Sequencing Solutions Technical Note

November 2020

How To…

Use MagNA Pure 24 and MagNA Pure 96 Sample Eluates with KAPA HyperPrep and KAPA HyperPlus Kits

Applications
Human whole-genome sequencing
Human exome sequencing
Targeted sequencing

Products
MagNA Pure 24 System
MagNA Pure 96 System
KAPA HyperPrep Kit
KAPA HyperPlus Kit
KAPA Library Quantification Kit

1. Overview

Commonly used next-generation sequencing (NGS) library preparation methods employ T4 DNA ligase to join sequencer-specific adapters to the ends of double-stranded DNA fragments (input DNA), thereby providing the pre-defined sequences necessary for the amplification and qPCR-based quantification of library molecules, as well as for clonal amplification and multiplexed sequencing. For optimal library construction efficiency, input DNA should be of a high quality (free of single-stranded molecules and molecular damage that cannot be repaired by the enzymes typically utilized in the library construction process). Furthermore, input DNA should be chemically compatible with the enzymatic processes used in NGS library construction.

The output of many nucleic extraction methods was not originally optimized for NGS applications, and employ high elution temperatures and/or buffers that are not compatible with library construction enzymes. Several protocols for the Roche MagNA Pure 24 and MagNA Pure 96 Nucleic Acid Extraction Systems were optimized for NGS library construction, and have been validated for use with KAPA HyperPrep and KAPA HyperPlus library construction kits.

KAPA HyperPrep Kits utilize DNA fragmented biologically (e.g. cell-free DNA) or by mechanical or enzymatic means as the input into library construction. Fragmented DNA suspected of containing inhibitors, enzymes or buffer components carried over from the DNA extraction or fragmentation process may be purified or re-buffered using commonly available bead- or column-based DNA purification systems, prior to the first enzymatic step of the KAPA HyperPrep protocol.

The KAPA HyperPlus Kit employs the same core library construction protocol and chemistries as the KAPA HyperPrep Kit, but contains an integrated enzymatic fragmentation module. This allows for all the processes, from DNA fragmentation to adapter ligation to be completed in a single tube. The enzymatic fragmentation module (KAPA Frag) is sensitive to high levels of salts and Ethylenediaminetetraacetic acid (EDTA) in DNA extracts.

This Technical Note provides specific instructions for using DNA extracts generated with NGS-qualified MagNA Pure protocols as the input into the KAPA HyperPrep and KAPA HyperPlus protocol.

Specific instructions for the use of the MagNA Pure 24 Total NA Isolation Kit or MagNA Pure 96 DNA and Viral NA Small Volume Kit in combination with NGS-qualified methods are not included in this document, but may be found in the Instructions for Use.[1,2] Detailed protocols for the KAPA HyperPrep Kit may be found in the KAPA HyperPrep Kit Technical Data Sheet (v8.20 or later),[3] whereas detailed protocols for the KAPA HyperPlus Kit may be found in the KAPA HyperPlus Kit Technical Data Sheet (v6.20 or later).[4]

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3. REQUIRED REAGENTS

The following materials and devices are required for extraction using the MagNA Pure 24 or MagNA Pure 96 System and are supplied by Roche:

If using the MagNA Pure 24 instrument, the following materials and devices are required:

- MagNA Pure 24 Instrument (Roche PN: 07290519001)
- MagNA Pure 24 Total NA Isolation Kit (Roche PN: 07658036001)
- MagNA Pure 24 MGP Set (Roche PN: 07806361001)
- MagNA Pure 24 Processing Cartridge (Roche PN: 07345577001)
- MagNA Pure 24 Processing Tip Park / Piercing Tool (Roche PN: 07345585001)
- MagNA Pure 24 Piercing Tool (Roche PN: 07534205001)
- MagNA Pure Tip 1000 µL (Roche PN: 06241620001)
- MagNA Pure Sealing Foil (Roche PN: 06241638001)
- MagNA Pure Tube 2.0 mL (Roche PN: 07857551001)
- FrameStrip® Tube Strip with flat caps – Low Profile (Roche PN: 07345593001)
- FrameStrip® Tube Strip with flat caps – High Profile (Roche PN: 07652275001)

If extracting cfNA from plasma, the following reagent set is required in addition to the above:

- MagNA Pure cfNA Buffer Set (Roche PN: 07794398001)

If using the MagNA Pure 96 instrument, the following materials and devices are required:

- MagNA Pure 96 Instrument (Roche PN: 06541089001)
- MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche PN: 06543588001)
- MagNA Pure 96 System Fluid (Internal) (Roche PN: 06430112001)
- MagNA Pure 96 System Fluid (External) (Roche PN: 06640729001)
- MagNA Pure 96 Filter Tips (1000 µl) (Roche PN: 06241620001)
- MagNA Pure 96 Processing Cartridge (Roche PN: 06241603001)
- MagNA Pure 96 Output Plate (Roche PN: 06241611001)
- MagNA Pure 96 Internal Control Tube (Roche PN: 06374905001)
- MagNA Pure 96 Sealing Foil (Roche PN: 06241638001)

The following reagents are supplied by Roche for the KAPA HyperPrep workflow:

