

Collibri™ PCR-free ES DNA Library Prep Kit for Illumina®

USER GUIDE

for use with:

Illumina® next generation sequencing (NGS) platforms

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For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](https://www.thermofisher.com/symbols-definition).

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A.0	3 May 2019	New user guide.

The information in this guide is subject to change without notice.

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Product information

Product description

Invitrogen™ Collibri™ PCR-free 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 (100 ng–500 ng) starting from intact DNA.

In the Collibri™ PCR-free ES DNA Library Prep Kits 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 2 hours, 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™ PCR-free 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™ PCR-free ES DNA Library Prep Kits, see “Technology overview” on page 11.



Product specifications

Assay time	~110 minutes on average
Hands-on time	~30 minutes on average
Sample type	<ul style="list-style-type: none">• Low complexity dsDNA (bacteria/phage DNA)• High complexity dsDNA (Mammalian, Mouse, Human, Rat, Plant)
Sample input amount	100 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	<ul style="list-style-type: none">• 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, Set A–D)
System compatibility	iSeq™, Hi-Seq™ 1000, Hi-Seq™ 1500, Hi-Seq™ 2000, Hi-Seq™ 2500, Hi-Seq™ 3000, Hi-Seq™ 4000, Hi-Seq™ X, MiSeq™, MiniSeq™, NextSeq™ 500, NextSeq™ 550, NovaSeq™ 6000
Sequencing application	Whole-genome sequencing (WGS)



Kit contents and storage

Kit configurations

The Colibri™ PCR-free 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 Colibri™ DNA CD (Combinatorial Dual) or UD (Unique Dual) Indexes.

Kit configuration	Kit size	DNA index type ^[1]	Cat. No.
Colibri™ PCR-free ES DNA Library Prep Kit	24 preps	CD	A38545024
	96 preps		A38603096
	24 preps	UD Set A (1–24)	A38602024
		UD Set B (25–48)	A43602024
		UD Set C (49–72)	A43603024
		UD Set D (73–96)	A43604024
	96 preps	UD Set A–D (1–96)	A38608196
Colibri™ PCR-free ES DNA Library Prep Core Kit	24 preps	Without	A38545024W
	96 preps		A38603096W

^[1] CD = combinatorial dual indexes, UD = unique dual indexes

Note: Kits containing PCR amplification module that support library preparation from 1 ng of input DNA (Cat. Nos. A38605024, A38607096, A38606024, A43605024, A43606024, A43607024, A38607196, A38605024W and A38607096W) are available from Thermo Fisher Scientific For more information, see www.thermofisher.com.

Kit configuration ^[1]	Kit size	Type	Cat. No.
Colibri™ DNA Library Cleanup module	24 preps	Cleanup	A43190024
Colibri™ DNA Library Cleanup module	96 preps	Cleanup	A38584096
Colibri™ PCR-free ES DNA Library Prep module	24 preps	Fragmentation, End Repair/A-tailing step, Ligation, PCR	A38575024
Colibri™ PCR-free ES DNA Library Prep module	96 preps	Fragmentation, End Repair/A-tailing step, Ligation, PCR	A38577096
Colibri™ DNA CD Indexes module	24 preps	Indexing	A38586024
Colibri™ DNA UD Indexes (Set A, 1-24) module	24 preps	Indexing	A38587024



(continued)

Kit configuration ^[1]	Kit size	Type	Cat. No.
Collibri™ DNA UD Indexes (Set B, 25-48) module	24 preps	Indexing	A43348024
Collibri™ DNA UD Indexes (Set C, 49-72) module	24 preps	Indexing	A43350024
Collibri™ DNA UD Indexes (Set D, 73-96) module	24 preps	Indexing	A43351024
Collibri™ DNA CD Indexes module	96 preps	Indexing	A39123096
Collibri™ DNA UD Indexes (Set A-D, 1-96) module	96 preps	Indexing	A39123196

^[1] CD = combinatorial dual indexes, UD = unique dual indexes

Note: Kits containing PCR amplification module that support library preparation from 1 ng of input DNA (Cat. Nos. A38579024 and A39097096) are available from Thermo Fisher Scientific. For more information, go to www.thermofisher.com.

Kit components and storage

Upon receipt, immediately store the Collibri™ PCR-free 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™ PCR-free 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
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



(continued)

Component	Cap/reagent color	24 preps	96 preps
Colibri™ 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)

^[2] Unique Dual-Indexed Adaptors (UD)

^[3] For the Adaptor index sequences and plate layouts, see Appendix B, “Adaptor index sequences and plate layouts” (Appendix B, “Adaptor index sequences and plate layouts”).

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](https://www.thermofisher.com). "MLS" indicates that the material is available from [fisherscientific.com](https://www.fisherscientific.com) or another major laboratory supplier.

