invitrogen

Invitrogen™ Collibri™ ES DNA Library Prep Kit for Illumina™

USER GUIDE

- For use with Illumina™ next-generation sequencing (NGS) platforms
- With enzymatic fragmentation
- With library amplification

Catalog Numbers A38605024, A38606024, A43605024, A43606024, A43607024, A38607096

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Revision history: MAN0018545

Revision	Date	Description
A.0	03 May 2019	New user guide.

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1. Product information

Product description

Invitrogen™ Collibri™ ES 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 (1 ng–500 ng) starting from intact DNA.

In the Collibri™ ES DNA library prep workflow, the enzymatic DNA fragmentation step is combined with dA-tailing in a one-vial protocol. Subsequent adaptor ligation reaction is carried out in the same vial followed by a size-selection step. Reduced number of sample transfer steps used in the workflow minimizes handling errors, and saves time and valuable sample. The entire library prep takes less than 3 hours with PCR, and does not require expensive equipment to shear DNA mechanically.

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 or sequencing results.

The Collibri™ ES 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™ ES DNA Library Prep Kits, see "Technology overview", page 6.

Product specifications

Assay time	~160 minutes on average
Hands-on time	~45 minutes on average
Sample type	• Low complexity dsDNA (bacteria/phage DNA) • High complexity dsDNA (Mammalian, Mouse, Human, Rat, Plant)
Sample input amount	1 ng– 500 ng of intact DNA
Sample input quality	Double-stranded DNA with A_{260}/A_{280} ratio of 1.7–2.0
Fragment size range	150 bp– 850 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	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™ ES 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™ ES DNA Library Prep Kit	24 preps	CD	A38605024
	96 preps	CD	A38607096
	24 preps	UD Set A (1–24)	A38606024
		UD Set B (25–48)	A43605024
		UD Set C (49–72)	A43606024
		UD Set D (73–96)	A43607024

[1] CD: Combinatorial Dual, UD: Unique Dual.

Note: PCR-free kits without the PCR amplification module that support library preparation from 100 ng to 500 ng of input DNA (Cat. Nos. A38545024, A38602024, A43602024, A43603024, A43604024, and A38603096) are available from Thermo Fisher Scientific. For more information, go to **thermofisher.com**.

Kit components and storage

Upon receipt, immediately store the Collibri™ ES 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	24 preps	96 preps
Collibri™ ES DNA Library Prep Kit (Store at –20°C)			
5X Fragmentation and dA-tailing Enzyme Mix	White ○	250 µL	1 mL
10X Fragmentation and dA-tailing Buffer	Blue ●	125 µL	500 µL
7X Ligation Master Mix for ES	Red ●	250 µL	1 mL
2X Library Amplification Master Mix for ES	Blue ●	1.25 mL	2 × 1.25 mL
Primer Mix	Yellow ●	500 µL	2 × 500 µL
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[1] or UD[2] Indexes[3] (Store at –20°C)			
Dual Index Adaptors (7 µM)	—	10 µL/well (24 wells)	10 µL/well (96 wells)

[1] Combinatorial Dual-Indexed Adaptors (CD) are available with Catalog Nos. A38605024, A38607096.

[2] Unique Dual-Indexed Adaptors (UD) are available with Catalog Nos. A38606024, A43605024, A43606024, A43607024.

[3] For the Adaptor index sequences and plate layouts, see "Appendix B: Adaptor index sequences and plate layouts" (page 34).

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
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
96-well 0.2-mL PCR plates	MLS
Nuclease-free 1.5-mL tubes, such as Eppendorf™ DNA LoBind™ Tubes	Eppendorf, 022431021
0.2-mL or 0.5-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
10 mM Tris-HCl buffer, pH 7.5–8.5	MLS
Ethanol 96–100%, molecular biology grade	MLS

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

Item	Source
(Optional) Qubit™ 4 Fluorometer[2]	Q33226
(Optional) Qubit™ DNA HS Assay Kit	Q32854
Invitrogen™ Collibri™ Library Quantification Kit	A38524100, A38524500

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

Technology overview

The Collibri™ ES DNA Library Prep Kit provides a fast and fully enzymatic procedure for library construction starting from intact DNA. The kit combines the DNA fragmentation, dA-tailing, and adaptor ligation steps into a convenient one-tube protocol (Figure 1).

DNA fragmentation, dA-tailing, and adaptor ligation

First, DNA is fragmented and a single dA-overhang is added at the 3'-end of each strand. Next, 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™ ES 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 34 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 using the Cleanup magnetic particles (included in the kit) while preserving high library yields.

PCR amplification

PCR amplification step is performed with Collibri™ Library Amplification Master Mix and is suitable for DNA sample amounts ranging between 1 ng and 500 ng.

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.

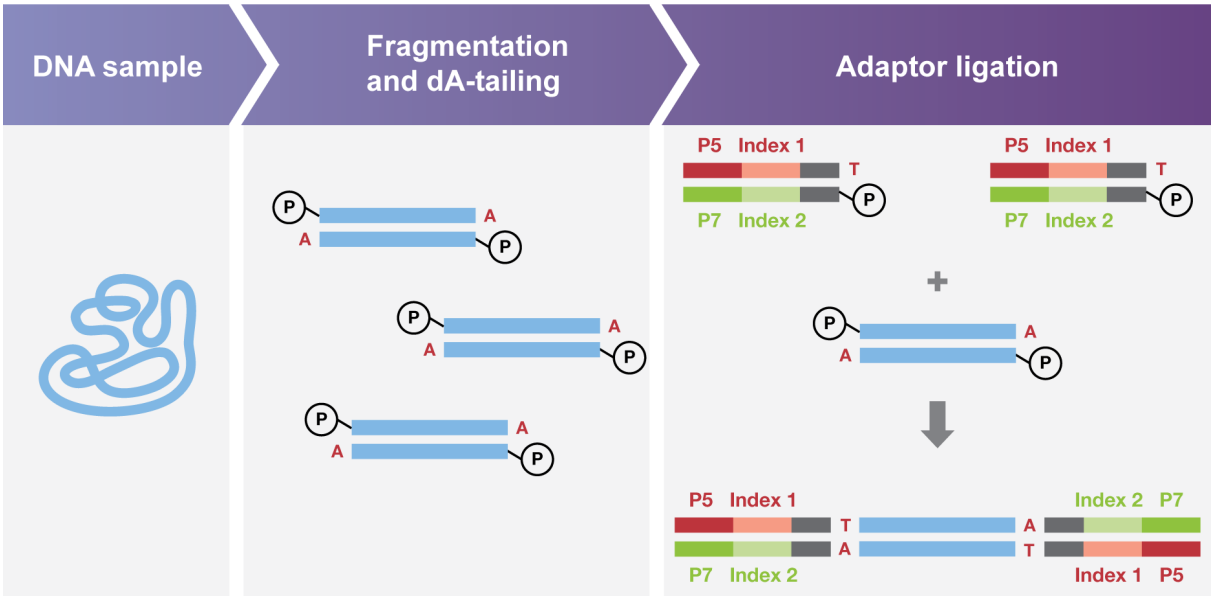


Figure 1 Simplified schematic representation of technology used in the Collibri™ ES DNA Library Prep Kit

