequencing.

Before you begin

- Read the entire protocol before beginning. Take into account the safe stopping points where you can store the samples frozen at –20°C, and plan your workflow accordingly.
- Use good laboratory practices to minimize cross-contamination of nucleic acid products. Use filtered pipette tips and, if possible, perform library construction in a separate area or room.
- Ensure that the Collibri™ PCR-free PS DNA Library Prep Kit components have been fully thawed on ice and thoroughly mixed before use.
- Keep all enzyme components on ice as long as possible during handling.
- Reaction mixtures prepared from the enzyme mixes (2X End Conversion Master Mix and 7X Ligation Master Mix for PS) are very viscous and require special attention during pipetting. Pipet viscous solutions slowly, and ensure complete mixing of the reaction mixture by vortexing or pipetting up and down several times as indicated in the protocol.
- Perform all library cleanup steps using 1.5-mL Eppendorf™ DNA LoBind™ Tubes (Eppendorf™, Cat. No. 022431021).
- You can safely pause the library construction process after the completion of post-ligation cleanup or size selection. Safe stopping points are marked accordingly in the protocol.
- Purified, adaptor-ligated library DNA can be stored at 2°C to 8°C for 1–2 weeks or at –20°C for one month. When possible, minimize the number of freeze-thaw cycles.

End conversion

Overview

This section describes the end-repair and dA-tailing of the input DNA to prepare it for ligation with Illumina™-compatible NGS adaptors. The Collibri™ PCR-free PS DNA Library Prep Kit combines the end-repair of input DNA and the addition of 3' dA-overhangs in a single one-vial reaction.

Required materials

Components from the Collibri™ PCR-free PS DNA Library Prep Kit:

- 2X End Conversion Master Mix for PS

Other materials and equipment:

- 10 mM Tris-HCl Buffer, pH 7.5–8.5
- 1.5-mL Eppendorf™ DNA LoBind™ Tubes
- 0.2-mL sterile, thin-wall PCR tubes
- Vortex mixer
- Microcentrifuge
- Thermal cycler with a heated lid set to 99°C
- Ice or cooling block set to 4°C

Before you begin

- Thaw the reaction components on ice.
- Before use, mix the 2X End Conversion Master Mix by vortexing, then briefly centrifuge to collect all the droplets at the bottom of the tube. Keep on ice.
- Dilute the fragmented DNA in 10 mM Tris-HCl, pH 7.5–8.5, if needed.

End-repair DNA fragments and add dA-tails

1. On ice or a cooling rack, assemble the End conversion reaction for each DNA sample in a sterile 0.2-mL thin-wall PCR tube. Add the reagents in the order given.

Component	Volume
10 mM Tris-HCl, pH 7.5–8.5	to 50 µL
Fragmented DNA (250 ng–1 µg)[1] (clear ○)	X µL
2X End Conversion Master Mix (blue ●)	25 µL
Total volume (light blue mixture ●):	**50 µL**

[1] Use ≥500 ng–1 µg for size-selected libraries.

2. Keep the mixture on ice or cooling block. Mix the contents by vortexing for 3–5 seconds, then centrifuge briefly to collect the liquid at the bottom of the tube.

3. Incubate the mixture in a thermal cycler with the heated lid set to 99°C and programmed as outlined in the following table.

IMPORTANT! Heated lid set to 99°C is required for this step.

Step	Temperature	Time
End repair	20°C	5 minutes
dA-tailing	65°C	10 minutes
Hold	4°C	Hold

4. When the thermocycler program is complete and the sample block has cooled to 4°C, **immediately** remove the samples and place them on ice.

IMPORTANT! Proceed immediately to the next step, "Dual-Indexed Adaptor ligation" (page 17).

Dual-Indexed Adaptor ligation

Overview

This section describes the ligation of the Illumina™-compatible NGS adaptors to end-converted DNA fragments.

Note that the color of the reaction mixture changes as each reaction component is added. Mixing the Dual-Indexed Adaptors (yellow) with the end-converted DNA fragments (blue) produces a green mixture. If the correct amount of the Ligation Master Mix (red) is added to this mixture, the final ligation reaction becomes purple.

Required materials

Components from the Collibri™ PCR-free PS DNA Library Prep Kit:

- 7X Ligation Master Mix for PS

Components from the Collibri™ CD or UD Indexes:

- Collibri™ Dual-Indexed Adaptor plate

Other materials and equipment:

- End conversion reaction mixture (from step 4, page 16)
- Microcentrifuge
- Thermomixer or Thermal Cycler with a heated lid (see "Required materials not supplied", page 4)
- Ice or cooling block set to 4°C

Before you begin

Before use, mix the 7X Ligation Master Mix for PS by vortexing thoroughly, then briefly centrifuge to collect all the droplets at the bottom of the tube. Keep on ice.

Ligate the adaptors

1. Remove the seal from the wells of the 24-well or 96-well Collibri™ Dual-Indexed Adaptor plate that you plan to use, then transfer 10 µL of Dual-Indexed Adaptor from one well to each 50 µL end-converted DNA sample (from step 4, page 16). Use a new adaptor for each DNA sample. Keep the Adaptor-DNA mixture on ice.

Component	Volume
End conversion reaction mixture (light blue 🔵)	50 µL
Dual-Indexed Adaptor (yellow 🟡)	10 µL
Total volume (green mixture 🟢):	**60 µL**

IMPORTANT! Keep track of the indexes from each adaptor well used for each DNA sample.

2. Seal the used wells of the Collibri™ Dual-Indexed Adaptor plate with Easy-Peal™ seal (provided with the kit) cut to the appropriate size and shape, then store the unused adaptors frozen at –20°C. The Collibri™ Dual-Indexed Adaptor plate is stable for at least 10 freeze-thaw cycles.

Note: Do **not** reuse the same adaptor wells.

3. To prepare the ligation reaction mix, add the 7X Ligation Master Mix for PS to the Adaptor-DNA mixture on ice (from step 1, page 17), then mix well by vortexing.

Component	Volume
Adaptor-DNA mixture from step 1 (green ●)	60 µL
7X Ligation Master Mix for PS (red ●)	10 µL
Total volume (purple mixture ●):	**70 µL**

IMPORTANT! Observe the color change as each reaction component is added. If the correct component is added, the ligation mix should be purple.

4. Incubate the ligation reaction mixture at 20°C for **30 minutes** in a thermomixer or thermocycler with heated lid off.

IMPORTANT! Ensure that the lid has cooled and is not heated after the previous run.

5. Proceed to "Post-ligation cleanup or size-selection of Adaptor-ligated library", page 19.

Note: You can store the Adaptor-ligated DNA samples at –20°C. However, this can result in lower yields.

Post-ligation cleanup or size selection of Adaptor-ligated library

Overview

This section describes bead-based post-ligation purification of adaptor-ligated DNA library.

- Removal of fragments smaller than 150 bp is referred as "One-Sided Size Selection" (or simply "Cleanup") (page 20).
- Collection of fragments in the range of 150–850 bp requires "Double-Sided Size Selection" (or simply "Size selection") (page 22).