- KAPA HyperPrep Kit
    - Roche PN: 07962312001 (8 libraries)
    - Roche PN: 07962339001 (8 libraries; PCR-free)
    - Roche PN: 07962347001 (24 libraries)
    - Roche PN: 07962355001 (24 libraries; PCR-free)
    - Roche PN: 07962363001 (96 libraries)
    - Roche PN: 07962371001 (96 libraries; PCR-free)

The following reagents are supplied by Roche for the KAPA HyperPlus workflow:

- KAPA HyperPlus Kit
    - Roche PN: 07962380001 (8 libraries)
    - Roche PN: 07962398001 (8 libraries; PCR-free)
    - Roche PN: 07962401001 (24 libraries)
    - Roche PN: 07962410001 (24 libraries; PCR-free)
    - Roche PN: 07962428001 (96 libraries)
    - Roche PN: 07962436001 (96 libraries; PCR-free)

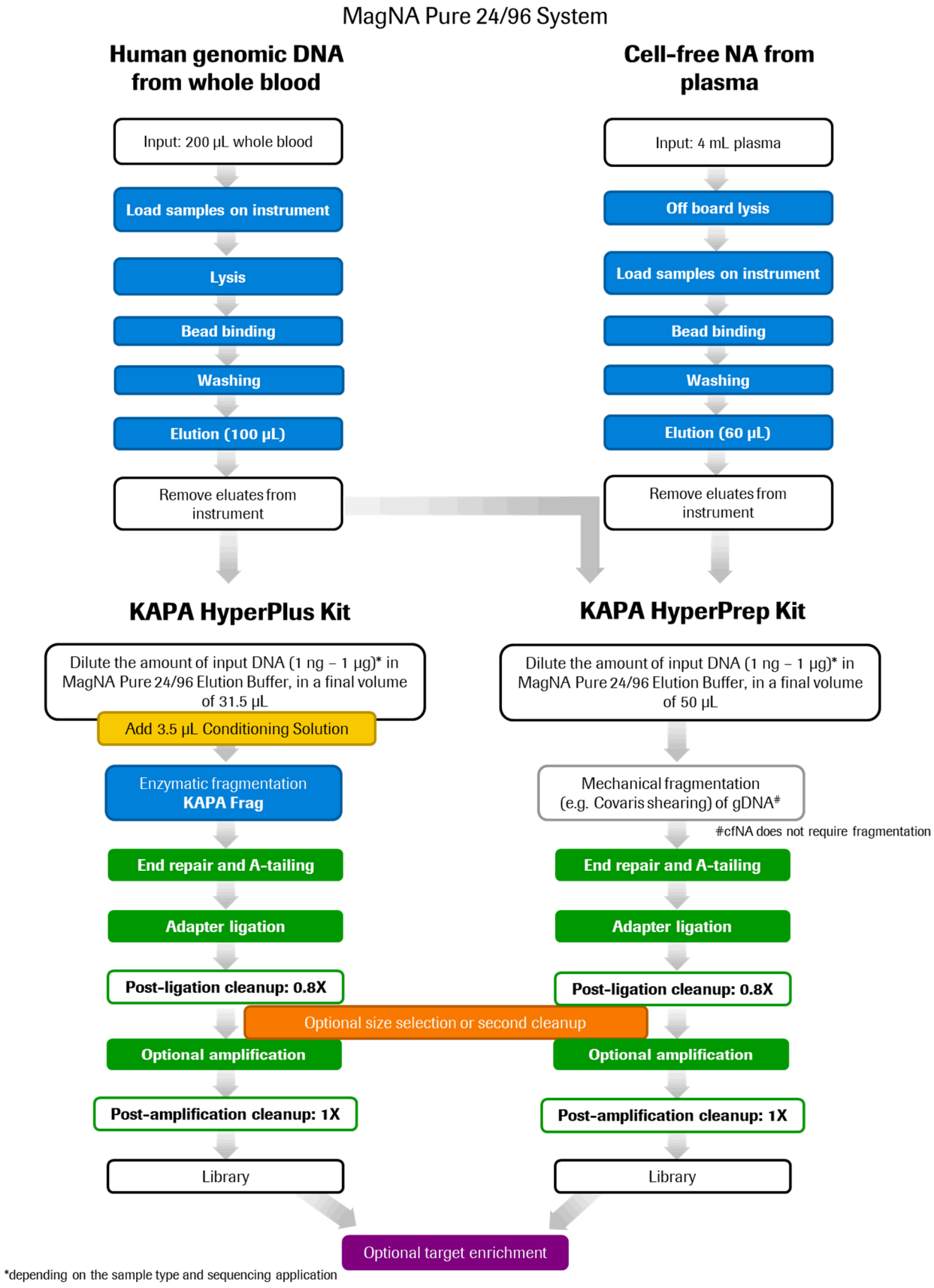
The following ancillary reagents are supplied by Roche for the KAPA HyperPrep and HyperPlus workflow:

- KAPA Unique Dual-Indexed Adapter Kit (Roche PN: 08861919702)
- KAPA HyperPure Beads
    - Roche PN: 08963851001 (60 mL)
    - Roche PN: 08963843001 (30 mL)
    - Roche PN: 08963835001 (5 mL)
    - Roche PN: 08963860001 (450 mL)
    - Roche PN: 08963878001 (4 x 60 mL)
- KAPA Pure Beads
    - Roche PN: 07983298001 (60 mL)
    - Roche PN: 07983280001 (30 mL)
    - Roche PN: 07983271001 (5 mL)
- KAPA Library Quantification Kit (500 reactions)
    - Universal qPCR Master Mix (Roche PN: 07960140001)
    - ABI Prism qPCR Master Mix (Roche PN: 07960204001)
    - Bio-Rad iCycler qPCR Master Mix (Roche PN: 07960255001)
    - ROX Low qPCR Master Mix (Roche PN: 07960336001)
    - qPCR Master Mix optimized for LightCycler® 480 instrument (Roche PN: 07960298001)

The following equipment and plastic ware are not supplied by Roche but are required for this workflow:

- Thermocyclers (for standard PCR and qPCR)
- PCR tubes/96-well PCR plates (0.2 mL)
- Magnetic block e.g DynaMag 96 Side Magnet (ThermoFisher Scientific Catalog number: 12331D)
- Pipettes
- Low DNA-binding microtubes (1.5 mL)

4. WORKFLOW


MagNA Pure 24/96 System
Human genomic DNA from whole blood
Input: 200 µL whole blood
Load samples on instrument
Lysis
Bead binding
Washing
Elution (100 µL)
Remove eluates from instrument
Cell-free NA from plasma
Input: 4 mL plasma
Off board lysis
Load samples on instrument
Bead binding
Washing
Elution (60 µL)
Remove eluates from instrument
KAPA HyperPlus Kit
Dilute the amount of input DNA (1 ng – 1 µg)* in MagNA Pure 24/96 Elution Buffer, in a final volume of 31.5 µL
Add 3.5 µL Conditioning Solution
Enzymatic fragmentation KAPA Frag
End repair and A-tailing
Adapter ligation
Post-ligation cleanup: 0.8X
Optional amplification
Post-amplification cleanup: 1X
Library
KAPA HyperPrep Kit
Dilute the amount of input DNA (1 ng – 1 µg)* in MagNA Pure 24/96 Elution Buffer, in a final volume of 50 µL
Mechanical fragmentation (e.g. Covaris shearing) of gDNA#
#cfNA does not require fragmentation
End repair and A-tailing
Adapter ligation
Post-ligation cleanup: 0.8X
Optional amplification
Post-amplification cleanup: 1X
Library
Optional size selection or second cleanup
Optional target enrichment
*depending on the sample type and sequencing application

5. PREPARE INPUT MATERIAL

In relation to this technical note, two types of nucleic acids (NA) may be extracted with NGS-qualified methods using the MagNA Pure 24 and 96 Nucleic Extraction Systems:

a) Human genomic DNA (gDNA) from whole blood

b) Cell-free nucleic acids (cfNA or cfDNA) from plasma

The following automated extraction protocols have been optimized and qualified for NGS library preparation using KAPA HyperPrep or KAPA HyperPlus Kits:

System*	Sample Input	Protocol Name	MagNA Pure Kit	Output Volume & Nucleic Acid	Recommended KAPA library prep kit
MagNA Pure 24	200 µL whole blood	hgDNA ds 200 (no pretreatment of samples required)	MagNA Pure 24 Total NA Isolation Kit	100 µL gDNA	KAPA HyperPrep or KAPA HyperPlus
	4 mL plasma	cfNA ds 4000 hp (pretreatment of samples required)		60 µL cfNA	KAPA HyperPrep*
MagNA Pure 96	200 µL whole blood	DNA Blood SV (no pretreatment of samples required)	MagNA Pure 96 DNA and Viral NA Small Volume Kit	100 µL gDNA	KAPA HyperPrep or KAPA HyperPlus
	4 mL plasma	cfDNA ds 4000 (pretreatment of samples required)		100 µl cfNA	KAPA HyperPrep*

***Cell-free NA is already fragmented and does not require enzymatic or mechanical fragmentation.**

A specific protocol for the MagNA Pure 24 Total NA Isolation Kit can be found in the following document:

- MagNA Pure 24 Total NA Isolation Kit Instructions for Use (v05 or later).[1] Roche Document Number: 08100128001

A specific protocol for the MagNA Pure 96 DNA and Viral NA Small Volume Kit can be found in the following document:

- MagNA Pure 96 DNA and Viral NA Small Volume Kit Instructions for Use (v08 or later).[2] Roche Document Number: 06671942001

a) Extraction of human genomic DNA from whole blood

MagNA Pure 24 System

Use fresh or, if previously frozen, thawed whole blood without any pretreatment.

Notes:

- If the white blood cell (WBC) count is above 1 x 10^7 blood cells/mL, dilute the whole blood with phosphate-buffered saline (PBS) prior to use to avoid clumping of magnetic glass particles (MGP). Ensure that the WBC are homogenously distributed.
- Ensure that there are no clots in anticoagulated whole blood samples.
- For further instructions on loading the samples and removing the samples after the run, refer to the MagNA Pure 24 Total NA Isolation Kit Instructions for Use.[1]

MagNA Pure 96 System

Use fresh or, if previously frozen, thawed whole blood without any pretreatment.