2. Methods

Workflow

Figure 2 and Figure 3 illustrate the Collibri™ ES DNA Library Prep Kit workflow to construct sequencing-ready DNA fragment libraries for whole-genome DNA sequencing.

Figure 2 Collibri™ ES DNA Library Prep Kit workflow

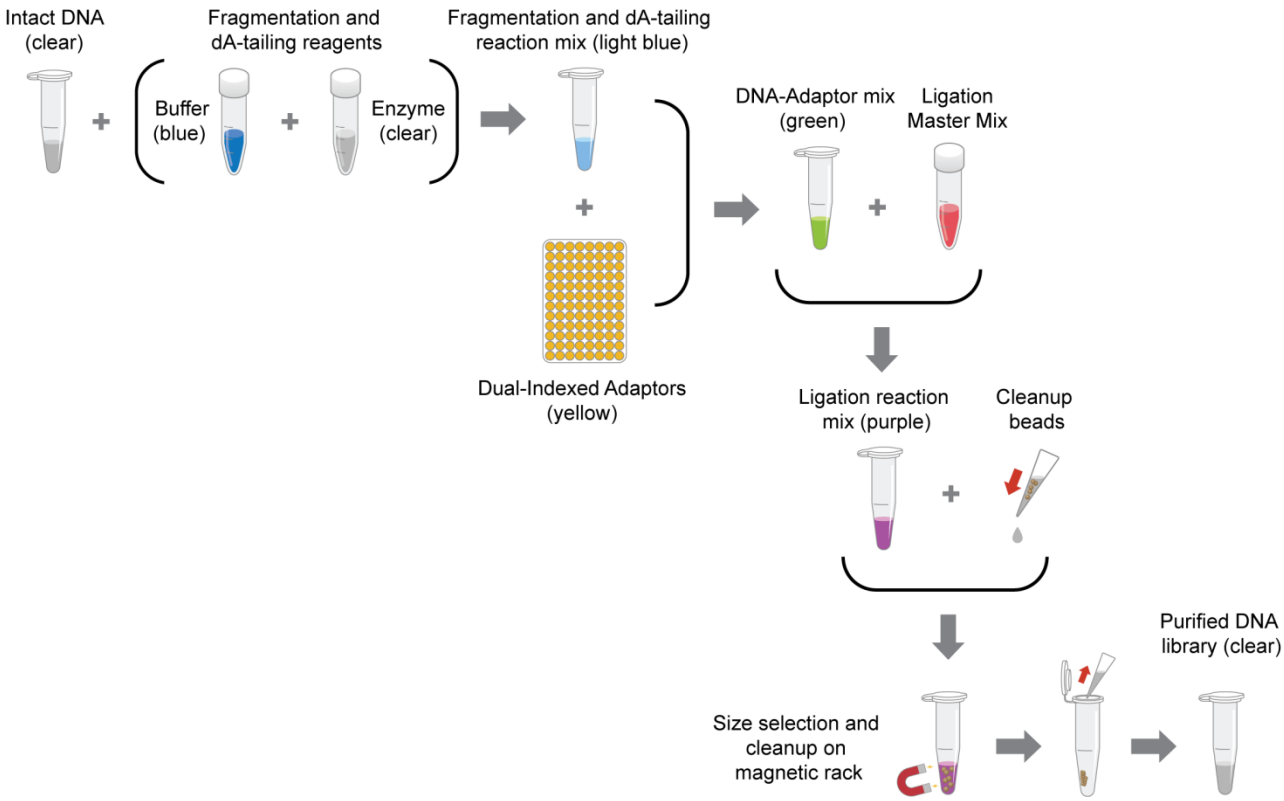


Figure 3 Collibri™ ES DNA Library Prep Kit components are colored with inert dyes to provide a visual control of the proper workflow progress – reaction mix changes color in every step to ensure that right component is added.

Important procedural guidelines

Input DNA requirements

"Input" typically refers to the amount of DNA used in the fragmentation and dA-tailing reaction. DNA input recommendations for library construction workflows 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	1 ng–500 ng	

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 double-stranded DNA dissolved in 10 mM Tris (pH 7.5–8.5) buffer.
- The enzymatic fragmentation reaction is very sensitive to the presence of EDTA in the DNA sample, which is usually introduced via elution buffers used in the final stages of the DNA purification process. Ensure that your input DNA does not contain any EDTA before proceeding with the fragmentation step.
- If the DNA contains EDTA, we recommend that you perform magnetic bead purification to ensure consistent and optimal results. For the recommended protocol, see "Remove EDTA from DNA samples", page 14.
- Use 10 mM Tris-HCl buffer, pH 7.5–8.5 to dilute the DNA.

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

- The DNA fragment size distribution after enzymatic fragmentation depends on the fragmentation reaction time. Evaluate the quality of fragmented DNA by agarose gel electrophoresis or with the Agilent™ High Sensitivity DNA Kit using the Agilent™ 2100 Bioanalyzer™ (or an equivalent instrument) (Figure 4, page 9).

Note: The standard fragmentation parameters provided in the Collibri™ ES DNA Library Prep Kit protocols apply to the fragmentation of high-quality genomic DNA. You can modify the fragmentation time to meet the specific requirements of your experiments to achieve the desired results.