Required materials

Components from the Collibri™ DNA Library Cleanup Kit:

- DNA Cleanup Beads
- Wash Buffer (diluted with 96% ethanol)
- Elution Buffer

Other materials and equipment:

- Dual Index Adaptor-ligated DNA library (from step 4, page 18)
- 96% ethanol, molecular biology grade (used for diluting the Wash Buffer before first use)
- 1.5-mL Eppendorf™ DNA LoBind™ Tubes or 96-well plate
- Microcentrifuge
- Magnetic rack (see "Required materials not supplied", page 4)

Before you begin

- Ensure that the appropriate volume of 96% ethanol (as noted on the bottle) was added to the Wash Buffer before first use.
- Ensure that the DNA Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the DNA Cleanup Beads to completely resuspend the magnetic beads in the solution.

Option A: Post-ligation cleanup

Purify the adaptor-ligated library

1. Mix the Dual Index Adaptor-ligated DNA library (70 µL) with **45 µL** of Cleanup Beads by vortexing until you have obtained a homogeneous suspension.
2. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.

   IMPORTANT! Do **not** extend the binding step to more than 5 minutes. Over-incubation can result in greater amount of adaptor and adaptor dimers in the final library.

3. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.

   Note: The time required for the complete capture of the cleanup beads can vary depending on the reaction vessel and the magnet used. Optimize the capture time accordingly.

4. Keeping the reaction tube in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all the supernatant is removed.

   Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube on the magnet again.

5. Keeping the reaction tube on the magnet, add **200 µL** of Wash Buffer (pre-mixed with ethanol), then incubate for **30 seconds** at room temperature.

   IMPORTANT! Do **not** resuspend the magnetic beads in Wash Buffer.

6. Carefully remove and discard the supernatant using a pipette.
7. Repeat steps 5–6.
8. To remove the residual ethanol, briefly centrifuge the reaction tube, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
9. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

   IMPORTANT! Do **not** over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

10. Remove the tube from the magnetic rack, add **70 µL** of Elution Buffer, then vortex to mix thoroughly.
11. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.
12. Add **52 µL** of fresh DNA Cleanup Beads directly to the bead suspension in Elution Buffer, then mix by vortexing until you have obtained a homogeneous suspension.
13. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.

14. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes**.
15. Keeping the reaction tube in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all the supernatant is removed.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the bottom of the tube on the magnet again.

16. Keeping the reaction tube on the magnet, add **200 µL** of Wash Buffer (pre-mixed with ethanol), then incubate for **30 seconds** at room temperature.

    IMPORTANT! Do **not** resuspend the magnetic beads in Wash Buffer.

17. Carefully remove and discard the supernatant using a pipette.
18. Repeat steps 16–17.
19. To remove the residual ethanol, briefly centrifuge the reaction tube, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
20. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

    IMPORTANT! Do **not** over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

21. Remove the tube from the magnetic rack, add **25 µL** of Elution Buffer, then vortex to mix thoroughly.
22. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.
23. Place the tube in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
24. Without removing the tube from the magnetic rack, collect **22–23 µL** of the supernatant to a new sterile tube for storage.

    Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the bottom of the tube on the magnet again.

STOPPING POINT. Store the eluted DNA library at 4°C for up to 1–2 weeks or at –20°C for long-term storage, or immediately proceed to "Verify the size distribution and quality of prepared DNA libraries", page 28.

Option B: Post-ligation double-sided size selection

Overview

This section describes the optional bead-based size selection of the Dual Index Adaptor-ligated DNA sample after the ligation step. During the procedure, smaller and longer library fragments are removed from the adaptor-ligated DNA sample to generate a library with the desired fragment size distribution.

We recommend that you perform size selection if the average DNA fragment size is larger than the desired target insert size. If the average fragment size of your sample is smaller than the desired target insert size, we do not recommend size selection (see Figure 6).

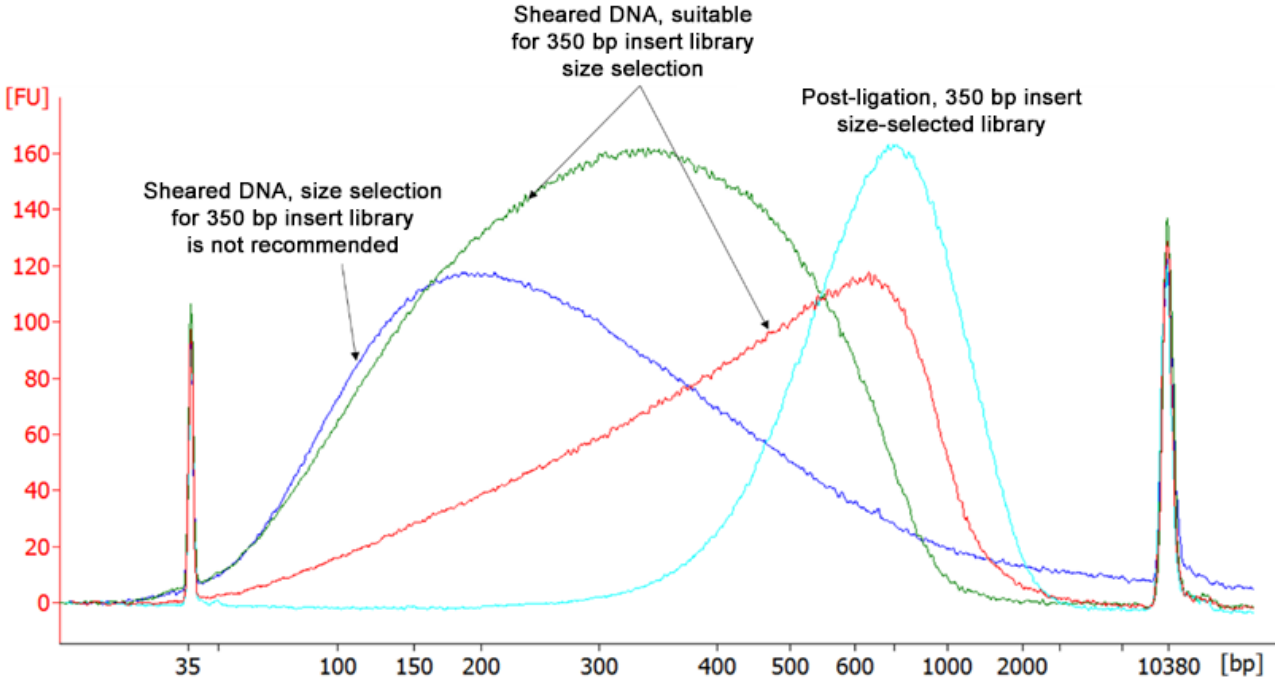


Figure 6 Criteria for performing size selection for a library with a desired target insert size of 350 bp. For samples with average fragment size of >350 bp, size selection with a target insert size of 350 bp is recommended. If the average fragment size of the sample is <350 bp, size selection is not recommended and the cleanup protocol (page 20) should be used instead.

IMPORTANT! Recommended conditions for bead-based size selection depend on the desired fragment size distribution of the DNA library. See Table 2 (page 23) for the appropriate volume of DNA cleanup beads to use for the desired library size.