Notes:

- If the white blood cell (WBC) count is above 1 x 10^7 blood cells/mL, dilute the whole blood with PBS prior to use to avoid clumping of MGP. Ensure that the WBC are homogenously distributed.
- Ensure that there are no clots in anticoagulated whole blood samples.
- For further instructions on loading the samples and removing the samples after the run, refer to the MagNA Pure 96 DNA and Viral NA Small Volume Kit Instructions for Use.[2]

b) Extraction of cell-free NA from plasma

Use the MagNA Pure cfNA Buffer Set when isolating cfNA.

A specific protocol for the MagNA Pure cfNA Buffer Set can be found in the following document:

- MagNA Pure cfNA Buffer Set Instructions for Use (v01).[3] Roche Document Number: 08107092001

MagNA Pure 24 and 96 System

Notes:

- Before purifying cfNA, centrifuge the samples for 5 to 10 min at 1000 to 1900 x g. Avoid transferring any of the pellet.
- Avoid introducing foam/bubbles during all pipetting steps.

Pretreatment steps for extraction of cfNA from plasma when using MagNA Pure 24 or 96 Systems:

1. In a fresh tube compatible with the MagNA Pure 24 or 96 sample rack, add 0.4 mL of Proteinase K. Add the 4 mL plasma sample into the tube containing Proteinase K, mix gently, and incubate at 37°C for 20 min.
2. Depending on the number of samples to be processed, prepare the cfNA buffer mix in bulk by mixing Cell-Free Nucleic Acid Enhancement Buffer (CELB) and Isopropanol (IPA) in that order, in an appropriately sized container as per table below. Cap and mix gently by inversion. The solution is stable for a maximum of 2 hours.

Reagent	Volume (mL)
CELB	3.5 mL
IPA	0.6 mL
cfNA Buffer mix (CELB + IPA)	**4.1 mL**

3. Add 4 mL of the cfNA Buffer mix to the tube containing 4.4 mL plasma sample mixed with Proteinase K.
4. Mix thoroughly by manually dispensing and aspirating the liquid approximately 8 times to produce a homogeneous mixture.
5. **Immediately** load the tubes containing the lysate onto the sample rack. Load the sample rack into the instrument.
6. For further instructions on loading the samples and removing the samples after the run, refer to the MagNA Pure 24 Total NA Isolation Kit Instructions for Use[1] or MagNA Pure 96 DNA and Viral NA Small Volume Kit Instructions for Use.[2]

Notes:

- Do not store the lysate.
- If bubbles form, they may be removed by aspiration into a pipette tip held near the side of the tube just above the surface of the liquid. Alternatively, bubbles may be removed by capping tubes and centrifuging at 2000 x g for 1 min.

6. QUALITY CONTROL OF MAGNA PURE ELUATES/INPUT DNA

Recommended Quality Control Steps

- Performing quality control on the input DNA is strongly recommended at this stage.
- Recommended QC assays:
  - Electrophoretic analysis to determine quality of input DNA
  - Fluorometric analysis to quantify input DNA

Quality control of the MagNA Pure eluate (extracted DNA) is strongly recommended prior to NGS library construction. Extracted 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 (**Figure 1**, **Figure 2** and **Figure 3**). 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), TapeStation instrument (Agilent Technologies), or Fragment Analyzer (Advanced Analytical) instrument to assess DNA quality.

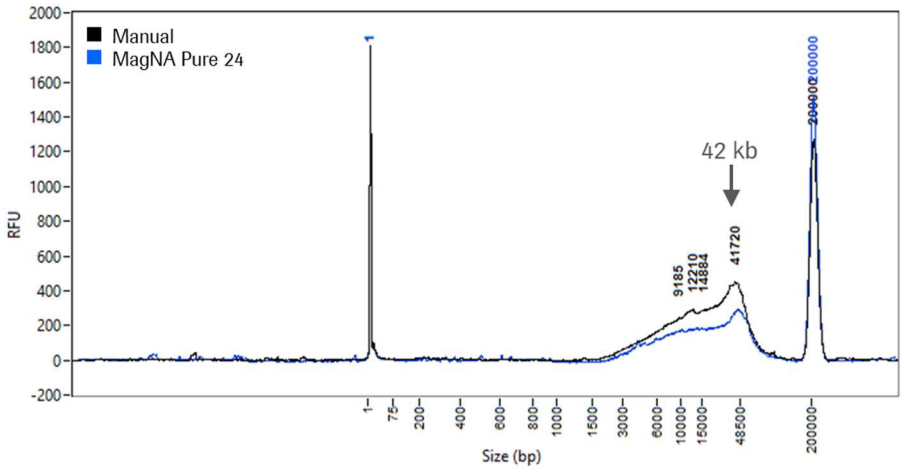


Figure 1: Expected electrophoretic profile of genomic DNA extracted from whole blood using an NGS-qualified method for the MagNA Pure 24. hgDNA was extracted from 200 µL whole blood using a manual method (QIAamp DNA Blood Mini Kit; Qiagen; black) and the MagNA Pure 24 System with the MagNA Pure 24 Total NA Isolation Kit (blue) using the *hgDNA ds 200* protocol. Eluted hgDNA was analyzed using the Fragment Analyzer and Genomic DNA 50 kbp Analysis Kit (Advanced Analytical). The DNA eluates contained intact hgDNA with an average molecular weight of ~40 kbp. The absence of observable low-molecular weight fragments indicates that minimal DNA damage occurred during extraction.

