KAPABIOSYSTEMS

Technical Data Sheet

KAPA HyperPlus Kit

KR1145 – v5.19

This Technical Data Sheet provides product information and a detailed protocol for the KAPA HyperPlus Kit.

This document applies to KAPA HyperPlus Kits (07962380001, 07962401001 and 07962428001), and KAPA HyperPlus Kits for PCR-free workflows (07962398001, 07962410001 and 07962436001).

Contents

Quick Notes

- This kit provides a versatile, streamlined DNA fragmentation and library construction protocol. Libraries for Illumina sequencing may be prepared from a wide range of DNA samples and inputs (1 ng – 1 µg) in 1.5 – 3 hrs.
- The one-tube DNA fragmentation and library construction chemistry improves library yield and quality, particularly for FFPE and low-input libraries.

Kapa/*Roche* Kit Codes and Components

KK8510 *07962380001* **KK8511*** *07962398001* 8 libraries	KAPA Frag Enzyme	100 µL
	KAPA Frag Buffer (10X)	50 µL
	Conditioning Solution	580 µL
	End Repair & A-Tailing Buffer	70 µL
	HyperPrep ERAT Enzyme Mix	30 µL
	HyperPlus ERAT Enzyme Mix**	30 µL
	Ligation Buffer	300 µL
	DNA Ligase	100 µL
	KAPA HiFi HotStart ReadyMix (2X)*	250 µL
	Library Amplification Primer Mix (10X)*	50 µL
KK8512 *07962401001* **KK8513*** *07962410001* 24 libraries	KAPA Frag Enzyme	270 µL
	KAPA Frag Buffer (10X)	140 µL
	Conditioning Solution	580 µL
	End Repair & A-Tailing Buffer	210 µL
	HyperPrep ERAT Enzyme Mix	90 µL
	HyperPlus ERAT Enzyme Mix**	90 µL
	Ligation Buffer	900 µL
	DNA Ligase	300 µL
	KAPA HiFi HotStart ReadyMix (2X)*	690 µL
	Library Amplification Primer Mix (10X)*	138 µL
KK8514 *07962428001* **KK8515*** *07962436001* 96 libraries	KAPA Frag Enzyme	1.27 mL
	KAPA Frag Buffer (10X)	640 µL
	Conditioning Solution	580 µL
	End Repair & A-Tailing Buffer	930 µL
	HyperPrep ERAT Enzyme Mix	400 µL
	HyperPlus ERAT Enzyme Mix**	400 µL
	Ligation Buffer	3.8 mL
	DNA Ligase	1.26 mL
	KAPA HiFi HotStart ReadyMix (2X)*	3.0 mL
	Library Amplification Primer Mix (10X)*	600 µL

*07962398001, 07962410001, and 07962436001 are available for PCR-free workflows, and do not contain library amplification reagents. **KAPA HyperPlus End Repair & A-Tailing (ERAT) Enzyme Mix (enhanced formulation to improve performance) is only compatible with the HyperPlus Workflow.

Quick Notes Continued

- The protocol is easy to automate. Generous reagent excesses are supplied in 96-reaction kits to accommodate automated liquid handling.
- This kit contains all the reagents for DNA fragmentation, library construction, and high efficiency and low bias library amplification except for adapters and beads. KAPA HyperPure Beads or KAPA Pure Beads (jointly referred to hereafter as KAPA cleanup beads) are recommended for this application. KAPA cleanup beads and KAPA Adapters are sold separately. Kits without an amplification module are available for PCR-free workflows.
- **The Process Workflow** provides an overview of the DNA fragmentation and library construction process. Two workflows are available for the End Repair and A-tailing step — see **Important Parameters** for further guidelines. **Appendix 1** provides a size selection protocol. **Appendix 2** contains guidelines for optimization of fragmentation.
- If your input DNA contains EDTA, please consult the **Important Parameters: Input DNA** and additional guidelines in **Appendix 2**.

Product Description

The KAPA HyperPlus Kit provides a versatile, streamlined DNA fragmentation and library construction protocol for the rapid preparation of libraries for Illumina sequencing. The one-tube chemistry and protocol improves the efficiency and consistency of library construction, and yields libraries of similar or better quality than those produced with the KAPA HyperPrep Kit from Covaris-sheared DNA. It outperforms tagmentation-based workflows in terms of robustness, flexibility and sequence coverage and uniformity.

The workflow combines enzymatic steps and employs minimal bead-based cleanups, thereby reducing sample handling and overall library preparation time to 1.5 – 3 hrs. The kit contains all of the enzymes and reaction buffers required for:

1. enzymatic fragmentation to produce dsDNA fragments;
2. end repair and A-tailing to produce end-repaired, 5'-phosphorylated, 3'-dA-tailed dsDNA fragments;
3. adapter ligation, during which dsDNA adapters with 3'-dTMP overhangs are ligated to 3'-dA-tailed molecules; and
4. library amplification (optional), which employs high-fidelity, low-bias PCR to amplify library fragments carrying appropriate adapter sequences on both ends.

The kit provides a single concentrated buffer and a single enzyme mixture for enzymatic fragmentation, a single concentrated buffer and two enzyme mixes for End Repair and A-tailing, a single concentrated buffer and a single enzyme mixture for adapter ligation. The kit does not include adapters or beads required for reaction cleanups. KAPA cleanup beads and KAPA Adapters are sold separately.

In order to maximize sequence coverage uniformity, it is critical to minimize library amplification bias. KAPA HiFi DNA Polymerase is designed for low-bias, high-fidelity PCR, and is the reagent of choice for NGS library amplification.[1,2,3,4] KAPA HyperPlus Kits include KAPA HiFi HotStart ReadyMix (2X), a ready-to-use PCR mix comprising all the components for library amplification—except primers and template. Kits also include KAPA Library Amplification Primer Mix (10X), designed for the high-efficiency amplification of Illumina libraries flanked by adapters containing the P5 and P7 flow cell sequences. Kits without the amplification module (07962398001, 07962410001, and 07962436001) are available for PCR-free workflows. They may also be combined with the KAPA HiFi HotStart Real-Time Library Amplification Kit (07959028001) for more precise control over library amplification.

1. Oyola, S.O., et al., *BMC Genomics* **13**, 1 (2012).
2. Quail, M.A., et al., *Nature Methods* **9**, 10 (2012).
3. Quail, M.A., et al., *BMC Genomics* **13**, 341 (2012).
4. Ross, M.G., et al., *Genome Biology* **14**, R51 (2013).

Product Applications

KAPA HyperPlus Kits are ideally suited for low- and high-throughput NGS library construction workflows that require DNA fragmentation, end repair, A-tailing, adapter ligation and library amplification (optional). Kits are designed for library construction from a wide range of sample types and inputs (1 ng – 1 µg), and are compatible with complex, genomic DNA; low-complexity samples such as small viral genomes, plasmids, cDNA and long amplicons; and low-quality DNA such as FFPE samples.

The entire workflow is automation-friendly and may be incorporated into workflows for a wide range of NGS applications, including:

- whole-genome, shotgun sequencing
- whole exome or targeted sequencing, using SeqCap EZ, Agilent SureSelect, Illumina TruSeq, or IDT xGen Lockdown Probes, or other hybridization capture systems
- RNA-seq (starting with cDNA).

Product Specifications

Shipping and Storage

The enzymes provided in this kit are temperature sensitive, and appropriate care should be taken during shipping and storage. KAPA HyperPlus Kits are shipped on dry ice or ice packs, depending upon country of destination. Upon receipt, immediately store enzymes and reaction buffers at -15°C to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Always ensure that KAPA HyperPlus Kit components have been fully thawed and thoroughly mixed before use. The End Repair & A-Tailing Buffer and Ligation Buffer may contain precipitates when thawed at 2°C to 8°C. These buffers must be thawed at room temperature and vortexed thoroughly before use. KAPA HiFi HotStart ReadyMix (2X) contains isostabilizers and may not freeze completely, even when stored at -15°C to -25°C. Nevertheless, always ensure that the ReadyMix is fully thawed and thoroughly mixed before use. Reaction master mixes prepared from the enzymes and buffers for fragmentation, end repair and A-tailing, as well as for ligation, are very viscous and require special attention during pipetting. Keep all enzyme components and master mixes on ice as long as possible during handling and preparation.