Required materials

Components from the Collibri™ DNA Library Cleanup Kit:

- DNA Cleanup Beads
- Wash Buffer (diluted with 96% ethanol)
- Elution Buffer

Other materials and equipment:

- Dual Index Adaptor-ligated DNA sample (from step 4, page 18)
- 1.5-mL Eppendorf™ DNA LoBind™ Tubes or 96-well plate
- Microcentrifuge
- Magnetic rack (see "Required materials not supplied", page 4)

Before you begin

- Ensure that the appropriate volume of 96% ethanol (as noted on the bottle) was added to the Wash Buffer before first use.
- Ensure that the DNA Cleanup Beads, Wash Buffer, and Elution Buffer are at room temperature.
- Gently vortex the DNA Cleanup Beads to completely resuspend the magnetic beads in the solution.

Important procedural guidelines

- **The following size selection protocol is for libraries with 350 bp inserts only.** To select for libraries with different size fragment inserts, see Table 2 for the appropriate volume of cleanup beads to use.
- To obtain a population of shorter or longer fragment sizes in your library, you can further optimize the size selection protocol by varying the volume of cleanup beads used in the size selection steps (see "Optimize bead-based size selection", page 26).

Table 2 Recommended conditions for bead-based size selection of libraries.

Insert size	Volume of DNA Cleanup Beads		
	Buffer exchange	First binding	Second binding
200 bp	60 µL	65 µL	20 µL
350 bp	60 µL	45 µL	20 µL
550 bp	60 µL	35 µL	20 µL

Perform size selection

Initial cleanup

1. Mix the Dual Index Adaptor-ligated DNA sample (70 µL) with **60 µL** of DNA Cleanup Beads by vortexing until you have obtained a homogeneous suspension.
2. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.

> IMPORTANT! Do **not** extend the binding step to more than 5 minutes. Over-incubation can result in greater amount of adaptor and adaptor dimers in the final library.

3. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.

   Note: Time required for the complete capture of the cleanup beads can vary depending on the reaction vessel and the magnet used. Optimize the capture time accordingly.

4. Keeping the reaction tube in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all the supernatant is removed.

   Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube on the magnet again.

5. Keeping the reaction tube on the magnet, add **200 µL** of Wash Buffer (pre-mixed with ethanol), then incubate for **30 seconds** at room temperature.

   IMPORTANT! Do **not** resuspend the magnetic beads in Wash Buffer.

6. Carefully remove and discard the supernatant using a pipette.
7. Repeat steps 5–6.
8. To remove the residual ethanol, briefly centrifuge the tube, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
9. Keeping the reaction tube on the magnet, air dry the magnetic beads for **2 minutes** at room temperature or until there are no droplets of Wash Buffer left on the walls of the tube.
10. Remove the tube from the magnetic rack, add **100 µL** of Elution Buffer, then vortex to mix thoroughly.
11. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.
12. Place the tube in the magnetic rack for **2–3 minutes** or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
13. Without removing the tube from the magnetic rack, transfer the supernatant (i.e., the eluate) into a new 1.5-mL Eppendorf™ DNA LoBind™ tube using a pipette.

First binding

14. Add **45 µL** of fresh DNA Cleanup Beads directly to the eluate, then mix by vortexing until you have obtained a homogeneous suspension.

    IMPORTANT! To select for libraries with different size fragment inserts, see Table 2 (page 23) for the appropriate volume of DNA cleanup beads to use.

15. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.

    IMPORTANT! Do **not** extend the binding step to more than 5 minutes.

16. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.
17. Keeping the reaction tube in the magnetic rack, carefully remove and transfer all supernatant to a clean tube for the second size selection binding step.

> **Note:** Do **not** transfer the magnetic beads. If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube on the magnet again.

Second binding

18. Add **20 µL** of fresh DNA Cleanup Beads to the transferred supernatant, then mix by vortexing until you have obtained a homogeneous suspension

> **IMPORTANT!** To select for libraries with different size fragment inserts, see Table 2 (page 23) for the appropriate volume of cleanup beads to use.

19. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.
20. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.
21. Keeping the reaction tube in the magnetic rack, carefully remove and discard the supernatant using a pipette. Ensure that all the supernatant is removed.

> **Note:** If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the side of the tube on the magnet again.

22. Keeping the reaction tube on the magnet, add **200 µL** of Wash Buffer (pre-mixed with ethanol), then incubate for **30 seconds** at room temperature.

> **IMPORTANT!** Do **not** resuspend the magnetic beads in Wash Buffer.

23. Carefully remove and discard the supernatant using a pipette.
24. Repeat steps 21–22.
25. To remove the residual ethanol, briefly centrifuge the tubes, place it back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
26. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

> **IMPORTANT!** 1 minute is usually sufficient for air drying, but ensure that there are no droplets of ethanol left on the walls of the tube. Do **not** over-dry by prolonged incubation for more than 5 minutes. Over-drying significantly decreases the elution efficiency.

27. Remove the tube from the magnetic rack, add **25 µL** of Elution Buffer, then vortex to mix thoroughly.
28. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.
29. Place the tube in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
30. Without removing the tube from the magnetic rack, collect **22–23 µL** of the supernatant to a new sterile tube for storage.

Note: If the pellet of magnetic beads was disturbed, mix the sample and let the beads settle to the bottom of the tube on the magnet again.

STOPPING POINT. Store the eluted DNA library at 4°C for 1–2 weeks or at –20°C for long-term storage, or immediately proceed to "Verify the size distribution and quality of prepared DNA libraries", page 28.

Optimize bead-based size selection

To obtain a population of shorter or longer fragment sizes in your library, you can vary the ratio of the volume of DNA Cleanup Beads to the volume of the DNA at the start of each binding step in the size selection procedure (see Figure 7, page 27).

Note that the volume of cleanup beads required for the second binding step is calculated relative to the volume of the DNA-containing supernatant transferred after the first binding, and not to the volume of the DNA at the start of the size selection procedure.

