KAPABIOSYSTEMS

Technical Data Sheet

KAPA LTP Library Preparation Kit

Illumina® Platforms

KR0453 – v6.17

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

This document applies to KAPA LTP Library Preparation Kits (07961863001 and 07961880001), KAPA LTP Library Preparation Kits for PCR-free workflows (07961871001 and 07961898001) and KAPA PEG/NaCl Kit (07961928001).

Contents

Kapa/*Roche* Kit Codes and Components		
KK8230 *07961863001* (8 libraries) **KK8231*** *07961871001* (8 libraries)	End Repair Buffer (10X) End Repair Enzyme Mix A-Tailing Buffer (10X) A-Tailing Enzyme Ligation Buffer (5X) DNA Ligase PEG/NaCl Solution KAPA HiFi HotStart ReadyMix (2X)* Library Amplification Primer Mix (10X)*	100 µL 50 µL 50 µL 30 µL 100 µL 50 µL 5 mL 250 µL 50 µL
KK8232 *07961880001* (48 libraries) **KK8233*** *07961898001* (48 libraries)	End Repair Buffer (10X) End Repair Enzyme Mix A-Tailing Buffer (10X) A-Tailing Enzyme Ligation Buffer (5X) DNA Ligase PEG/NaCl Solution KAPA HiFi HotStart ReadyMix (2X)* Library Amplification Primer Mix (10X)*	500 µL 250 µL 250 µL 150 µL 500 µL 250 µL 20 mL 1.25 mL 250 µL
KK8236 *07961928001* (48 libraries)	PEG/NaCl Solution	20 mL

*KK8231 and KK8233 are available for PCR-free workflows and do not contain library amplification reagents.

Quick Notes

- This kit is designed for the construction of DNA libraries from a wide range of DNA samples and inputs (10 ng – 5 µg) for Illumina sequencing.
- The optimized “with-bead” protocol improves the efficiency of library construction.
- Kits contain all the reagents for library construction and high-efficiency, low-bias library amplification, except for adapters and beads. PEG/NaCl Solution required for “with-bead” reaction cleanups is provided. KAPA Pure Beads (KK8000, KK8001, KK8002) 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 and options for size selection. The protocol provided in this document is a generic prototype, and may require additional optimization.
- The **KAPA NGS Library Preparation Technical Guide** (available at **www.kapabiosystems.com**) contains more detailed information about library construction parameters, and may facilitate protocol development and optimization.

Product Description

The KAPA LTP Library Preparation Kit is designed for the construction of libraries for Illumina sequencing, starting from fragmented, double-stranded DNA (dsDNA). The kit contains all of the enzymes and reaction buffers required for the following steps of library construction:

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

Efficient, cost-effective reaction cleanups and higher library yields are achieved through implementation of the "with-bead" strategy developed at The Broad Institute of MIT and Harvard and Foundation Medicine.[1] The kit includes PEG/NaCl Solution for this purpose. Adapters and beads required for cleanups after end repair and library amplification are not included. KAPA Pure Beads (KK8000, KK8001, KK8002) 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.[2,3,4,5] KAPA LTP Library Preparation 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 (KK8231 and KK8233) 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 Kits (KK2801 and KK2802) for the amplification of bisulfite-converted libraries.

1. Fisher, S., et al., *Genome Biology* **12**, R1 (2011).
2. Oyola, S.O., et al., *BMC Genomics* **13**, 1 (2012).
3. Quail, M.A., et al., *Nature Methods* **9**, 10 (2012).
4. Quail, M.A., et al., *BMC Genomics* **13**, 341 (2012).
5. Ross, M.G., et al., *Genome Biology* **14**, R51 (2013).

Product Applications

KAPA LTP Library Preparation Kits are ideally suited for low-throughput, manual 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 (10 ng – 5 µg). For small genomes, cell-free/circulating tumor DNA and lower complexity samples such as ChIP DNA, amplicons or cDNA (for RNA-seq), successful library construction has been achieved from lower inputs (~100 pg or more).

The protocol may be incorporated into workflows for a wide range of NGS applications, including:

- whole-genome shotgun sequencing
- whole exome or targeted sequencing, using Roche® NimbleGen™ SeqCap™ EZ, Agilent SureSelect, Illumina TruSeq®, IDT xGen™ Lockdown™ Probes, or other hybridization capture systems
- ChIP-seq
- RNA-seq (starting with cDNA)
- methyl-seq (in combination with the KAPA HiFi HotStart Uracil+ ReadyMix for library amplification)

Specific guidelines for the construction of libraries for target capture using the Roche NimbleGen SeqCap EZ system may be found in the **Appendix** (p. 17).

