Roche

Sequencing Solutions Technical Note

February 2018

How To…

Construct PCR-free human whole-genome shotgun libraries using the KAPA HyperPrep Kit

Applications
Human Whole-genome Sequencing

Products
KAPA HyperPrep Kit (PCR-free)
KAPA Pure Beads
KAPA Dual-Index Adapters
KAPA Library Quantification Kit

1. Overview

Routine human whole-genome sequencing (WGS) on production-scale Illumina instruments requires highly efficient, streamlined and flexible library preparation protocols. With the KAPA HyperPrep Kit and accessory products, Roche offers a complete library preparation solution that is highly suitable for high-throughput human WGS.

The KAPA HyperPrep protocol requires fragmented DNA as input. It combines enzymatic steps and employs minimal bead-based cleanups to reduce sample handling and overall library preparation time to 2 – 3 hr. The core library construction workflow consists of an *end repair and A-tailing step*, which produces end-repaired, 5'-phosphorylated, 3'-dA-tailed dsDNA fragments; and an *adapter ligation step,* during which full-length double-stranded DNA (dsDNA) adapters with 3'-dTMP overhangs are ligated to 3'-dA-tailed molecules.

Since Covaris shearing yields relatively broad fragment size distributions in the optimal range for human WGS, size selection is inevitable when preparing libraries for this application. With the flexible KAPA HyperPrep Kit, tunable size selection with KAPA Pure Beads may be performed at different stages of the protocol: directly after DNA fragmentation, thus prior to the end repair/A-tailing reaction (post-fragmentation), or after the adapter ligation cleanup step (post-ligation).

This Technical Note provides a detailed protocol for the construction of DNA libraries for human WGS using the KAPA HyperPrep Kit. Two strategies for the preparation of PCR-free libraries, using either post-fragmentation or post-ligation size selection, are covered. Library construction metrics and sequencing results generated with these methods may be found in an accompanying Application Note.[1]

2. TABLE OF CONTENTS

3. REQUIRED REAGENTS

The following reagents are supplied by Roche:

- KAPA HyperPrep Kit (PCR-Free)
  - Roche PN: 07962339001 (8 libraries)
  - Roche PN: 07962355001 (24 libraries)
  - Roche PN: 07962371001 (96 libraries)
- KAPA Dual-Indexed Adapters (Roche PN: 08278555702)
- KAPA Pure Beads
  - Roche PN: 07983298001 (60 mL)
  - Roche PN: 07983280001 (30 mL)
  - Roche PN: 07983271001 (5 mL)
- Library Quantification Kit for Illumina platforms
  - Roche PN: 07960140001 (Universal qPCR Master Mix)
  - Roche PN: 07960204001 (ABI Prism qPCR Master Mix)
  - Roche PN: 07960255001 (Bio-Rad iCycler qPCR Master Mix)
  - Roche PN: 07960336001 (ROX Low qPCR Master Mix)
  - Roche PN: 07960298001 (qPCR Master Mix optimized for LightCycler 480)

The following reagents are not supplied by Roche, and must be sourced from general laboratory stocks or third-party suppliers:

- Freshly prepared 80% ethanol
- Elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5 at 25°C)
- Nuclease-free water

The following equipment and plastic ware are required for this workflow:

- Covaris E220 or S220 instrument
- Covaris microTUBE AFA Fiber 6x16mm with Pre-Slit Snap-Cap
- Thermocyclers (for standard and qPCR)
- PCR tubes/96-well PCR plates (0.2 mL)
- qPCR tubes/plates
- Magnetic block e.g DynaMag 96 Side Magnet (ThermoFisher Scientific Catalog number: 12331D)
- Pipettes
- Low DNA-binding microtubes (1.5 mL)