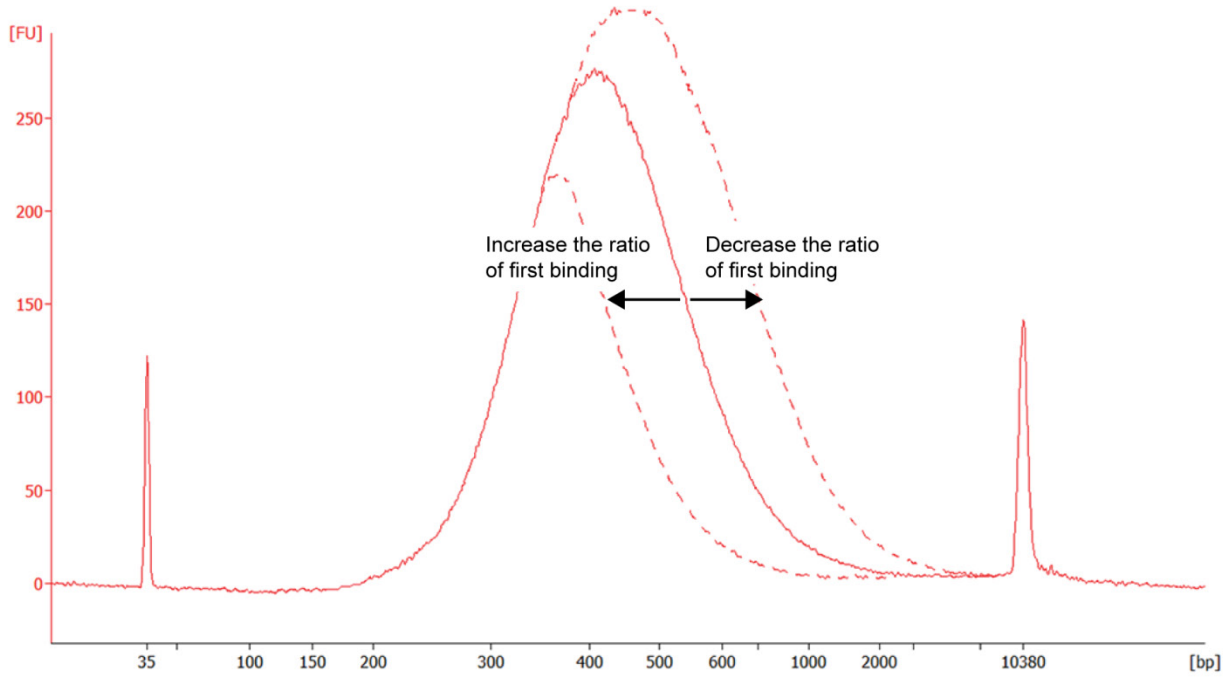
To optimize the ratio of the cleanup bead volume to obtain the desired fragment size distribution for your library, refer to Table 3.

Table 3 Recommended actions to obtain a population of shorter or longer fragment size libraries.

Upper size limit	Modification	Lower size limit	Modification
Increase	Decrease the ratio for the first binding	Increase	Decrease the ratio for the second binding[1]
Decrease	Increase the ratio for the first binding	Decrease	Increase the ratio for the second binding [1]

[1] The volume of DNA Cleanup Beads required for the second binding step is calculated relative to the volume of the DNA-containing supernatant transferred after the first binding, and not to the volume of the DNA at the start of the size selection procedure. The second binding should be performed with ~0.15X volume of Cleanup Beads. To increase the amount of DNA recovered, you can use ≥0.2X volume of DNA cleanup beads for the second binding. However, this can result in the recovery of smaller library fragments and/or a broader size distribution.

(A) Size modulation during First binding



(B) Size modulation during Second binding

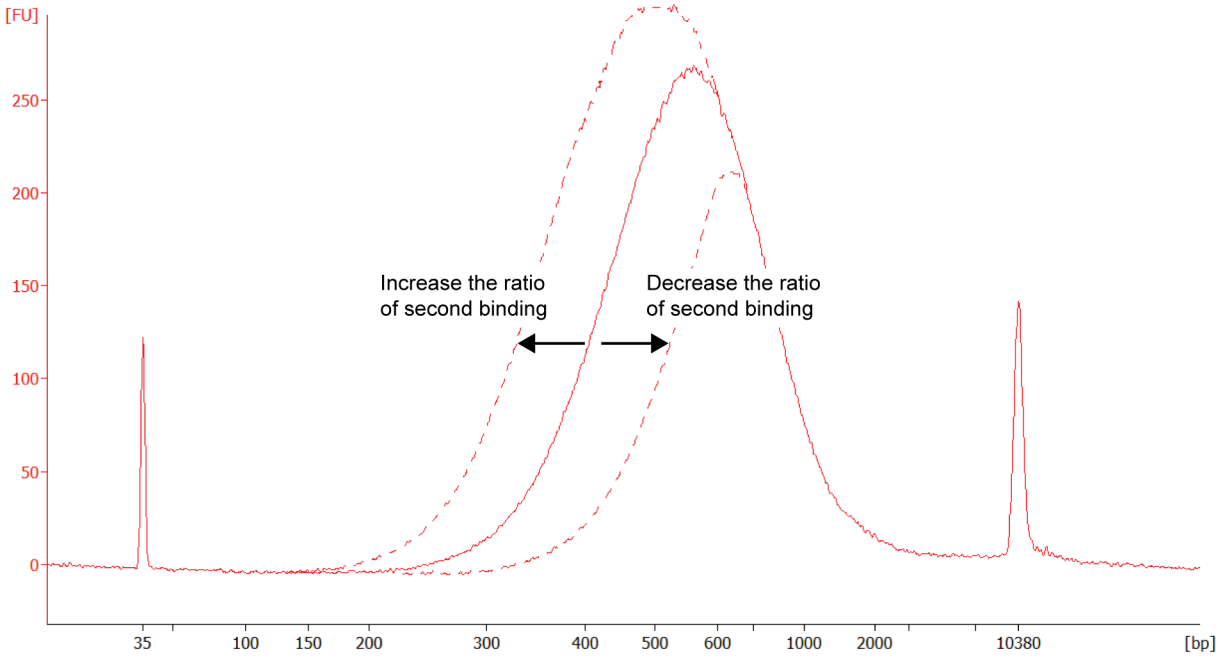


Figure 7 Varyfing the ratio of DNA Cleanup Beads-to-DNA volume at the start of the **(A)** first and **(B)** second binding steps results in shorter or longer fragment size libraries.

Verify the size distribution and quality of prepared DNA libraries

Overview

Verify the size distribution and quality of prepared DNA library by performing capillary electrophoresis analysis on Agilent™ 2100 Bioanalyzer instrument (or any similar instrument) using the Agilent™ High Sensitivity DNA Kit.

Required materials

- Agilent™ 2100 Bioanalyzer™ instrument (Agilent, Cat. No. G2938A)
- Agilent™ High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Nuclease-free water

Analyze the size distribution of the amplified library

1. Remove 1 µL from each prepared DNA library and dilute it 2–5-fold in nuclease-free water.
2. Analyze 1 µL of the diluted DNA library using the appropriate chip on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit.
3. Using the 2100 Expert software, perform a smear analysis to determine the average library length using a size range of 150–1000 bp. Check for the expected size distribution of library fragments and for the absence of residual Adaptor or Adaptor dimers peaks near 140 bp.

Note: For instructions on how to perform the smear analysis, refer to the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Agilent, Pub. No. G2946-90004).

Expected results

It is normal that PCR-free library fragment sizes that are measured on the Agilent™ Bioanalyzer instrument are substantially larger than could be expected. This is due to atypical migration of fragments on the chip caused by the structural features of Illumina™ Adaptors that are ligated to both ends of the DNA fragments.

For a typical Agilent™ 2100 Bioanalyzer trace of size-selected libraries, see Figure 8 (page 29).

STOPPING POINT. You can store the purified DNA libraries at 4°C for 1–2 weeks. For longer term, store the library at –20°C until ready for sequencing.