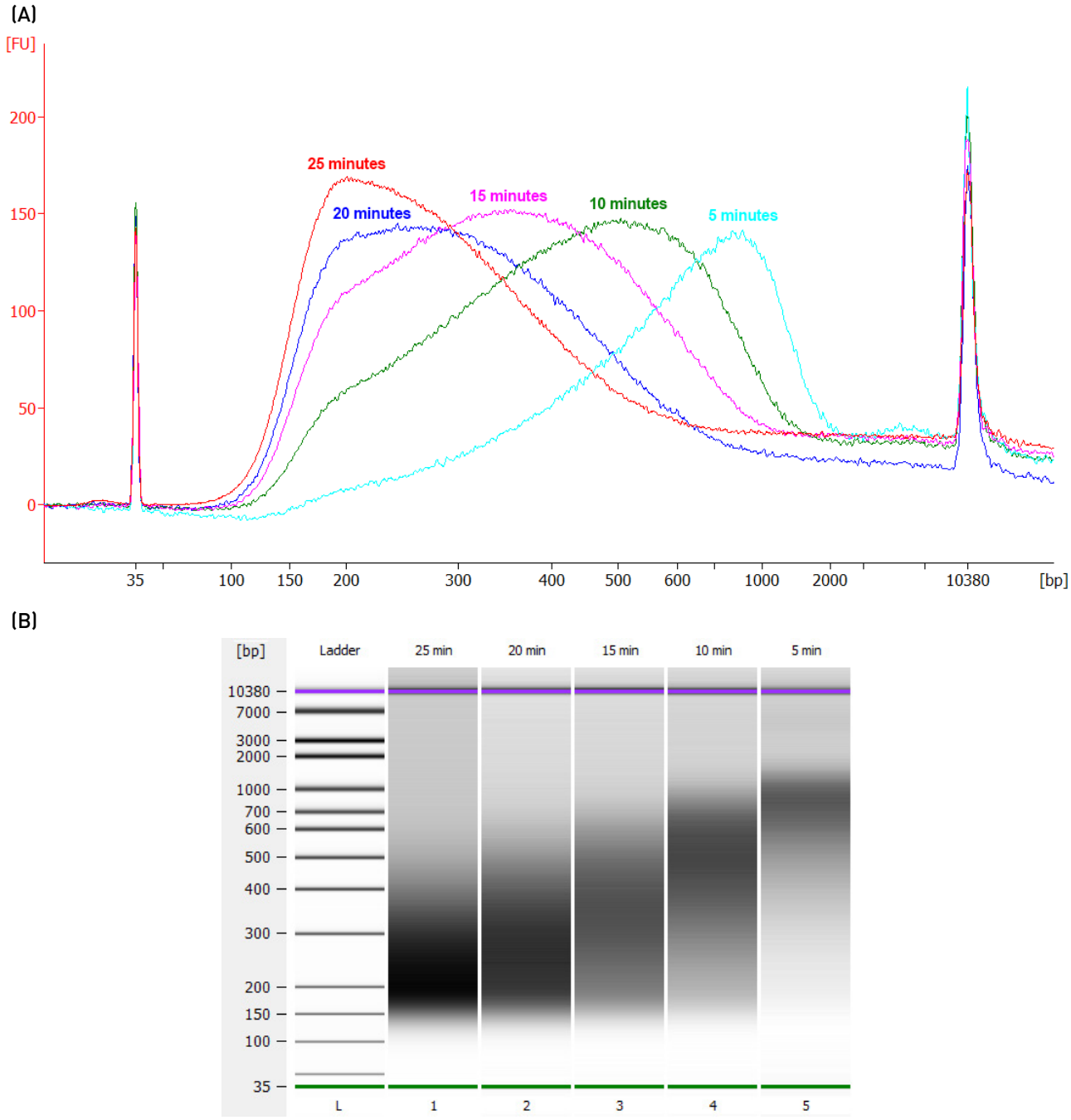


Figure 4 Enzymatic fragmentation reactions were performed for 25, 20, 15, 10, and 5 minutes, then analyzed with the Agilent™ High Sensitivity DNA Kit and run on an Agilent™ 2100 Bioanalyzer instrument. **(A)** Trace view of the analysis results illustrate the dependence of DNA fragment size distribution on fragmentation time. Peaks at 35 bp and 10380 bp represent low and high-molecular weight markers. **(B)** The results of the same enzymatic fragmentation reactions 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™ ES 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 34).
- 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 library cleanup

- Post-ligation library cleanup 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™ ES DNA Library Prep Kit) eliminates unused adaptors and adaptor dimers very efficiently. Therefore, the library prep workflow requires only a post-ligation size selection 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 makes the beads 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 library DNA (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 before PCR 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.
- PCR amplification step eliminates Y-shape structure of Adaptors and all Adaptor-ligated molecules are fully double-stranded. Therefore electrophoretic analysis of amplified libraries is more accurate to evaluate size distribution.
- 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 the Collibri™ Library Quantification Kit for qPCR-based quantification of prepared libraries before sequencing.

