invitrogen

Collibri™ PS DNA Library Prep Kits for Whole-Exome Sequencing

USER GUIDE

Catalog Numbers A38614196, A38612024, A38614096, A38613024, A43611024, A43612024, A43613024, A38612024W, A38614096W

Publication Number MAN0025533

Revision A.0

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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

Revision history: Pub. No. MAN0025533

Revision	Date	Description
A.0	9 December 2021	New manual for Collibri™ PS DNA Library Prep Kits.

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1 Product information

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

This manual is used to generate exome libraries from human genomic DNA for sequencing on Illumina® next generation sequencing (NGS) systems. Invitrogen™ Collibri™ PS DNA Library Prep Kits for Illumina® are designed for the construction of high-efficiency DNA fragment libraries. Collibri™ PS DNA Library Prep Kits are compatible with Twist Human Core Exome Hybridization Kits.

Kit contents and storage

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

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

Component	Cap/reagent color[1]	Without Library Amplification[2]		With Library Amplification[3]	
		24 preps	96 preps	24 preps	96 preps
Collibri™ PS DNA Library Prep Kit (Store at –20°C)					
2X End conversion Master Mix	Blue	600 µL	2 × 1.2 mL	600 µL	2 × 1.2 mL
7X Ligation Master Mix	Red	250 µL	1 mL	250 µL	1 mL
2X Platinum™ SuperFi™ Library Amplification Master Mix	Blue	—	—	1.25 mL	2 × 1.25 mL
Primer Mix	Yellow	—	—	500 µL	2 × 500 µL

(continued)

Component	Cap/reagent color[1]	Without Library Amplification[2]		With Library Amplification[3]	
		24 preps	96 preps	24 preps	96 preps
Collibri™ DNA Library Cleanup Kit (Store at 2°C to 8°C) IMPORTANT! Do not freeze.)					
DNA Cleanup Beads	Orange	10 mL	30 mL	10 mL	30 mL
Wash Buffer (Concentrated)	Blue	4.5 mL	18 mL	4.5 mL	18 mL
Elution Buffer	White	5 mL	20 mL	5 mL	20 mL
Collibri™ DNA Combinatorial Dual (CD) or Unique Dual (UD) Indexed Adapters (if included) (Store at –20°C)					
Dual-Index Adaptors (7 µM)	—	10 µL/well (24 wells)	10 µL/well (96 wells)	10 µL/well (24 wells)	10 µL/well (96 wells)

[1] In the Collibri™ PS DNA Library Prep Kit, the cap colors match the color of the reagent in the vial. However, this is not the case for caps and reagents in the Collibri™ Library Cleanup Kit.

[2] PCR-free Cat. Nos.: A38608024, A38610096, A38609024, A43608024, A43609024, A43610024, A38615196, A38608024W, A38610096W

[3] Invitrogen™ Collibri™ Library Amplification Master Mix with (A38540050, A38540250) and without Primer Mix (A38539050, A38539250) can be bought separately.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Thermal cycler with heated lid, such as: • VeritiPro™ 96-well Thermal Cycler • ProFlex™ 96-well PCR System • ProFlex™ 3 × 32-well PCR System	 • A48141 • 4484075 • 4484073
Agilent™ 2100 Bioanalyzer™ instrument[1]	Agilent™ G2938A
Agilent™ High Sensitivity DNA Kit	Agilent™ 5067-4626
Covaris™ M220 Focused-ultrasonicator™	Covaris™ 4482277

(continued)