Item	Source
Thermal cycler with heated lid, such as: <ul style="list-style-type: none"> 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 	<ul style="list-style-type: none"> 4375786 4484075 4484073 thermofisher.com
Agilent™ 2100 Bioanalyzer™ instrument ^[1]	Agilent™, G2938A
Agilent™ High Sensitivity DNA Kit ^[1]	Agilent™, 5067-4626
Magnetic rack, such as: <ul style="list-style-type: none"> Invitrogen™ DynaMag™-2 Magnet (for 1.5-mL tubes) Invitrogen™ DynaMag™-96 Side Magnet (for PCR strips or 96-well 0.2-mL plates) 	<ul style="list-style-type: none"> 12321D 12331D
Benchtop microcentrifuge	MLS



(continued)

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

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

^[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™ PCR-free 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.

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'-dTTP overhangs are added to each end of the 3'-dA-tailed DNA molecules.

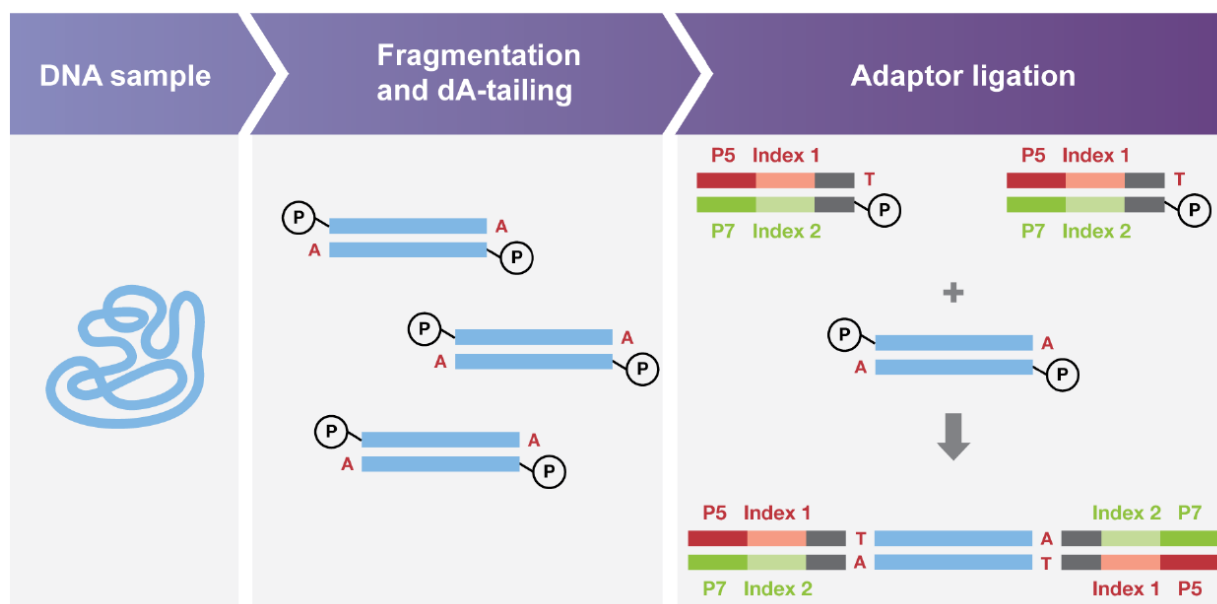


Figure 1 Simplified schematic representation of technology used in the Collibri™ PCR-free ES DNA Library Prep Kit

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 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 “Adaptor index sequences” on page 38 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.



Library quantification

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



Workflow

Figure 2 and Figure 3 and illustrates workflow to construct sequencing-ready DNA fragment libraries for whole-genome DNA sequencing.