4. Workflow Overview

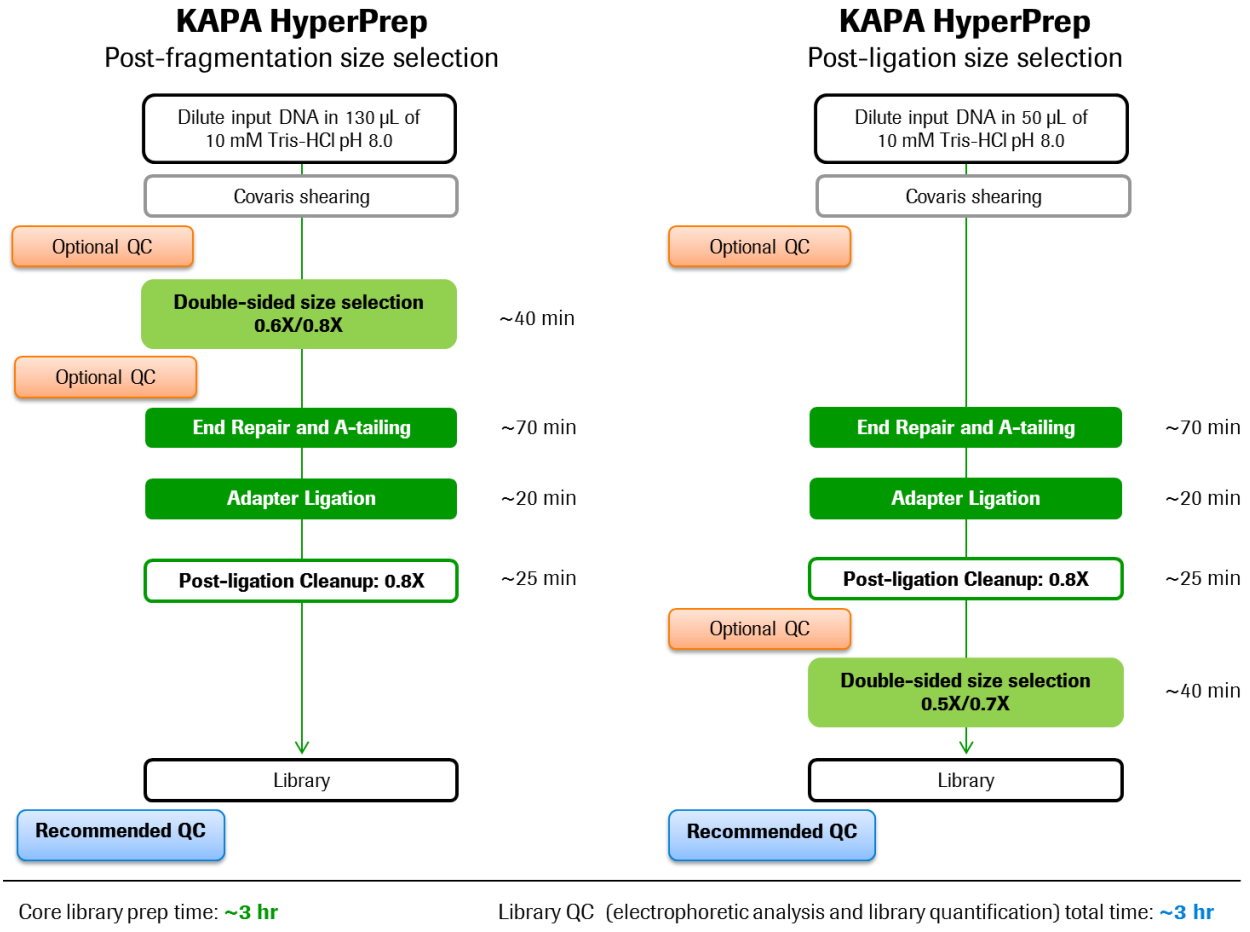

Figure 1: PCR-free KAPA HyperPrep workflows for human WGS. Input DNA (500 ng) is sheared using a Covaris instrument. Detailed shearing parameters for a Covaris E220 or S220 instrument are provided. Optimal shearing parameters for other instruments will have to be optimized. Size selection may be performed at different stages of the HyperPrep workflow: directly after DNA fragmentation (prior to the end repair/A-tailing reaction), or after adapter ligation (after the post-ligation cleanup). Optional QC steps may be performed where indicated, and described throughout ***Sections 6 – 7***. Core library prep times do not include DNA quantification, Covaris shearing or library QC (quantification and size distribution assessment). These workflows require approximately 3 hours to complete in our hands, but may require more or less time, depending on specific skills and laboratory conditions.

5. QUALITY CONTROL OF INPUT DNA

Quality control of input DNA is strongly recommended. DNA may be quantified using an Invitrogen Qubit Fluorometer and Qubit quantitation assays (ThermoFisher Scientific), or the Quant-iT PicoGreen dsDNA Assay Kits (ThermoFisher Scientific). These assays are designed to provide an accurate concentration of double-stranded DNA (dsDNA) molecules in the sample. An electrophoretic profile of the DNA is also useful to confirm DNA quality prior to fragmentation. Agarose gel electrophoresis is sufficient for this purpose. Alternatively, a genomic DNA (gDNA) assay kit may be used in conjunction with a LabChip GX, GXII or GX Touch instrument (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), or Fragment Analyzer (Advanced Analytical) instrument to assess DNA quality.

QUALITY CONTROL 1 (Recommended)

- Performing quality control of the sheared DNA is strongly recommended at this stage.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
  - Electrophoretic analysis to determine quality of input DNA
  - Fluorometric analysis to quantify input DNA

6. LIBRARY CONSTRUCTION PROTOCOLS

Post-Fragmentation (PF) Size Selection Library Construction Protocol

This protocol describes library construction using the KAPA HyperPrep Kit with size selection after Covaris shearing (post-fragmentation) and is based on the protocol outlined in the KAPA HyperPrep Kit Instructions for Use (KR0961 v6.17 or later).[2] Optimal shearing parameters for Covaris instruments other than the E220 and S220 will have to be empirically determined.

