



KAPA Hyper Prep Kit

KR0961 – v6.17

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

This document applies to the KAPA Hyper Prep Kits (07962312001, 07962347001 and 07962363001), and to the KAPA Hyper Prep Kits for PCR-free workflows (07962339001, 07962355001 and 07962371001).

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Kapa/Roche Kit Codes and Components		
KK8500 07962312001	End Repair & A-Tailing Buffer	60 µL
	End Repair & A-Tailing Enzyme	25 µL
KK8501* 07962339001	Ligation Buffer	245 µL
	DNA Ligase	80 µL
	KAPA HiFi HotStart ReadyMix (2X)*	250 µL
8 libraries	Library Amplification Primer Mix (10X)*	50 µL
KK8502 07962347001	End Repair & A-Tailing Buffer	210 µL
	End Repair & A-Tailing Enzyme	90 µL
KK8503* 07962355001	Ligation Buffer	900 µL
	DNA Ligase	300 µL
	KAPA HiFi HotStart ReadyMix (2X)*	690 µL
24 libraries	Library Amplification Primer Mix (10X)*	138 µL
KK8504 07962363001	End Repair & A-Tailing Buffer	930 µL
	End Repair & A-Tailing Enzyme	400 µL
KK8505* 07962371001	Ligation Buffer	3.8 mL
	DNA Ligase	1.26 mL
	KAPA HiFi HotStart ReadyMix (2X)*	3.0 mL
96 libraries	Library Amplification Primer Mix (10X)*	600 µL

*KK8501, KK8503, and KK8505 are available for PCR-free workflows, and do not contain library amplification reagents.

Quick Notes
<ul style="list-style-type: none"> • This kit provides a versatile, streamlined DNA library construction protocol. Libraries for Illumina® sequencing may be prepared from a wide range of DNA samples and inputs (1 ng – 1 µg) in 2 – 3 hrs, with minimal hands-on time. DNA input is appropriately fragmented double-stranded DNA. • The novel one-tube chemistry improves library yield and quality, particularly for FFPE and low-input samples. • The protocol is easy to automate. Generous reagent excesses are supplied in 96-reaction kits to accommodate the dead volumes required for automated liquid handling. • Kits contain all the reagents for library construction and high-efficiency, low-bias library amplification, except for adapters and beads. KAPA Pure 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 library construction process. Appendix 1 provides protocols for bead-based, double-sided size selection. Appendix 2 provides details for integration with the SeqCap EZ workflow.

Product Description

The KAPA Hyper Prep Kit provides a versatile, streamlined protocol for the rapid construction of libraries for Illumina sequencing from fragmented, double-stranded DNA (dsDNA). The novel chemistry and streamlined, one-tube protocol improves the efficiency and consistency of library construction across a wide range of sample types and inputs.

The workflow combines enzymatic steps and employs minimal bead-based cleanups, thereby reducing sample handling and overall library preparation time to 2 – 3 hrs. The kit contains all of the enzymes and reaction buffers required for:

1. end repair and A-tailing, which produces end-repaired, 5'-phosphorylated, 3'-dA-tailed dsDNA fragments;
2. adapter ligation, during which dsDNA adapters with 3'-dTTP overhangs are ligated to 3'-dA-tailed molecules;
3. 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 each of the two library construction steps. This offers the best combination of product stability, convenience and efficiency. Adapters and beads required for cleanups after adapter ligation and library amplification are not included. KAPA Pure 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 Hyper Prep 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 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 (KK8501, KK8503, and KK8505) are available for PCR-free workflows. They may also be combined with KAPA HiFi Real-time Library Amplification Kits (KK2701 and KK2702), or with KAPA HiFi HotStart Uracil+ ReadyMix (KK2801 and KK2802) for the amplification of bisulfite-converted libraries.

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 Hyper Prep Kits are ideally suited for low- and high-throughput NGS library construction workflows that require 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, cell-free/circulating tumor DNA and low-quality DNA such as FFPE samples. For small genomes, cell-free/circulating tumor DNA and lower complexity samples such as ChIP DNA, amplicons or cDNA (for RNA-seq), library construction from lower inputs (~100 pg or more) may be attempted.