Quality Control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo- and endonuclease activities, and meet strict requirements with respect to DNA contamination.

Please contact Technical Support for more information at sequencing.roche.com/support.

Important Parameters

Library construction workflows must be tailored and optimized to accommodate specific experimental designs, sample characteristics, sequencing applications and equipment. The protocol provided in this document is generic, and reaction parameters may be adjusted as required to optimize performance, efficiency and cost-effectiveness.

In addition to the information in this section, please consult the **KAPA NGS Library Preparation Technical Guide** (available on request from Technical Support at sequencing.roche.com/support) for further guidelines when designing or optimizing your library construction workflow.

Automated Library Construction

The KAPA HyperPlus workflow described in this document is designed to be "automation friendly" and can be performed manually, or in a semi- or fully-automated fashion using a suitable automated liquid handling platform. In addition to increased sample throughput, automation may be expected to provide additional advantages such as improved reproducibility and process control. However, automation may result in slightly lower yields and/or different size distributions when compared with manual library construction performed by a skilled, experienced and attentive technician. Most often, these discrepancies can be minimized through careful selection of hardware and plasticware, and optimization of liquid handling parameters.

Kapa Biosystems does not supply automated liquid handling equipment, but collaborates with automation solution providers and customers to develop and qualify optimized automated methods for our kits, for liquid handling platforms routinely used in NGS library construction. Please contact your instrument vendor or visit sequencing.roche.com/support if you are interested in using the KAPA HyperPlus Kit with your particular automated liquid handling system.

When attempting to develop an automated KAPA HyperPlus method, please keep the following in mind:

- Reaction components for enzymatic reactions should be combined into master mixes, rather than dispensed separately. The enzymatic fragmentation master mix must be made fresh, and actively cooled at 4°C. In contrast, the master mixes for end repair and A-tailing, as well as for adapter ligation, are stable for ≤24 hrs at room temperature, and do not have to be actively cooled during automated library construction.
- Master mixes for fragmentation, the end repair and A-tailing reaction, as well as for adapter ligation, are highly viscous, and require careful optimization of pipetting parameters.
- Due to the strong 3'→5' exonuclease activity of KAPA HiFi HotStart DNA Polymerase, PCR master mixes with primers should preferably not be left on-deck for long periods of time, particulary if they are not actively cooled. Prepare library amplification master mixes freshly before use, or dispense primer mixes separately from the KAPA HiFi HotStart ReadyMix.
- Since an excess (5 – 20%) of each reagent master mix will be required, generous reagent overages are included in 96-reaction kits. The appropriate excess for other reagents (adapters, beads, 80% ethanol and elution buffer) varies from one liquid handling system to another.
- Enzymatic fragmentation reactions must be set up in a plate that is actively cooled to 4°C, and moved to a second, pre-equilibrated Peltier device or on-deck thermocycler for the fragmentation incubation (typically performed at 37°C). After fragmentation, immediately return the plate to 4°C, add end repair and A-tailing reagents at 4°C, and move the plate to an on- or off-deck cycler for the 65°C incubation without delay.
- Incubations at temperatures above 50°C must be performed in a thermocycler with a heated lid.
- This protocol has been optimized for 96-well PCR plates with a maximum working volume of ~200 µL. Plates with larger working volumes or deep-well plates may be used to accommodate larger reaction volumes if needed.
- Always use plastics that are certified to be nuclease-free. Low DNA-binding plastics are recommended. When selecting the most appropriate plasticware for your workflow, consider compatibility with:
  - the plate gripper and other components of your liquid handling system;
  - the magnet used during bead manipulations;
  - Peltier devices or thermocyclers used for reaction incubations and/or library amplification.
- Design automated methods in a manner that ensures the highest consistency across all 12 columns of 96-well working plates, and eliminates all possible sources of sample-to-sample and environmental contamination. Consider performing pre- and post-PCR steps on dedicated instruments, if available.

Safe Stopping Points

The library construction process, from enzymatic fragmentation to final library, can be performed in 1.5 to 3 hrs—depending on experience, the number of samples being processed, and whether or not library amplification is performed. If necessary, the protocol may be paused safely after completion of the **Post-ligation Cleanup** (step 4.17; the end of the protocol for PCR-free workflows).

Purified, adapter-ligated library DNA may be stored at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C for ≤1 month before amplification, target capture and/or sequencing. To avoid degradation, always store DNA in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5) when possible, and minimize the number of freeze-thaw cycles.

Input DNA

- This protocol is suitable for fragmentation and library construction from 1 ng – 1 µg of double-stranded DNA. Please refer to Table 1 for recommended inputs of different types of DNA, for different sequencing applications.

Table 1. Recommended inputs into library construction

Application	Sample type	Recommended input
WGS	Complex gDNA (high quality)	50 ng – 1 µg
Target capture (WES, custom panels)	Complex gDNA (high quality)	10 ng – 1 µg
WGS, target capture	FFPE DNA	≥50 ng (quality dependent)
WGS	Microbial DNA	1 ng – 1 µg
WGS (PCR-free)	High-quality DNA	≥50 ng (no SS)* ≥200 ng (w/SS)*
Targeted sequencing	Long amplicons	≥1 ng
RNA-seq	Full-length/ unfragmented cDNA	≥1 ng

*SS = size selection; results in the loss of 60 – 95% of DNA, irrespective of whether a bead- or gel-based technique is used.

- The proportion of fragmented DNA that is successfully converted to adapter-ligated molecules decreases as input is reduced. Table 2 summarizes the expected conversion rates for different DNA input ranges. These figures apply to high-quality DNA, and may be lower for DNA of lower quality, e.g., FFPE samples. Workflows with additional cleanups or size selection prior to adapter ligation are also likely to result in a lower yield of adapter-ligated molecules.

Table 2. Expected conversion rates for DNA input ranges

DNA input	Expected conversion rate
1 – 10 ng	5 – 20%
11 – 100 ng	10 – 50%
>100 ng	50 – 100%

- ***The enzymatic fragmentation reaction is very sensitive to the presence of EDTA, which must be removed or neutralized prior to fragmentation.*** EDTA in DNA preparations is usually introduced via elution buffers used in the final stages of the DNA extraction or purification process.
- Removal of EDTA from DNA samples prior to fragmentation is recommended to ensure consistent results. This may be achieved by means of a 3X bead-based cleanup with KAPA cleanup beads. Please refer to the relevant **Technical Data Sheet** (KR1705 or KR1245) for a detailed DNA cleanup protocol. For optimal fragmentation results, elute DNA in 10 mM Tris-HCl (pH 8.0 - 8.5) after the cleanup.
- DNA isolated from blood samples has been reported to contain inhibitors, which can affect the efficiency of fragmentation. Performing a 3X bead-based cleanup prior to fragmentation is recommended.
- Bead-based cleanups to remove EDTA from FFPE DNA samples may not yield comparable results. Recovery of FFPE DNA may be low, and not always proportional to DNA quality. For FFPE DNA, neutralization of EDTA with the Conditioning Solution (see below) is recommended as a first approach.
- If a DNA cleanup is not feasible, the inhibitory effect of the EDTA can be mitigated by the inclusion of Conditioning Solution at the appropriate final concentration in the fragmentation reaction.
- To facilitate reaction setup, the Conditioning Solution is pre-diluted to the appropriate working concentration as outlined in Table 3, and a fixed volume (5 µL) is included in the fragmentation reaction. ***Please note that dilution of the Conditioning Solution is based on the final concentration of EDTA in the fragmentation reaction*** (once input DNA has been diluted in a volume of 50 µL), and not on the EDTA concentration in the DNA preparation.