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 LTP Library Preparation 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. The PEG/NaCl Solution must be protected from light, and stored at 2°C to 8°C for short-term use (≤2 months), or at -15°C to -25°C. 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 LTP Library Preparation Kit components have been fully thawed and thoroughly mixed 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. PEG/NaCl Solution does not freeze at -15°C to -25°C, but should be equilibrated to room temperature and thoroughly mixed before use. 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 **kapabiosystems.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 **kapabiosystems.com/support** for further guidelines when designing or optimizing your library construction workflow.

Reaction Setup

This kit is intended for low-throughput, manual library construction. However, the protocol is designed to be "automation friendly" to facilitate the transition to automation should throughput requirements increase over time.

Please keep the following in mind, especially when using the kit for the first time:

- To enable a streamlined "with-bead" strategy, reaction components for enzymatic reactions must be combined into master mixes, rather than dispensed separately. It is recommended that master mixes be kept on ice during reaction setup, even though these mixes are stable for ≤24 hrs at room temperature.
- Due to the strong 3'→5' exonuclease activity of KAPA HiFi HotStart DNA Polymerase, PCR master mixes with primers should preferably not be kept at room temperature for long periods of time. Prepare library amplification master mixes freshly before use, keep them on ice, and/or dispense primer mixes separately from the KAPA HiFi HotStart ReadyMix.
- An excess (5 – 10%) of each master mix will be required. The appropriate excess for other reagents (adapters, beads, PEG/NaCl Solution, 80% ethanol and elution buffer) varies.
- Incubations at temperatures above 50ºC must be performed in a thermocycler with a heated lid.
- Libraries may be prepared in standard reaction vessels, including 1.5 mL microtubes, PCR tubes, strip tubes or PCR plates.
- 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 magnet used during bead manipulations;
  - vortex mixers and centrifuges, where appropriate; and
  - heating blocks or thermocyclers used for reaction incubations and/or library amplification.

Safe Stopping Points

The library construction process, from end repair to final, amplified library can be performed in 4 – 8 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 any of the bead cleanup steps, as outlined below:

- After **End Repair Cleanup** (step 3), resuspend the washed beads in 20 µL of A-Tailing Buffer (1X) (without enzyme), and store the reactions at 4°C for ≤24 hrs.
- After **A-tailing Cleanup** (step 5), resuspend the washed beads in 20 µL of Ligation Buffer (1X) (without enzyme or adapter), and store the reactions at 4°C for ≤24 hrs.
- After **1st Post-ligation Cleanup** (step 7), resuspend the washed beads in the appropriate volume of 10 mM Tris-HCl (pH 8.0 – 8.5) as outlined in step 7.14, and store the reactions at 4°C for ≤24 hrs.

DNA solutions containing cleanup beads must not be frozen, and beads must not be stored dry, as this is likely to damage the beads and result in sample loss. To resume the library construction process, centrifuge the reaction vessels briefly to recover any condensate, and add the remaining components required for the next enzymatic reaction in the protocol (see Tables 6B and 7B on p. 11). If the protocol was paused after **1st Post-ligation Cleanup** (step 7), continue directly with the **2nd Post-ligation Cleanup** (step 8), **Double-sided Size Selection** (step 9), or size selection using an alternative method.

Purified, adapter-ligated library DNA may be stored at 4°C for 1 – 2 weeks, or at -20°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 10 ng – 5 µg 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 for recommended inputs of different types of DNA for different sequencing applications.
- "Input" typically refers to the input into the end repair reaction. If DNA was quantified before fragmentation and/or fragmented DNA was subjected to cleanup or size selection prior to end repair, 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.