Covaris Shearing

1. Prepare a Covaris E220 or S220 instrument as per the manufacturer's instructions.
2. Dilute input DNA to 3.85 ng/µL and transfer 130 µL into a Covaris microTUBE AFA Fiber 6x16mm with Pre-Slit Snap-Cap.
3. Shear using the following settings:
    a. Duty factor: 5%
    b. Peak incident power: 175 W
    c. Time: 50 s
    d. Cycles per burst: 200
    e. Power mode: frequency sweeping
    f. Temperature of water bath: 6°C
4. Transfer the total volume of sheared DNA to a new plate/tube(s) and proceed to ***Post-Fragmentation Double-sided Size Selection.***

QUALITY CONTROL 2 (Optional)

- Performing quality control of the sheared DNA is recommended at this stage.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
    - Electrophoretic analysis to determine fragment size and distribution
    - Fluorometric analysis to quantify sheared DNA
- The Covaris shearing settings provided above are expected to yield DNA with the fragment size distribution depicted in ***Figure 2***.

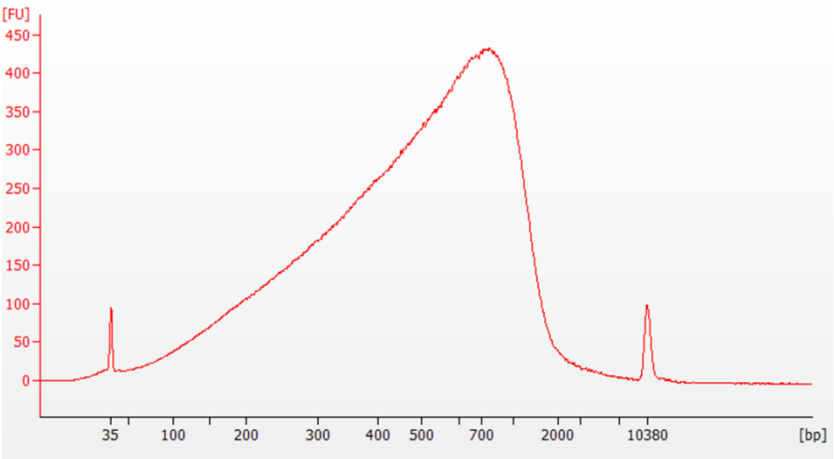

Figure 2: Expected size distribution profile of fragmented DNA sheared using a Covaris E220 instrument. Human gDNA was diluted to 3.85 ng/µL and a volume of 130 µL sheared in a Covaris microTUBE AFA Fiber 6x16mm with Pre-Slit Snap-Cap using the following parameters: Duty factor: 5%; Peak incident power: 175 W; Time: 50 s; Cycles per burst: 200; Power mode: frequency sweeping, and Temperature of water bath: 6°C. For size determination, a 1/5 dilution of each sample was analyzed using an Agilent 2100 Bioanalyzer instrument.

Post-Fragmentation Double-Sided Size Selection

1. Perform the first size cut (to exclude large DNA fragments) by adding 0.6X volume of KAPA Pure Beads to the sample:

Component	Volume
DNA sample	130 µL
KAPA Pure Beads	78 µL
Total Volume	**208 µL**

2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3. Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully transfer 200 µL of supernatant (containing DNA fragments smaller than those that were intentionally excluded) to a new plate/tube(s). It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) with beads to which the unwanted, large DNA fragments are bound.
6. Perform the second size cut by adding 0.2X volume of KAPA Pure Beads to the supernatant from the first size cut:

Component	Volume
Supernatant from first size cut	200 µL
KAPA Pure Beads	26 µL
Total Volume	**226 µL**

7. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
8. Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
9. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
10. Carefully remove and discard the supernatant.

11. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
12. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
13. Carefully remove and discard the ethanol.
14. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
15. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
16. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
17. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    Caution: over-drying the beads may result in reduced yield.
18. Remove the plate/tube(s) from the magnet.
19. Thoroughly resuspend the beads in 55 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).
20. **Important note:** Incubate the plate/tube(s) at 37°C for 10 min to elute DNA off the beads.
21. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
22. Transfer 50 µL of the clear supernatant to a new plate/tube(s) and proceed to ***End Repair and A-tailing.***

QUALITY CONTROL 3 (Optional)

- Performing quality control of the size selected, sheared DNA is recommended at this stage.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
  - Electrophoretic analysis to determine fragment size and distribution
  - Fluorometric analysis to quantify size selected, sheared DNA
- The expected electrophoretic profile for size selected, sheared DNA using the suggested Covaris settings and 0.6X – 0.8X bead-to-sample ratio is depicted in ***Figure 3***.