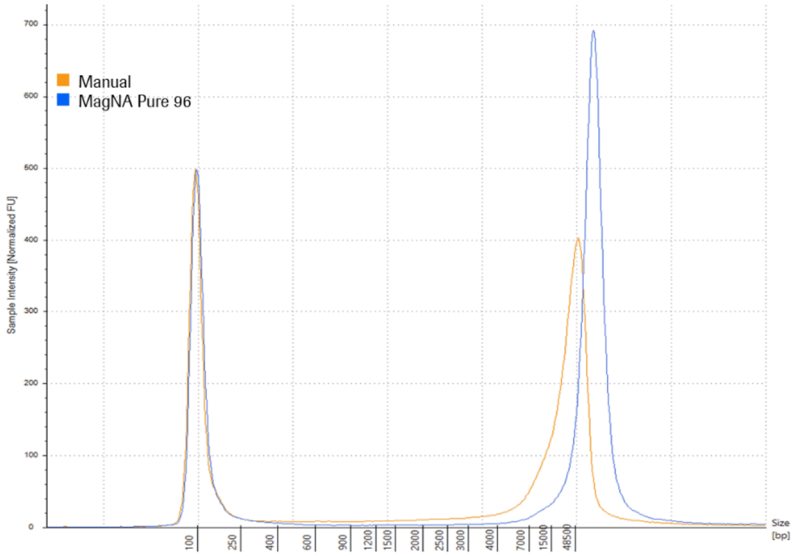


Figure 2: Expected electrophoretic profile of genomic DNA extracted from whole blood using an NGS-qualified method for the MagNA Pure 96 Systems. hgDNA was extracted from 200 µL whole blood using a manual method (QIAamp DNA Blood Mini Kit; Qiagen; orange) or MagNA Pure 96 System with the MagNA Pure 96 DNA and Viral NA Small Volume Kit and *DNA Blood SV* protocol (blue). Eluted hgDNA was analyzed using the Agilent TapeStation System and Genomic DNA ScreenTape assay (Agilent). The DNA eluates contained intact hgDNA with an average molecular weight of ~40 kbp. The absence of observable low-molecular weight fragments indicates that minimal DNA damage occurred during extraction.

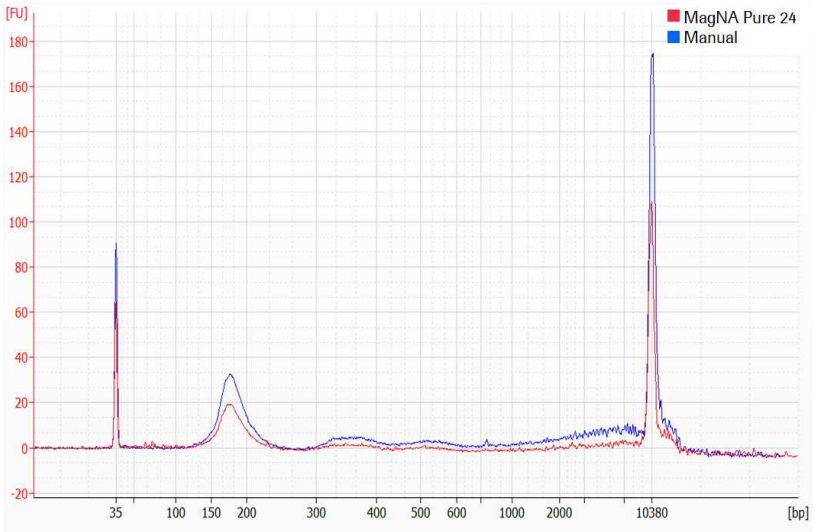


Figure 3: Expected electrophoretic profile of cfNA extracted from plasma using the MagNA Pure 24 System. cfNA was extracted from 4 mL plasma using a manual method (AVENIO cfNA Isolation Kit; blue) or the MagNA Pure 24 System with the MagNA Pure 24 Total NA Isolation Kit (red). The *cfNA ds 4000 hp* protocol was used. Eluted cfNA was analyzed using the Agilent 2100 Bioanalyzer Instrument and the Agilent High Sensitivity DNA Kit. The peak between 150 and 250 bp represents cfNA.

7. PREPARE THE SAMPLE LIBRARY

The sample library may be prepared following one of two workflows:

a) KAPA HyperPrep Kit – mechanical fragmentation

b) KAPA HyperPlus Kit – enzymatic fragmentation

a) Library Preparation using the KAPA HyperPrep Kit

The KAPA HyperPrep Kit is a versatile, streamlined solution for DNA library preparation that is compatible with DNA that is already fragmented (biologically; or through mechanical or enzymatic methods that yield discrete DNA fragments, excluding those employing tagmentation). Please refer to the fragmentation guidelines provided by the manufacturer of your fragmentation equipment or reagent.