Table 1. Recommended inputs into library construction

Application	Sample type	Recommended input
WGS	Complex gDNA (high quality)	50 ng – 5 µg
Target capture (WES, custom panels)	Complex gDNA (high quality)	10 ng – 5 µg
WGS, target capture	FFPE DNA	≥100 ng (quality dependent)
WGS, target capture	Cell-free/circulating tumor DNA	≥1 ng
WGS	Microbial DNA	1 ng – 1 µg
WGS (PCR-free)	High-quality DNA	≥100 ng (no SS)* ≥500 ng (w/SS)*
ChIP-seq	ChIP DNA	≥100 pg
Targeted sequencing	Amplicons	≥100 pg
RNA-seq	cDNA	≥10 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 input DNA that is successfully converted to adapter-ligated molecules decreases as input is reduced. When starting library construction (end repair) with >100 ng fragmented DNA, 15 – 40% of input DNA is typically converted to adapter-ligated molecules, whereas conversion rates range between 0.5% and 15% for libraries constructed from 100 pg – 100 ng DNA. These figures apply to high-quality DNA and may be significantly lower for DNA of lower quality, e.g., FFPE samples. Workflows that contain additional bead-based cleanups or size selection prior to library amplification 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 reaction. If fragmented DNA is not subjected to a bead-based cleanup or size selected prior to end repair, 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 LTP Library Preparation 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 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. Please refer to the **KAPA Frag Technical Data Sheet** for a detailed protocol and information related to enzymatic fragmentation reaction parameters.
- In some circumstances it may be convenient to fragment input DNA in KAPA End Repair Buffer (1X), in which case the end repair reaction setup should be adjusted accordingly. Please contact Technical Support at **kapabiosystems.com/support** for more information.

End Repair and A-tailing Cleanup

- This protocol provides for 1.7X – 1.8X cleanups after end repair and A-tailing. This ratio of PEG/NaCl Solution-to-sample volume will retain DNA fragments longer than ~75 bp. If you wish to retain very small DNA fragments and/or improve recovery, the PEG/NaCl Solution-to-sample ratio can be increased to 2X – 3X for all cleanups prior to adapter ligation.
- When performing library construction in standard PCR plates, tubes or strip tubes, a 3X cleanup after end repair is not possible, as the maximum working volume per well is usually ~200 µL. To achieve a 3X cleanup after end repair, libraries must be prepared in 500 µL PCR tubes or 1.5 mL microtubes, or the end repair reaction must be scaled down. Please contact Technical Support at **kapabiosystems.com/support** if your workflow or sample type requires modified bead-based cleanups.

Adapter Design and Concentration

- KAPA Adapters are recommended for use with the KAPA LTP Library Preparation Kit. However, the kit is also compatible with non-indexed, single-indexed, and dual-indexed adapters that are routinely used in Illumina TruSeq®, Roche® NimbleGen™ SeqCap™ EZ, Agilent® SureSelect and other similar library construction and target capture workflows. Custom adapters that are of similar design and are compatible with "TA-ligation" of dsDNA may also be used, remembering that this may impact library construction efficiency. For assistance with adapter compatibility and ordering, please contact Technical Support at **kapabiosystems.com/support**

Table 2. Recommended adapter concentrations

Insert DNA per 70 µL end repair reaction	Recommended adapter concentration for DNA sheared to an average size of:					
	175 bp		350 bp		500 bp	
	Stock	Final	Stock	Final	Stock	Final
3 – 5 µg	60 µM	6 µM	30 µM	3 µM	21 µM	2.1 µM
1 µg	20 µM	2 µM	10 µM	1 µM	7 µM	700 nM
500 ng	10 µM	1 µM	5 µM	500 nM	3.5 µM	350 nM
100 ng	2 µM	200 nM	1 µM	100 nM	700 nM	70 nM
10 ng	200 nM	20 nM	100 nM	10 nM	70 nM	7 nM
1 ng	20 nM	2 nM	10 nM	1 nM	7 nM	700 pM

- Adapter concentration affects ligation efficiency, as well as adapter and adapter-dimer carry-over during post-ligation cleanups. 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 50:1, making it unnecessary to adjust adapter stock concentrations to accommodate moderate variations in DNA input or fragment length. As a general guideline, an adapter:insert molar ratio of ~10:1 is recommended for inputs ≥100 ng. This translates to different final adapter concentrations for libraries with different size distributions (see Table 2).
- An adapter:insert molar ratio >10:1 may be beneficial for low-input applications and challenging samples such as FFPE, cell-free/circulating tumor, and ChIP DNA. Adapter:insert molar ratios up to ~100:1 may be evaluated, but the top end of this range could result in unacceptable levels of adapter-dimer.
- 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.
- While a single bead-based cleanup removes most unligated adapter and adapter-dimer, a second cleanup is recommended to fully eliminate adapter species from the library. The amount of adapter and adapter-dimer carried through the first cleanup is dependent on the adapter concentration in the ligation reaction.
- If no post-ligation size selection is carried out, two consecutive 1X bead-based cleanups are recommended after ligation.
- The volume in which washed beads are resuspended after the post-ligation cleanup(s) 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 procedure described in the **Library Construction Protocol** (step 9) beads must be resuspended in 100 µL of elution buffer.