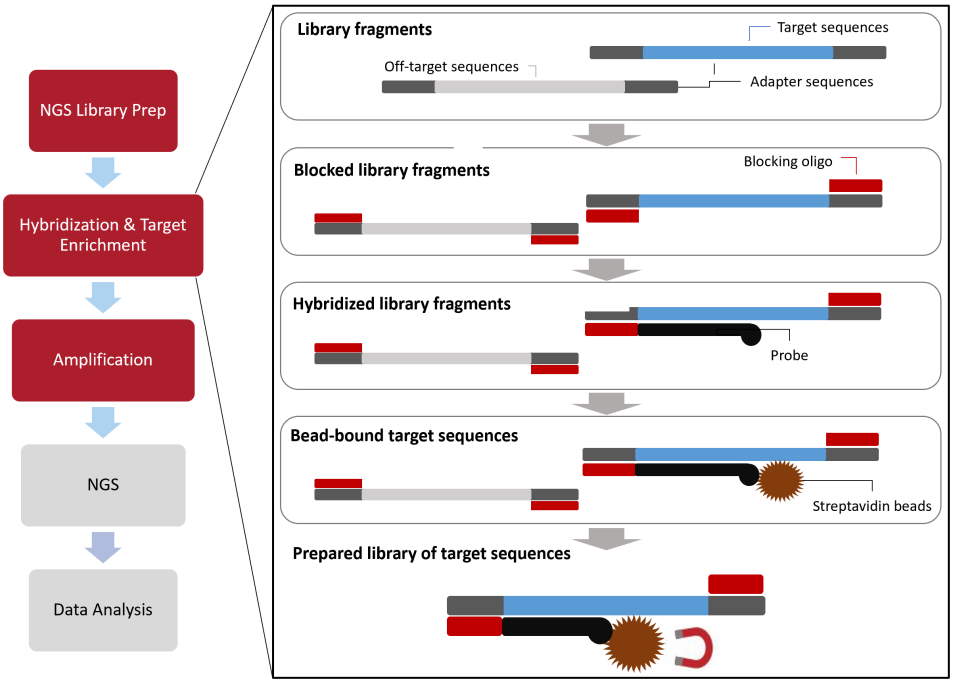
Item	Source
Magnetic rack, such as: • Invitrogen™ DynaMag™-2 Magnet (for 1.5-mL tubes) • Invitrogen™ DynaMag™-96 Side Magnet (for PCR strips or 96-well 0.2-mL plates)	• 12321D • 12331D
Benchtop microcentrifuge	**MLS**
Vortex mixer	**MLS**
Heating block and/or thermomixer	**MLS**
Nuclease-free 1.5-mL tubes, such as Eppendorf™ DNA LoBind™ Tubes	Eppendorf™, 022431021
0.2-mL thin-wall PCR tubes or plates	**MLS**
Cooling rack for 0.2-mL PCR tubes/plates	**MLS**
Calibrated single-channel or multi-channel pipettes (1 µL– 1,000 µL)	**MLS**
Nuclease-free pipette tips	**MLS**
Disposable gloves	**MLS**
One of the following Tris buffers: • 10 mM Tris-HCl buffer, pH 7.5–8.5 • TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) • Low TE buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA)	**MLS**
Ethanol 96–100%, molecular biology grade	**MLS**
(*Optional*) Qubit™ 4 Fluorometer[2]	Q33226
(*Optional*) Qubit™ DNA HS Assay Kit	Q32854

[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. The Qubit™ 2.0 Fluorometer is supported, but it is no longer available for purchase.

Technology overview

The Collibri™ PS 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.



2 Procedural guidelines

Input DNA requirements

The recommended DNA input for WES library prep is 50 ng of purified human gDNA. This protocol includes DNA fragmentation by physical DNA shearing. The alternative protocol includes enzymatic DNA fragmentation.

Guidelines for DNA quality

- The success of WES DNA library preparation and reliable DNA sequencing results strongly depend on the quality, quantity, and input of 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 Qubit™ dsDNA HS Assay with the Qubit™ 4 Fluorometer (or a similar instrument).
- For high-quality gDNA purification from various human biological samples, use specialized commercial kits.

Guidelines for DNA fragmentation

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

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

- For optimal results, we recommend using 150–200 bp fragmented DNA.
- Evaluate the quality of fragmented DNA by agarose gel electrophoresis or using the Agilent™ 2100 Bioanalyzer™ Instrument (or similar instrument).

Guidelines for adapter ligation

- Indexed adapters 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 following Illumina® multiplexing guidelines.
- Depending on the Collibri™ PS DNA Library Prep Kit, the Collibri™ Dual-Indexed Adaptor plate contains a set of either 24 or 96 adapters, each carrying two 8-nucleotide indexes (barcodes).
- Collibri™ Dual-Indexed Adaptors are supplied in fully-skirted PCR plates, which are sealed with non-pierceable, non-porous, Easy-Peal™ seal to minimize cross-contamination during handling. Each well contains 10 µL of adaptor at a concentration of 7 µM, 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. Use new, sterile pipette tips for every well of the adaptor plate.
- To ensure equal read distribution when multiplexing libraries, carefully quantify and normalize individual libraries before pooling. We recommend using the Collibri™ Library Quantification Kit (Cat. No. A38524100, A38524500) as the preferred qPCR-based method for accurate and reproducible quantification of molecules being sequenced.