Figure 2 Collibri™ PCR-free ES DNA Library Prep Kit workflow

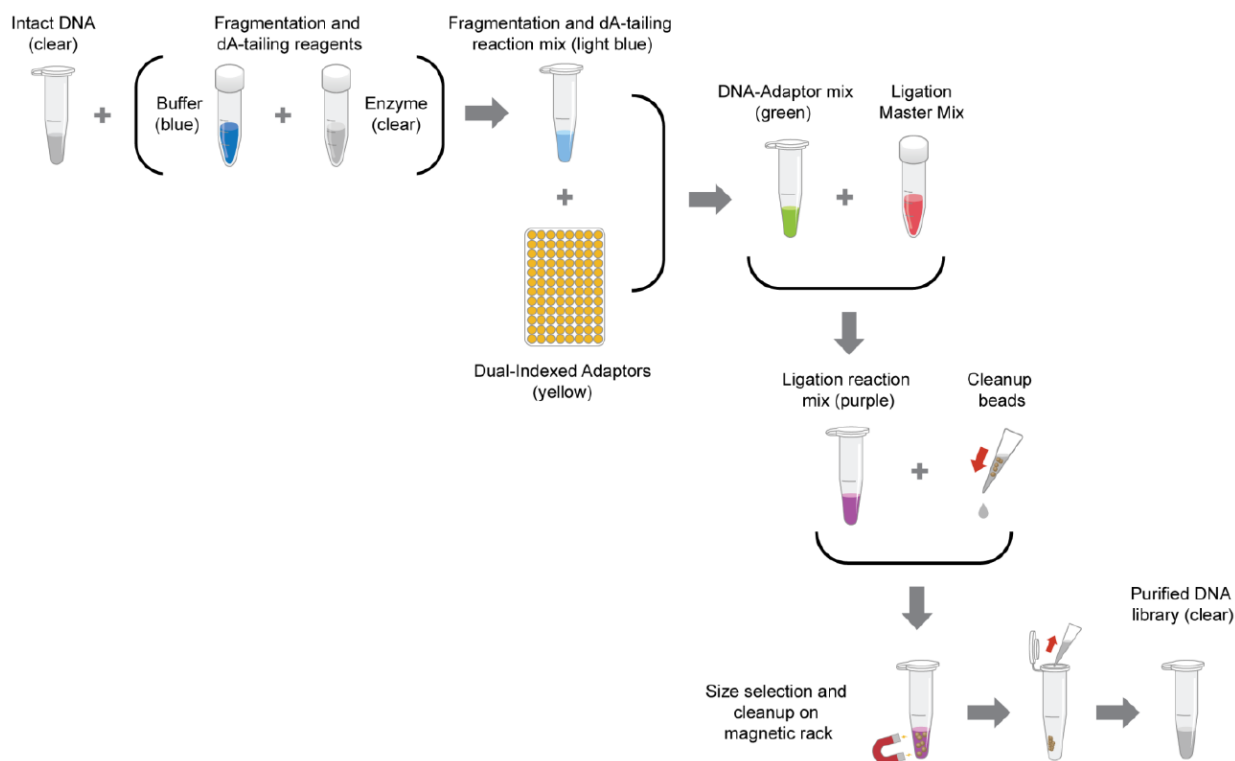


Figure 3 Collibri™ PCR-free ES DNA Library Prep Kit

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 the correct 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 PCR-free and amplified library construction workflows are listed in Table 1 on page 14.

Table 1 Input DNA requirements

	PCR-free library	
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	100 ng–500 ng ^[1]	500 ng

^[1] 100 ng input DNA is sufficient to prepare PCR-free libraries of ≥4 nM concentration. For higher concentration PCR-free libraries, use 500 ng 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) (“Required materials not supplied” on page 9).
- 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” on page 21.
- 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 on page 16).