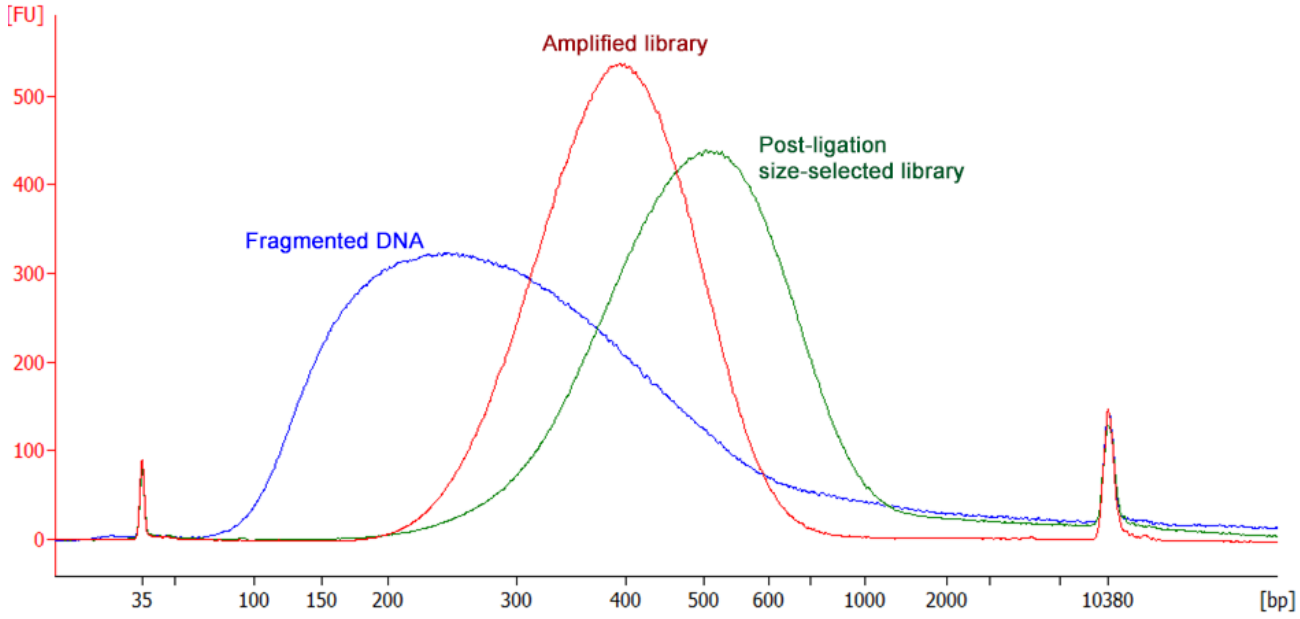


Figure 5 500 ng input DNA was enzymatically fragmented for 20 minutes and libraries were prepared using the Collibri™ ES DNA Library Prep Kit. Aliquots of the sample were collected at each stage of the library prep process and electrophoregrams were generated on an Agilent™ 2100 Bioanalyzer™ instrument.

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™ ES 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 (5X Fragmentation and dA-tailing Enzyme Mix and 7X Ligation Master Mix for ES) 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 size select and the post-amplification cleanup steps. These 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.

Removal of EDTA from DNA samples (if needed)

Overview

The enzymatic fragmentation reaction is very sensitive to the presence of EDTA in the DNA sample, which is usually introduced via elution buffers used in the final stages of the DNA purification process. If you suspect that your input DNA contains EDTA, perform the purification procedure described in this section.

Required materials

Components from the Collibri™ DNA Library Cleanup Kit:

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

Other materials and equipment:

- 1.5-mL Eppendorf™ DNA LoBind™ Tubes
- Vortex mixer
- Magnetic rack (see "Required materials not supplied", page 4)
- Microcentrifuge

Before you begin

- Equilibrate the DNA Cleanup Beads, Wash Buffer, and Elution Buffer to room temperature before use.
- Thoroughly resuspend the DNA Cleanup Beads by pipetting up and down several times or by vortexing before use.
- Ensure that appropriate volume of 96% ethanol (as noted on the bottle) was added to the Wash Buffer before first use.

Remove EDTA from DNA samples

1. Mix the sample DNA with twice the volume of DNA Cleanup Beads and vortex until you obtain a homogeneous suspension. If there are droplets on the tube wall, briefly centrifuge to collect all the droplets at the bottom of the tube.
2. Incubate for **5 minutes** at room temperature.
3. Place the tube in the magnetic rack for at least **2 minutes** or until the beads have formed a tight pellet.
4. Keeping the 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 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 and 6.
8. To remove the residual ethanol, briefly centrifuge the tubes, place them back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.

9. Keeping the tube on the magnet, air dry the magnetic particles for **2 minutes** 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 DNA yield.

10. Remove the tube from the magnetic rack, then add the desired volume of Elution Buffer (use at least 10 µL) directly to the pellet to disperse the beads. Mix the suspension thoroughly by pipetting up and down or vortexing.
11. Briefly centrifuge the tube, then place it back in the magnetic rack for at least **2 minutes** or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.
12. Keeping the tube on the magnet, collect and transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ Tube without disturbing the pellet.

    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.

    IMPORTANT! The supernatant contains the eluted DNA. **Do not discard.**

STOPPING POINT. (*Optional*) Store the DNA at –20°C.

Fragmentation and dA-tailing of input DNA

Overview

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

Required materials

Components from the Collibri™ ES DNA Library Prep Kit:

- 5X Fragmentation and dA-tailing Enzyme Mix
- 10X Fragmentation and dA-tailing Buffer

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 80–85°C (see "Required materials not supplied", page 4)
- Ice or cooling block set to 4°C

Before you begin

- Thaw the reaction components on ice.
- Before use, mix the 5X Fragmentation and dA-tailing Enzyme Mix by inverting the tube several times, then briefly centrifuge to collect all the droplets at the bottom of the tube. Keep on ice.
- Mix the 10X Fragmentation and dA-tailing Buffer by vortexing to avoid any localized concentrations, then briefly centrifuge to collect all the droplets at the bottom of the tube. Keep on ice.
- Dilute the intact genomic DNA in 10 mM Tris-HCl, pH 7.5–8.5, if needed.
- Set incubation program on thermal cycler before mixing reaction components so that incubation block and heating lid would have required temperature when reaction mixtures are ready.

Fragment the DNA and add dA-tails

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

IMPORTANT! Do not scale up reaction volumes or prepare master mixes.

Component	Volume
10 mM Tris-HCl, pH 7.5–8.5	to 40 µL
Double-stranded intact DNA (1 ng–500 ng)	X µL
10X Fragmentation and dA-tailing Buffer (blue 🔵)	5 µL
Total volume (light blue mixture 🔵):	**40 µL**

2. Mix the contents vigorously by vortexing for 5 seconds, then centrifuge briefly to collect the liquid at the bottom of the tube.

3. Add 5X Fragmentation and dA-tailing Enzyme Mix to the sample.

Component	Volume
Buffer-DNA mixture from step 1 (light blue mixture 🔵)	40 µL
5X Fragmentation and dA-tailing Enzyme Mix (clear ○)	10 µL
Total volume (light blue mixture 🔵):	**50 µL**

4. Mix the sample by rapidly pipetting up and down (avoid creating bubbles) until the mixture becomes homogeneous, then centrifuge briefly to collect the liquid at the bottom of the tube. Place the samples back on ice or a cooling rack.

IMPORTANT! Ensure that the reaction components are mixed well. Incomplete mixing results in uneven DNA fragmentation.

5. Incubate the mixture in a thermal cycler with the heated lid set to 80–85°C, the block pre-cooled to 4°C, and programmed as outlined in the following table.