The protocol 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 Roche SeqCap EZ, Agilent SureSelect, Illumina TruSeq, or IDT xGen Lockdown Probes, or other hybridization capture systems
- ChIP-seq
- RNA-seq (starting with cDNA)
- methyl-seq (in combination with KAPA HiFi HotStart Uracil+ ReadyMix for library amplification).

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 Hyper Prep Kits are shipped on dry ice or ice packs, depending on the destination country. 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 Hyper Prep Kit components have been fully thawed and thoroughly mixed before use. The KAPA Hyper Prep 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 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 at sequencing.roche.com/support for more information.

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** and/or contact Technical Support at sequencing.roche.com/support for further guidelines when designing or optimizing your library construction workflow.

Automated Library Construction

The library construction protocol 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 Technical Support at sequencing.roche.com/support if you are interested in using the KAPA Hyper Prep Kit with your particular automated liquid handling system.

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

- Reaction components for enzymatic reactions should be combined into master mixes, rather than dispensed separately. Master mixes are stable for ≤ 24 hrs at room temperature, and do not have to be actively cooled during automated library construction.
- Master mixes for 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, particularly 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.
- Incubations at temperatures above 50°C must be performed in a thermocycler with a heated lid.
- This protocol has been optimized with 96-well PCR plates with a maximum working volume of ~200 μ L. 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; and
 - 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 end repair and A-tailing to final, amplified library can be performed in 2 – 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 safely paused after completion of the **Post-ligation Cleanup** (step 3.17).

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. Library amplification products may be stored in a similar way, but the post-amplification cleanup should be performed as soon as possible. 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 and Fragmentation

- This protocol is suitable for library construction from 1 ng – 1 μ g of appropriately fragmented double-stranded DNA. However, libraries can be prepared from lower input amounts if the sample copy number is sufficient to ensure the requisite coverage and complexity in the final library. Please refer to Table 1 on the next page 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, target capture	Cell-free/circulating tumor DNA	≥100 pg
WGS	Microbial DNA	1 ng – 1 µg
WGS (PCR-free)	High-quality DNA	≥50 ng (no SS)* ≥200 ng (w/SS)*
ChIP-seq	ChIP DNA	≥100 pg
Targeted sequencing	Amplicons	≥100 pg
RNA-seq	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

- “Input” typically refers to the input into the end repair and A-tailing reaction. If DNA was quantified before fragmentation, and/or fragmented DNA was subjected to cleanup or size selection prior to end repair and A-tailing, the actual input into library construction may be significantly lower. This should be taken into account when evaluating the efficiency of the process and/or during optimization of library amplification cycle number.
- The proportion of fragmented DNA that is successfully converted to adapter-ligated molecules decreases as input is reduced. When starting library construction with >100 ng fragmented DNA, 25 – 50% of input DNA is typically converted to adapter-ligated molecules, whereas conversion rates range between 1% and 25% for libraries constructed from 100 pg – 100 ng DNA. These figures apply to high-quality DNA, and may be lower for DNA of lower quality, e.g., FFPE samples. Workflows with additional bead-based cleanups or size selection prior to adapter ligation are likely to result in a lower yield of adapter-ligated molecules.
- DNA preparations containing high concentrations of EDTA, other chelating agents or salts may inhibit the end repair and A-tailing reaction. If fragmented DNA is not subjected to a bead-based cleanup or size selection prior to library construction, DNA should be fragmented in 10 mM Tris-HCl (pH 8.0 – 8.5) + 0.1 mM EDTA. Fragmentation in water is not recommended. For DNA cleanup protocols please refer to the **KAPA Pure Bead Technical Data Sheet**.
- The KAPA Hyper Prep Kit is compatible with all mechanical and enzymatic fragmentation methods that are commonly used in NGS library construction workflows, except for tagmentation. Please refer to the fragmentation guidelines provided by the manufacturer of your fragmentation equipment or reagent of choice.

The KAPA Frag Kit for Enzymatic Fragmentation is highly recommended, particularly if you prefer not to use mechanical shearing, or do not have access to specialized equipment. KAPA HyperPlus Library Preparation Kits combine the KAPA Frag and the KAPA Hyper Prep chemistries in a streamlined, one-tube fragmentation/library construction protocol that offers the highest library yields from your available sample. Please visit www.sequencing.roche.com for more information.