Table 3. Conditioning Solution dilutions for DNA samples containing EDTA

Final EDTA concentration in 50 µL rxn	Dilution factor	Volume of conditioning solution (per 100 µL)	Volume of PCR-grade water (per 100 µL)
0.02 – 0.05 mM	32.0	3.1 µL	96.9 µL
0.1 mM	15.4	6.5 µL	93.5 µL
0.2 mM	7.4	13.5 µL	86.5 µL
0.3 mM	4.8	21.0 µL	79.0 µL
0.4 mM	3.3	30.0 µL	70.0 µL
0.5 mM	2.6	38.8 µL	61.2 µL
0.6 mM	2.2	46.5 µL	53.5 µL
0.7 mM	1.8	56.0 µL	44.0 µL
0.8 mM	1.6	64.0 µL	36.0 µL
0.9 mM	1.4	72.0 µL	28.0 µL
1.0 mM	1.3	80.0 µL	20.0 µL

- Prepare a minimum of 100 µL of diluted Conditioning Solution (as indicated in Table 3), or calculate the volume needed using the following formula:

  (number of reactions x 5 µL) + 10% excess

- ***The addition of Conditioning Solution to fragmentation reactions will lead to suboptimal results if your DNA does not contain EDTA, or if the final concentration of the Conditioning Solution is not matched to the final EDTA concentration in the reaction.***
- Please refer to **Appendix 2: Optimization of Fragmentation Parameters** (p. 16) for more guidelines regarding the processing of EDTA-containing DNA samples.

Fragmentation Parameters

- The fragmentation parameters provided in the **Library Construction Protocol** (step 1, p. 12) apply to the fragmentation of high-quality genomic DNA.
- The degree of fragmentation (mode size and size distribution of DNA fragments) is controlled by fragmentation time and temperature, and both factors may be modulated to achieve the desired results.
- DNA quality impacts the fragmentation of FFPE DNA. The guidelines in the **Library Construction Protocol** (step 1, p. 12) are a good starting point for FFPE samples with a Q129/Q41 ratio of ~0.4 or higher (as determined with the KAPA hgDNA Quantification and QC Kit). However, slightly longer fragmentation times may improve results for lower-quality FFPE samples. Longer fragmentation times typically increase the proportion of input DNA converted to fragments in the 150 – 250 bp range, reduce residual high-molecular weight DNA, and correlate with higher yields during library construction.
- Standard fragmentation parameters may result in over-fragmentation of low-complexity samples, such as small viral genomes, plasmids, long amplicons and cDNA. For these sample types, the fragmentation time may have to be reduced to 5 min or less to achieve the desired mode fragment length. This makes control over the reaction difficult, particularly when a large number of samples are processed manually. To enable more robust and reproducible results, the fragmentation temperature may be decreased (to 30°C or 25°C) to reduce enzymatic activity, thus increasing the time needed to achieve the desired fragment length.
- Please refer to **Appendix 2** (p. 16) for guidelines on how to systematically optimize fragmentation parameters for your specific samples.
- Different devices (e.g., a thermocycler vs. heating block, or different Peltier devices integrated into automated liquid handling systems) may not yield identical fragmentation profiles for the same sample and input, and fragmentation times may have to be modified slightly when switching between devices. The relative impact of the device used for the fragmentation incubation is likely to be less significant as fragmentation time increases.

End Repair & A-Tailing Enzyme Mix Selection

- This protocol has been validated for use with either the HyperPrep End Repair & A-Tailing Enzyme Mix (existing chemistry) or the HyperPlus End Repair & A-Tailing Enzyme Mix (enhanced chemistry). The formulation of the HyperPlus End Repair & A-Tailing Enzyme Mix has been modified to improve performance for more sensitive assays.
- The HyperPlus workflow does not change depending on the End Repair & A-Tailing Enzyme Mix selected. Either option can be used in the existing workflow.
- For all new applications, it is recommended to use the HyperPlus End Repair & A-Tailing Enzyme Mix (enhanced chemistry). For existing validated workflows, it is recommended to perform a side-by-side comparison using the two enzyme mixes and determine which one is most suitable for the specific application.

Adapter Design and Concentration

- KAPA Adapters are recommended for use with the KAPA HyperPlus Kit. However, the kit is also compatible with other full-length adapter designs wherein both the sequencing and cluster generation sequences are added during the ligation step, such as those routinely used in SeqCap EZ, TruSeq (Illumina) and SureSelect XT2 (Agilent) kits, and other similar library construction workflows. Custom adapters that are of similar design and are compatible with “TA-ligation” of dsDNA may also be used, remembering that custom adapter designs may impact library construction efficiency. For assistance with adapter compatibility and ordering, please contact Technical Support at sequencing.roche.com/support.
- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over during the post-ligation cleanup. The optimal adapter concentration for your workflow represents a compromise between the above factors and cost.
- Ligation efficiency is robust for adapter:insert molar ratios ranging from 10:1 to >200:1, making it unnecessary to adjust adapter stock concentrations to accommmodate moderate variations in DNA input or fragment length. Please refer to Table 4 for the recommended adapter concentrations for different DNA inputs.

Table 4. Recommended adapter concentrations for libraries constructed from 1 ng – 1 µg input DNA*

Input DNA	Adapter stock concentration	Adapter:insert molar ratio	Input DNA	Adapter stock concentration	Adapter:insert molar ratio
1 µg	15 µM	10:1	25 ng	7.5 µM	200:1
500 ng	15 µM	20:1	10 ng	3 µM	200:1
250 ng	15 µM	40:1	5 ng	1.5 µM	200:1
100 ng	15 µM	100:1	2.5 ng	750 nM	200:1
50 ng	15 µM	200:1	1 ng	300 nM	200:1

*Adapter:insert molar ratio calculations are based on a mode DNA fragment length of 200 bp, and will be higher for longer DNA fragments, or slightly lower for DNA fragmented to a mode size <200 bp. The lower adapter:insert molar ratios recommended for inputs >100 ng represent a fair compromise between library construction efficiency and cost; higher library yields will be achieved if a higher adapter concentration is used.

- High adapter:insert molar ratios (>200:1) are beneficial for low-input applications. When optimizing workflows for DNA inputs ≤25 ng, two or three adapter concentrations should be evaluated: try the recommended adapter concentration (Table 4), as well as one or two additional concentrations in a range that is 2 – 10 times higher than the recommended concentration.
- Adapter quality has an impact on the effective concentration of adapter available for ligation. Always source the highest quality adapters from a reliable supplier, dilute and store adapters in a buffered solution with the requisite ionic strength, and avoid excessive freezing and thawing of adapter stock solutions.
- To accommodate different adapter concentrations within a batch of samples processed together, it is best to vary the concentrations of adapter stock solutions, and dispense a fixed volume (5 µL) of each adapter. The alternative (using a single stock solution, and dispensing variable volumes of adapter into ligation reactions) is not recommended.

Post-ligation Processing

- It is important to remove unligated adapter and/or adapter-dimer molecules from the library prior to library amplification or cluster generation.
- The KAPA HyperPlus chemistry reduces adapter-dimer formation, and enables efficient elimination of unused adapter and adapter-dimer with a single post-ligation cleanup. The optimal bead to DNA ratio for libraries prepared from fragmented dsDNA with a mode fragment length in the range of 150 – 350 bp is 0.8X. This ratio may be modified to accommodate libraries prepared from longer DNA fragments or custom adapter designs, or to shift the mode fragment length of adapter-ligated molecules.
- The volume in which washed beads are resuspended after the post-ligation cleanup should be adjusted to suit your chosen workflow:
  - If proceeding directly to library amplification, determine the appropriate final volume in which to elute the library DNA, keeping in mind that you may wish to divert and/or reserve some of this library material for archiving and/or QC purposes. Since a 50 µL library amplification reaction can accommodate 20 – 24 µL template DNA, an elution volume of ~25 µL is recommended.
  - If proceeding with size selection, elute the library DNA in a volume appropriate for the size selection method of choice. For the double-sided size selection protocol described in **Appendix 1**, beads must be resuspended in 55 µL of elution buffer.
- A second post-ligation cleanup (using a 1X or different bead-to-DNA ratio) may be performed if post-ligation or post-amplification analysis reveals unacceptable levels of adapter and/or adapter-dimer carry-over after the first cleanup. A second cleanup may be particularly beneficial when libraries are prepared in PCR-free workflows for direct sequencing on Illumina instruments that employ patterned flow cells. The sample volume should be adjusted (with elution buffer) to at least 50 µL for a second post-ligation cleanup. The adapter concentration may also be optimized to eliminate carry-over of adapter and/or adapter-dimer (and eliminate the need for a second post-ligation cleanup). However, keep in mind that library construction is most efficient when high adapter:insert molar ratios are used.