IMPORTANT! Heated lid is required for this step (set to 80–85°C).

Step	Temperature	Time
Pre-cool the block	4°C	As required
Fragmentation	37°C	See Table 2
dA-tailing	65°C	10 minutes
Hold	4°C	Hold

Table 2 Recommended fragmentation time and optimization range to attain the desired fragment size

Fragment size	Fragmentation time at 37°C	
	Recommended	Optimization range
150–300 bp	20 minutes	20–30 minutes
300–500 bp	10 minutes	10–20 minutes
500–700 bp	5 minutes	5–10 minutes

6. 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 18).

Dual-Indexed Adaptor ligation

Overview

This section describes the ligation of the Illumina™-compatible NGS adaptors to the fragmented and dA-tailed DNA sample.

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

Required materials

Components from the Collibri™ ES DNA Library Prep Kit:

- 7X Ligation Master Mix for ES

Components from the Collibri™ CD or UD Indexes

- Collibri™ Dual-Indexed Adaptor plate

Other materials and equipment:

- Fragmented and dA-tailed DNA samples (from step 6, page 17)
- Microcentrifuge
- Thermomixer or Thermal cycler with a heated lid off (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 ES 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 fragmented and dA-tailed DNA sample (from step 6, page 17), using a new adaptor for each DNA sample. Keep the Adaptor-DNA mixture on ice.

Component	Volume
Fragmented, dA-tailed DNA sample (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 ES to the Adaptor-DNA mixture on ice (from step 1, page 18), then mix well by vortexing.

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

IMPORTANT! Observe the color change as each reaction component is added. If the appropriate 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 the heated lid off.

IMPORTANT! Do not use a thermocycler with heated lid. Ligase is a thermo-sensitive enzyme and it can be inactivated if the temperature increases above 25°C, resulting in lower library yields.

5. Proceed to "Post-ligation double-sided size selection" (page 20).

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

Post-ligation double-sided size selection

Overview

This section describes bead-based size selection of the Dual Index Adaptor-ligated DNA sample. 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.

IMPORTANT! Recommended conditions for bead-based size selection depend on the desired fragment size distribution of the DNA library. See Table 3 for the appropriate volume of 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 19)
- 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.
- Vortex thoroughly 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 3 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 23).

Table 3 Recommended conditions for bead-based size selection of libraries.

Insert size	Volume of DNA Cleanup Beads		
	Initial cleanup	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. If there are droplets on the tube wall, briefly centrifuge to collect all the droplets at the bottom of the tube.
2. 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. Place the mixture 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 tubes, place them 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 ethanol left on the walls of the tube.
10. Remove the tube from the magnetic rack, add **105 µL** of Elution Buffer, then mix the suspension thoroughly by pipetting up and down or vortexing. If there are droplets on the tube wall, briefly centrifuge to collect all the droplets at the bottom of the tube.
11. Incubate for **1 minute** at room temperature.
12. 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.
13. Without removing the tube from the magnetic rack, collect **100 µL** of the supernatant (i.e., the eluate) into a new 1.5-mL Eppendorf™ DNA LoBind™ Tube.

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. If there are droplets on the tube wall, briefly centrifuge to collect all the droplets at the bottom of the tube.

    IMPORTANT! The volume of cleanup beads given is for libraries with 350 bp inserts. To select for libraries with different size fragment inserts, see Table 3 (page 20) for the appropriate volume of cleanup beads to use.

15. Incubate for **5 minutes** at room temperature.

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

16. Place the mixture 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. Do **not** discard the supernatant.

    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. If there are droplets on the tube wall, briefly centrifuge to collect all the droplets at the bottom of the tube.

    IMPORTANT! To select for libraries with different size fragment inserts, see Table 3 (page 20) for the appropriate volume of cleanup beads to use.

19. Incubate for **5 minutes** at room temperature.
20. Place the mixture 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 them 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 mix the suspension thoroughly by pipetting up and down or vortexing. If there are droplets on the tube wall, centrifuge them down to the bottom.
28. 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 the next step:

- To PCR amplify your prepared library, see page 25.
- To evaluate the yield and size distribution of your library, see page 30.

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 6, page 24).

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.

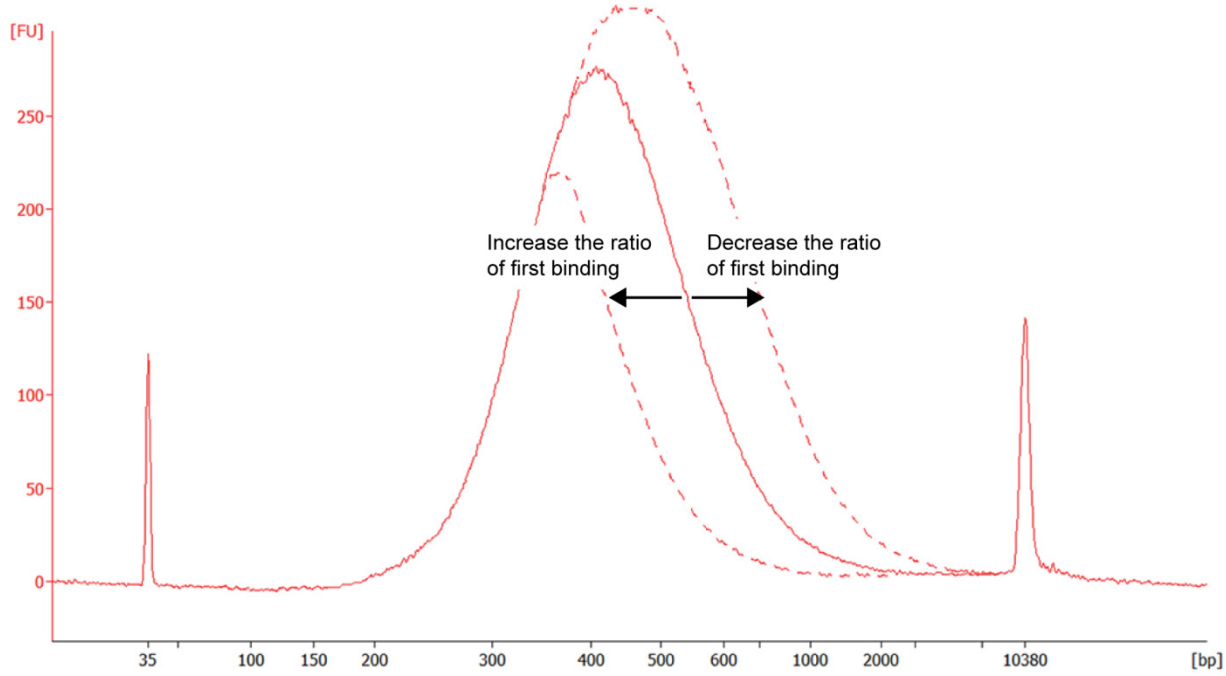
To optimize the ratio of the cleanup bead volume to obtain the desired fragment size distribution for your library, see Table 4.