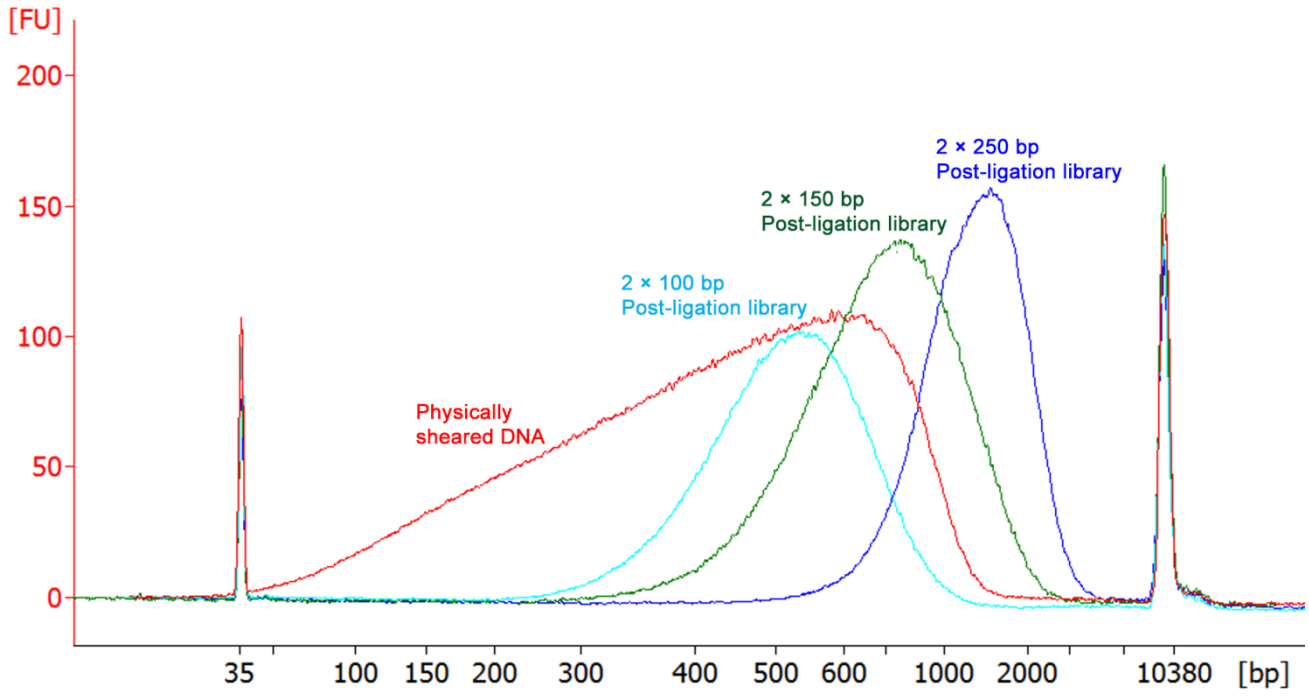


Figure 8 Typical Agilent™ 2100 Bioanalyzer trace of libraries using the Collibri™ PCR-free PS DNA Library Prep Kit. Libraries were prepared using 500 ng of physically sheared dsDNA, then size-selected. Peaks at 35 bp and 10,380 bp represent low and high-molecular weight markers.

Next steps

Quantify the prepared library by qPCR

We strongly recommend that you perform qPCR quantification of prepared libraries using the Invitrogen™ Collibri™ Library Quantification Kit (available separately from Thermo Fisher Scientific, Cat. Nos. A38524100, A38524500) before proceeding to sequencing.

Typical sequencing-ready library concentration obtained using the Collibri™ PCR-free PS DNA Library Prep Kit depends on the amount of input DNA and the insert size. Yield is not indicative of library quality, and libraries below 1,000 pM can still provide good quality sequences.

Sequence the prepared library

Denature, dilute, and load the libraries according to the standard guidelines appropriate for the Illumina™ NGS platform you are using.

Appendix A: Troubleshooting

<table>
<tr><th>Observation</th><th>Possible cause</th><th>Recommended action</th></tr>
<tr><td rowspan="3">Low DNA library yield</td><td>Improperly fragmented DNA.</td><td>DNA should be fragmented to DNA fragments of appropriate size before ligation to Illumina™-compatible adaptors. The adaptor-ligated library is then size-selected based on the selected target read length:
<table>
<tr><th>Target insert size</th><th>Average library size[1]</th><th>Target read length</th></tr>
<tr><td>~200 bp</td><td>~500 bp</td><td>200-base read library</td></tr>
<tr><td>~350 bp</td><td>~1000 bp</td><td>300-base read library</td></tr>
<tr><td>~550 bp</td><td>~2000 bp</td><td>500-base read library</td></tr>
</table>
[1] On Agilent™ 2100 Bioanalyzer instrument.
It is normal that PCR-free library fragment sizes as measured on the Bioanalyzer instrument are substantially larger than expected. This is due to atypical migration of fragments on the chip caused by the structural features of Illumina™ Adaptors that are ligated to both ends of the DNA fragments.
Optimize your DNA shearing protocol to generate the DNA fragments of correct median insert size.</td></tr>
<tr><td>Low DNA quality.</td><td>The quality of the input DNA has a significant impact on the yield of the resulting library. The DNA samples must be free of contaminating proteins, RNA, organic solvents, and salts to ensure optimal conditions for the activity of enzymes used for library preparation.
For samples with unknown DNA quality, we highly recommend that you re-purify your input DNA. High-quality DNA can be obtained using commercial DNA purification kits.</td></tr>
<tr><td>Too small amount of input DNA used for PCR-free library preparation.</td><td>• For libraries constructed without the size selection step, 500 ng of input DNA usually generates sufficient amount of Illumina™-compatible 300-base read library to use directly for sequencing without amplification.
Single stranded DNA, RNA, or free nucleotides can interfere with accurate quantification of purified DNA, and if too low of an amount of dsDNA is used for the reaction, an insufficient amount of prepared library could be obtained.
• Refer to Table 1 (page 9) for the recommended amount of input DNA for the preparation of PCR-free NGS library.</td></tr>
</table>