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

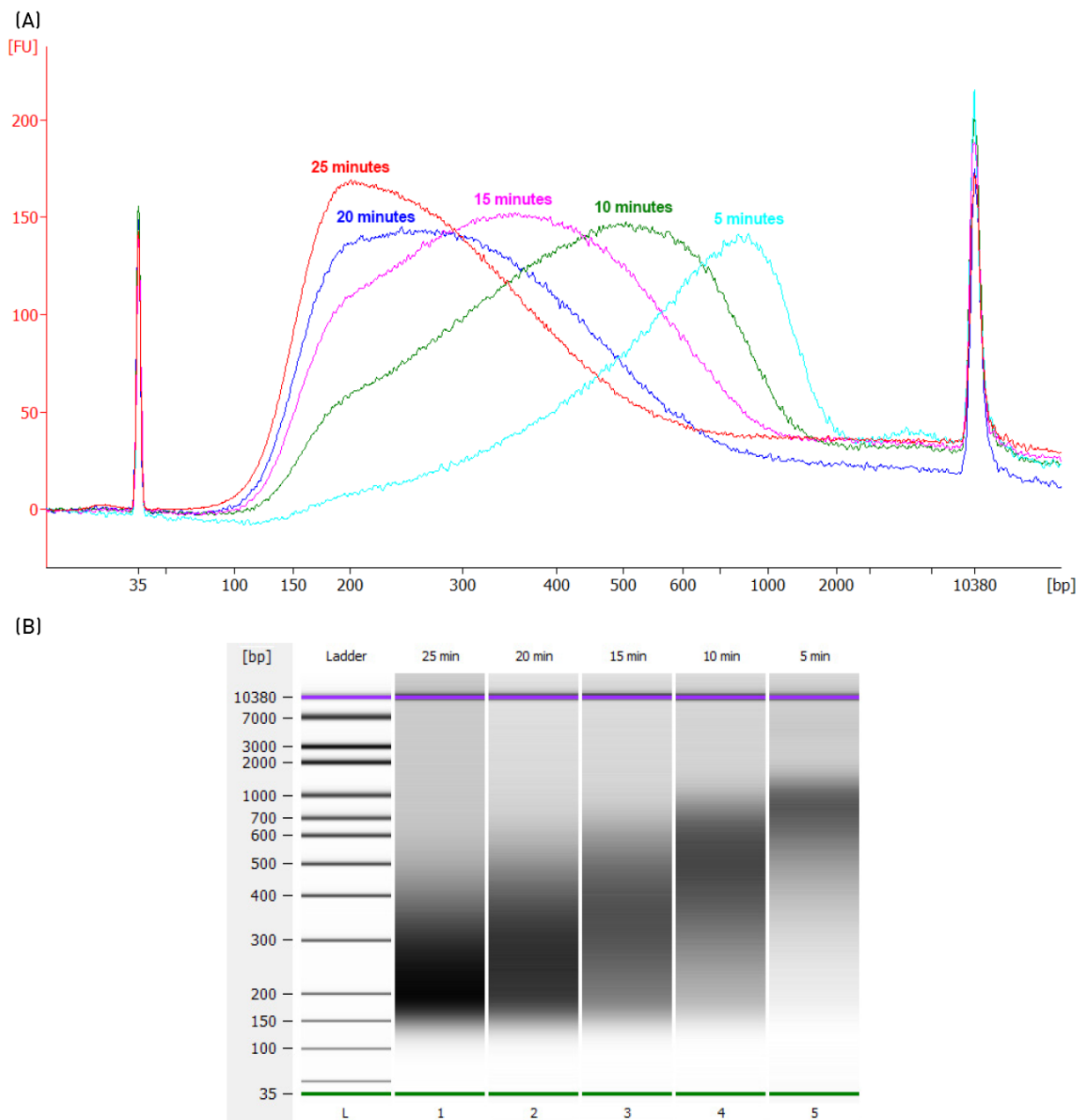


Figure 4 Enzymatic fragmentation reactions were performed for 25, 20, 15, 10, and 5 minutes, then analyzed with the Agilent™ High Sensitivity DNA Kit and run on an Agilent™ 2100 Bioanalyzer™ instrument. (A) Trace view of the analysis results illustrate the dependence of DNA fragment size distribution 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 Colibri™ PCR-free ES DNA Library Prep Kit, the Colibri™ 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, see [page 38](#).
- Colibri™ 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).
- Colibri™ 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 Colibri™ 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 Colibri™ DNA Library Cleanup Kit (included in the Colibri™ PCR-free 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 in 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.
- 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.

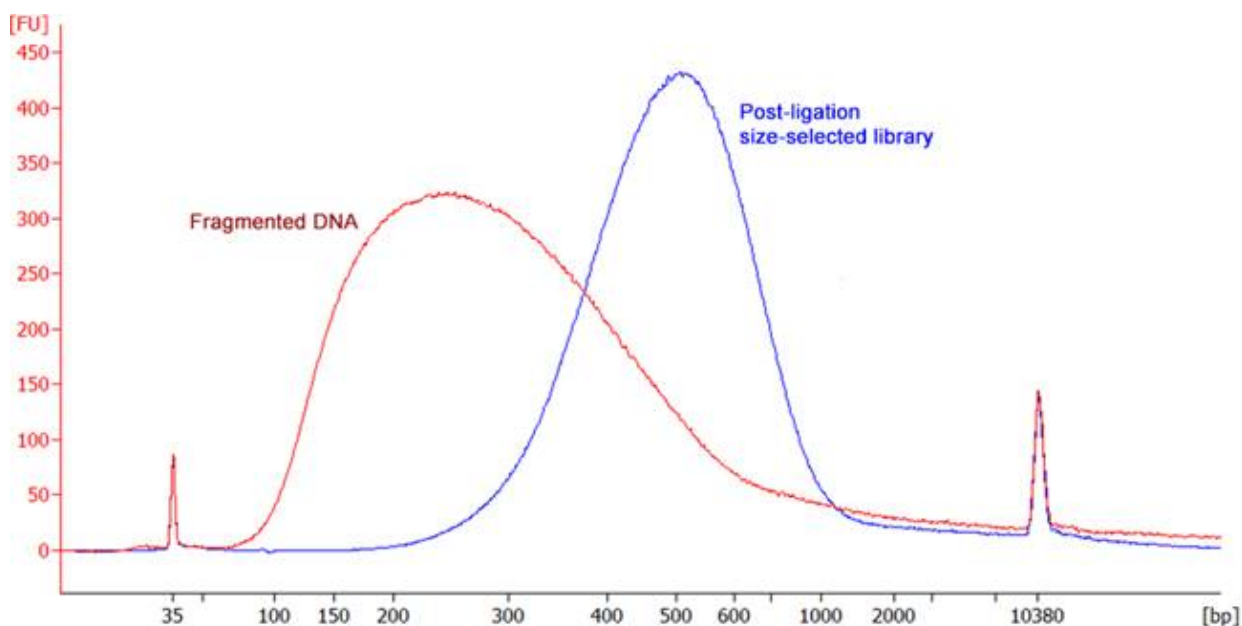


Figure 5 Input DNA fragmented and library preparation

500 ng input DNA was enzymatically fragmented for 20 minutes and libraries were prepared using the Collibri™ PCR-free ES DNA Library Prep Kit. Aliquots of the sample were collected at each stage of the library prep process and electropherograms were generated on an Agilent™ 2100 Bioanalyzer™ instrument. Note: Size-selected library appears longer than expected due to Y-shaped adaptors.