Reaction Cleanups

- This protocol has been validated for use with either KAPA Pure Beads (KK8000, KK8001, KK8002), or Agencourt® AMPure® XP (Beckman Coulter®). Solutions and conditions for DNA binding and size selection may differ if other beads are used.
- Cleanups should be performed in a timely manner to ensure that enzymatic reactions do not proceed beyond optimal incubation times.
- 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 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 to completely capture beads varies according to the reaction vessel and magnet used. It is important to not 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 4°C for 1 – 2 weeks, or for long-term storage at -20°C. The long-term stability of library DNA at -20°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 may be integrated in the KAPA LTP Library Preparation Kit Workflow.
- Size selection may be carried out at different points in the overall workflow, for example:
  - prior to end repair of fragmented DNA;
  - before library amplification—as outlined in **Library Construction Protocol** (p. 10); or
  - after library amplification.
- The double-sided size selection procedure described in the **Library Construction Protocol** (step 9) is designed for selection of adapter-ligated fragments approximately 250 – 450 bp in length. Please consult the **KAPA NGS Library Preparation Technical Guide** or contact Technical Support at **kapabiosystems.com/support** if you wish to select a different range of fragment lengths.
- 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.
- Ligation Buffer (5X) contains a high concentration 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 1X bead-based cleanup prior to performing bead- or electrophoresis-based size selection.
- Over-amplification of libraries often results in the observation of secondary, higher molecular weight peaks when amplified libraries are analyzed electrophoretically. 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/or 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 KAPA HiFi HotStart ReadyMix (2X), 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.
- Library Amplification Primer Mix (10X) is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix (2X). 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 **kapabiosystems.com/support** for guidelines on the formulation of user-supplied library amplification primers.

- To achieve optimal 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 – 2 µM each. For libraries constructed from ≥100 ng input DNA, the highest final concentration (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 4°C for short-term use, or as single-use aliquots at -20°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 **Library Construction Protocol (**step 10)—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 library from 1 ng – 1 µg of input DNA

Input DNA (into end repair)	Number of cycles required to generate	
	100 ng library	1 µg library
1 µg	0 – 1*	2 – 3
500 ng	0 – 1*	3 – 4
250 ng	1 – 3*	4 – 6
100 ng	3 – 4	6 – 8
50 ng	4 – 5	8 – 9
25 ng	5 – 7	9 – 11
10 ng	8 – 10	11 – 13
5 ng	9 – 11	13 – 15
2.5 ng	11 – 13	15 – 17
1 ng	14 – 16	18 – 20

*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** for a calculator to assist with these calculations. Please note 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 streamlines the workflow and reduces overall library preparation time by about 1 hr. The high conversion efficiency achievable with the KAPA LTP Library Preparation Kit enables PCR-free workflows from as little as 100 ng of input DNA. KAPA LTP Library Preparation Kits without amplification reagents (KK8231 and KK8233) are available for PCR-free workflows.

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 (2X) and Library Amplification Primer Mix (10X), and library quantification with the qPCR-based KAPA Library Quantification Kit.