Table 4 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 DNA Cleanup Beads. To increase the amount of DNA recovered, you can use ≥0.2X volume of 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

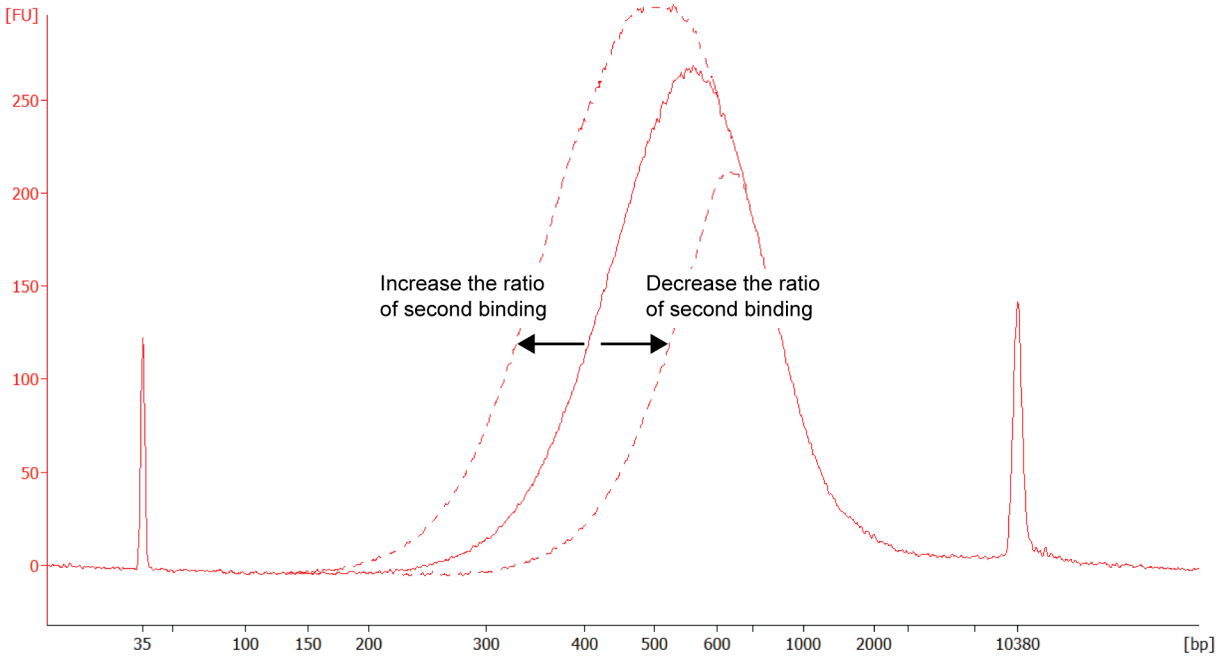


Figure 6 Varying the ratio of DNA Cleanup Beads-to-DNA volume at the start of the **(A)** first and **(B)** second binding steps results in shorter or longer fragment size libraries.

PCR amplify the library

Overview

This section describes the PCR-based amplification of the size-selected DNA library. PCR-based library amplification is normally required if the large amounts of libraries are needed for downstream applications.

Note that the color of the reaction mixture changes as each reaction component is added. Mixing the eluted DNA library (clear) with the 2X Library Amplification Master Mix (blue) and the Primer Mix (yellow) produces a green PCR mixture.

Required materials

Use components from the Collibri™ ES DNA Library Prep Kit with Library Amplification:

- 2X Library Amplification Master Mix
- Primer Mix

> **Note:** The components that are listed above are included in the kits with Library Amplification (Cat. Nos. A38605024, A38606024, A43605024, A43606024, A43607024, A38607096).
>
> These components are also available as the Invitrogen™ Collibri™ Library Amplification Master Mix (2X) with Primer Mix (Cat. Nos. A38540050, A38540250) from Thermo Fisher Scientific (**thermofisher.com**).

Other materials and equipment:

- Adaptor-ligated and size-selected DNA library (from step 30, page 23)
- 0.2-mL sterile, thin-wall PCR tubes
- Thermal cycler with the heated lid set to 105°C (see "Required materials not supplied", page 4)
- Ice or cooling block set to 4°C

Before you begin

Thaw the 2X Library Amplification Master Mix and the Primer Mix on ice. After the reagents have thawed, mix thoroughly by vortexing to prevent localized concentrations of reagent components, then return to ice until ready to use.

Amplify the DNA library

1. Transfer 20 µL of the DNA library (from step 30, page 23) into a sterile thin-wall 0.2-mL PCR tube on ice, then add the following reagents in the given order.

Component	Volume
DNA library, Adaptor-ligated and size-selected (clear ○)	20 µL
2X Library Amplification Master Mix (blue ●)	25 µL
Primer Mix (yellow ●)	5 µL
Total volume (green mixture ●):	**50 µL**

2. Vortex the PCR mixture (3–5 seconds) to mix, then centrifuge it briefly to collect all the droplets at the bottom.

3. Run the reactions in a thermal cycler with the lid temperature set to 105°C:

Stage	Number of cycles[1]	Temperature	Time
Activate the enzyme	1 cycle	98°C	30 seconds
Denature	3–4 cycles for 100 ng of input DNA 6–8 cycles for 10 ng of input DNA 10–12 cycles for 1 ng of input DNA	98°C	15 seconds
Anneal		60°C	30 seconds
Extend		72°C	30 seconds
Final extension	1 cycle	72°C	1 minute
Hold	1 cycle	4°C	Hold

[1] The number of PCR cycles depends on the starting amount of DNA (i.e., input DNA).

4. After the PCR is completed, proceed with the post-amplification cleanup (see "Purify the amplified DNA libraries", page 27).