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 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, 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. 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” on page 9)
- 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 μ L 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 step 5 and step 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 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 DNA yield.

10. Remove the tube from the magnetic rack, then add the desired volume of Elution Buffer (use at least 10 μ L) 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 on page 22.
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™ PCR-free 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™ PCR-free 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” on page 9)
- 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 (100 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 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 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

- 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” on page 26.



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™ PCR-free 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 4 on page 24)
- Thermomixer or Thermal cycler with a heated lid off (see “Required materials not supplied” on page 9)
- Microcentrifuge
- 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), 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 on page 26), then mix well by vortexing.

Component	Volume
Adaptor-DNA mixture from step 1 on page 26 (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” (“Post-ligation double-sided size selection” on page 28).

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 initial DNA input amount and the desired fragment size distribution of the DNA library. See Table 3 on page 29 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 on page 27)
- 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” on page 9)

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 the thoroughly 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 28).



- **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 μ L	65 μ L	20 μ L
350 bp	60 μ L	45 μ L	20 μ L
550 bp	60 μ L	35 μ L	20 μ L

- 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” on page 32).

Perform size selection

Initial cleanup

1. Mix the Dual Index Adaptor-ligated DNA sample (70 μ L) with 60 μ L 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 μ L 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 on page 29–6 on page 29.
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 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 2–3 minutes or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step on page 30.
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

1. 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 on page 29 (Recommended conditions for bead-based size selection of libraries on page 29) for the appropriate volume of cleanup beads to use.

2. Incubate for 5 minutes at room temperature.

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

3. Place it in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.
4. Keeping the reaction tube in the magnetic rack, carefully remove and transfer all supernatant to a clean tube for the second size selection step. 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

1. 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 for the appropriate volume of cleanup beads to use.

2. Incubate for 5 minutes at room temperature.
3. Place it in the magnetic rack for 2 minutes or until the beads have formed a tight pellet.



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 μ L 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 21 on page 31–22 on page 31.
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! 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.

10. Remove the tube from the magnetic rack, add 25 μ L 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 on page 31.
13. Without removing the tube from the magnetic rack, collect 22–23 μ L 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 “Verify the size distribution and quality of prepared DNA libraries” on page 34



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-Figure 7).

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 Figure 6 and Figure 7.

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.