Observation	Possible cause	Recommended action
Low DNA library yield (continued)	Cleanup and/or size selection protocols for Adaptor removal were not carefully followed.	• Strictly follow the cleanup protocol and use exact volumes of the DNA Cleanup Beads. • Before each use, vortex the DNA Cleanup Beads thoroughly so that the beads are fully resuspended in solution. • Ensure that residual ethanol from wash steps is removed and air-dried. Remaining ethanol reduces DNA library yields. • To ensure the best DNA yields, do not lose any DNA cleanup beads during the procedures and do not shorten incubation times described. • Perform all size selection steps using 1.5-mL Eppendorf™ LoBind™ Tubes (Eppendorf™, Cat. No. 022431021). • Use well-calibrated pipettes.
Adaptor contamination	Cleanup and/or size selection protocols were not carefully followed.	• Briefly centrifuge the tube to collect the droplets at the bottom before placing it in the magnetic rack. Wait for 2–3 minutes or until the beads have formed a tight pellet. • If the pellet of magnetic particles was disturbed while removing supernatant, mix the sample and let the beads settle to the magnet again.
Size selected library is outside the range of interest	Atypical PCR-free library fragment migration on the Agilent™ 2100 Bioanalyzer.	It is normal that PCR-free library fragment sizes as measured on the Bioanalyzer instrument are substantially larger than expected. This is due to atypical migration of fragments on the chip caused by the structural features of Illumina™ Adaptors that are ligated to both ends of the DNA fragments. **Note:** Expected migration of the DNA library fragments that correspond to the expected size (the median fragment size plus 140 bp of ligated 65–70 bp Adaptors at both ends) is observed only if the DNA Library is PCR-amplified following ligation step.
	Improper fragmentation of DNA sample.	• Ensure that your DNA fragmentation protocol generates the DNA fragments close to the correct median insert size. • We recommend that you perform size selection if the average DNA fragment size in your sample is larger than the desired median insert size. For example, for a library with a desired median insert size of 350 bp, we recommend size selection targeting 350 bp median insert size, if your fragmented DNA sample has an average fragment size of >350 bp (see Figure 6, page 22).

Observation	Possible cause	Recommended action
Size selected library is outside the range of interest (continued)	Size selection protocol was not carefully followed.	Size selection protocol is extremely sensitive to the volume of DNA Cleanup Beads used. Make sure to add the correct volumes of the cleanup beads and add the components in the order described. Refer to Table 2 (page 23) for the appropriate volume of DNA cleanup beads to use for the desired library size.

Appendix B: Adaptor index sequences and plate layouts

Adaptor index sequences

Index sequences used for CD adaptors

Index sequences used for Combinatorial Dual-Indexed Adaptors (CD) in Collibri™ PCR-free PS DNA Library Prep Kits are listed in Table 4.

Indexes D501–D508 and D701–D712 correspond to the respective Illumina™ adaptor indexes.

24-prep and 96-prep CD adaptor plate layouts are shown in Tables 9–10 (page 38).

Table 4 Indexes used in Collibri™ PCR-free PS DNA Library kits to generate Combinatorial Dual-Indexed (CD) Adaptors.

D70X index name	i7 Bases for entry on sample sheet	D50X index name	i5 bases for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	i5 bases for entry on sample sheet (MiniSeq™, NextSeq™, iSeq™, HiSeq™ 3000/4000, HiSeq™ X)[1]
D701	ATTACTCG	D501	TATAGCCT	AGGCTATA
D702	TCCGGAGA	D502	ATAGAGGC	GCCTCTAT
D703	CGCTCATT	D503	CCTATCCT	AGGATAGG
D704	GAGATTCC	D504	GGCTCTGA	TCAGAGCC
D705	ATTCAGAA	D505	AGGCGAAG	CTTCGCCT
D706	GAATTCGT	D506	TAATCTTA	TAAGATTA
D707	CTGAAGCT	D507	CAGGACGT	ACGTCCTG
D708	TAATGCGC	D508	GTACTGAC	GTCAGTAC
D709	CGGCTATG	—	—	—
D710	TCCGCGAA	—	—	—
D711	TCTCGCGC	—	—	—
D712	AGCGATAG	—	—	—

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Index sequences used for UD adaptors

Index sequences used for Unique Dual-Indexed Adaptors (UD) in Collibri™ PCR-free PS DNA Library Prep Kits are listed in Tables 5–8 (pages 34–37).

Plate layouts of 24-prep UD adaptor Sets A–D are shown in Tables 11–14 (pages 39–40).

Table 5 Indexes used in Collibri™ DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set A.

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
Set A			
UDI001	CCGCGGTT	AGCGCTAG	CTAGCGCT
UDI002	TTATAACC	GATATCGA	TCGATATC
UDI003	GGACTTGG	CGCAGACG	CGTCTGCG
UDI004	AAGTCCAA	TATGAGTA	TACTCATA
UDI005	ATCCACTG	AGGTGCGT	ACGCACCT
UDI006	GCTTGTCA	GAACATAC	GTATGTTC
UDI007	CAAGCTAG	ACATAGCG	CGCTATGT
UDI008	TGGATCGA	GTGCGATA	TATCGCAC
UDI009	AGTTCAGG	CCAACAGA	TCTGTTGG
UDI010	GACCTGAA	TTGGTGAG	CTCACCAA
UDI011	TCTCTACT	CGCGGTTC	GAACCGCG
UDI012	CTCTCGTC	TATAACCT	AGGTTATA
UDI013	CCAAGTCT	AAGGATGA	TCATCCTT
UDI014	TTGGACTC	GGAAGCAG	CTGCTTCC
UDI015	GGCTTAAG	TCGTGACC	GGTCACGA
UDI016	AATCCGGA	CTACAGTT	AACTGTAG
UDI017	TAATACAG	ATATTCAC	GTGAATAT
UDI018	CGGCGTGA	GCGCCTGT	ACAGGCGC
UDI019	ATGTAAGT	ACTCTATG	CATAGAGT
UDI020	GCACGGAC	GTCTCGCA	TGCGAGAC
UDI021	GGTACCTT	AAGACGTC	GACGTCTT
UDI022	AACGTTCC	GGAGTACT	AGTACTCC
UDI023	GCAGAATT	ACCGGCCA	TGGCCGGT
UDI024	ATGAGGCC	GTTAATTG	CAATTAAC

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Table 6 Indexes used in Collibri™ DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set B.

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
Set B			
UDI025	ACTAAGAT	AACCGCGG	CCGCGGTT
UDI026	GTCGGAGC	GGTTATAA	TTATAACC
UDI027	CTTGGTAT	CCAAGTCC	GGACTTGG
UDI028	TCCAACGC	TTGGACTT	AAGTCCAA
UDI029	CCGTGAAG	CAGTGGAT	ATCCACTG
UDI030	TTACAGGA	TGACAAGC	GCTTGTCA
UDI031	GGCATTCT	CTAGCTTG	CAAGCTAG
UDI032	AATGCCTC	TCGATCCA	TGGATCGA
UDI033	TACCGAGG	CCTGAACT	AGTTCAGG
UDI034	CGTTAGAA	TTCAGGTC	GACCTGAA
UDI035	AGCCTCAT	AGTAGAGA	TCTCTACT
UDI036	GATTCTGC	GACGAGAG	CTCTCGTC
UDI037	TCGTAGTG	AGACTTGG	CCAAGTCT
UDI038	CTACGACA	GAGTCCAA	TTGGACTC
UDI039	TAAGTGGT	CTTAAGCC	GGCTTAAG
UDI040	CGGACAAC	TCCGGATT	AATCCGGA
UDI041	ATATGGAT	CTGTATTA	TAATACAG
UDI042	GCGCAAGC	TCACGCCG	CGGCGTGA
UDI043	AAGATACT	ACTTACAT	ATGTAAGT
UDI044	GGAGCGTC	GTCCGTGC	GCACGGAC
UDI045	ATGGCATG	AAGGTACC	GGTACCTT
UDI046	GCAATGCA	GGAACGTT	AACGTTCC
UDI047	GTTCCAAT	AATTCTGC	GCAGAATT
UDI048	ACCTTGGC	GGCCTCAT	ATGAGGCC

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Table 7 Indexes used in Collibri™ DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set C.