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™ Library Cleanup Kit efficiently eliminates unused adaptors, primers, and adaptor dimers.
- 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, ensure the cleanup beads are resuspended in the solution by pipetting up and down or vortexing. When properly resuspended, the bead solution should have a uniform color with no visible clumping on the walls or at the bottom of the tube.
- To ensure optimal DNA recovery, it is crucial 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 confirm you are not aspirating any beads when discarding 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.

IMPORTANT! Cleanup beads must be entirely submerged during the wash steps.

- Remove all traces of ethanol before proceeding with subsequent reactions. However, over-drying the beads can make them difficult to resuspend, which can result in considerable DNA loss.
- The volume of Elution Buffer used to elute the DNA library depends on the downstream workflow and is indicated in the protocol.
- 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 normalization of DNA library concentration

- This manual provides an alternative method for normalizing DNA library concentration prior to exome enrichment, which includes Qubit-based normalization and pooling DNA libraries.
- See “Qubit-based normalization of libraries concentration” on page 18.

Guidelines for target hybridization

The Collibri™ PS DNA Library Prep Kit is compatible with Twist Human Core Exome Hybridization Kits.

Guidelines for evaluating DNA library construction

- Verify the size distribution of the prepared DNA library via electrophoresis using the Agilent™ High Sensitivity DNA Kit on the Agilent™ 2100 Bioanalyzer™ Instrument (or similar) to conduct the analysis.
- To achieve the highest quality sequencing data, it is essential to create optimal cluster densities across the flow cell. Optimizing cluster densities requires accurate DNA library quantification. The most reliable quantification methods are based on qPCR.
- The Collibri™ Library Quantification Kit is recommended for qPCR-based quantification of prepared libraries before sequencing.

3 Methods

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™ PS DNA Library Prep Kit components have been fully thawed on ice and thoroughly mixed before use.
- Keep all enzyme components on ice as much as possible during handling.
- Reaction mixtures prepared from the enzyme mixes (2X End Conversion Master Mix, 7X Ligation Master Mix for PS, and 2X Library Amplification Master Mix) are very viscous and require special attention during pipetting. Pipette viscous solutions slowly and ensure the complete mixing of the reaction mixture by vortexing or pipetting up and down several times as indicated in the protocol.
- You can safely pause the library construction process after the completion of post-ligation cleanup, the post-amplification cleanup steps. These safe stopping points are marked accordingly in the protocol.
- Enriched WES libraries can be stored at –20°C. Use Nuclease-free 1.5-mL tubes, such as Eppendorf™ DNA LoBind™ Microcentrifuge Tubes (or equivalent) for long term storage. When possible, minimize the number of freeze-thaw cycles.

Preparation of single or 8-indexed libraries

Overview

This section describes the DNA fragmentation by physical DNA shearing. 1-8 gDNA samples could be used for one exome enrichment reaction.

Before you begin

This protocol has been optimized using a Covaris™ model E220 instrument and 130 µL Covaris™ microTUBE for shearing 50 µL DNA samples to a target DNA fragment size of 150 to 200 bp. See the manufacturer's recommendations for use of other Covaris™ instruments or sample holders to achieve the same target DNA fragment size.

Fragment the DNA

1. Check that the water in the Covaris™ tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.
2. Check that the water covers the visible glass part of the tube.
3. On the instrument control panel, push the **Degas** button. Degas the instrument for least **30 minutes** before use, or according to the manufacturer's recommendations.
4. Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.
5. Transfer the 50-µl DNA sample into a Covaris™ microTUBE, using a tapered pipette tip to slowly transfer the sample through the pre-split septa of the cap.
6. Spin the microTUBE for **30 seconds** to collect the liquid and to remove any bubbles from the bottom of the tube.
7. Secure the microTUBE in the tube holder and shear the DNA with the settings.