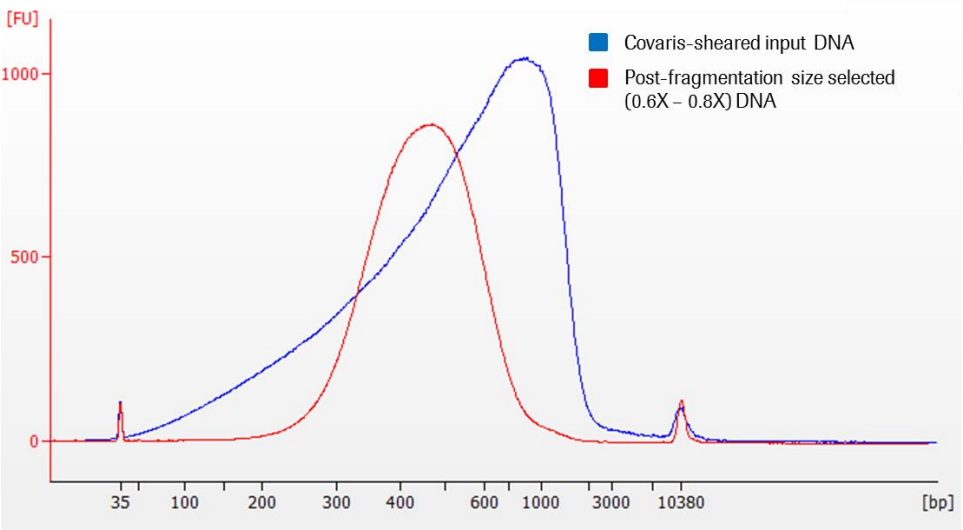

Figure 3: Expected size distribution profile of Covaris-sheared DNA subjected to a 0.6X – 0.8X size selection. Input DNA was sheared using Covaris E220 instrument (blue curve) as described above. Fragmented DNA was subjected to a 0.6X – 0.8X double-sided size selection using KAPA Pure Beads, to generate the size-selected DNA represented by the red curve. For size determination, samples were analyzed without dilution using an Agilent 2100 Bioanalyzer instrument.

End Repair and A-tailing

1. Assemble each end repair and A-tailing reaction in a tube or well of a PCR plate as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End Repair & A-tailing Buffer	7 µL
End Repair & A-tailing Enzyme Mix	3 µL
Total Volume	**60 µL**

* The buffer and enzyme mix should preferably be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hr at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

2. Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.
3. Incubate in a thermocycler programmed as outlined below:

Step	Temp	Time
End Repair and A-tailing	20°C	30 min
	65°C*	30 min
HOLD	4°C**	∞

* A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C, instead of the usual ~105°C.
** If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

4. Proceed immediately to ***Adapter Ligation.***

Adapter Ligation

1. In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

Component	Volume
End repair and A-tailing reaction product	60 µL
KAPA Dual-Indexed Adapters (15 µM)	5 µL
PCR-Grade water*	5 µL
Ligation Buffer*	30 µL
DNA Ligase*	10 µL
Total Volume	**110 µL**

* The water, buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hr at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

2. Mix thoroughly and centrifuge briefly.
3. Incubate at 20°C for 15 min.
4. Proceed immediately to ***Post-Ligation Cleanup***

Post-Ligation Cleanup

1. In the same plate/tube(s), perform a 0.8X bead-based cleanup by combining the following:

Component	Volume
Adapter ligation reaction product	110 µL
KAPA Pure Beads	88 µL
Total Volume	**198 µL**

2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3. Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.
4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    Caution: over-drying the beads may result in reduced yield.
13. Remove the plate/tube(s) from the magnet.
14. Thoroughly resuspend the beads in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).
15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer the clear supernatant to a new plate/tube(s) and proceed with ***library quantification and quality control.***

QUALITY CONTROL 4 (Recommended)

- Performing quality control of the final, sequence-ready, adapter-ligated library is strongly recommended.
- Refer to ***Section 7:*** *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
  - Electrophoretic analysis to determine final library size and distribution
  - qPCR quantification of adapter-ligated library
- The expected electrophoretic profile for a post-fragmentation size selected (using a 0.6X – 0.8X bead-to-sample ratio) DNA library is depicted in ***Figure 4***.

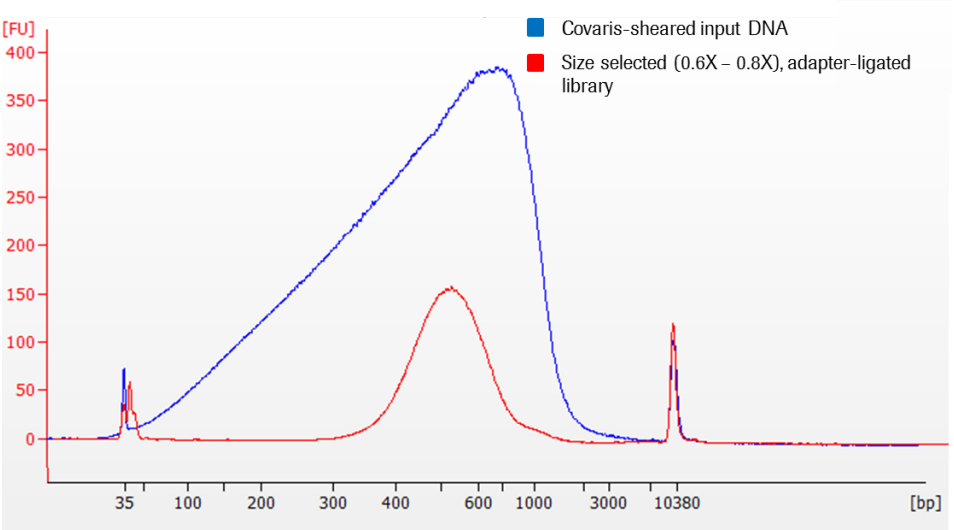

Figure 4: Expected size distribution of post-fragmentation size selected KAPA HyperPrep libraries. Input DNA (blue curve) was sheared using the Covaris E220 instrument as described above. Fragmented DNA was subjected to 0.6X – 0.8X double-sided size selection prior to end repair/A-tailing (see Fig 3). An aliquot (20%) of the adapter-ligated library (red curve) was amplified for five cycles for reliable fragment length determination (see ***Section 7***, **Evaluating the Success of Library Construction** for further details). For size determination, a 1/5 dilution of each sample was analyzed using an Agilent 2100 Bioanalyzer instrument.