Reaction Cleanups

- This protocol has been validated for use with either KAPA HyperPure Beads and KAPA Pure Beads (collectively referred to hereafter as KAPA cleanup beads) or Agencourt AMPure XP (Beckman Coulter). Solutions and conditions for DNA binding and size selection may differ if other beads are used.
- Observe all the storage and handling recommendations for KAPA cleanup beads or AMPure XP. Equilibration to room temperature is essential to achieve specified size distribution and yield of libraries.
- Beads will settle gradually; always ensure that they are fully resuspended before use.
- ***To ensure optimal DNA recovery, it is critical that the DNA and the KAPA cleanup beads are thoroughly mixed*** (by vortexing or extensive up-and-down pipetting) before the DNA binding incubation.
- Bead incubation times are guidelines only, and may be modified/optimized according to current protocols, previous experience, specific equipment and samples in order to maximize library construction efficiency and throughput.
- The time required for complete capture of beads varies according to the reaction vessel and magnet used. It is important not to discard or transfer any beads with the removal or transfer of supernatant. Capture times should be optimized accordingly.
- The volumes of 80% ethanol used for bead washes may be adjusted to accommodate smaller reaction vessels and/or limited pipetting capacity, but it is important that the beads are entirely submerged during the wash steps. ***Always use freshly prepared 80% ethanol.***
- It is important to remove all ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, resulting in a dramatic loss of DNA. With optimized aspiration of ethanol, drying of beads for 3 – 5 min at room temperature should be sufficient. ***Drying of beads at 37°C is not recommended.***
- Where appropriate, DNA should be eluted from beads in elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions. However, libraries constructed for hybridization target capture that require drying of DNA prior to probe hybridization must be eluted and stored in PCR-grade water. Purified

DNA in elution buffer should be stable at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C for long-term storage. The long-term stability of library DNA at -15°C to -25°C depends on a number of factors, including library concentration. Always use low DNA-binding tubes for long-term storage, and avoid excessive freezing and thawing.

Size Selection

- Size selection requirements vary widely for different sequencing applications. If required, any commonly used bead- or gel-based size selection technique may be integrated in the KAPA HyperPlus workflow.
- Size selection may be carried out at different points in the overall workflow, for example after the post-ligation cleanup, or after library amplification.
- The standard protocol (pp. 12 – 14) does not include size selection. Please refer to **Appendix 1** (p. 15) for a detailed double-sided size selection protocol.
- Size selection inevitably leads to a loss of sample material. These losses can be dramatic (60 – 95%), and may significantly increase the number of amplification cycles required to generate sufficient material for the next step in the process (capture or sequencing). The potential advantages of one or more size selection steps in a library construction workflow should be weighed against the potential loss of library complexity, especially when input DNA is limited. A well-optimized fragmentation protocol, especially for shorter insert libraries and/or read lengths, may eliminate the need for size selection, thereby simplifying the library construction process and limiting sample losses.
- The Ligation Buffer contains high concentrations of PEG 6000, which will interfere with efficient size selection and can affect the efficiency of other size selection techniques if not removed. If size selection is performed after ligation, it is important to perform at least one bead-based cleanup prior to performing bead- or electrophoresis-based size selection.
- Over-amplification typically results in the observation of secondary, higher molecular weight peaks in electrophoretic profiles of amplified libraries. These higher molecular weight peaks are artifacts of the analysis, and typically contain authentic library molecules of the appropriate length. To eliminate these artifacts, optimization of library amplification reaction parameters (cycle number and primer concentration), rather than post-amplification size selection, is recommended. Please refer to the next subsection for more information.

Library Amplification

- KAPA HiFi HotStart, the enzyme provided in the KAPA HiFi HotStart ReadyMix, is an antibody-based hotstart formulation of KAPA HiFi DNA Polymerase—a B-family DNA polymerase engineered for increased processivity and high fidelity. KAPA HiFi HotStart has 5'→3' polymerase and 3'→5' exonuclease (proofreading) activity, but no 5'→3' exonuclease activity. The strong 3'→5' exonuclease activity results in superior accuracy during DNA amplification. The error rate of KAPA HiFi HotStart is 2.8 x 10^{-7} errors/base, equivalent to 1 error in 3.5 x 10^{6} nucleotides incorporated.
- The KAPA Library Amplification Primer Mix (10X) is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix. The primer mix is suitable for the amplification of all Illumina libraries flanked by the P5 and P7 flow cell sequences. Primers are supplied at a 10X concentration of 20 µM each, and have been formulated as described below. User-supplied primer mixes may be used in combination with incomplete or custom adapters. Please contact Technical Support at sequencing.roche.com/support for guidelines on the formulation of user-supplied library amplification primers.
- To achieve the highest amplification efficiency and avoid primer depletion, it is critical to use an optimal concentration of high quality primers. Primers should be used at a final concentration of 0.5 – 4 µM each. For libraries constructed from ≥100 ng input DNA, a final concentration of at least 2 µM of each primer is recommended.
- Library amplification primers should be HPLC-purified and modified to include a phosphorothioate bond at the 3'-terminal of each primer (to prevent degradation by the strong proofreading activity of KAPA HiFi HotStart DNA Polymerase). Always store and dilute primers in a buffered solution (e.g., 10 mM Tris-HCl, pH 8.0 – 8.5), and limit the number of freeze-thaw cycles. To achieve the latter, store primers at 2°C to 8°C for short-term use, or as single-use aliquots at -15°C to -25°C.
- In library amplification reactions (set up according to the recommended protocol), primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to substrate depletion, subsequent rounds of DNA denaturation and annealing result in the separation of complementary DNA strands, followed by imperfect annealing to non-complementary partners. This presumably results in the formation of so-called “daisy chains” or “tangled knots”, comprising large assemblies of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they typically comprise library molecules of the desired length, which are individualized during denaturation prior to cluster amplification or probe hybridization. Since

these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes. qPCR-based library quantifications methods, such as the KAPA Library Quantification assay, quantify DNA by denaturation and amplification, thereby providing an accurate measure of the amount of adapter-ligated molecules in a library—even if the library was over-amplified.

- Please refer to the KAPA NGS Library Preparation Technical Guide (available on request from Technical Support at sequencing.roche.com/support) for a more detailed discussion of factors that can affect the efficiency of library amplification, and the impact of over-amplification on library quantification.
- Excessive library amplification can result in other unwanted artifacts such as amplification bias, PCR duplicates, chimeric library inserts and nucleotide substitutions. The extent of library amplification should therefore be limited as much as possible, while ensuring that sufficient material is generated for QC and downstream processing (e.g., target capture or sequencing).
- If cycled to completion (***not recommended***), one 50 µL library amplification PCR—performed as described in the **Library Construction Protocol** (step 5)—can produce 8 – 10 µg of amplified library. To minimize over-amplification and its associated, undesired artifacts, the number of amplification cycles should be tailored to produce the optimal amount of final library required for downstream processes. This is typically in the range of 250 ng – 1.5 µg. Table 5 provides recommended cycle numbers for libraries prepared from high-quality input DNA, to obtain approximately 100 ng or 1 µg of amplified library.

Table 5. Recommended cycle numbers to generate 100 ng or 1 µg of amplified DNA from 1 ng – 1 µg of input DNA

Input into library construction	Number of cycles required to generate	
	100 ng library	1 µg library
1 µg	0*	0 – 1*
500 ng	0*	2 – 3
250 ng	0 – 1*	3 – 5
100 ng	0 – 2*	5 – 6
50 ng	3 – 5	7 – 8
25 ng	5 – 6	8 – 10
10 ng	7 – 9	11 – 13
5 ng	9 – 11	13 – 14
2.5 ng	11 – 13	14 – 16
1 ng	13 – 15	17 – 19

*When using incomplete adapters, a minimum number of amplification cycles (1 – 3) may be required to complete adapter sequences for the next step in the process (target capture or sequencing), irrespective of whether a sufficient amount of library is available after ligation. The number of cycles needed depends on the specific adapter and amplification primer design.