Adapter Design and Concentration

- KAPA Adapters are recommended for use with the KAPA Hyper Prep 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 TruSeq (Illumina), SeqCap EZ (Roche) 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 visit 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 accommodate moderate variations in DNA input or fragment length. Please refer to Table 2 on the next page for the recommended adapter concentrations for different DNA inputs.
- 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 2), 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.

Table 2. Recommended adapter concentrations for libraries constructed from 1 ng – 1 µg input DNA*

Fragmented DNA per 50 µL ER & AT reaction	Adapter stock concentration	Adapter:insert molar ratio	Fragmented DNA per 50 µL ER & AT reaction	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.

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 Hyper Prep 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 to shift the mode fragment length of the population 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 an 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 the **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 the carry-over of adapter and/or adapter-dimer (and obviate 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 Pure 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 Pure 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 Pure 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 based on 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 target capture must be eluted and stored in PCR-grade water to facilitate drying of DNA prior to probe hybridization.

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 Hyper Prep workflow.
- Size selection may be carried out at several points in the overall workflow, for example:
 - prior to end repair and A-tailing of fragmented DNA;
 - after the post-ligation cleanup; or
 - after library amplification.
- The standard protocol (pp. 11 – 12) does not include size selection. Please refer to **Appendix 1** for detailed double-sided size selection protocols.
- 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 a high concentration of PEG 6000, which will interfere with efficient double-sided 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 the 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 hot start formulation of KAPA HiFi DNA Polymerase—a novel 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 2.8×10^{-7} errors/base, equivalent to 1 error in 3.5×10^6 nucleotides incorporated.
- The 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). 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 quantification 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** 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 4), 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 3 provides recommended cycle numbers for libraries prepared from high-quality input DNA, to obtain approximately 100 ng or 1 µg of amplified library.

Table 3. Recommended cycle numbers to generate 100 ng or 1 µg of amplified DNA from 1 ng – 1 µg of input DNA

Input DNA (into ER and AT)	Number of cycles required to generate	
	100 ng library	1 µg library
1 µg	0*	1 – 2*
500 ng	0*	2 – 4
250 ng	0 – 2*	4 – 6
100 ng	2 – 3*	6 – 7
50 ng	3 – 5	7 – 8
25 ng	5 – 7	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 4 for the number of cycles recommended to obtain approximately 1 µg of DNA from 0.5 – 500 ng of adapter-ligated DNA, or contact Technical Support at kapabiosystems.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.

Table 4. Theoretical number of cycles required to obtain approximately 1 µg of amplified library DNA from 0.5 – 500 ng of adapter-ligated library DNA*

Amount of adapter-ligated DNA in amplification reaction	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 KAPA Library Amplification Primer Mix, and library quantification with the qPCR-based KAPA Library Quantification Kit.

- 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 <2 hrs. The high conversion efficiency achievable with the KAPA Hyper Prep Kit enables PCR-free workflows from as little as 50 ng of input DNA. KAPA Hyper Prep Kits without amplification reagents (KK8501, KK8503, and KK8505) are available for PCR-free workflows.

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.
- 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 Hyper Prep 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. 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 orientation for cluster amplification and sequencing, and
 - measurements are not affected by library over-amplification—see **Important Parameters: Library Amplification** (p. 6).
- 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 (end repair, A-tailing and 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.