Post-Ligation (PL) Size Selection Library Construction Protocol

This protocol describes library construction using the KAPA HyperPrep Kit with size selection after adapter ligation and is based on the protocol outlined in the KAPA Hyper Prep Kit Instructions for Use (KR0961 v6.17 or later).[2] Optimal shearing parameters for Covaris instruments other than the E220 and S220 will have to be empirically determined.

Covaris Shearing

1. Prepare a Covaris E220 or S220 instrument as per the manufacturer's instructions.
2. Dilute input DNA to 10 ng/µL and transfer 50 µL into a Covaris microTUBE AFA Fiber 6x16mm with Pre-Slit Snap-Cap.
3. Shear using the following settings:
    a. Duty factor: 5%
    b. Peak incident power: 175 W
    c. Time: 50 s
    d. Cycles per burst: 200
    e. Power mode: frequency sweeping
    f. Temperature of water bath: 6°C
4. Transfer the total volume of sheared DNA to a new plate/tube(s) and proceed to ***End Repair and A-tailing*.**

QUALITY CONTROL 2 (Optional)

- Performing quality control of the sheared DNA is recommended at this stage.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
    - Electrophoretic analysis to determine fragment size and distribution
    - Fluorometric analysis to quantify sheared DNA
- The Covaris shearing settings given above are expected to yield DNA with the fragment size distribution depicted in ***Figure 5***.

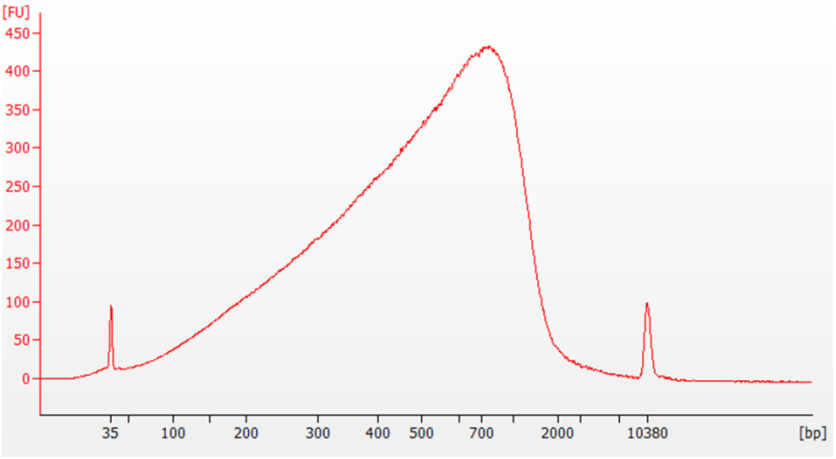

Figure 5: Expected size distribution profile of fragmented DNA sheared using a Covaris E220 instrument. Human gDNA was diluted to 10 ng/µL and a volume of 50 µL sheared in a Covaris MicroTUBE AFA Fiber 6x16mm with Pre-Slit Snap-Cap using the following parameters: Duty factor: 5%; Peak incident power: 175 W; Time: 50 s; Cycles per burst: 200; Power mode: frequency sweeping, and Temperature of water bath: 6°C. For size determination, a 1/5 dilution of each sample was analyzed using an Agilent 2100 Bioanalyzer instrument.

End Repair and A-tailing

1. Assemble each end repair and A-tailing reaction in a tube or well of a PCR plate as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End Repair & A-tailing Buffer	7 µL
End Repair & A-tailing Enzyme Mix	3 µL
Total Volume	**60 µL**

* The buffer and enzyme mix should preferably be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hr at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

2. Vortex gently and spin down briefly. Return the plate/tube(s) to ice.
3. Proceed immediately to the next step.
4. Incubate in a thermocycler programmed as outlined below:

Step	Temp	Time
End Repair and A-tailing	20°C	30 min
	65°C	30 min
HOLD	4°C	∞

* A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C, instead of the usual ~105°C.
** If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

5. Proceed immediately to ***Adapter Ligation.***

Adapter Ligation

1. In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

Component	Volume
End repair and A-tailing reaction product	60 µL
KAPA Dual-Indexed Adapters (15 µM)	5 µL
PCR-Grade water*	5 µL
Ligation Buffer*	30 µL
DNA Ligase*	10 µL
Total Volume	**110 µL**

* The water, buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hr at room temperature, for ≤3 days at 4°C, and for ≤4 weeks at -20°C.