- The quantification of adapter-ligated libraries (prior to library amplification) can greatly facilitate the optimization of library amplification parameters, particularly when a library construction workflow is first established. With the KAPA Library Quantification Kit, the amount of template DNA (adapter-ligated molecules) available for library amplification can be determined accurately. From there, the number of amplification cycles needed to achieve a specific yield of amplified library can be predicted theoretically. Please refer to Table 6 for the number of cycles recommended to obtain approximately 1 µg of DNA from 500 pg – 500 ng of adapter-ligated DNA, or contact Technical Support at sequencing.roche.com/support regarding a calculator designed to assist with these calculations. Please note that the actual optimal number of amplification cycles may be 1 – 3 cycles higher or lower, depending on the sample type and size distribution of the input DNA.
- Depending on the amount of library material required for your application, it may be possible to omit library amplification. In such cases, it is important to ensure that your adapters are designed to support sample indexing (where required), cluster amplification and sequencing. Omitting library amplification further streamlines the workflow and reduces overall library preparation time to ≤1.5 hrs. The high conversion efficiency achievable with the KAPA HyperPlus Kit enables PCR-free workflows from as little as 50 ng of input DNA. KAPA HyperPlus Kits without amplification reagents (07962398001, 07962410001, and 07962436001) are available for PCR-free workflows.

Table 6. Theoretical number of cycles required to obtain approximately 1 µg of amplified library DNA from 500 pg – 500 ng of adapter-ligated library DNA*

Amount of adapter-ligated DNA in amplification rxn	Number of cycles required to generate 1 µg of library DNA
500 ng	1 – 2
100 ng	3 – 4
50 ng	5 – 6
10 ng	7 – 8
5 ng	8 – 9
1 ng	11 – 12
500 pg	12 – 13

*Guidelines are based on amplification with KAPA HiFi HotStart ReadyMix and the Library Amplification Primer Mix, and library quantification with the qPCR-based KAPA Library Quantification Kit.

Evaluating the Success of Library Construction

- Your specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for the next step in the process (e.g., target capture or sequencing), as well as for library QC and archiving purposes.
- While it is possible to remove aliquots of the fragmentation reaction product for analysis in the integrated fragmentation/library construction workflow, it is most productive to assess the outcome

of fragmentation once the entire KAPA HyperPlus workflow has been completed—for the following reasons:

- It is difficult and disruptive to process low-volume aliquots in a way that is fully representative of the final library.
- Fragmentation profiles for low-input samples (1 – 10 ng into fragmentation) may not be informative, even when high-sensitivity assays are used.
- The final size distribution of libraries prepared from FFPE samples is typically smaller than expected based on the size distribution after fragmentation and adapter length. This is a common phenomenon, attributable to the inability of high-fidelity DNA polymerases used in library amplification to efficiently amplify damaged DNA, particularly templates that contain deaminated or oxidized bases.

- Please refer to **Appendix 2** (p. 16) for guidelines on how to systematically optimize fragmentation parameters for your specific samples.
- The size distribution of pre-capture or final libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII, or GX Touch (PerkinElmer), Bioanalyzer or TapeStation (Agilent Technologies), Fragment Analyzer (Advanced Analytical) or similar instrument is recommended over conventional gels. Typical electrophoretic profiles for libraries prepared with the KAPA HyperPlus Kit are given in Figure 1 on the next page.
- Please note that libraries prepared with "forked" adapters in PCR-free workflows will appear to have a longer than expected mode fragment length, and/or may display a broad or bimodal size distribution when analyzed electrophoretically (see Figure 1). The difference in overall appearance and fragment size distribution of an unamplified vs. the corresponding amplified library varies, and depends on the adapter design and electrophoretic system used. To accurately determine the size distribution of an unamplified library, an aliquot of the library may be subjected to a few cycles of amplification prior to electrophoretic analysis, to ensure that all adapter-ligated molecules are fully double-stranded. Alternatively, size information may be obtained by electrophoretic analysis of library quantification products generated with the KAPA Library Quantification Kit (see below).
- KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated using the KAPA HyperPlus workflow. These kits employ primers based on the Illumina flow cell oligos, and can be used to quantify libraries that:
  - are ready for flow-cell amplification, and/or
  - were constructed with full-length adapters, once ligation has been completed, i.e., after the post-ligation cleanup, after the (pre-capture) amplification cleanup, or before/after post-ligation or post-amplification size selection.

  The KAPA Library Quantification Kit provides the only reliable means for quantifying libraries at different stages of the workflow, and libraries produced in PCR-free workflows, as:
  - it only quantifies those molecules with two adapters in the correct configuation for cluster amplification and sequencing, and
  - measurements are not affected by library over-amplification (see **Important Parameters: Library Amplification**, p. 7).
- Once a library construction workflow has been optimized, and consistently yields the desired amount of amplified library of the requisite size distribution, it is typically not necessary to perform in-process quality control. However, qPCR-based quantification of libraries after the **Post-ligation Cleanup** (prior to **Library Amplification**) can provide useful data for optimization or troubleshooting. Quantification at this stage allows you to assess the efficiency of:
  - the core library construction process (fragmentation to ligation), by determining the percentage of input DNA converted to adapter-ligated molecules, and
  - library amplification with the selected number of cycles, based on the actual amount of template DNA used in the PCR.

  The availability of quantification data before and after library amplification allows the two major phases of the library construction process to be evaluated and optimized independently to achieve the desired yield of amplified library.
- If size selection is performed at any stage, qPCR quantification before and after size selection may also be helpful to define the relative benefit of size selection, and to determine the loss of material associated with the process.
- Electrophoretic evaluation of libraries after the post-ligation cleanup/before library amplification may be informative, but remember that the apparent mode fragment length and size distribution will be inaccurate due to the retardation of non-complementary adapter regions, as outlined above and illustrated in Figure 1.