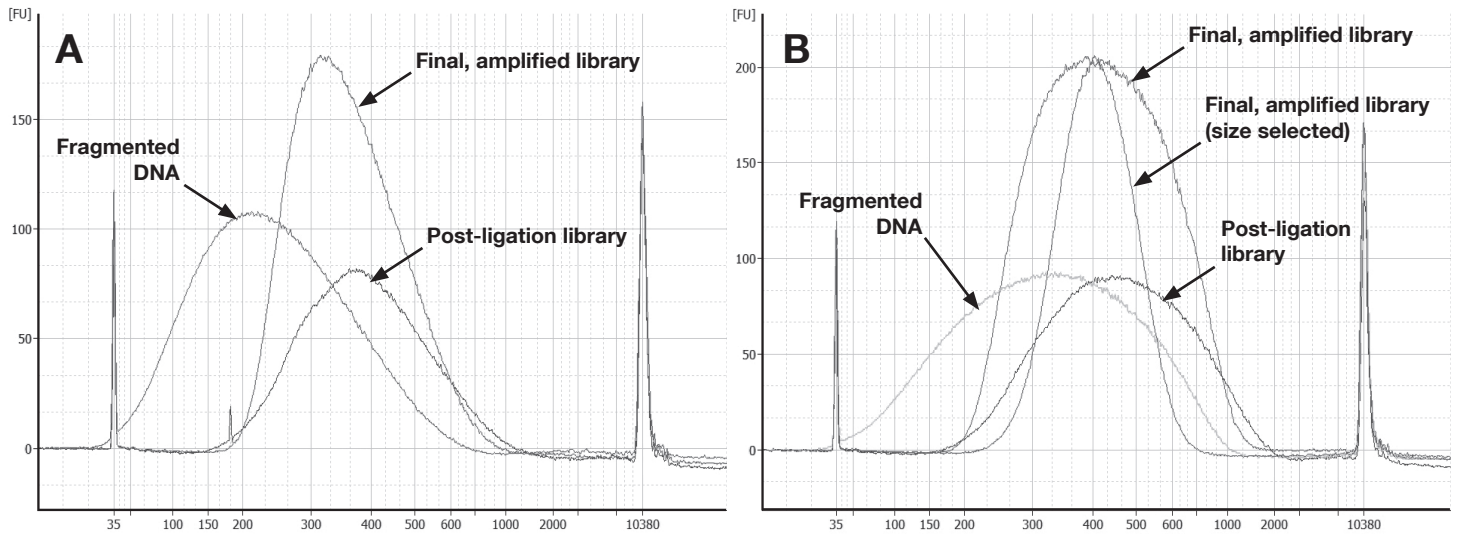


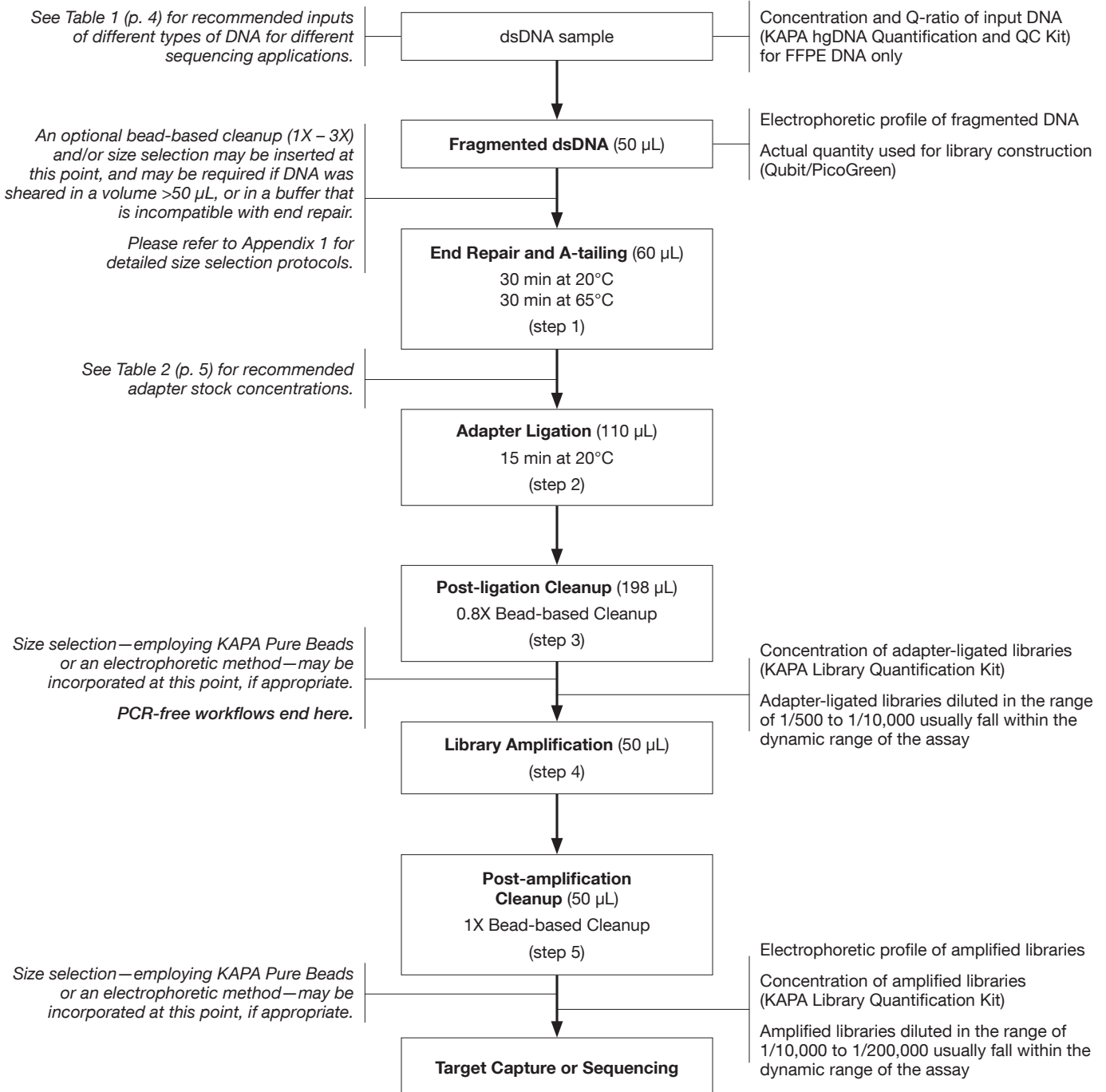
Figure 1. Examples of libraries prepared with the KAPA Hyper Prep Kit