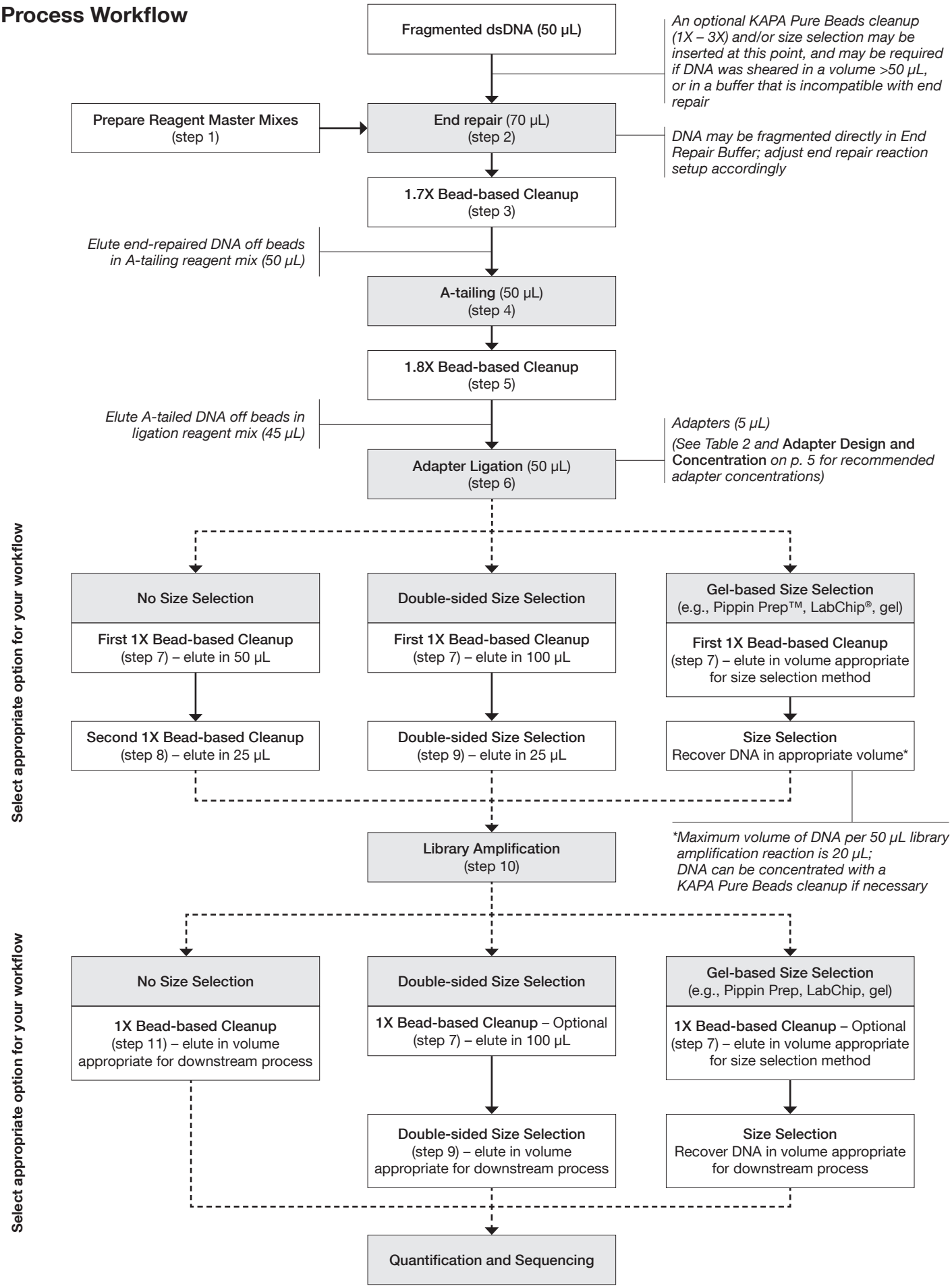
Evaluating the Success of Library Construction

- 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 enrichment or sequencing), as well as for QC and archiving purposes.
- The size distribution of 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.
- 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. 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 adapters 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 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 cleanups (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.
  - 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.

Process Workflow



Library Construction Protocol

1. Reagent Preparation

For maximum stability and shelf-life, the enzyme preparations and concentrated reaction buffers for end repair, A-tailing and ligation are supplied separately in KAPA LTP Library Preparation Kits. For a streamlined "with-bead" protocol, a reagent master mix is prepared for each of these enzymatic steps, as outlined in Tables 5, 6, and 7. Additional reagents required for the KAPA LTP Library Preparation protocol are listed in Table 8.

Master mixes may be constituted with varying proportions of the total final water requirement. In the examples given in the tables below, all of the required water is included in each master mix, allowing the entire reaction mix to be dispensed in a single pipetting step (after cleanups, beads are resuspended directly in master mix for the next enzymatic step).

At safe stopping points, some or all of the water and/or reaction buffer may be added to the beads, for storage at 4°C for ≤24 hrs. To resume library construction, prepare the master mix with the remaining volume of water (if applicable) and reaction buffer, and the required volume of enzyme. Recommendations on how to formulate master mixes after safe stopping points are provided in Tables 6B and 7B.

Master mixes for end repair, A-tailing, and ligation may be prepared immediately before use, or stored for ≤1 week at 4°C, or ≤3 months at -20°C. Master mixes stored at -20°C are stable through three freeze-thaw cycles.