Setting	High-quality DNA
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	280 seconds
Bath Temperature	2°C to 8°C

8. After completing the shearing step(s), put the Covaris™ microTUBE back into the loading and unloading station.
9. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.
10. Transfer the sheared DNA sample (approximately 50 µL) to a 96-well plate or strip tube sample well. Keep the samples on ice.
11. After transferring the DNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in step 10.

IMPORTANT! It is important to avoid loss of input DNA at this step, especially for low-abundance DNA samples. Visually inspect the microTUBE to ensure that all of the sample has been transferred. If droplets remain in the microTUBE, repeat step 11.

End conversion

Overview

This section describes the end-repair and dA-tailing of the input DNA to prepare it for ligation with Illumina®-compatible NGS adaptors. The Collibri™ PS DNA Library Prep Kit combines the end-repair of input DNA and the addition of 3' dA-overhangs in a single one-vial reaction. 1-8 gDNA samples could be used for one exome enrichment reaction.

Before you begin

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

End-repair DNA fragments and add dA-tails

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

Component	Volume
10 mM Tris-HCl, pH 7.5–8.5	to 50 µL
Fragmented DNA (50 ng) (clear ◯)	X µL
2X End conversion Master Mix (blue ●)	25 µL
Total volume (light blue mixture ●):	**50 µL**

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

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

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

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

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

IMPORTANT! Proceed immediately to the next step, "Dual-indexed adaptor ligation" on page 15.

Dual-indexed adaptor ligation

Overview

This section describes the ligation of the Illumina®-compatible NGS adaptors to end-converted DNA fragments. For optimal results we recommend following Illumina® indexed adaptors multiplexing guidelines.

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

Before you begin

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

Ligate the adaptors

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

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

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

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

Note:

- The Collibri™ Dual-Indexed Adaptor plate is stable for a minimum of 10 freeze-thaw cycles.
- **Do not** re-use the same adaptor wells.

3. To prepare the ligation reaction mix, add the 7X Ligation Master Mix for PS to the Adaptor-DNA mixture on ice, then mix the contents vigorously by vortexing.

Component	Volume
Adaptor-DNA mixture (green 🟢)	60 µL
7X Ligation Master Mix for PS (red 🔴)	10 µL
Total volume (purple mixture 🟣):	**70 µL**

IMPORTANT! Observe the color change as each reaction component is added. If the 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 cleanup" on page 16.

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

Post-ligation cleanup

Overview

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

Before you begin

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

Purify the adaptor-ligated library

1. Mix the Dual Index Adaptor-ligated DNA library (70 µL) (from step 5) with 56 µL of DNA Cleanup Beads by vortexing until you have obtained a homogeneous suspension.

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

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

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

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

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

   Note: If the pellet of magnetic beads was disturbed, mix the sample, then 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 step 5 and step 6.

8. To remove the residual ethanol, briefly centrifuge the reaction tube and place it back in the magnetic rack then carefully remove any remaining supernatant with a pipette without disturbing the pellet.

9. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

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

10. Remove the tube from the magnetic rack, add 70 µL of Elution Buffer, then vortex to mix thoroughly.

11. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.

12. Add 70 µL of fresh DNA Cleanup Beads directly to the bead suspension in Elution Buffer, then mix by vortexing until you have obtained a homogeneous suspension.

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

14. Briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.

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

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

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

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

17. Carefully remove and discard the supernatant using a pipette.

18. Repeat step 16 and step 17.

19. To remove the residual ethanol, briefly centrifuge the reaction tube, then place it back in the magnetic rack and carefully remove any remaining supernatant with a pipette without disturbing the pellet.

20. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

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

21. Remove the tube from the magnetic rack, add 25 µL of Elution Buffer, then vortex to mix thoroughly.

22. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.

23. Place the tube in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet. Wait for the solution to clear before proceeding to the next step.

24. Without removing the tube from the magnetic rack, collect 22–23 µL of the supernatant to a new sterile tube for storage.

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

STOPPING POINT Store the eluted DNA library at 4°C for up to 1–2 weeks or at –20°C for long-term storage, or immediately proceed to the next step, “Qubit-based normalization of libraries concentration” on page 18.

Qubit-based normalization of libraries concentration

Overview

This section describes the PCR-based amplification of the purified adaptor-ligated DNA library for 1-8-plex whole exome hybridization reaction.

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

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 24) into a sterile thin-wall 0.2-mL PCR tube on ice, then add the following reagents in the given order.