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
Set C			
UDI049	ATATCTCG	ATCTTAGT	ACTAAGAT
UDI050	GCGCTCTA	GCTCCGAC	GTCGGAGC
UDI051	AACAGGTT	ATACCAAG	CTTGGTAT
UDI052	GGTGAACC	GCGTTGGA	TCCAACGC
UDI053	CAACAATG	CTTCACGG	CCGTGAAG
UDI054	TGGTGGCA	TCCTGTAA	TTACAGGA
UDI055	AGGCAGAG	AGAATGCC	GGCATTCT
UDI056	GAATGAGA	GAGGCATT	AATGCCTC
UDI057	TGCGGCGT	CCTCGGTA	TACCGAGG
UDI058	CATAATAC	TTCTAACG	CGTTAGAA
UDI059	GATCTATC	ATGAGGCT	AGCCTCAT
UDI060	AGCTCGCT	GCAGAATC	GATTCTGC
UDI061	CGGAACTG	CACTACGA	TCGTAGTG
UDI062	TAAGGTCA	TGTCGTAG	CTACGACA
UDI063	TTGCCTAG	ACCACTTA	TAAGTGGT
UDI064	CCATTCGA	GTTGTCCG	CGGACAAC
UDI065	ACACTAAG	ATCCATAT	ATATGGAT
UDI066	GTGTCGGA	GCTTGCGC	GCGCAAGC
UDI067	TTCCTGTT	AGTATCTT	AAGATACT
UDI068	CCTTCACC	GACGCTCC	GGAGCGTC
UDI069	GCCACAGG	CATGCCAT	ATGGCATG
UDI070	ATTGTGAA	TGCATTGC	GCAATGCA
UDI071	ACTCGTGT	ATTGGAAC	GTTCCAAT
UDI072	GTCTACAC	GCCAAGGT	ACCTTGGC

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Table 8 Indexes used in Collibri™ DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set D.

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, HiSeq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, HiSeq™ 3000/4000, HiSeq™ X) [1]
Set D			
UDI073	CAATTAAC	CGAGATAT	ATATCTCG
UDI074	TGGCCGGT	TAGAGCGC	GCGCTCTA
UDI075	AGTACTCC	AACCTGTT	AACAGGTT
UDI076	GACGTCTT	GGTTCACC	GGTGAACC
UDI077	TGCGAGAC	CATTGTTG	CAACAATG
UDI078	CATAGAGT	TGCCACCA	TGGTGGCA
UDI079	ACAGGCGC	CTCTGCCT	AGGCAGAG
UDI080	GTGAATAT	TCTCATTC	GAATGAGA
UDI081	AACTGTAG	ACGCCGCA	TGCGGCGT
UDI082	GGTCACGA	GTATTATG	CATAATAC
UDI083	CTGCTTCC	GATAGATC	GATCTATC
UDI084	TCATCCTT	AGCGAGCT	AGCTCGCT
UDI085	AGGTTATA	CAGTTCCG	CGGAACTG
UDI086	GAACCGCG	TGACCTTA	TAAGGTCA
UDI087	CTCACCAA	CTAGGCAA	TTGCCTAG
UDI088	TCTGTTGG	TCGAATGG	CCATTCGA
UDI089	TATCGCAC	CTTAGTGT	ACACTAAG
UDI090	CGCTATGT	TCCGACAC	GTGTCGGA
UDI091	GTATGTTC	AACAGGAA	TTCCTGTT
UDI092	ACGCACCT	GGTGAAGG	CCTTCACC
UDI093	TACTCATA	CCTGTGGC	GCCACAGG
UDI094	CGTCTGCG	TTCACAAT	ATTGTGAA
UDI095	TCGATATC	ACACGAGT	ACTCGTGT
UDI096	CTAGCGCT	GTGTAGAC	GTCTACAC

[1] Sequencing on the MiniSeq™, NextSeq™, HiSeq™ 3000/4000, and HiSeq™ X systems follow a different dual-indexing workflow than other Illumina™ systems, which require the reverse complement of the i5 index adaptor sequence.

Adaptor plate layouts

Note: Colors of the borders in the plate layouts provided bellow match the colors of the plates containing individual adaptor sets.

Combinatorial Indexed Adaptor Sets:

Table 9 Collibri™ 96-prep Combinatorial Indexed **(CD)** Adaptor plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703	501/704	501/705	501/706	501/707	501/708	501/709	501/710	501/711	501/712
B	502/701	502/702	502/703	502/704	502/705	502/706	502/707	502/708	502/709	502/710	502/711	502/712
C	503/701	503/702	503/703	503/704	503/705	503/706	503/707	503/708	503/709	503/710	503/711	503/712
D	504/701	504/702	504/703	504/704	504/705	504/706	504/707	504/708	504/709	504/710	504/711	504/712
E	505/701	505/702	505/703	505/704	505/705	505/706	505/707	505/708	505/709	505/710	505/711	505/712
F	506/701	506/702	506/703	506/704	506/705	506/706	506/707	506/708	506/709	506/710	506/711	506/712
G	507/701	507/702	507/703	507/704	507/705	507/706	507/707	507/708	507/709	507/710	507/711	507/712
H	508/701	508/702	508/703	508/704	508/705	508/706	508/707	508/708	508/709	508/710	508/711	508/712

Table 10 Collibri™ 24-prep Combinatorial Indexed **(CD)** Adaptor plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703	—	—	—	—	—	—	—	—	—
B	501/704	501/705	501/706	—	—	—	—	—	—	—	—	—
C	501/707	501/708	501/709	—	—	—	—	—	—	—	—	—
D	501/710	501/711	501/712	—	—	—	—	—	—	—	—	—
E	502/701	502/702	502/703	—	—	—	—	—	—	—	—	—
F	502/704	502/705	502/706	—	—	—	—	—	—	—	—	—
G	502/707	502/708	502/709	—	—	—	—	—	—	—	—	—
H	502/710	502/711	502/712	—	—	—	—	—	—	—	—	—

Unique Dual Indexed Adaptor Sets:

Table 11 Collibri™ 24-prep Unique Indexed **(UD)** Adaptor **Set A** plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017	—	—	—	—	—	—	—	—	—
B	UDI002	UDI010	UDI018	—	—	—	—	—	—	—	—	—
C	UDI003	UDI011	UDI019	—	—	—	—	—	—	—	—	—
D	UDI004	UDI012	UDI020	—	—	—	—	—	—	—	—	—
E	UDI005	UDI013	UDI021	—	—	—	—	—	—	—	—	—
F	UDI006	UDI014	UDI022	—	—	—	—	—	—	—	—	—
G	UDI007	UDI015	UDI023	—	—	—	—	—	—	—	—	—
H	UDI008	UDI016	UDI024	—	—	—	—	—	—	—	—	—