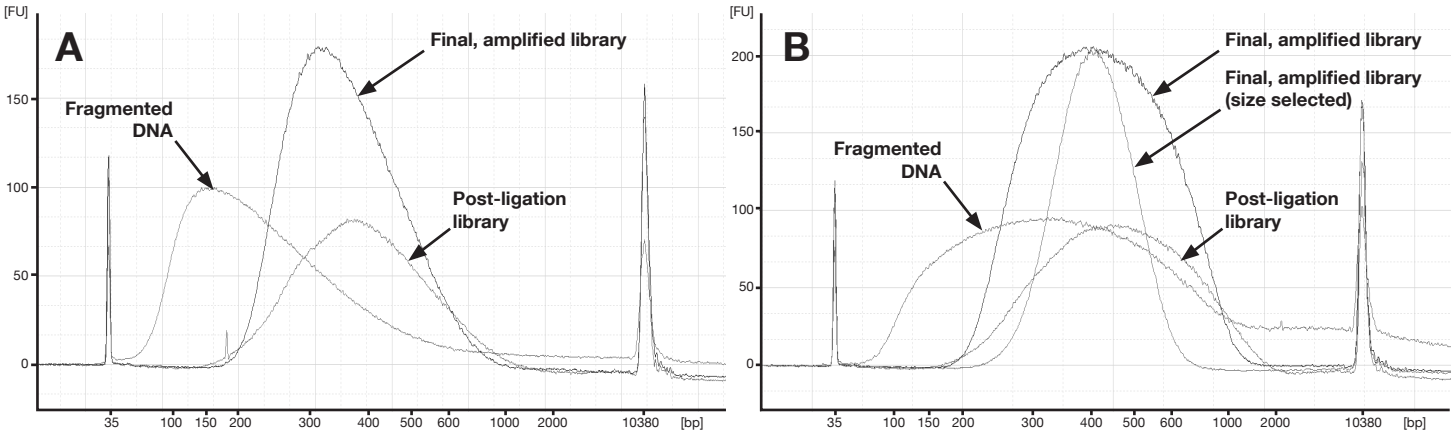


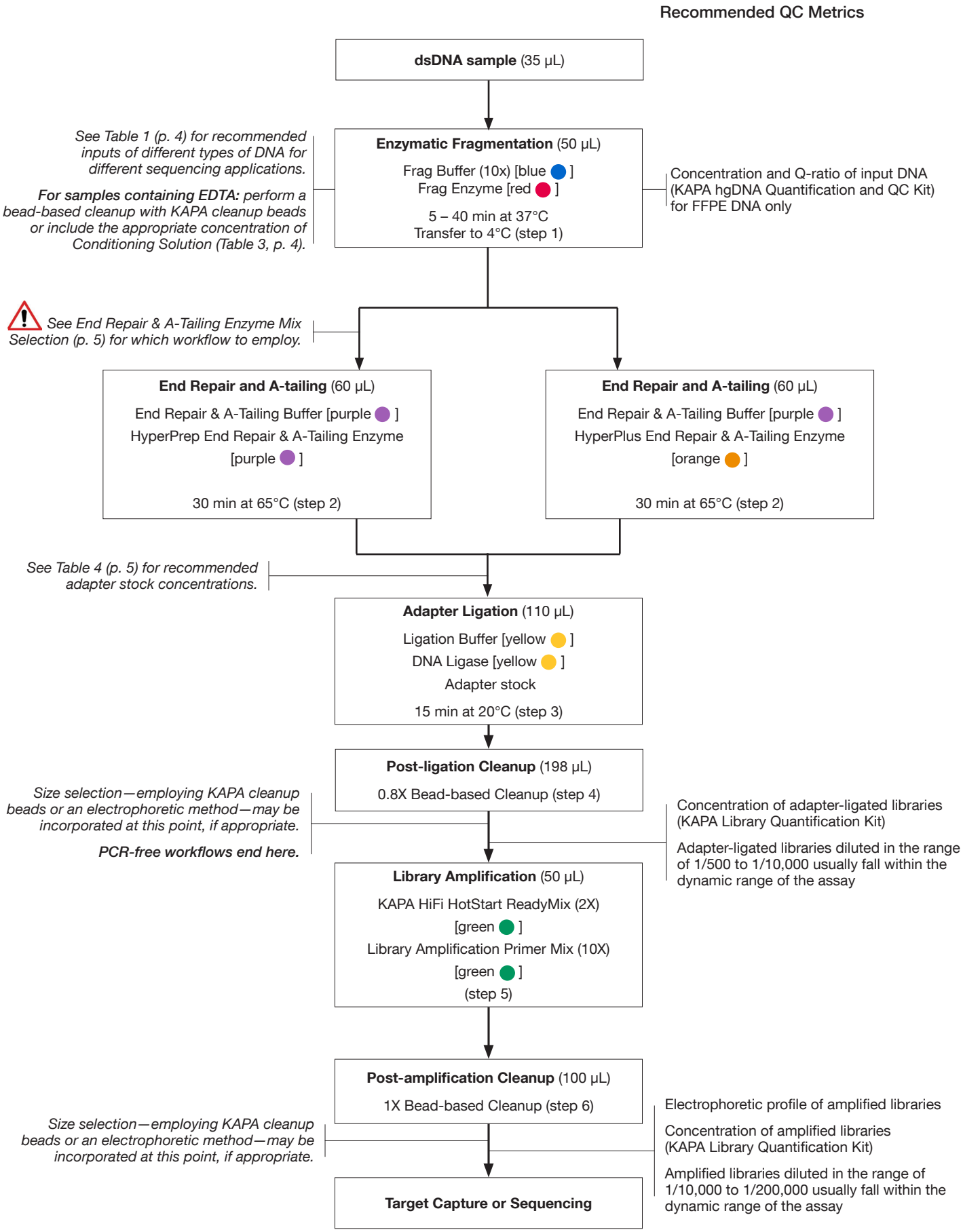
Figure 1. Examples of libraries prepared with the KAPA HyperPlus Kit

Input DNA (100 ng high-quality human genomic DNA) was fragmented at 37°C for 30 min (**A**) or 10 min (**B**), to achieve a mode fragment length of ~150 bp or ~350 bp, respectively. Libraries were prepared as described in the **Library Construction Protocol** (pp. 12 – 14), using the recommended adapter:insert molar ratio. Larger-insert libraries (**B**) were prepared in duplicate. One library was subjected to double-sided size selection after the Post-ligation Cleanup, as described in **Appendix 1: Size Selection** (p. 15), whereas the other was not. Electropherograms were generated with a Bioanalyzer 2100 High Sensitivity DNA Kit. DNA concentrations were normalized prior to analysis and are not reflective of the actual DNA concentrations at different stages of the process. Electrophoretic profiles for fragmented DNA were generated in a "standalone" workflow, whereby the protocol was terminated after fragmentation, and reaction products purified using a 2X bead-based cleanup.

After ligation, the non-complementary ends of full-length, "forked" adapters retard the migration of library fragments in gel matrices, leading to a larger than expected size distribution. The difference between the actual and apparent mode fragment length of unamplified, adapter-ligated libraries depends on the adapter design and electrophoretic system used, and can be much more pronounced than observed here. Size selection results in a much narrower final library size distribution, but at the cost of a significant amount of library material.

KAPA HyperPlus Kit

Process Workflow



Library Construction Protocol

Notes:

- First-time users should refer to **Appendix 2: Optimization of Fragmentation Parameters** (p. 16) before trying this kit, as standard fragmentation parameters may not result in the optimal size distribution for libraries prepared from your specific DNA samples. Precious samples should not be used when evaluating this kit. Instead, parameters should be optimized with a non-precious, bulk DNA sample that is representative of the actual samples to be processed.
- ***If your DNA samples contain EDTA,*** please consult the **Appendix 2: Handling of DNA Samples Containing EDTA** (p. 16), as well as **Important Parameters: Input DNA** (p. 4) before starting this protocol.
- This protocol does not include size selection. Please refer to **Appendix 1** (p. 15) for a detailed double-sided size selection protocol that may be included after ligation or after amplification.
- Always ensure that KAPA cleanup beads are fully equilibrated to room temperature and fully resuspended before use.

1. Enzymatic Fragmentation

If the DNA samples contain EDTA, perform a 3X bead-based cleanup with KAPA cleanup beads to remove EDTA prior to fragmentation. Please refer to the relevant **Technical Data Sheet** (KR1705 or KR1245) for a detailed DNA cleanup protocol.

Alternatively, prepare a sufficient volume of appropriately diluted Conditioning Solution (5 µL per DNA sample, plus excess). Refer to Table 2 (p. 4) for guidelines on the dilution of the Conditioning Solution.

1.1 Dilute the amount of dsDNA to be used for library construction as follows:

- If the DNA preparation **does not** contain EDTA, dilute in 10 mM Tris-HCl (pH 8.0 – 8.5) in a total of 35 µL
- If the DNA preparation **does** contain EDTA, dilute in the EDTA-containing buffer in which samples are currently suspended, in a total of 30 µL. To each reaction with 30 µL of EDTA-containing DNA, add 5 µL of diluted Conditioning Solution.

1.2 Mix by gentle vortexing or pipetting up and down.

1.3 Assemble each fragmentation reaction **on ice** by adding the components in this order:

Component	Volume
Double-stranded DNA (with Conditioning Solution, if needed)	35 µL
KAPA Frag Buffer (10X)*	5 µL
KAPA Frag Enzyme*	10 µL
Total volume:	**50 µL**

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

1.4 Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.

1.5 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	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** (p. 16) for guidelines on how to optimize fragmentation time and temperature. If incubation times longer than the recommended range are needed, samples likely contain inhibitors which impact the fragmentation efficiency. Bead-based DNA cleanup, prior to fragmentation, is recommended over longer fragmentation times.

1.6 Transfer reactions to ice, and proceed immediately to **End Repair and A-tailing** (step 2).

2. End Repair and A-tailing

Two End Repair & A-Tailing Enzyme Mixes are provided for the End Repair and A-tailing step — see **Important Parameters** for further guidelines.