Purify the amplified DNA libraries

Overview

This section describes post-amplification cleanup of the DNA library using the DNA Cleanup Beads. You do not need to perform this cleanup procedure if you have not PCR-amplified your DNA library after the size selection procedure.

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:

- PCR-amplified DNA library (from step 4, page 26)
- 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 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.
- Vortex thoroughly the DNA Cleanup Beads to completely resuspend the magnetic beads in the solution.

Purify the amplified DNA library

Perform all cleanup steps at room temperature.

1. Mix the amplified DNA library (50 µL) (from step 4, page 26) with **40 µL** of DNA Cleanup Beads by vortexing until you have obtained a homogeneous suspension. If there are droplets on the tube wall, centrifuge them down to the bottom.
2. Incubate for **5 minutes** at room temperature.

   IMPORTANT! Do **not** extend the binding step to more than 5 minutes. Over-incubation can result in lower DNA yields.

3. Place the mixture 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 tube on the magnet, 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. Remove the tube from the magnetic rack, add **50 µL** of Elution Buffer, then vortex to mix thoroughly.
6. Incubate for **1 minute** at room temperature.
7. Add **50 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. If there are droplets on the tube wall, centrifuge them down to the bottom.
8. Incubate for **5 minutes** at room temperature.
9. Place the mixture in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.
10. Keeping the tube on the magnet, 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.

11. Keeping the 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.

12. Carefully remove and discard the supernatant using a pipette.
13. Repeat steps 11–12.
14. To remove the residual ethanol, briefly centrifuge the tubes, place them back in the magnetic rack, then carefully remove any remaining supernatant with a pipette without disturbing the pellet.
15. Keeping the 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.

16. Remove the tube from the magnetic rack, add **25 µL** of Elution Buffer, then mix the suspension thoroughly by pipetting up and down or vortexing. If there are droplets on the tube wall, centrifuge them down to the bottom.
17. Incubate for **1 minute** at room temperature.
18. 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.

19. Without removing the tube from the magnetic rack, transfer **22–23 µL** of the supernatant (i.e., the eluate) to a new tube for storage.

    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.

20. Proceed to the assessment of the DNA library size and yield (see "Verify the size distribution and quality of prepared DNA libraries", page 30).

STOPPING POINT. After purification, you can store the amplified DNA library 4°C for 1–2 weeks. For longer term, store the library at –20°C until ready for sequencing.

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 (i.e., purified and amplified DNA from step 19, page 28), and dilute it 5–10-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 120 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).

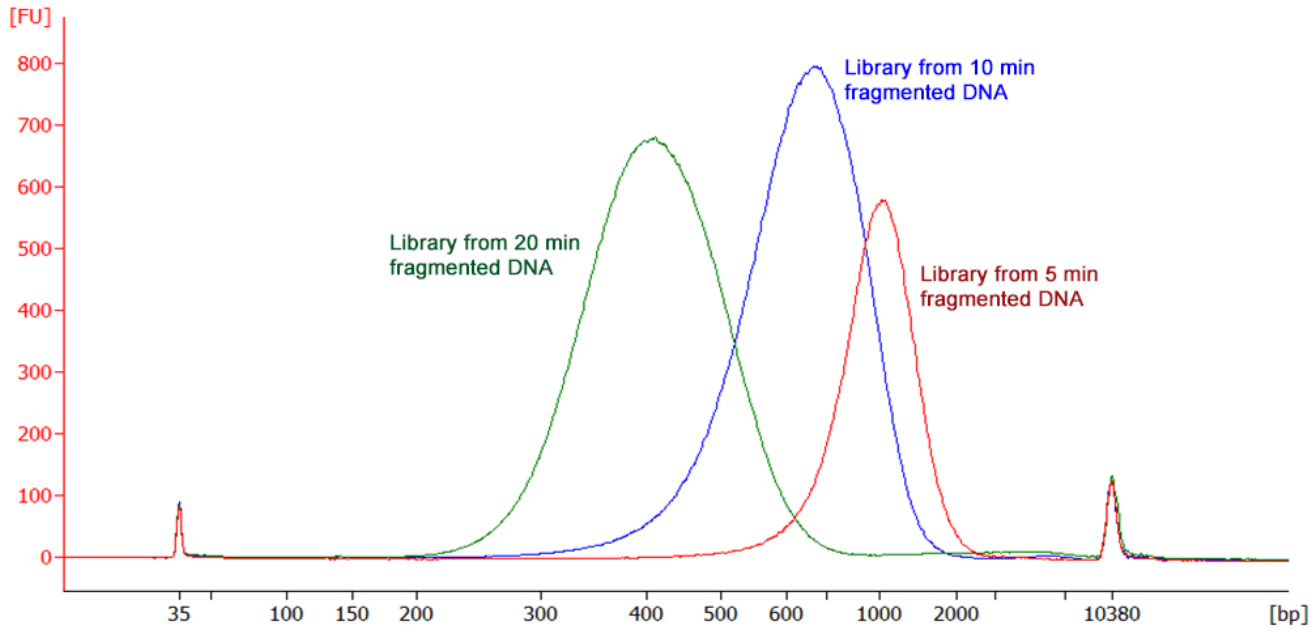


Figure 7 Typical Agilent™ 2100 Bioanalyzer trace of libraries prepared using the Collibri™ ES DNA Library Prep Kit. Libraries were prepared using 1 ng input DNA fragmented for 5, 10, and 20 minutes, adaptor-ligated, then size-selected and PCR-amplified following the protocol described here. Peaks at 35 bp and 10380 bp represent low and high molecular weight markers.

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

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™ ES 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. If more sequencable material is needed, optimize the number of PCR cycles to obtain the desired yield.