Input DNA (100 ng high-quality human genomic DNA) was Covaris-sheared to a mode fragment size of ~200 bp (A) or ~300 bp (B), respectively. Libraries were prepared as described in the [Library Construction Protocol](#) (pp. 11 – 12), 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](#), 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.

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.

Process Workflow

Recommended QC Metrics



Library Construction Protocol

Note: This protocol does not include size selection. Please refer to **Appendix 1** for detailed double-sided size selection protocols.

1. End Repair and A-tailing

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

Component	Volume
Fragmented, double-stranded DNA	50 μ L
End Repair & A-Tailing Buffer*	7 μ L
End Repair & A-Tailing Enzyme Mix*	3 μ L
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.

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

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

*A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C, instead of the usual $\sim 105^\circ\text{C}$.

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

- 1.4 Proceed immediately to **Adapter Ligation** (step 2).

2. Adapter Ligation

- 2.1 Dilute adapter stocks to the appropriate concentration, as outlined in Table 2 (p. 5).
- 2.2 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 μ L
Adapter stock (concentration as required)	5 μ L
PCR-grade water*	5 μ L
Ligation Buffer*	30 μ L
DNA Ligase*	10 μ L
Total volume:	110 μL

*The water, buffer and ligase enzyme 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 4°C, and for ≤ 4 weeks at -20°C.

- 2.3 Mix thoroughly and centrifuge briefly.

- 2.4 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.

- 2.5 Proceed immediately to the next step.

3. Post-ligation Cleanup

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

Component	Volume
Adapter ligation reaction product	110 μ L
KAPA Pure Beads	88 μ L
Total volume:	198 μL

- 3.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.
- 3.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.
- 3.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 3.5 Carefully remove and discard the supernatant.
- 3.6 Keeping the plate/tube(s) on the magnet, add 200 μ L of 80% ethanol.
- 3.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.
- 3.8 Carefully remove and discard the ethanol.
- 3.9 Keeping the plate/tube(s) on the magnet, add 200 μ L of 80% ethanol.
- 3.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.
- 3.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
- 3.12 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.**
- 3.13 Remove the plate/tube(s) from the magnet.
- 3.14 Thoroughly resuspend the beads:
- in 25 μ L of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with **Library Amplification** (step 4), or
 - in 55 μ L of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with double-sided size selection (**Appendix 1**).

- 3.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
- 3.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 3.17 Transfer the clear supernatant to a new plate/tube(s):
- to proceed with **Library Amplification** (step 4), transfer 20 μL of supernatant, or
 - to proceed with double-sided size selection (**Appendix 1**), transfer 50 μL of supernatant.

4. Library Amplification

Note: Please refer to **Important Parameters: Library Amplification** (pp. 6 – 7) and the **KAPA NGS Library Preparation Technical Guide** for more information on optimizing library amplification.