2. Mix thoroughly and centrifuge briefly.
3. Incubate at 20°C for 15 min.
4. Proceed immediately to ***Post-Ligation Cleanup.***

Post-Ligation Cleanup

1. In the same plate/tube(s), perform a 0.8X bead-based cleanup by combining the following:

Component	Volume
Adapter ligation reaction product	110 µL
KAPA Pure Beads	88 µL
Total Volume	**198 µL**

2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3. Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.
4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
8. Carefully remove and discard the ethanol.
9. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    Caution: over-drying the beads may result in reduced yield.
13. Remove the plate/tube(s) from the magnet.
14. Thoroughly resuspend the beads in 55 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with double-sided size selection.
15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer 50 µL of the clear supernatant to a new plate/tube(s) and proceed with ***Post-Ligation Double-Sided Size Selection.***

QUALITY CONTROL 4 (Optional)

- Performing quality control of the adapter-ligated library, prior to size selection is recommended at this stage.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
  - qPCR quantification of adapter-ligated library

Post-Ligation Double-Sided Size Selection

1. Perform the first size cut (to exclude large DNA library molecules) by adding 0.5X volume of KAPA Pure Beads to the DNA:

Component	Volume
DNA to be size-selected	50 µL
KAPA Pure Beads	25 µL
Total Volume	**75 µL**

2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3. Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully transfer 70 µL of supernatant (containing library molecules smaller than those that were intentionally excluded) to a new plate/tube(s). It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) with beads to which the unwanted, large library molecules are bound.
6. Perform the second size cut by adding 0.2X volume of KAPA Pure Beads to the supernatant from the first size cut:

Component	Volume
Supernatant from first size cut	75 µL
KAPA Pure Beads	10 µL
Total Volume	**85 µL**

7. Mix thoroughly by vortexing and/or pipetting up and down multiple times.
8. Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
9. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
10. Carefully remove and discard the supernatant.
11. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
12. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
13. Carefully remove and discard the ethanol.
14. Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
15. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
16. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
17. Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated.

    Caution: over-drying the beads may result in reduced yield.
18. Remove the plate/tube(s) from the magnet.
19. Thoroughly resuspend the beads in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).
20. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
21. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
22. Transfer the clear supernatant to a new plate/tube(s) and proceed with ***library quantification and quality control***.

QUALITY CONTROL 5 (Recommended)

- Performing quality control of the final, sequence-ready, adapter-ligated library is strongly recommended.
- Refer to ***Section 7***: *Evaluating the Success of Library Construction* and ***Table 1***.
- Recommended QC assays:
  - Electrophoretic analysis to determine final library size and distribution
  - qPCR quantification of size selected, adapter-ligated library
- The expected electrophoretic profile for a post-ligation size selected DNA library using a 0.5X – 0.7X bead-to-sample ratio is depicted in ***Figure 6***.

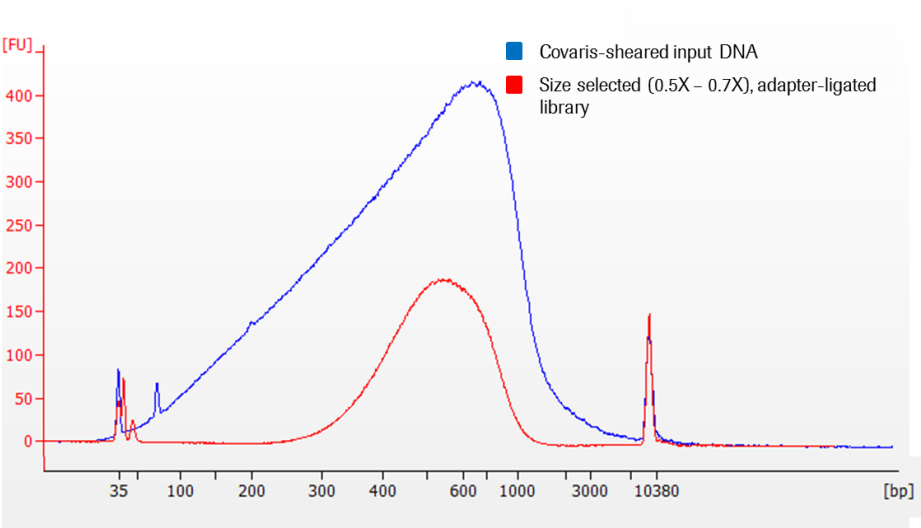

Figure 6: Expected size distribution of post-ligation size selected KAPA HyperPrep libraries. Input DNA (blue curve) was sheared using the Covaris E220 instrument as described above and used as input into end repair/A-tailing reaction. Adapter-ligated libraries were subjected to 0.5X - 0.7X double-sided size selection. An aliquot (20%) of the adapter-ligated library (red curve) was amplified for five cycles for reliable fragment length determination (see ***Section 7***, **Evaluating the Success of Library Construction** for further details). For size determination, a 1/5 dilution of each sample was analyzed using an Agilent 2100 Bioanalyzer instrument.