2.1 In the same plate/tube(s) in which enzymatic fragmentation was performed, assemble each End Repair and A-tailing reaction as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End Repair & A-Tailing Buffer*	7 µL
HyperPrep/HyperPlus ERAT 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 hrs at room temperature, for ≤3 days at 2°C to 8°C, and for ≤4 weeks at -15°C to -25°C.

**Use either the HyperPrep ERAT Enzyme Mix (existing chemisty) or the HyperPlus ERAT Enzyme Mix (enhanced chemistry).

2.2 Vortex gently and spin down briefly. Return the reaction plate/tube(s) to ice. Proceed immediately to the next step.

2.3 Incubate in a thermocycler programmed as outlined below. A heated lid is required for this step. If possible, set the temperature of the heated lid to ~85°C (instead of the usual 105°C).

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

*Both the fragmentation and end repair enzymes are inactivated at 65°C. When reactions are set up according to recommendations, additional fragmentation should be negligible. The brief period of end repair is sufficient for enzymatically fragmented DNA.

**If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

2.4 Proceed immediately to **Adapter Ligation** (step 3).

3. Adapter Ligation

3.1 Dilute adapter stocks to the appropriate concentration, as outlined in Table 4 (p. 5).

3.2 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
Adapter stock (diluted as per Table 4 on p. 5)	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 hrs at room temperature, for ≤3 days at 2°C to 8°C, and for ≤4 weeks at -15°C to -25°C.

3.3 Mix thoroughly and centrifuge briefly.

3.4 Incubate at 20°C for 15 min.

Note: to achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time to a maximum of 4 hrs at 20°C or overnight at 2°C to 8°C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly.

3.5 Proceed immediately to **Post-ligation Cleanup** (step 4).

4. Post-ligation Cleanup

4.1 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 cleanup beads	88 µL
Total volume:	**198 µL**

4.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

4.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

4.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

4.5 Carefully remove and discard the supernatant.

4.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4.8 Carefully remove and discard the ethanol.

4.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

4.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4.12 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.***

4.13 Remove the plate/tube(s) from the magnet.

4.14 Resuspend the beads:

- in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with **Library Amplification** (step 5), or
- in 55 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with double-sided size selection (**Appendix 1**).

4.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

4.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

4.17 Transfer the clear supernatant to a new plate/tube(s):

- to proceed with **Library Amplification** (step 5), transfer 20 µL of supernatant, or
- to proceed with double-sided size selection (**Appendix 1**), transfer 50 µL of supernatant.

5. Library Amplification

Note: Please refer to **Important Parameters: Library Amplification** (p. 7) and the **KAPA NGS Library Preparation Technical Guide** (available on request from Technical Support at sequencing.roche.com/support) for more information on optimizing library amplification.

5.1 Assemble each library amplification reaction as follows:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Library Amplification Primer Mix (10X)*	5 µL
Adapter-ligated library	20 µL
Total volume:	**50 µL**

*Or other, suitable 10X library amplification primer mix.The recommended final concentration of each primer in the library amplification reaction is 0.5 – 4 µM. Also refer to **Important Parameters: Library Amplification** (p. 7).

5.2 Mix thoroughly and centrifuge briefly.

5.3 Amplify using the following cycling protocol:

Step	Temp	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Minimum number required for optimal amplification (**Table 5** or **6**)
Annealing*	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

*Optimization of the annealing temperature may be required for non-standard (i.e., other than Illumina TruSeq) adapter/primer combinations.

5.4 Proceed directly to **Post-amplification Cleanup** (step 6).

6. Post-amplification Cleanup

6.1 In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining the following:

Component	Volume
Library amplification reaction product	50 µL
KAPA cleanup beads	50 µL
Total volume:	**100 µL**

6.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

6.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

6.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

6.5 Carefully remove and discard the supernatant.

6.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

6.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

6.8 Carefully remove and discard the ethanol.

6.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

6.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

6.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

6.12 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.***

6.13 Remove the plate/tube(s) from the magnet.

6.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) or PCR-grade water.

Note: If proceeding with a second post-amplification cleanup, or double-sided size selection (**Appendix 1**), resuspend the beads in 55 µL of elution buffer.

6.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

6.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

6.17 Transfer the clear supernatant to a new plate/tube(s) and proceed with size selection (refer to **Appendix 1**), library QC, target capture or sequencing, as appropriate. Store purified, amplified libraries at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C.

Appendix 1: Size Selection

Any commonly used size selection technique (e.g., the double-sided size selection described here, or an electrophoretic method) may be integrated into the KAPA HyperPlus library construction workflow. Size selection should preferably be carried out after the post-ligation cleanup, or after library amplification. Whether or not size selection is performed, which technique is used, and at what stage of the library construction process it is performed, depends on the nature of the sample, input into library construction, and the sequencing application and read length. For more information on size selection, refer to **Important Parameters: Size Selection** (p. 7).

The double-sided size selection protocol outlined in this appendix is designed for the selection of library molecules (**inclusive of adapter**) in the range of 250 – 450 bp. To obtain a population of shorter or longer molecules, the protocol may be modified as follows:

Upper size limit	Modification	Lower size limit	Modification
Increase	Decrease the ratio of the first cut	Increase	Decrease the ratio of the second cut*
Decrease	Increase the ratio of the first cut	Decrease	Increase the ratio of the second cut*

*The second size cut should be performed with at least 0.2 volumes of KAPA cleanup beads reagent. Please note that the volume of KAPA cleanup beads needed for the second cut is calculated relative to the volume of the DNA at the start of the size selection procedure, not the volume of the DNA-containing supernatant transferred after the first cut. DNA recovery is dramatically reduced if the difference between first and second cuts is less than ~0.2 volumes. To increase the amount of DNA recovered, >0.2 volumes of KAPA cleanup beads may be used for the second cut, but note that this may result in the recovery of smaller library fragments and/or a broader size distribution. For more information on double-sided size selection, please refer to the **KAPA NGS Library Preparation Technical Guide** (available on request from Technical Support at sequencing.roche.com/support).

A1.1 Perform the first (0.7X) size cut (to exclude library molecules larger than ~450 bp) by combining the following:

Component	Volume
DNA to be size selected	50 µL
KAPA cleanup beads	35 µL
Total volume per well/tube:	**85 µL**

A1.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

A1.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind library molecules larger than ~450 bp to the beads.

A1.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

A1.5 Carefully transfer ~80 µL of supernatant containing library molecules smaller than ~450 bp to a new plate/tube. It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) with the beads to which library molecules larger than ~450 bp were bound.

A1.6 Perform the second size cut (0.9X), to retain library molecules >250 bp) by combining the following:

Component	Volume
Supernatant from first size cut	80 µL
KAPA cleanup beads	10 µL
Total volume per well/tube:	**90 µL**

A1.7 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

A1.8 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind library molecules larger than ~250 bp to the beads.

A1.9 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

A1.10 Carefully remove and discard the supernatant, which contains library molecules smaller than ~250 bp.

A1.11 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

A1.12 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

A1.13 Carefully remove and discard the ethanol.

A1.14 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

A1.15 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

A1.16 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

A1.17 Dry the beads for 3 – 5 min at room temperature, or until all of the ethanol has evaporated. ***Caution: over-drying the beads may result in reduced yield.***

A1.18 Remove the plate/tube(s) from the magnet.

A1.19 Thoroughly resuspend the beads in the required volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5).

A1.20 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

A1.21 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

A1.22 Transfer the clear supernatant with size-selected DNA to a new plate/tube(s) and proceed with the next step in your workflow, or store DNA at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C.

Appendix 2: Optimization of Fragmentation Parameters

Fragmentation guidelines provided in the **Library Construction Protocol: Enzymatic Fragmentation** (step 1) may not result in the optimal library size distribution for your specific DNA samples. For this reason, precious samples should not be used when evaluating the KAPA HyperPlus Kit for the first time. Instead, fragmentation parameters should be optimized in the context of the KAPA HyperPlus workflow, using a non-precious, bulk DNA sample that is representative of the actual samples to be processed.

The information in this Appendix should be considered during the experimental design for your evaluation of the KAPA HyperPlus Kit.