Component	Volume
Adaptor-ligated DNA library (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	Temperature	Time
Activate the enzyme	1 cycle	98°C	30 seconds
Denature	10 cycles for 50 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

4. After the PCR is completed, proceed with the post-amplification cleanup. See “Purification of the amplified DNA libraries” on page 19.

Purification of the amplified DNA libraries

Overview

This section describes post-amplification cleanup of the DNA library using the DNA Cleanup Beads.

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.
- Gently vortex the DNA Cleanup Beads to completely resuspend the magnetic beads in the solution.
- Perform all cleanup steps at room temperature.

Purify the amplified DNA library

1. Mix the amplified DNA library (50 µL) with 55 µL of DNA Cleanup Beads by vortexing until you have obtained a homogenous suspension.
2. Briefly centrifuge the tube containing the amplified DNA library and bead mixture to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.

   IMPORTANT! Do not extend the binding step to more than 5 minutes. Over-incubation can result in lower DNA yields.
3. Briefly centrifuge the tube to collect all the droplets at the bottom, then place it in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.

   Note: Time required for the complete capture of the cleanup beads can vary depending on the reaction vessel and the magnet used. Optimize the capture time accordingly.
4. Keeping the reaction tube in the magnetic rack, then 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, then 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. Add 65 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.
7. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **5 minutes** at room temperature.
8. If the mixture was disturbed briefly centrifuge the tube to collect all the droplets at the bottom, then place the tube in the magnetic rack for **2 minutes** or until the beads have formed a tight pellet.
9. Keeping the reaction tube in the magnetic rack, then 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, then allow the beads to form a tight pellet on the magnet again.
10. 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.
11. Carefully remove and discard the supernatant using a pipette.
12. Repeat step 10 and step 11.

13. To remove the residual ethanol, briefly centrifuge the reaction tube, then place it back in the magnetic rack and carefully remove any remaining supernatant with a pipette without disturbing the pellet.

14. Keeping the reaction tube on the magnet, air dry the magnetic beads for **1 minute** at room temperature or until there are no droplets of ethanol left on the walls of the tube.

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

15. 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.

16. Briefly centrifuge the tube to collect all the droplets at the bottom, then incubate for **1 minute** at room temperature.

17. 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.

18. 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, then let the beads settle to the side of the tube on the magnet again.

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 exome enrichment.

Verification of quality and quantity of prepared pre-capture 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 and Qubit™ dsDNA HS Assay Kit.

Analyze size distribution of amplified library

1. Remove 1 µL from each prepared DNA library (i.e., purified and amplified DNA from step 18), then dilute it 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 Agilent™ 2100 Bioanalyzer™ Expert software, perform a smear analysis to determine the average library length using a size range of 150–1000 bp.
   - See Figure 1 for an ideal Agilent™ 2100 Bioanalyzer™ trace. If the DNA is high quality, the electropherogram should show a peak positioned between 300 to 400 bp, indicating the average DNA fragment size.

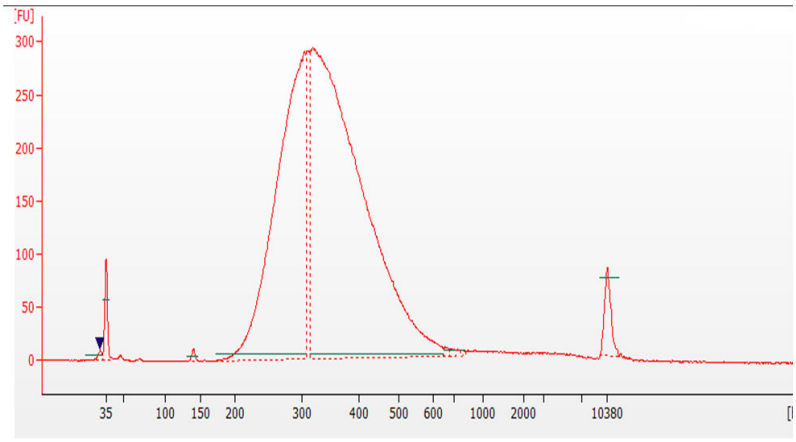


Figure 1 Pre-capture library prepared from a high-quality gDNA sample analyzed using High Sensitivity DNA assay on Agilent™ 2100 Bioanalyzer™.