If DNA fragments were generated by PCR, or by using an **enzymatic fragmentation** reagent from another supplier, a 3X bead-based cleanup is required before proceeding with **End repair and A-tailing.** Following mechanical fragmentation (e.g. if a Covaris instrument was used), no cleanup is typically required, unless the buffer in which the DNA was extracted or fragmented is suspected to contain components that may inhibit the End repair and A-tailing reaction.

When an NGS-qualified MagNA Pure 24 method or MagNA Pure 96 method is used to extract DNA for the KAPA HyperPrep workflow, the required amount of input DNA (1 ng – 1 µg, depending on the sample type and sequencing application), is simply diluted in 50 mM Tris-HCl buffer (pH 8.5) or MagNA Pure 24/96 Elution Buffer, to a final volume of 50 µL. Please refer to the KAPA HyperPrep Kit Instructions for Use (v8.20 or later)[4], to proceed directly with **End Repair and A-tailing**; **Adapter Ligation**; **Post-ligation Cleanup**; **Library Amplification** and **Post-amplification Cleanup**.

Recommended Quality Control (QC) Steps

- Performing QC at several stages of the KAPA HyperPrep workflow is recommended. Performing QC of the final, sequencing-ready, adapter-ligated library is **strongly** recommended. ***Refer to Section 8:*** *Evaluating the Success of Library Construction*
- Recommended QC assays:
  - After Covaris-shearing/mechanical fragmentation of the input DNA
    - Electrophoretic analysis to confirm fragment size and distribution
    - Fluorometric analysis to quantify fragmented DNA
  - After the post-ligation bead cleanup
    - Electrophoretic analysis to determine final library size and distribution (if preparing PCR-free libraries)
    - qPCR quantification of adapter-ligated, sequencing-ready library
  - After library amplification and bead cleanup (if performed)
    - Electrophoretic analysis to determine final library size and distribution
    - qPCR quantification of amplified, sequencing-ready library

b) Library Preparation using the KAPA HyperPlus Kit

The KAPA HyperPlus Kit provides a streamlined, single-tube library construction workflow with integrated, low-bias enzymatic fragmentation.

Enzymatic Fragmentation

IMPORTANT! The KAPA HyperPlus Kit contains an enzymatic fragmentation module (KAPA Frag) which is sensitive to high levels of salt or EDTA in nucleic acid input. For gDNA extracted with the NGS-compatible MagNA Pure 24 or MagNA Pure 96 method, the Conditioning Solution included in the KAPA HyperPlus Kit is required to balance the chemical composition of the MagNA Pure elution buffer. Prior to enzymatic fragmentation, prepare a sufficient volume of 1/10-diluted KAPA Conditioning Solution (3.5 µL per DNA sample, plus excess), to include in the enzymatic fragmentation reaction.

Alternatively, a 3X bead-based buffer exchange using KAPA HyperPure Beads may be performed. Once the MagNA Pure 24/96 elution buffer has been replaced by 10 mM Tris-HCl, the KAPA Conditioning Solution may be omitted from the reaction.

1. If required, dilute the amount of input DNA (1 ng – 1 µg, depending on the sample type and sequencing application) to be used for library construction in 50 mM Tris-HCl (pH 8.5) or MagNA Pure 24/96 Elution Buffer, to a final volume of 31.5 µL.
2. Mix by pipetting up and down.
3. Assemble each fragmentation reaction on ice by adding the components in this order:

Component	Volume
Double-stranded DNA (diluted in MagNA Pure 24/96 Elution Buffer if needed)	31.5 µL
Conditioning Solution (**1/10 dilution**)[1]	3.5 µL
KAPA Frag Buffer (10X)[2]	5 µL
KAPA Frag Enzyme[2]	10 µL
Total Volume:	**50 µL**

[1] The Conditioning Solution should be added separately to each individual sample.

[2] The KAPA Frag Buffer and KAPA Frag Enzyme may be pre-mixed and kept on ice prior to reaction setup, and dispensed as a single solution. Please note that the volume of buffer is less than the volume of enzyme in this reaction.

4. Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.
5. Incubate in a thermocycler, pre-cooled to 4°C and programmed as outlined below. A heated lid is not required for this step. If used, set the temperature of the heated lid to ≤50°C.

Step	Temp	Time
Pre-cool block	4°C	N/A
Fragmentation	37°C	Variable – see table below*
HOLD	4°C	∞

Mode fragment length	Incubation time at 37°C*	Optimization range
600 bp	5 min	3 – 10 min
350 bp	10 min	5 – 20 min
200 bp	20 min	10 – 25 min
150 bp	30 min	20 – 40 min

*These parameters are a good starting point for high-quality genomic DNA. Please refer to **Appendix 2: Optimization of Fragmentation Parameters** in the **KAPA HyperPlus Kit Technical Data Sheet (v6.20 or later)**[5] for guidelines on how to optimize fragmentation time and temperature if needed.

6. **Transfer reactions to ice, and proceed immediately** to **End Repair and A-tailing** as described in the KAPA HyperPlus Kit Instructions for Use (v6.20 or later).[5] Continue with the library preparation protocol as outlined in the Instructions for Use from this step onwards.