Table 12 Collibri™ 24-prep Unique Indexed **(UD)** Adaptor **Set B** plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI025	UDI033	UDI041	—	—	—	—	—	—	—	—	—
B	UDI026	UDI034	UDI042	—	—	—	—	—	—	—	—	—
C	UDI027	UDI035	UDI043	—	—	—	—	—	—	—	—	—
D	UDI028	UDI036	UDI044	—	—	—	—	—	—	—	—	—
E	UDI029	UDI037	UDI045	—	—	—	—	—	—	—	—	—
F	UDI030	UDI038	UDI046	—	—	—	—	—	—	—	—	—
G	UDI031	UDI039	UDI047	—	—	—	—	—	—	—	—	—
H	UDI032	UDI040	UDI048	—	—	—	—	—	—	—	—	—

Table 13 Collibri™ 24-prep Unique Indexed **(UD)** Adaptor **Set C** plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI049	UDI057	UDI065	—	—	—	—	—	—	—	—	—
B	UDI050	UDI058	UDI066	—	—	—	—	—	—	—	—	—
C	UDI051	UDI059	UDI067	—	—	—	—	—	—	—	—	—
D	UDI052	UDI060	UDI068	—	—	—	—	—	—	—	—	—
E	UDI053	UDI061	UDI069	—	—	—	—	—	—	—	—	—
F	UDI054	UDI062	UDI070	—	—	—	—	—	—	—	—	—
G	UDI055	UDI063	UDI071	—	—	—	—	—	—	—	—	—
H	UDI056	UDI064	UDI072	—	—	—	—	—	—	—	—	—

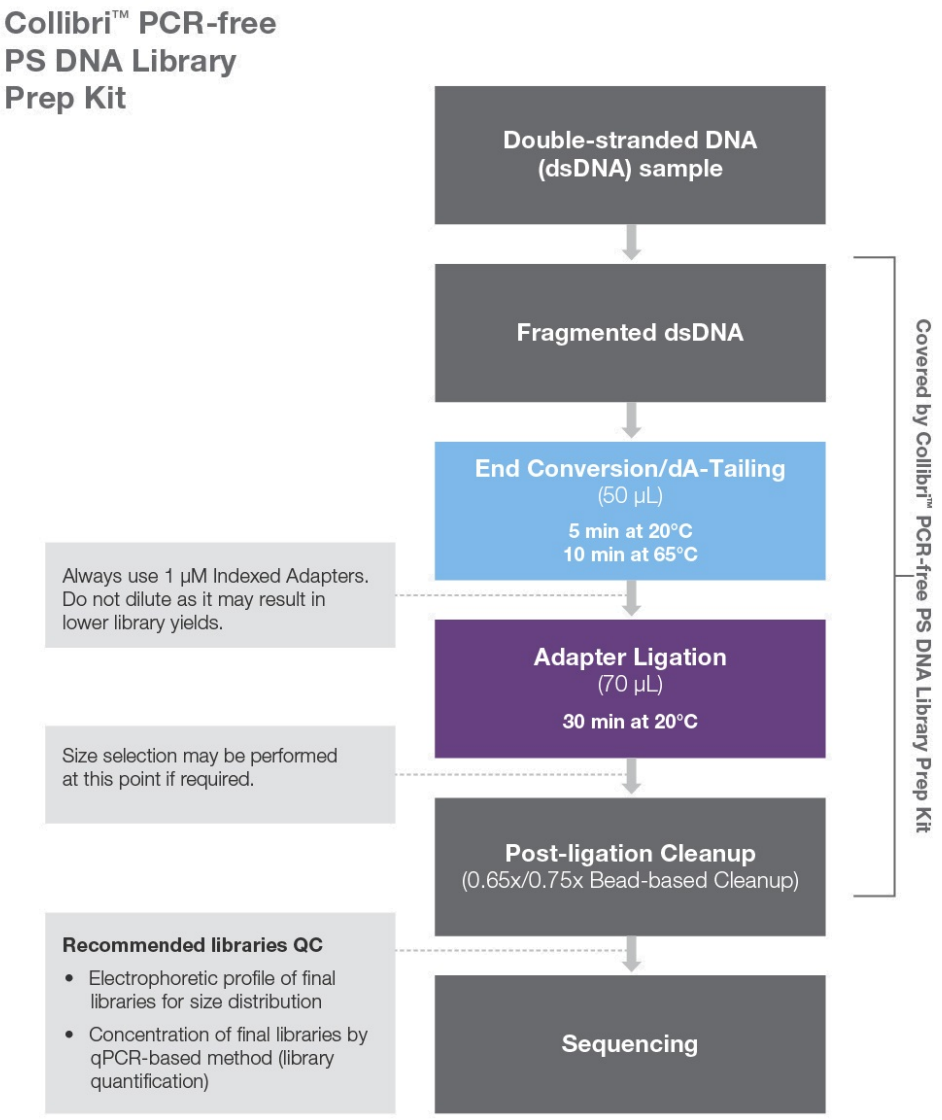
Table 14 Collibri™ 24-prep Unique Indexed **(UD)** Adaptor **Set D** plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI073	UDI081	UDI089	—	—	—	—	—	—	—	—	—
B	UDI074	UDI082	UDI090	—	—	—	—	—	—	—	—	—
C	UDI075	UDI083	UDI091	—	—	—	—	—	—	—	—	—
D	UDI076	UDI084	UDI092	—	—	—	—	—	—	—	—	—
E	UDI077	UDI085	UDI093	—	—	—	—	—	—	—	—	—
F	UDI078	UDI086	UDI094	—	—	—	—	—	—	—	—	—
G	UDI079	UDI087	UDI095	—	—	—	—	—	—	—	—	—
H	UDI080	UDI088	UDI096	—	—	—	—	—	—	—	—	—

Table 15 Collibri™ 96-prep Unique Indexed **(UD)** Adaptor **Set A–D** plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI001	UDI009	UDI017	UDI025	UDI033	UDI041	UDI049	UDI057	UDI065	UDI073	UDI081	UDI089
B	UDI002	UDI010	UDI018	UDI026	UDI034	UDI042	UDI050	UDI058	UDI066	UDI074	UDI082	UDI090
C	UDI003	UDI011	UDI019	UDI027	UDI035	UDI043	UDI051	UDI059	UDI067	UDI075	UDI083	UDI091
D	UDI004	UDI012	UDI020	UDI028	UDI036	UDI044	UDI052	UDI060	UDI068	UDI076	UDI084	UDI092
E	UDI005	UDI013	UDI021	UDI029	UDI037	UDI045	UDI053	UDI061	UDI069	UDI077	UDI085	UDI093
F	UDI006	UDI014	UDI022	UDI030	UDI038	UDI046	UDI054	UDI062	UDI070	UDI078	UDI086	UDI094
G	UDI007	UDI015	UDI023	UDI031	UDI039	UDI047	UDI055	UDI063	UDI071	UDI079	UDI087	UDI095
H	UDI008	UDI016	UDI024	UDI032	UDI040	UDI048	UDI056	UDI064	UDI072	UDI080	UDI088	UDI096

Appendix C: Process workflow



Appendix D: Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL),* 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organisation (WHO), *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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