7. Evaluating the Success of Library Construction

Quality control (QC) may be performed at several stages during the library construction workflow (Table 1): before/after Covaris shearing; before/after size selection and after adapter ligation and cleanup. Most of these are optional QC steps, but provide valuable information relating to the size, concentration and/or quality of the DNA fragments. These QC steps also serve as checkpoints, should troubleshooting of the workflow be necessary.

Library Size Distribution

The size distribution of final, adapter-ligated libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII or GX Touch (PerkinElmer) instrument, Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer (Advanced Analytical) instrument or similar instrument is recommended over conventional gels.

In PCR-free workflows, it is difficult to obtain an accurate average library size from routinely-used electrophoretic systems, as molecules flanked by adapters with long single-stranded portions migrate anomalously in gel matrices employed in these systems (thereby appearing to be longer than they truly are).

The difference in overall appearance and library size distribution of an unamplified vs. the corresponding amplified library varies, and depends on the adapter design and electrophoretic system used. Thus electrophoretic evaluation of libraries after the post-ligation cleanup may be informative, but the apparent mode fragment length and size distribution will be inaccurate due to the retardation of non-complementary adapter regions, as illustrated in ***Figure 7***.

Since the average fragment size is imperative for qPCR quantification of PCR-free libraries, several easy workarounds to address this problem are described below:

- **Recommended:** Amplify an aliquot (20%) of the PCR-free library for a few cycles prior to assessment using electrophoretic analysis. Amplification will render all molecules fully double-stranded, and yield a reliable size determination from the electrophoretic assay. KAPA Library Amplification Kits, which contain KAPA HiFi DNA polymerase, are recommended for this purpose.[4]
- Use the average length of the fragmented DNA plus the total length of two adapters (usually ~120 bp) as an estimate for the average library fragment size in concentration calculations.
- Subject the product of the library quantification reaction to electrophoretic analysis. The library quantification reaction is performed for 35 cycles, and will contain artefacts generated in later cycles impacted by reagent depletion—but the main peak will provide a good indication of the average fragment size of the quantified library.

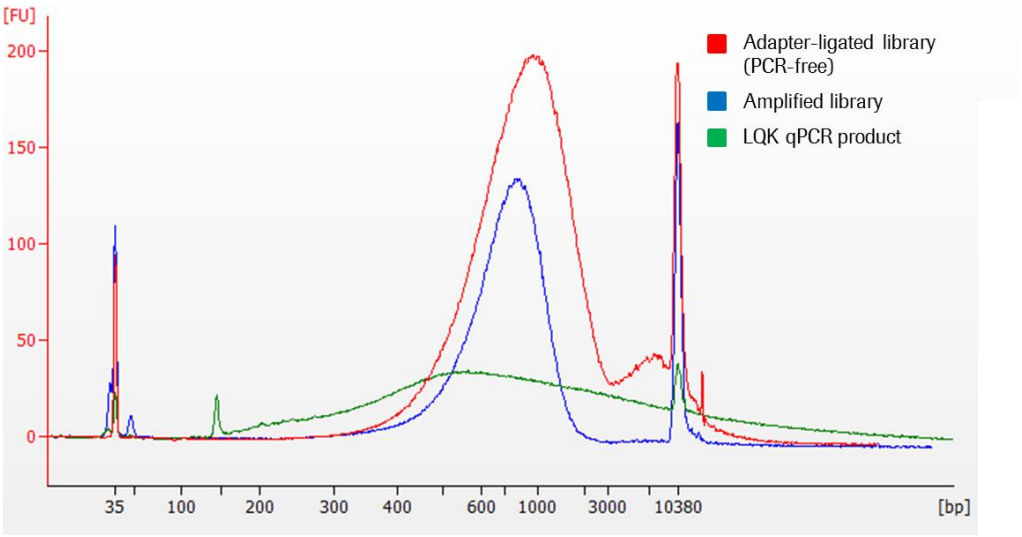

Figure 7: Methods for determining the true average fragment size of human WGS libraries produced in PCR-free workflows, for qPCR-based library concentration calculations. Libraries were prepared from gDNA sheared to a mode fragment length of ~650 bp with the KAPA HyperPrep Kit, as described in **Post-Ligation (PL) Size Selection Library Construction Protocol**. Unamplified libraries (red curve) have a significantly longer apparent average fragment length, due to the anomalous migration of inserts flanked by adapters with single-stranded terminals. Amplification for five cycles of an aliquot (20%) of a PCR-free library results in reliable fragment length determination (blue curve). The product of the library quantification assay (green curve) may provide a reasonable estimate if amplification of a part of the library for the purpose analysis is not feasible. For size determination, a 1/5 dilution of the amplified library was analyzed using an Agilent 2100 Bioanalyzer instrument. The adapter-ligated library and qPCR product were analyzed without dilution.