Table 5. End repair

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
End repair master mix:			
Water	8 µL	67 µL	403 µL
End Repair Buffer (10X)	7 µL	59 µL	353 µL
End Repair Enzyme Mix	5 µL	42 µL	252 µL
Total master mix volume:	**20 µL**	**168 µL**	**1008 µL**
Final reaction composition:	**Per reaction**		
Fragmented DNA	50 µL		
End repair master mix	20 µL		
Total reaction volume:	**70 µL**		

Table 6A. A-tailing (uninterrupted protocol)

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
A-tailing master mix:			
Water	42 µL	353 µL	2117 µL
A-Tailing Buffer (10X)	5 µL	42 µL	252 µL
A-Tailing Enzyme	3 µL	25 µL	151 µL
Total master mix volume:	**50 µL**	**420 µL**	**2520 µL**
Final reaction composition:	**Per reaction**		
Beads with end-repaired DNA			
End repair master mix	50 µL		
Total reaction volume:	**50 µL**		

Table 6B. A-tailing (with safe stopping point)

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
A-Tailing Buffer (1X) for safe stopping point:			
Water	18 µL	151 µL	907 µL
A-Tailing Buffer (10X)	2 µL	17 µL	101 µL
Total buffer volume:	**20 µL**	**168 µL**	**1008 µL**
Component:	**1 library**	**8 libraries** *Inc. 5% excess*	**48 libraries** *Inc. 5% excess*
A-tailing master mix after safe stopping point:			
Water	24 µL	202 µL	1,210 µL
A-Tailing Buffer (10X)	3 µL	25 µL	151 µL
A-Tailing Enzyme	3 µL	25 µL	151 µL
Total master mix volume:	**30 µL**	**252 µL**	**1512 µL**
Final A-tailing reaction composition (when protocol is resumed):	**Per reaction**		
Beads with DNA in A-Tailing Buffer (1X)	20 µL		
A-tailing master mix	30 µL		
Total reaction volume:	**50 µL**		

Table 7A. Adapter ligation (uninterrupted protocol)

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
Ligation master mix:			
Water	30 µL	252 µL	1512 µL
Ligation Buffer (5X)	10 µL	84 µL	504 µL
DNA Ligase	5 µL	42 µL	252 µL
Total master mix volume:	**45 µL**	**378 µL**	**2268 µL**
Final reaction composition:	**Per reaction**		
Beads with A-tailed DNA			
Adapter (<7 nM – 60 µM, as appropriate)	5 µL		
Ligation master mix	45 µL		
Total reaction volume:	**50 µL**		

Table 7B. Adapter ligation (with safe stopping point)

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
Ligation Buffer (1X) for safe stopping point:			
Water	16 µL	134 µL	806 µL
Ligation Buffer (5X)	4 µL	34 µL	202 µL
Total buffer volume:	**20 µL**	**168 µL**	**1008 µL**
Component:	**1 library**	**8 libraries** *Inc. 5% excess*	**48 libraries** *Inc. 5% excess*
Ligation master mix after safe stopping point:			
Water	14 µL	118 µL	706 µL
Ligation Buffer (5X)	6 µL	50 µL	302 µL
DNA Ligase	5 µL	42 µL	252 µL
Total master mix volume:	**25 µL**	**210 µL**	**1260 µL**
Final ligation reaction composition (when protocol is resumed):	**Per reaction**		
Beads with DNA in Ligation Buffer (1X)	20 µL		
Adapter (<7 nM – 60 µM, as appropriate)	5 µL		
Ligation master mix	25 µL		
Total reaction volume:	**50 µL**		

Table 8. Volumes of additional reagents required

Component:	1 library	8 libraries *Inc. 5% excess*	48 libraries *Inc. 5% excess*
PEG/NaCl Solution (provided in kit):			
A-tailing cleanup	90 µL	756 µL	4536 µL
First post-ligation cleanup	50 µL	420 µL	2520 µL
Second post-ligation cleanup/Double-sided size selection	60 µL	504 µL	3024 µL
Total volume required:	**200 µL**	**1.7 mL**	**10.1 mL**
Component:	**1 library**	**8 libraries** *Inc. 5% excess*	**48 libraries** *Inc. 5% excess*
KAPA Pure Beads (sold separately):			
End repair cleanup	120 µL	1008 µL	6048 µL
Double-sided size selection	20 µL	168 µL	1008 µL
Library amplification cleanup	50 µL	420 µL	2520 µL
Total volume required:	**190 µL**	**1.6 mL**	**9.6 mL**
Component:	**1 library**	**8 libraries** *Inc. 5% excess*	**48 libraries** *Inc. 5% excess*
80% ethanol (freshly prepared; not supplied):			
End repair cleanup	0.4 mL	3.36 mL	20.2 mL
A-tailing cleanup	0.4 mL	3.36 mL	20.2 mL
First post-ligation cleanup	0.4 mL	3.36 mL	20.2 mL
Second post-ligation cleanup/Double-sided size selection	0.4 mL	3.36 mL	20.2 mL
Library amplification cleanup	0.4 mL	3.36 mL	20.2 mL
Total volume required:	**2.0 mL**	**16.8 mL**	**101 mL**
Component:	**1 library**	**8 libraries** *Inc. 5% excess*	**48 libraries** *Inc. 5% excess*
Elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5; not supplied):			
Second post-ligation cleanup/Double-sided size selection	125 µL	1050 µL	6300 µL
Library amplification cleanup	25 µL	210 µL	1260 µL
Total volume required:	**150 µL**	**1260 µL**	**7560 µL**