QUALITY CONTROL (QC)

- Performing quality control at several stages of the KAPA HyperPlus workflow is recommended. Performing quality control of the final, sequencing-ready, adapter-ligated library is strongly recommended. ***Refer to Section 8***: *Evaluating the Success of Library Construction.*
- Recommended QC assays:
  - After the post-ligation bead cleanup
    - Electrophoretic analysis to determine final library size and distribution (if preparing PCR-free libraries)
    - qPCR quantification of adapter-ligated, sequencing-ready library
  - After library amplification and bead cleanup (if performed)
    - Electrophoretic analysis to determine final library size and distribution
    - qPCR quantification of amplified, sequencing-ready library

QC after enzymatic fragmentation: while it is possible to remove aliquots of the fragmentation reaction product for analysis in the integrated fragmentation/library construction workflow, this is not recommended as it is difficult to obtain a true assessment of DNA fragment size from such samples. The outcome of fragmentation should instead be evaluated in the context of the final library.

8. Evaluating the Success of Library Construction

Quality control (QC) may be performed at several stages during the library construction workflow (before/after Covaris shearing; before/after size selection (if performed), after adapter ligation and cleanup, and after amplification 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.

a) Library Insert Size Distribution

The size distribution of final, adapter-ligated library fragments 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 (**Figure 4**).

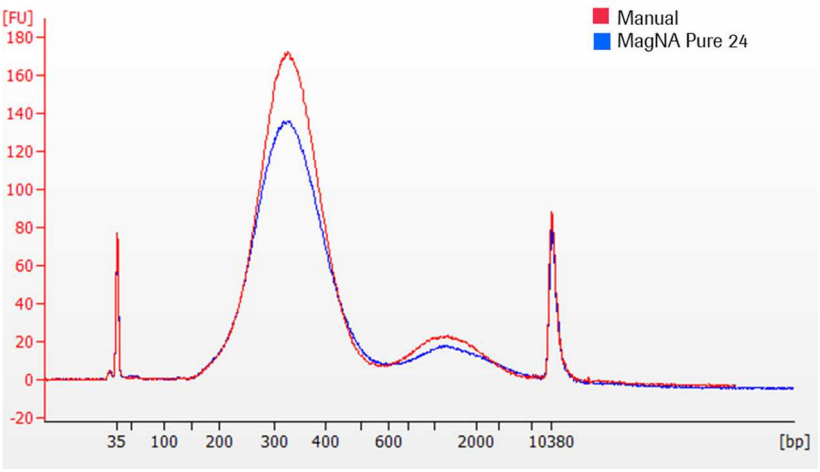
In PCR-free library construction workflows, such as those routinely employed for human whole-genome sequencing, it is difficult to obtain an accurate average library size from routinely-used electrophoretic systems, as molecules flanked by adapters with long single-stranded portions migrate anomalously 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** .

Since the average fragment size is needed for the calculation of library concentrations in qPCR-based quantification assays, several easy workarounds for PCR-free workflows are described below:

- **Recommended:** Amplify an aliquot (10 – 20%) of the PCR-free library for a few cycles prior to assessment with an electrophoretic assay. Amplification will render all molecules fully double-stranded, and yield a reliable size determination from the electrophoretic assay. KAPA HiFi HotStart Library Amplification Kits, which contain KAPA HiFi HotStart DNA polymerase, are recommended for this purpose.[6]
- 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. This approach is only feasible if the size selection parameters were optimized to preserve the size distribution of the fragmented DNA.
- 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. Since systematic under- or overestimation of library concentration is likely when using this approach, it is important to remember that the relationship between calculated library concentration and cluster density has to be determined empirically for each specific library prep workflow and sequencing system.

a



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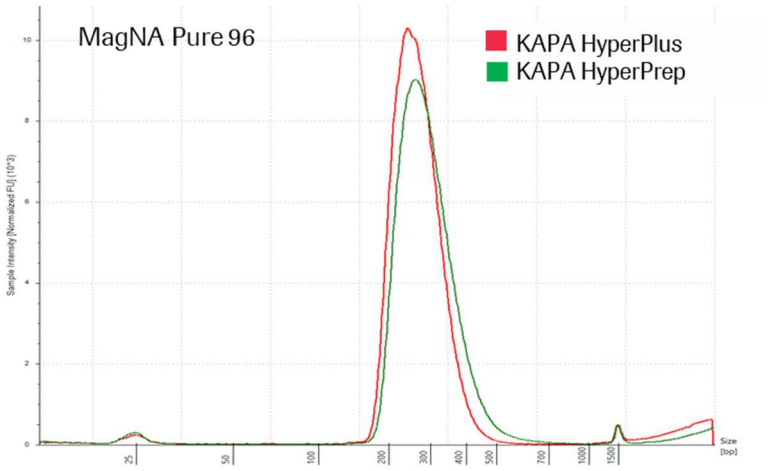

Figure 4: Examples of electrophoretic profiles of libraries generated using KAPA HyperPlus and KAPA HyperPrep.