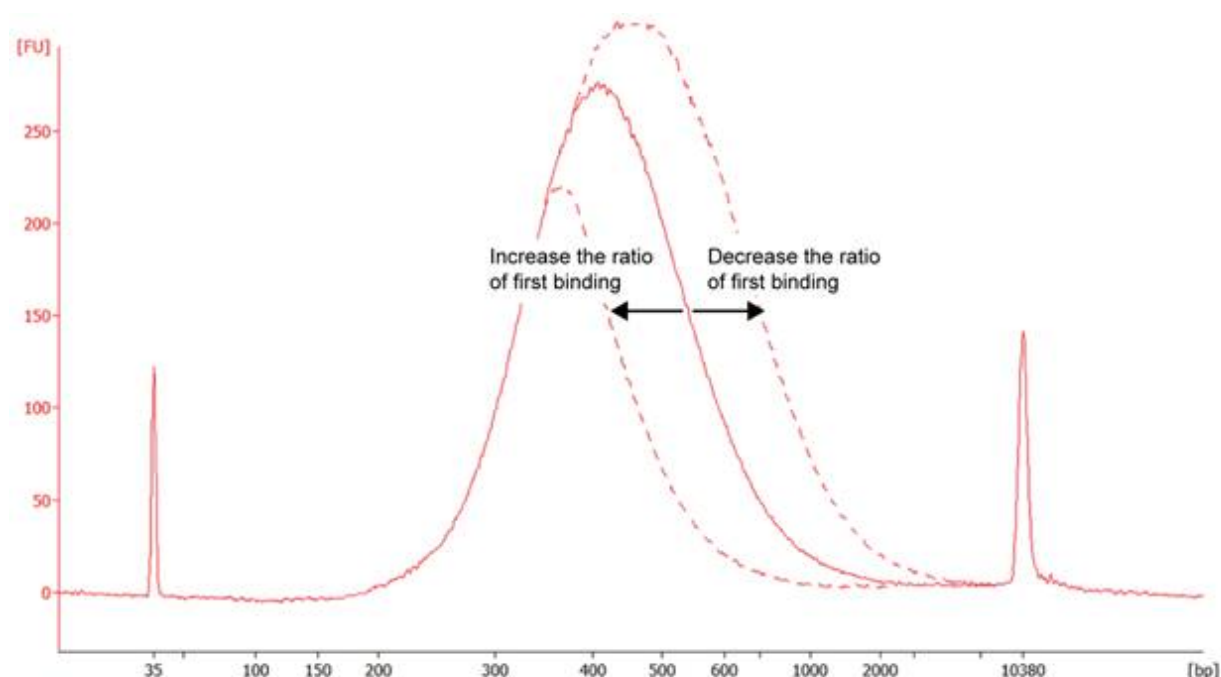


Figure 6 Size modulation during First binding

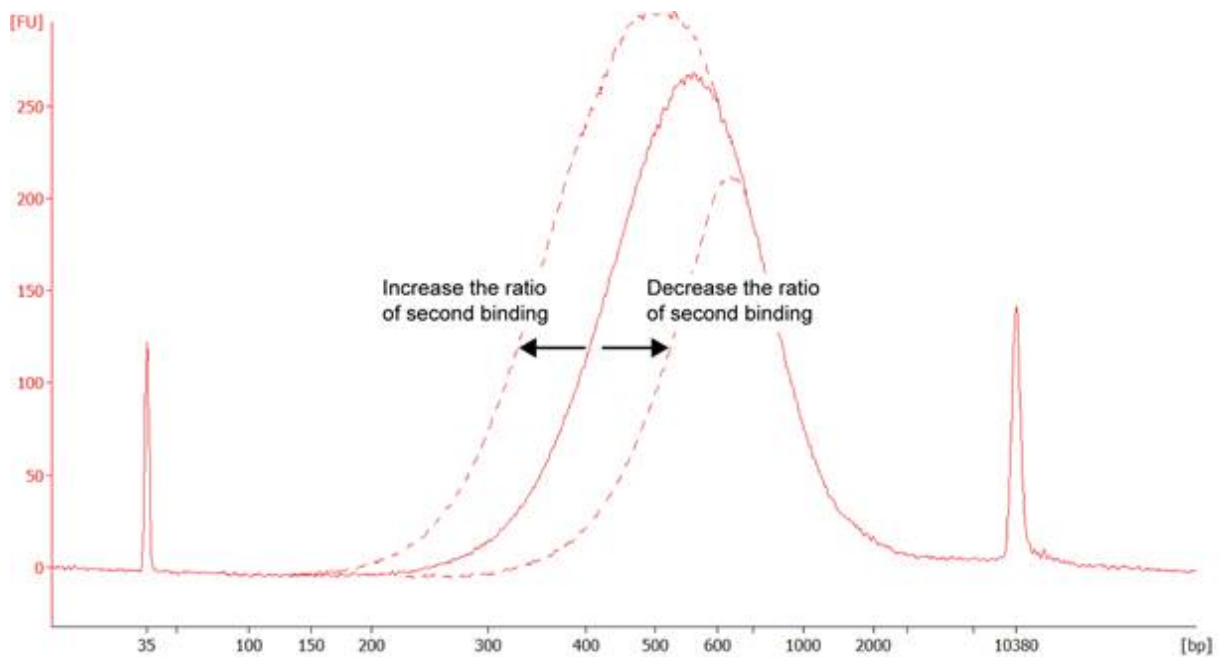


Figure 7 Size modulation during Second binding

Verifying the ratio of DNA Cleanup Beads-to-DNA volume at the start of the first and 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 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 120 bp.