- 4.1 Assemble each library amplification reaction as follows:

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

*Or another, 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. 6).

- 4.2 Mix thoroughly and centrifuge briefly.
- 4.3 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 3 or 4)
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.

- 4.4 Proceed directly to **Post-amplification Cleanup** (step 5).

5. Post-amplification Cleanup

- 5.1 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 Pure Beads	50 μL
Total volume:	100 μL

- 5.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.
- 5.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.
- 5.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 5.5 Carefully remove and discard the supernatant.
- 5.6 Keeping the plate/tube(s) on the magnet, add 200 μL of 80% ethanol.
- 5.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.
- 5.8 Carefully remove and discard the ethanol.
- 5.9 Keeping the plate/tube(s) on the magnet, add 200 μL of 80% ethanol.
- 5.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥ 30 sec.
- 5.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
- 5.12 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.**
- 5.13 Remove the plate/tube(s) from the magnet.
- 5.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) or PCR-grade water. **Always use PCR-grade water if proceeding to target capture.**
- 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.
- 5.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.
- 5.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 5.17 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 Hyper Prep library construction workflow. Size selection may be carried out at any one of several points in the workflow, for example:

- prior to end repair and A-tailing of fragmented DNA;
- 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**.

The double-sided size selection protocols outlined in this **Appendix** are designed for the selection of DNA fragments (**exclusive of adapter**) with a mode fragment length in the range of 150 – 350 bp. To obtain a population of shorter or longer molecules, protocols 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 2nd size cut should be performed with at least 0.2 volumes of KAPA Pure Beads. Please note that the volume of KAPA Pure Beads needed for the 2nd 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 1st and 2nd cuts is less than ~0.2 volumes. To increase the amount of DNA recovered, >0.2 volumes of KAPA Pure 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.

When size selecting adapter-ligated library DNA, keep in mind the long non-complementary arms of “forked” adapters ligated to DNA fragments affect size-dependent binding to KAPA Pure Beads, as well as the apparent fragment size determined with an electrophoretic method. For this reason, the double-sided size selection parameters for adapter-ligated libraries may have to be optimized to select for the appropriate size range. For more information on double-sided size selection, refer to the **KAPA NGS Library Preparation Technical Guide**, or contact Technical Support at sequencing.roche.com/support.

A1. Double-sided Size Selection Protocol

A1.1 Perform the first size cut (to exclude the unwanted, large DNA fragments or library molecules) by adding the appropriate volume of KAPA Pure Beads to the DNA, as follows:

Component	Fragmented dsDNA in 50 μ L (1.0X – 1.2X)	Fragmented dsDNA in 130 μ L (1.0X – 1.2X)	Adapter-ligated or amplified DNA (0.7X – 0.9X)*
DNA to be size-selected	50 μ L	130 μ L	50 μ L
KAPA Pure Beads	50 μ L	130 μ L	35 μ L
Total volume per well/tube:	100 μL	260 μL	85 μL

*For both adapter-ligated and amplified DNA, it is important to note that the adapters ligated to the insert DNA will increase the total fragment size by ~120 bp. For this reason, a lower ratio is required for the 1st cut.

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 the unwanted, large DNA fragments/library molecules 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 the required volume of supernatant (containing DNA fragments/library molecules smaller than those that were intentionally excluded) to a new plate/tube(s) (see table in section A1.6). It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) with beads to which the unwanted, large DNA fragments/library molecules are bound.

A1.6 Perform the second size cut by adding 0.2 volumes of KAPA Pure Beads to the supernatant from the first size cut, as follows:

Component	Fragmented dsDNA in 50 μ L (1.0X – 1.2X)	Fragmented dsDNA in 130 μ L (1.0X – 1.2X)	Adapter-ligated or amplified DNA (0.7X – 0.9X)
Supernatant from first size cut	95 μ L	255 μ L	80 μ L
KAPA Pure Beads	10 μ L	26 μ L	10 μ L
Total volume per well/tube:	105 μL	281 μL	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 the DNA fragments/library molecules that will eventually be retained 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 all unwanted, small DNA fragments/library molecules.
- A1.11 Keeping the plate/tube(s) on the magnet, add 200 μ L of 80% ethanol.
- A1.12 Incubate the plate/tube(s) on the magnet at room temperature for \geq 30 sec.
- A1.13 Carefully remove and discard the ethanol.
- A1.14 Keeping the plate/tube(s) on the magnet, add 200 μ L of 80% ethanol.
- A1.15 Incubate the plate/tube(s) on the magnet at room temperature for \geq 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 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.**
- 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), or PCR-grade water for capture libraries:
- For fragmented dsDNA, elute in \sim 55 μ L and use 50 μ L in the end repair and A-tailing reaction.
 - For adapter-ligated library DNA, either elute in \sim 25 μ L and use 20 μ L in the library amplification reaction, or elute in the volume required for the next step in your workflow (e.g., target capture or sequencing).
 - For amplified library DNA, elute in the volume required for the next step in your workflow (e.g., target capture or sequencing).
- 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: Library Construction Guidelines for the Roche SeqCap EZ Target Capture System

The KAPA Hyper Prep Kit may be used (instead of a KAPA HTP/LTP Library Preparation Kit) for the construction of pre-capture libraries in the SeqCap EZ workflow—to ensure a more streamlined protocol, shorter turnaround times and improved performance with low-input and challenging samples, such as FFPE and cell-free/free circulating tumor DNA.

Library Construction

Detailed instructions for specific steps or processes are included below.

DNA Input and Fragmentation

- The standard SeqCap EZ Library SR workflow specifies 100 ng of Covaris-sheared DNA as the input into library construction (the End Repair and A-tailing reaction).

Note: libraries may be constructed from lower inputs, but sequencing metrics are not guaranteed. To use the KAPA Hyper Prep Kit in a SeqCap EZ workflow with lower DNA inputs, please consult **Important Parameters** (pp. 3 – 9) or contact Technical Support at sequencing.roche.com/support for more information.

- Shear the input DNA to an average size of 180 – 220 bp using a Covaris instrument. If an optimized protocol is not available then start with the guidelines provided by the supplier of the Covaris instrument and fine-tune the parameters to yield the appropriate DNA fragmentation size distribution.

End Repair and A-tailing

- Follow instructions outlined in the **Library Construction Protocol: End Repair and A-tailing** (step 1).

Adapter Ligation

- Follow instructions outlined in the **Library Construction Protocol: Adapter Ligation** (step 2).
- Use 5 μ L of undiluted SeqCap Indexed Adapter per reaction.

Post-ligation Processing

- Perform the 0.8X post-ligation cleanup as outlined in the **Library Construction Protocol: Post-ligation Cleanup** (step 3). Resuspend the beads with adapter-ligated DNA in 55 μ L elution buffer, and recover 50 μ L of supernatant.
- Perform a 0.7X – 0.9X double-sided size selection, with KAPA Pure Beads as outlined in **Appendix 1** (p. 13).
- Resuspend beads with size-selected, adapter-ligated DNA in 22 –25 μ L elution buffer, and recover 20 μ L of supernatant.

Pre-capture Library Amplification (LM-PCR)

- Amplify 20 μ L of each pre-capture library, using the reaction setup and cycling parameters outlined in **Library Construction Protocol: Library Amplification** (step 4).
- Use 5 μ L of the KAPA Library Amplification Primer Mix (10X) supplied in the KAPA Hyper Prep Kit for each amplification reaction.
- Perform 9 cycles of pre-capture amplification.

Note: 9 cycles of pre-capture amplification should yield ≥ 1 μ g of pre-capture library (i.e., a sufficient amount to perform individual captures). If you are pooling libraries before capture, the amount of DNA needed per capture is ≥ 1 μ g/n, where n = the number of libraries per capture. The number of pre-capture amplification cycles may therefore be reduced to achieve the lowest possible duplication rates and highest library complexity prior to capture.

Cleanup after Pre-capture Library Amplification (LM-PCR)

- Perform a 1X bead-based cleanup of the pre-capture LM-PCR products, as outlined in **Library Construction Protocol: Post-amplification Cleanup** (step 5).

Quality Control of Pre-capture Libraries

- Please refer to the most recent version of the SeqCap EZ Library SR User's Guide for more details.

Target Enrichment

- Please refer to Chapters 5 – 8 of the latest version of the SeqCap EZ Library SR User's Guide to complete the workflow and generate sequencing-ready, enriched libraries.

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