- The appearance of a sharp low molecular weight (100–150 bp) peak can indicate the presence of adaptor dimers in the library. See Figure 2.

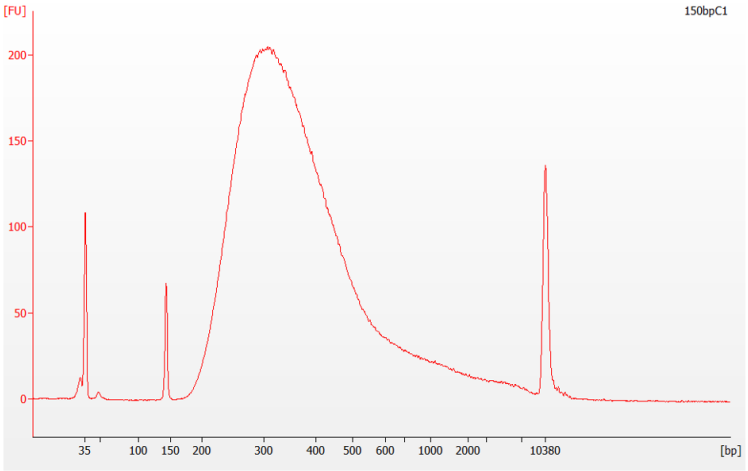


Figure 2 Pre-capture library with the presence of adaptor-dimers (second peak (~150 bp) from the left) prepared from a high-quality gDNA sample analyzed using High Sensitivity DNA assay on Agilent™ 2100 Bioanalyzer™.

- The appearance of a secondary high molecular weight (200-600 bp) peak can indicate the presence of single stranded DNA (ssDNA) fragments in the library. See Figure 2.

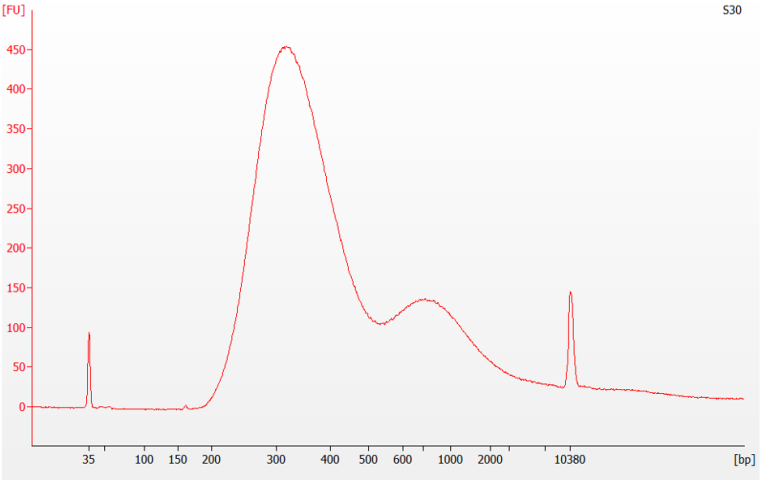


Figure 3 Pre-capture library with the presence of ssDNA peak (second peak (600-2000 bp) from the right) prepared from a high-quality gDNA sample analyzed using High Sensitivity DNA assay on Agilent™ 2100 Bioanalyzer™.

- It is acceptable to proceed to exome enrichment with library samples, whose electropherograms indicate a likely presence of adaptor dimers or secondary ssDNA (See Figure 2 and Figure 3).

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

Determine concentration of amplified library

Measure the concentration of the DNA library using the Qubit™ dsDNA HS Assay Kit with a Qubit™ Fluorometer. Ideally, the final concentration should be ≥50 ng/µL.

Note: The library preparation step may yield more material than needed for the next step. Excess product may be stored at –20°C for later use.

STOPPING POINT You can store the purified DNA libraries at 4°C for up to 1 month. For longer term, store at –20°C until ready for enrichment.

Determine concentration of amplified library

Measure the concentration of the DNA library using the Qubit™ dsDNA HS Assay Kit with a Qubit™ Fluorometer. Ideally, the final concentration should be ≥50 ng/µL.

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STOPPING POINT You can store the purified DNA libraries at 4°C for up to 1 month. For longer term, store at –20°C until ready for enrichment.

A 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, 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 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
  https://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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  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

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