a) The KAPA HyperPlus Kit was used to construct libraries from 100 ng of human gDNA extracted using either manual method (QIAamp DNA Blood Mini Kit; Qiagen; red) or by using NGS-qualified methods on the MagNA Pure 24 (blue). Samples were enzymatically fragmented for 20 minutes at 37°C. KAPA DI Adapters were utilized during adapter ligation. Samples were subjected to a 0.7X – 0.9X double- sided size selection using Agencourt Ampure XP (Beckman Coulter) before being amplified for 7 cycles using KAPA HiFi HotStart ReadyMix and KAPA Library Amplification Primer Mix (Illumina). For size determination, amplified libraries were analyzed using the Agilent 2100 Bioanalyzer Instrument and Agilent High Sensitivity DNA Kit.

b) The KAPA HyperPlus (red) and HyperPrep Kit (green) was used to construct libraries from 100 ng of human gDNA extracted using NGS-qualified methods on the MagNA Pure 96. Samples were enzymatically fragmented for 25 minutes at 37°C (HyperPlus) or subected to Covaris shearing (HyperPrep). KAPA DI Adapters were utilized during adapter ligation. Samples were amplified for 6 (HyperPlus) or 8 (HyperPrep) cycles using KAPA HiFi HotStart ReadyMix and KAPA Library Amplification Primer Mix (Illumina). Libraries underwent target enrichment using KAPA HyperChoice version of the SeqCap Sudden Cardiac Death Panel (610 kb). KAPA HyperCap Universal Enhancing Oligos were included in the hybridization. Enriched libraries were amplified for an additional 16 cycles using KAPA HiFi HotStart ReadyMix and Library Amplification Primer Mix. For size determination, amplified libraries were analyzed using the TapeStation System and D1000 ScreenTape Assay.

Note: Secondary peak between 700 – 2000 bp are so-called "daisy chains" or "tangled knots", comprising large assemblies of improperly annealed, partially double stranded, heteroduplex DNA. These may occur during library amplification reactions when primers are depleted before dNTPs. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries.

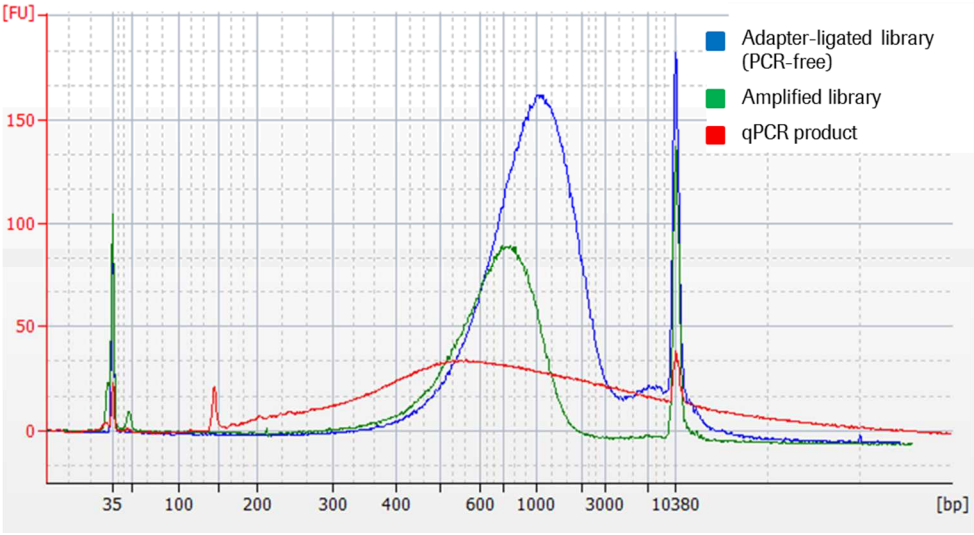

Figure 5: Methods for determining the true average fragment size of human WGS libraries produced in PCR-free workflows, for qPCR-based library concentration calculations. Libraries were prepared from gDNA sheared to a mode fragment length of ~650 bp with the KAPA HyperPrep Kit. Unamplified libraries (blue) 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 (green). The product of the library quantification assay (red) 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.

b) 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).[7] 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. 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.

9. ALTERNATIVES

- AMPure XP reagent (Beckman Coulter) may be used instead of KAPA cleanup beads for all bead cleanups in the context of research only.
- The MagNA Pure 24/96 elution buffer may be removed by performing a 3X bead-based cleanup with KAPA HyperPure Beads prior to fragmentation. In that event, please refer to KAPA HyperPlus Kit Instructions for Use (v6.20 or later); Roche Document Number KR1145 for a complete protocol. This protocol can be used as an alternative in the context of research use only.

10. REFERENCES

1. MagNA Pure 24 Total NA Isolation Kit Instructions for Use, v02 or later. Roche Document Number: 08100128001
2. MagNA Pure 96 DNA and Viral NA Small Volume Kit Instructions for Use, v08 or later. Roche Document Number: 06671942001
3. MagNA Pure cfNA Buffer Set Instructions for Use, v01 or later. Roche Document Number: 08107092001
4. KAPA HyperPrep Kit Instructions for Use, v8.20 or later. Roche Document Number KR0961
5. KAPA HyperPlus Kit Instructions for Use, v6.20 or later. Roche Document Number KR1145
6. KAPA HiFi HotStart Library Amplification Kit Illumina Platforms Technical Data Sheet, v9.19 or later. Roche Document Number KR0408
7. KAPA Library Quantification Kit Illumina Platforms Instructions for Use, v9.17 or later. Roche Document Number KR0405

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