2. End Repair Reaction Setup

2.1 Assemble each end repair reaction in a tube or well of a PCR plate as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End repair master mix (Table 5)	20 µL
Total volume:	**70 µL**

2.2 Mix and incubate at 20°C for 30 min.

2.3 Proceed immediately to **End Repair Cleanup** (step 3).

3. End Repair Cleanup

3.1 In the same plate/tube(s), perform a 1.7X bead-based cleanup by combining the following:

Component	Volume
End repair reaction product	70 µL
KAPA Pure Beads	120 µL
Total volume:	**190 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 µL 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 µL 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.

SAFE STOPPING POINT

Resuspend the beads in 20 µL of A-Tailing Buffer (1X) (Table 6B), cover the reaction and store at 4ºC for ≤24 hrs. Do not freeze the samples as this will damage the KAPA Pure Beads. When ready, proceed to **A-tailing After Safe Stopping Point** (step 4B).

4. A-tailing

A-tailing is performed either directly after **End Repair Cleanup**, or after **Safe Stopping Point**, where beads were resuspended in A-Tailing Buffer (1X) and stored at 4°C for ≤24 hrs. Depending on your chosen workflow, proceed with either **A-tailing Immediately** (step 4A) or **A-tailing After Safe Stopping Point** (step 4B).

4A. A-tailing Immediately

4A.1 In the plate/tube(s) containing end-repaired DNA and beads, assemble the A-tailing reaction as follows:

Component	Volume
Beads with DNA	–
A-tailing master mix (Table 6A)	50 µL
Total volume:	**50 µL**

4A.2 Thoroughly resuspend the beads by vortexing and/or pipetting up and down multiple times.

4A.3 Incubate the plate/tube(s) at 30°C for 30 min.

4B.4 Proceed immediately to **A-tailing Cleanup** (step 5).

4B. A-tailing After Safe Stopping Point

4B.1 To resume library preparation, combine the following reagents to perform A-tailing:

Component	Volume
Beads with DNA (in A-Tailing Buffer (1X))	20 µL
A-tailing master mix after Safe Stopping Point (Table 6B)	30 µL
Total volume:	**50 µL**

4B.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

4B.3 Incubate the plate/tube(s) at 30°C for 30 min.

4B.4 Proceed immediately to **A-tailing Cleanup** (step 5).

5. A-tailing Cleanup

5.1 In the same plate/tube(s), perform a 1.8X bead-based cleanup by combining the following:

Component	Volume
A-tailing reaction product	50 µL
PEG/NaCl Solution	90 µL
Total volume:	**140 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.

SAFE STOPPING POINT

Resuspend the beads in 20 µL of Ligation Buffer (1X) (Table 7B), cover the reaction and store at 4ºC for ≤24 hrs. Do not freeze the samples as this will damage the KAPA Pure Beads. When ready, proceed to **Adapter Ligation After Safe Stopping Point** (step 6B).

6. Adapter Ligation

Adapter ligation is performed either directly after **A-tailing Cleanup** (step 5) or after the **Safe Stopping Point**, where beads were resuspended in Ligation Buffer (1X) and stored at 4°C for ≤24 hrs. Depending on your chosen workflow, proceed with either **Adapter Ligation Immediately** (step 6A) or **Adapter Ligation After Safe Stopping Point** (step 6B).

6A. Adapter Ligation Immediately

6A.1 Dilute adapter stocks to the appropriate concentration, as outlined in Table 2 (p. 5).

6A.2 In the plate/tube(s) containing A-tailed DNA and beads, assemble the adapter ligation reaction as follows:

Component	Volume
Beads with DNA	–
Adapter (concentration as required)	5 µL
Ligation master mix (Table 7A)	45 µL
Total volume:	**50 µL**

6A.3 Thoroughly resuspend the beads by vortexing and/or pipetting up and down multiple times.

6A.4 Incubate at 20°C for 15 min.

6A.5 Proceed immediately to **1st Post-ligation Cleanup** (step 7).

6B. Adapter Ligation After Safe Stopping Point

6B.1 Dilute adapter stocks to the appropriate concentration, as outlined in Table 2 (p. 5).