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

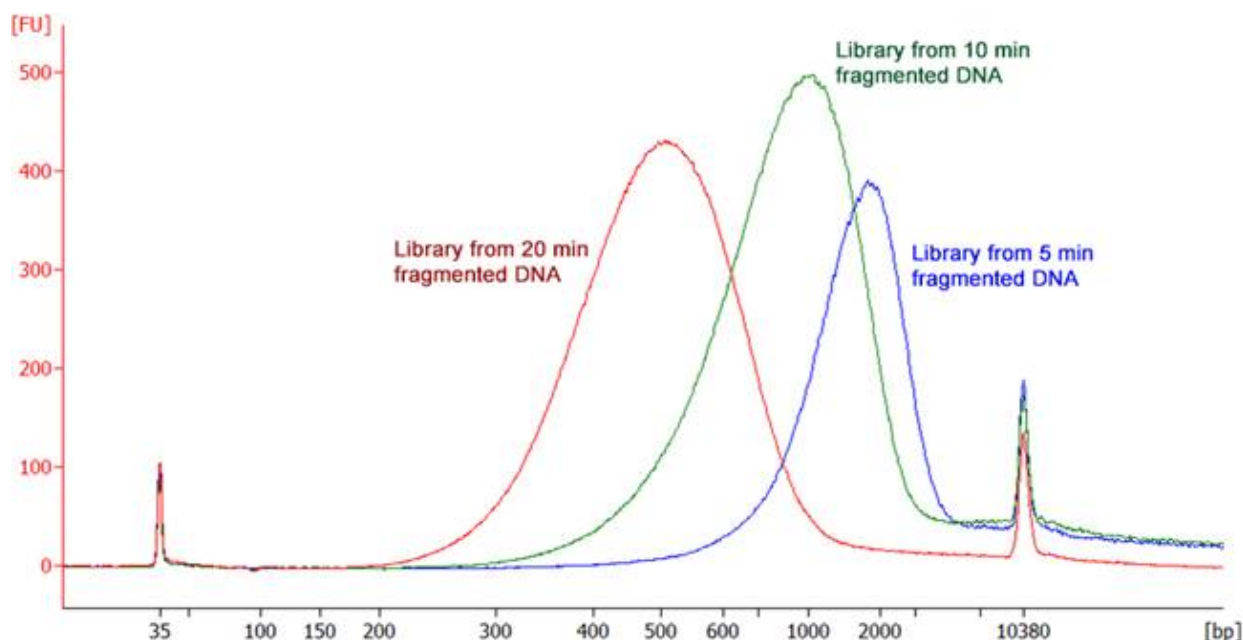


Figure 8 Typical Agilent™ 2100 Bioanalyzer™ trace of libraries prepared using the Collibri™ PCR-free ES DNA Library Prep Kit.

Libraries were prepared using 500 ng input DNA fragmented for 5, 10, and 20 minutes, adaptor-ligated, then size-selected. 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 up to 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.

Sequence the prepared library

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



Troubleshooting

Observation	Possible cause	Recommended action		
Low DNA library yield	Improperly fragmented DNA.	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:		
		Target insert size	Average library size ^[1]	Target read length
		~ 200 bp	~ 5000 bp	200-base read library
		~ 350 bp	~ 1000 bp	300-base read library
		~ 550 bp	~ 2000 bp	500-base read library
		Optimize your DNA shearing protocol to generate the DNA fragments of correct median insert size.		
	Low DNA quality.	<p>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.</p> <p>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.</p>		
	Too small amount of input DNA used for PCR-free library preparation.	<p>Usually 100 ng of input DNA generates sufficient amount of Illumina®-compatible 300-base read library to use directly for sequencing without amplification.</p> <p>However, 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. In such cases, we recommend increase DNA input amount.</p> <p>Refer to Table 1 (Table 1) for the recommended amount of input DNA amount for the preparation of PCR-free NGS libraries.</p>		

(continued)

Observation	Possible cause	Recommended action
Low DNA library yield	Cleanup protocols for Adaptor removal were not carefully followed.	<ul style="list-style-type: none"> 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.
Adaptor contamination	Cleanup protocol was not carefully followed.	<p>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.</p> <p>If the pellet of magnetic particles was disturbed while removing supernatant, mix the sample and let the beads settle to the magnet again.</p>
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 measured on the 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 Y-shaped Adaptors that are ligated to both ends of the DNA fragments.
	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.

[1] On Agilent™ 2100 Bioanalyzer instrument.



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 Figure 9–Figure 10.

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™, Hi-Seq™ 2000/2500)	i5 bases for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ 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 Hi-Seq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ 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 Colibri™ DNA Library Prep Kits are listed in Table 6–Table 9

Plate layouts of 24-prep UD adaptor Sets A–D are shown in Figure 11–Figure 14.

Table 6 Indexes used in Colibri™ 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™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ 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	GCTTGTC A	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	TTGGA CTC	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

Table 6 Indexes used in Colibri DNA library kits to generate Unique Dual-Indexed (UDI) adaptors – Set A. (continued)

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI021	GGTACCTT	AAGACGTC	GACGTCTT
UDI022	AACGTTCC	GGAGTACT	AGTACTCC
UDI023	GCAGAATT	ACCGGCCA	TGGCCGGT
UDI024	ATGAGGCC	GTTAATTG	CAATTAAC

^[1] Sequencing on the iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ 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 Colibri™ 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™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ 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	GCTTGTC A
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

Table 7 Indexes used in Colibri DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set B. (continued)

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
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 iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ 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 Colibri™ 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™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ 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

Table 8 Indexes used in Colibri DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set C. (continued)

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
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 iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ 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™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ 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	TCTCATTG	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	CCATTCTA
UDI089	TATCGCAC	CTTAGTGT	ACACTAAG
UDI090	CGCTATGT	TCCGACAC	GTGTCGGA
UDI091	GTATGTTC	AACAGGAA	TTCCTGTT
UDI092	ACGCACCT	GGTGAAGG	CCTTCACC
UDI093	TACTCATA	CCTGTGGC	GCCACAGG
UDI094	CGTCTGCG	TTCACAAT	ATTGTGAA

Table 9 Indexes used in Colibri DNA Library kits to generate Unique Dual Indexed (UDI) Adaptors – Set D. (continued)

UDI Adaptor name	P7 index	P5 index for entry on sample sheet (NovaSeq™, MiSeq™, Hi-Seq™ 2000/2500)	P5 index for entry on sample sheet (iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, Hi-Seq™ X) ^[1]
UDI095	TCGATATC	ACACGAGT	ACTCGTGT
UDI096	CTAGCGCT	GTGTAGAC	GTCTACAC

^[1] Sequencing on the iSeq™, MiniSeq™, NextSeq™, Hi-Seq™ 3000/4000, and Hi-Seq™ 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 match the colors of the plates containing individual adaptor sets.