Quantification of Input DNA

Although the KAPA Frag enzymatic fragmentation system is less sensitive to DNA input than tagmentation-based library construction methods, it is recommended that input DNA be quantified. The Qubit fluorometer is recommended for the quantification of high-quality DNA, whereas the KAPA hgDNA Quantification and QC Kit provides both concentration and quality information for FFPE DNA.

Handling of DNA Samples Containing EDTA

If the DNA samples contain EDTA, perform a 3X bead-based cleanup with KAPA cleanup beads to remove EDTA prior to fragmentation. Please refer to the relevant **Technical Data Sheet** (KR1705 or KR1245) for a detailed DNA cleanup protocol.

Alternatively, the Conditioning Solution may be used to neutralize EDTA prior to fragmentation. This strategy is recommended as a first approach for precious FFPE DNA samples of variable quality.

Since EDTA in DNA preparations is usually introduced via elution buffers used in the final stages of the DNA extraction or purification process, the concentration of EDTA is typically known (e.g., 1 mM for standard TE buffer and 0.1 mM for "low-EDTA" TE buffer). If this is the case, and your samples are of similar concentration (i.e., a constant volume of DNA is used for library construction), simply refer to Table 3 (p. 4) for the appropriate dilution of Conditioning Solution, and follow the **Library Construction Protocol** (step 1.1).

If you know the composition of the EDTA-containing buffer used for DNA purification, but your samples span a wide concentration range (i.e., variable volumes will be used to achieve the desired input into library construction), samples should be normalized in the same EDTA-containing buffer used for DNA purification.

For example:

- If your DNA samples are in TE buffer, and your input into library construction is 100 ng, dilute 100 ng of each sample into a final volume of 30 µL (i.e., to 3.33 ng/µL) using TE buffer.
- All samples will now contain the same final EDTA concentration once diluted to 50 µL for fragmentation. This concentration is:

  EDTA concentration in TE buffer x (30 µL/50 µL)

  = 1 mM x (30 µL/50 µL) = 0.6 mM
- Make a 2.2-fold dilution of the Conditioning Solution (as per Table 3 on p. 4), and follow the **Library Construction Protocol** (second half of step 1.1).

If you are unsure about the presence or concentration of EDTA in your DNA samples, remove the EDTA by performing a column- or bead-based purification or buffer exchange prior to enzymatic fragmentation, or follow the procedure outlined below:

- Set up a series of test reactions with the appropriate amount of input DNA, and different final concentrations of Conditioning Solution.
- Include at least one reaction with the same input of control DNA known to be EDTA-free. The control DNA should preferably be of the same type and quality as the test samples.
- Fragment the DNA using the appropriate parameters, as outlined in the **Library Construction Protocol: Enzymatic Fragmentation** (step 1). Complete the library construction process, and compare library size distributions for the test and control samples using an electrophoretic system (see **Important Parameters: Evaluating the Success of Library Construction**, p. 8).
- Titrate the final concentration of Conditioning Solution in the reaction until the test samples yield similar fragmentation profiles as the EDTA-free control sample, or until the desired library size distribution has been achieved.
- A two-step strategy may be the best. Start with 3 – 4 test samples covering a broad range of final Conditioning Solution concentrations, then perform a finer titration over a narrower concentration range.

Optimization of Fragmentation Time

The fragmentation guidelines in the **Library Construction Protocol: Enzymatic Fragmentation** (step 1) are a good starting point for high-quality genomic DNA. When evaluating the KAPA HyperPlus Kit for the first time, it is recommended that you proceed as follows:

- Set up at least three replicate reactions with the desired input of a non-precious, bulk sample that is representative of the actual samples to be processed.
- Select the most appropriate fragmentation time (for the desired mode fragment length) from the third table in the **Library Construction Protocol** (step 1.5, p. 12). Perform one reaction with that time, and one reaction each with either a slightly shorter or slightly longer fragmentation time within the optimization range. Increments of 3 – 5 min are recommended.

- Complete the library construction process, and evaluate the size distribution of the final libraries electrophoretically.
  - If the mode fragment length is too long, increase the fragmentation time in increments of 2 – 5 min until the optimal final library distribution is achieved.
  - If the mode fragment length is too short, reduce the fragmentation time in increments of 2 – 5 min until the optimal final library distribution is achieved.
- Further fine-tuning (plus or minus 1 – 2 min) may be necessary if the fragmentation time is relatively short (10 min or less). If this is the case, consider optimizing the fragmentation temperature (see below).

A similar strategy may be employed to optimize the fragmentation time for FFPE samples, remembering that lower quality samples may benefit from slightly longer fragmentation times. For FFPE samples:

- Set up 4 – 5 replicate reactions with the desired input of a non-precious, bulk sample that is representative of the actual samples to be processed. This sample may have to be generated by pooling a few individual samples.
- Select the fragmentation time corresponding to the desired mode fragment length from the third table in the **Library Construction Protocol** (step 1.5, p. 12). Use that as the minimum fragmentation time, and increase the incubation time at 37°C by 5 min for each additional replicate.
- Complete the library construction process, evaluate the size distribution of the final libraries, and fine-tune fragmentation time if needed, as described above.

With respect to FFPE samples, please note the following:

- Electrophoretic profiles of FFPE samples, generated during sample QC prior to fragmentation, are not always good predictors of library and sequence quality. Samples that appear to consist of high-molecular weight DNA may not yield libraries of significantly better quality than samples that appear to be degraded. The KAPA hgDNA Quantification and QC Kit provides a qPCR-based assay for assessment of FFPE DNA quality. Quality scores (Q-ratios) determined with this assay have been shown to correlate with the success of library construction.
- The mode fragment length of an amplified FFPE library is typically shorter than than expected based on the size distribution after fragmentation and adapter length. This is a common phenomenon, attributable to the inability of high-fidelity DNA polymerases used in library amplification to efficiently amplify damaged DNA, particularly templates that contain deaminated or oxidized bases. For this reason, it is not productive to try and optimize fragmentation parameters independently of the rest of the library construction process when using the KAPA HyperPlus workflow.

Optimization of Fragmentation Temperature

The standard fragmentation temperature is 37°C. If you are fragmenting high-quality genomic DNA, any other high-complexity DNA sample, or FFPE DNA to a mode fragment length <500 bp, it is unlikely that you will have to change or optimize the fragmentation temperature.

Low-complexity samples (e.g., small viral genomes, plasmids, long amplicons and cDNA) may, however, be over-fragmented at 37°C, even with short incubation times. The likelihood of over-fragmentation depends on the nature, molecular weight/length of the input DNA, the desired size distribution after fragmentation and, to a lesser degree, the DNA input into fragmentation. For example, 100 ng of a 1.8 kb PCR product will yield a similar mode fragment length (~300 bp) as 100 ng *E. coli* or human genomic DNA when fragmented at 37°C for 10 min, whereas 1 ng of a 1 kb PCR product will be fragmented to a mode size <250 bp using the same parameters.

To determine the optimal fragmentation parameters for low-complexity samples, or high-complexity samples when the desired mode fragment length is >500 bp:

- Set up four replicate reactions with a non-precious, bulk sample that is representative of the actual samples to be processed.
- Fragment two of the samples at 37°C, for 5 min and 10 min, respectively. Repeat these fragmentations with the other two samples, but at 30°C.
- Complete the library construction process, and evaluate the size distribution of the final libraries electrophoretically.
  - If the mode fragment length obtained with a 10 min incubation at 37°C is too long, continue optimizing (increasing) the fragmentation time at 37°C.
  - If the mode fragment length obtained with a 10 min incubation at 30°C is too long, but 5 min at 37°C resulted in over-fragmentation, continue optimizing (increasing) the fragmentation time at 30°C.
  - If a 5 min incubation at 30°C resulted in over-fragmentation, perform a second set of reactions (e.g., for 5 min, 10 min, 15 min, and 20 min) at 25°C, and fine-tune the fragmentation time if needed.

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Manufacturing, R & D
Cape Town, South Africa
Tel: +27 21 448 8200
Fax: +27 21 448 6503

Technical Support
sequencing.roche.com/support