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>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>~400 bp</td><td>200-base read library</td></tr><tr><td>~ 350 bp</td><td>~700 bp</td><td>300-base read library</td></tr><tr><td>~ 550 bp</td><td>~1000 bp</td><td>500-base read library</td></tr></table>[1] On Agilent™ 2100 Bioanalyzer instrument.
Optimize your DNA shearing protocol to generate the DNA fragments of correct median insert size.</td></tr>
<tr><td></td><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>Low DNA library yield (continued)</td><td>Cleanup protocols for Adaptor removal were not carefully followed.</td><td>• 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.
• To ensure the best DNA yields, do not lose any cleanup beads during the procedures and do not shorten incubation times described.
• Ensure that after Wash steps all remaining solution with ethanol is removed and dried. Remaining of ethanol reduces DNA library yields.
• Perform all size selection steps using 1.5-mL Eppendorf™ LoBind™ Tubes (Eppendorf™, Cat. No. 022431021).
• Use well-calibrated pipettes.</td></tr>
<tr><td>Adaptor contamination</td><td>Cleanup protocol was not carefully followed.</td><td>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.</td></tr>
</table>

Observation	Possible cause	Recommended action
Size selected library is outside the range of interest	Improper fragmentation of DNA sample.	Ensure that your DNA fragmentation protocol generates the DNA fragments close to the correct median insert size.
	Cleanup protocol was not carefully followed.	Cleanup protocols are 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.
	Over-amplification of DNA library.	Use as few amplification cycles as possible for library amplification (see recommendations on page 26). Over-amplification of DNA library can lead to large chimeric molecules, which are observed as a smear of larger fragments along with normal the DNA library peak when analyzed on the Agilent™ 2100 Bioanalyzer.

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™ DNA Library Prep Kits are listed in Table 5.

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 10–11 (page 39).

Table 5 Indexes used in Collibri™ 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™, 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™ DNA Library Prep Kits are listed in Tables 6–9 (pages 35–38).

Plate layouts of 24-prep UD adaptor Sets A–D are shown in Tables 12–15 (pages 40–41).

Table 6 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 (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 7 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 (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 8 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 (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 9 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 (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 10 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 11 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 12 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 13 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 14 Collibri™ 24-prep Unique Indexed **(UD)** Adaptor **Set C** plate layout

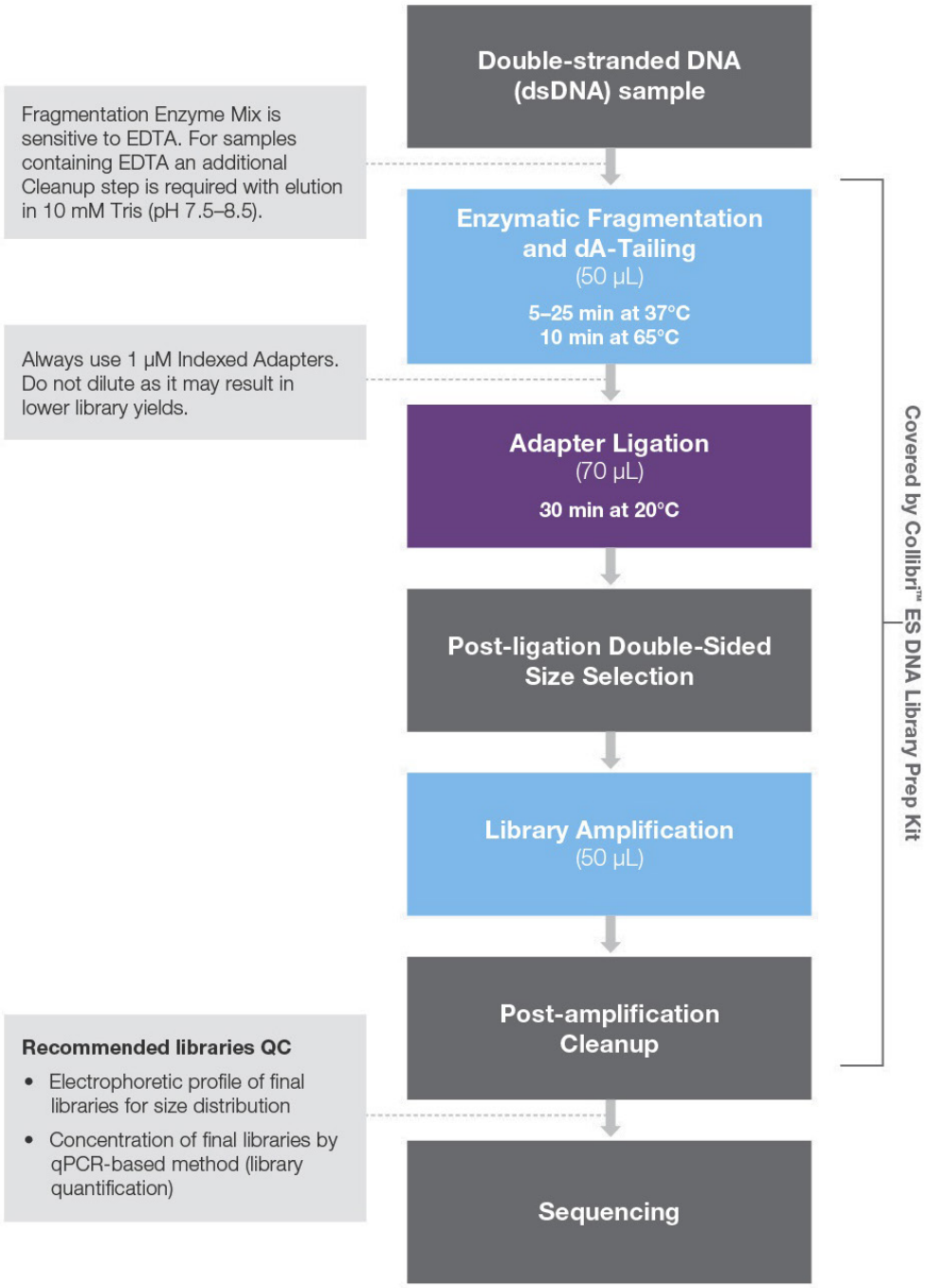
	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI049	UDI057	UDI065	—	—	—	—	—	—	—	—	—
B	UDI050	UDI058	UDI066	—	—	—	—	—	—	—	—	—
C	UDI051	UDI059	UDI067	—	—	—	—	—	—	—	—	—
D	UDI052	UDI060	UDI068	—	—	—	—	—	—	—	—	—
E	UDI053	UDI061	UDI069	—	—	—	—	—	—	—	—	—
F	UDI054	UDI062	UDI070	—	—	—	—	—	—	—	—	—
G	UDI055	UDI063	UDI071	—	—	—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

Table 15 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	—	—	—	—	—	—	—	—	—

Appendix C: Process workflow

Collibri™ ES DNA Library Prep Kit



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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