Combinatorial Indexed Adaptor Sets

	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

Figure 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	—	—	—	—	—	—	—	—	—
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	—	—	—	—	—	—	—	—	—

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

Unique Dual Indexed Adaptor Sets

	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	—	—	—	—	—	—	—	—	—

Figure 11 Colibri™ 24-prep Unique Indexed (UD) Adaptor Set A plate layout (Red)

	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	—	—	—	—	—	—	—	—	—

Figure 12 Colibri™ 24-prep Unique Indexed (UD) Adaptor Set B 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	—	—	—	—	—	—	—	—	—

Figure 13 Colibri™ 24-prep Unique Indexed (UD) Adaptor Set C 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	—	—	—	—	—	—	—	—	—

Figure 14 Colibri™ 24-prep Unique Indexed (UD) Adaptor Set 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

Figure 15 Colibri™ 96-prep Unique Indexed (UD) Adaptor Set A–D plate layout



Process workflow

Collibri™ PCR-free ES DNA Library Prep Kit

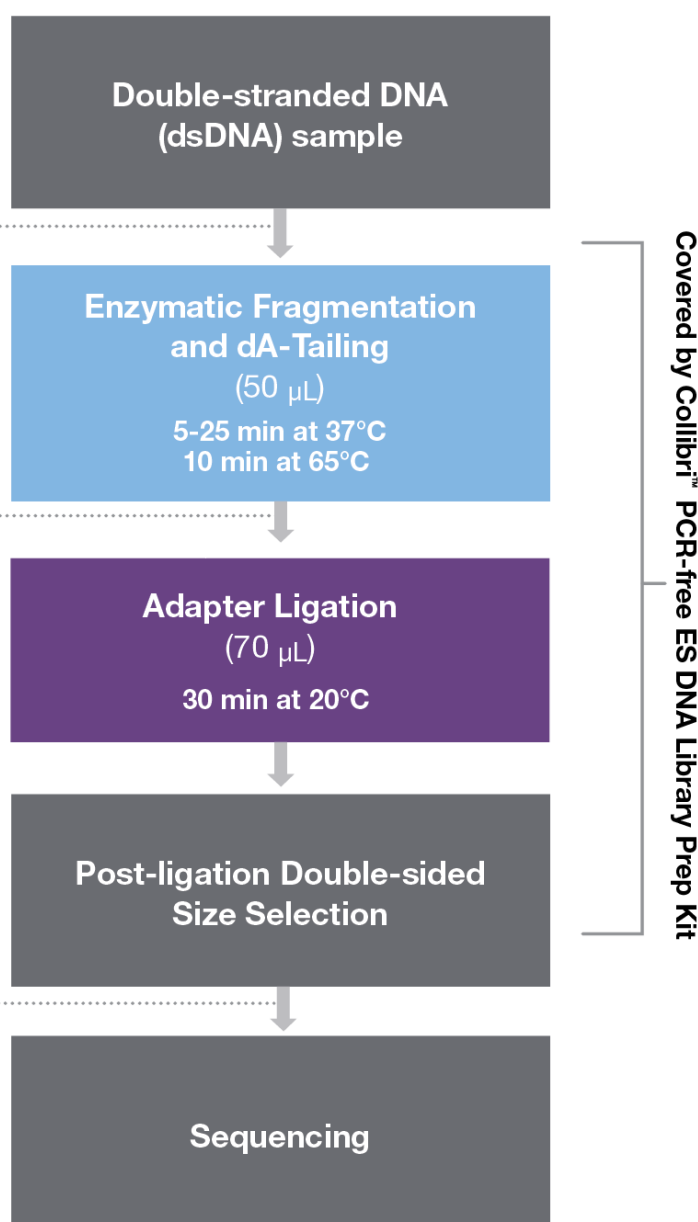
Collibri™ PCR-free ES DNA Library Prep Kit

Fragmentation Enzyme Mix is sensitive to EDTA. For samples containing EDTA and additional Cleanup step is required with elution in 10 mM Tris (pH 7.5-8.5).

Always use 1µM Indexed Adapters. Do not dilute as it may result in lower library yields.

Recommended libraries QC

- Electrophoretic profile of final libraries for size distribution
- Concentration of final libraries by qPCR-based method (library quantification)





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, and so on). To obtain SDSs, visit [thermofisher.com/support](https://www.thermofisher.com/support).

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 sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer 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 needed) 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.



WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.



WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety



WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, 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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
www.who.int/publications/i/item/9789240011311

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