Library Quantification

KAPA Library Quantification Kits are recommended for the absolute, qPCR-based quantification of human WGS libraries, particularly those produced in PCR-free workflows, flanked by the P5 and P7 Illumina flow cell oligo sequences. For a detailed protocol, please refer to the KAPA Library Quantification Kit for Illumina platforms Instructions for Use (KR0405 v9.17 or later).[3] Standard methods used for NGS library quantification have a number of disadvantages, particularly when used to quantify libraries produced in PCR-free workflows that do not include an enrichment step for sequencing-competent molecules. Most notably, fluorometry (as employed in Qubit/PicoGreen assays), spectrophotometry (on which the Nanodrop instrument is based) and electrophoretic methods (e.g. those performed using an Agilent Bioanalyzer or TapeStation instrument) measure total nucleic acid concentrations. In contrast, qPCR is inherently well-suited for NGS library quantification, as it measures only those library fragments that can serve as templates during cluster generation. Moreover, because qPCR is extremely sensitive, it allows for the quantification of dilute libraries and consumes only small amounts of library.

Libraries must be diluted to fall within the dynamic range of the assay, i.e., 20 – 0.0002 pM. Adapter-ligated libraries prepared using the workflows described typically require a dilution in the range of 1/500 to 1/10,000 to fall within the dynamic range of the assay. A KAPA Library Quantification Data Analysis template, designed for the analysis of NGS library quantification data generated with the KAPA Library Quantification Kit for Illumina platforms, is available from local Roche regional sales/support teams. Since an accurate assessment of the average library size is required for qPCR quantification of PCR-free libraries, we would recommend one of the workarounds described above (see **Library Size Distribution**).

QC step	Workflow	Workflow Step	Recommended QC metrics	Rationale
1	PF & PL*	Input DNA (prior to fragmentation)	Invitrogen Qubit Fluorometer and Qubit quantitation assays (ThermoFisher Scientific), or the Quant-iT PicoGreen dsDNA Assay Kits (ThermoFisher Scientific).	Provides accurate concentration of dsDNA molecules in the sample.
			Genomic DNA assay in conjunction with a LabChip GX, GXII or GX Touch instrument (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), or a Fragment Analyzer instrument (Advanced Analytical). Agarose gel electrophoresis may also be used.	An electrophoretic profile of the input DNA is recommended to confirm DNA quality (molecular weight) prior to fragmentation.
2	PF & PL	After Covaris shearing (post-fragmentation)	Invitrogen Qubit Fluorometer and Qubit quantitation assays (ThermoFisher Scientific), or the Quant-iT PicoGreen dsDNA Assay Kits (ThermoFisher Scientific).	Once DNA is fragmented, the output can be quantified to determine: a) DNA loss associated with mechanical fragmentation; and b) establish the actual input amount into library construction/size selection.
			LabChip GX, GXII or GX Touch instrument (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer instrument (Advanced Analytical)	An electrophoretic profile of the fragmented DNA confirms the fragment size and distribution.
3	PF	Post-fragmentation 0.6X - 0.8X size selection	Invitrogen Qubit Fluorometer and Qubit quantitation assays (ThermoFisher Scientific), or the Quant-iT PicoGreen dsDNA Assay Kits (ThermoFisher Scientific).	Quantification of fragmented, size-selected DNA allows for the determination of: a) DNA loss associated with size selection; and b) establish the actual input amount into library construction.
			LabChip GX, GXII or GX Touch instrument (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer instrument (Advanced Analytical)	An electrophoretic profile of the fragmented, size-selected input DNA allows for the verification of the final insert size range prior to library construction.
4	PF & PL	Post-ligation 0.8X cleanup	qPCR (KAPA Library Quantification Kits)	Accurate quantification of post-ligation DNA libraries is essential to ensure optimal pooling and utilization of sequencing capacity.
			LabChip GX, GXII or GX Touch (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer instrument (Advanced Analytical)	The size distribution of adapter-ligated final libraries should be confirmed with an electrophoretic method prior to sequencing. (See **Library size distribution** above).
5	PL	Post-ligation 0.5X - 0.7X size selection	qPCR (KAPA Library Quantification Kits)	Accurate quantification of post-ligation DNA libraries is essential to ensure optimal pooling and utilization of sequencing capacity. qPCR quantification before and after size selection may also be helpful to determine the loss of material associated with the process.
			LabChip GX, GXII or GX Touch (PerkinElmer), Bioanalyzer or TapeStation instrument (Agilent Technologies), Fragment Analyzer instrument (Advanced Analytical)	The size distribution of adapter-ligated final libraries should be confirmed with an electrophoretic method prior to sequencing. (See **Library size distribution** above).

Table 1: Recommended QC steps during library construction. * PF = post-fragmentation size selection; PL = post-ligation size selection

8. ALTERNATIVES

- AMPure XP reagent (Beckman Coulter) may be used instead of KAPA Pure Beads for all bead cleanups

9. REFERENCES

1. Appel et al. (2018). KAPA HyperPrep Kits offer a flexible, high efficiency library preparation solution for PCR-free human whole-genome sequencing. Roche Application Note, SEQ100056
2. KAPA HyperPrep Kit Instructions for Use, v6.17 or later. Roche document number KR0961
3. KAPA Library Quantification Kit Illumina Platforms Instructions for Use, v9.17 or later. Roche document number KR0405
4. KAPA Library Amplification Kit Illumina Platforms Instructions for Use, v8.17 or later. Roche document number KR0408

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