6B.2 To resume library preparation, combine the following reagents to perform adapter ligation:

Component	Volume
Beads with DNA (in Ligation Buffer (1X))	20 µL
Adapter (concentration as required)	5 µL
Ligation master mix (Table 7B)	25 µL
Total volume:	**50 µL**

6B.3 Thoroughly resuspend the beads by vortexing and/or pipetting up and down multiple times.

6B.4 Incubate at 20°C for 15 min.

6B.5 Proceed immediately to **1st Post-ligation Cleanup** (step 7).

7. 1st Post-ligation Cleanup

Depending on your requirements and chosen workflow, one or two post-ligation cleanups should be performed. Consult **Important Parameters: Post-ligation Processing** (p. 5) and the **KAPA NGS Library Preparation Technical Guide** for more information.

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

Component	Volume
Adapter ligation reaction product	50 µL
PEG/NaCl Solution	50 µL
Total volume:	**100 µL**

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

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

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

7.5 Carefully remove and discard the supernatant.

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

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

7.8 Carefully remove and discard the ethanol.

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

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

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

7.12 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.***

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

7.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5), and incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads. Recommended volumes are as follows:

- If proceeding to **2nd Post-ligation Cleanup** (step 8), resuspend the beads in 50 µL.
- If proceeding to **Double-sided Size Selection** (step 9), resuspend the beads in 100 µL, and omit the second post-ligation cleanup described below.
- **For a safe stopping point**, resuspend the beads in either 50 µL or 100 µL (as required), and store at 4°C for ≤24 hrs.

8. 2nd Post-ligation Cleanup

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

Component	Volume
Purified, adapter-ligated DNA with beads	50 µL
PEG/NaCl Solution	50 µL
Total volume:	**100 µL**

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

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

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

8.5 Carefully remove and discard the supernatant.

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

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

8.8 Carefully remove and discard the ethanol.

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

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

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

8.12 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.***

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

8.14 Thoroughly resuspend the beads in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Consult **Important Parameters: Post-ligation Processing** (p. 5) for more information on elution volumes.

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

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

8.17 Transfer the clear supernatant to a new plate/tube(s) and proceed with **Library Amplification** (step 10), QC, and/or sequencing, as appropriate.

9. Double-sided Size Selection (250 – 450 bp)

The double-sided size selection procedure described here is designed for selection of adapter-ligated fragments approximately 250 – 450 bp in length. Please consult the **KAPA NGS Library Preparation Technical Guide** or contact Technical Support at **kapabiosystems.com/support** if you wish to select a different range of fragment lengths.

9.1 In the same plate/tube(s) used for the first post-ligation cleanup, perform the first size cut (0.6X) by combining the following:

Component	Volume
Purified, adapter-ligated DNA with beads	100 µL
PEG/NaCl Solution	60 µL
Total volume:	**160 µL**

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

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

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

9.5 Carefully transfer 155 µL of supernatant containing library fragments smaller than ~450 bp to a new plate/tube(s). ***It is critical that no beads are transferred with the supernatant.***

9.6 Discard the plate/tube(s) with beads, to which library fragments larger than ~450 bp are bound.

9.7 Perform the second size cut (0.8X) by combining the following:

Component	Volume
Supernatant from first cut	155 µL
KAPA Pure Beads	20 µL
Total volume:	**175 µL**

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

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

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

9.11 Carefully remove and discard the supernatant, which contains library fragments smaller than ~250 bp.

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

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

9.14 Carefully remove and discard the ethanol.

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

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

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

9.18 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.***

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

9.20 Thoroughly resuspend the beads in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5). Consult **Important Parameters: Post-ligation Processing** (p. 5) for more information on elution volumes.

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

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

9.23 Transfer the clear supernatant containing size-selected DNA to a new plate/tube(s) and proceed with **Library Amplification** (step 10), QC, and/or sequencing, as appropriate.

10. Library Amplification Reaction Setup

Note: please refer to **Important Parameters: Library Amplification** (p. 6) and the **KAPA NGS Technical Guide** for more information on optimizing library amplification.

10.1 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 DNA	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 – 2 µM. Also refer to **Important Parameters: Library Amplification** (p. 6).

10.2 Mix thoroughly and centrifuge briefly.

10.3 Amplify using the following cycling protocol:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Minimum 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.

10.4 Proceed directly to **Library Amplification Cleanup** (step 11).

11. Library Amplification Cleanup

11.1 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**

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

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

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

11.5 Carefully remove and discard the supernatant.

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

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

11.8 Carefully remove and discard the ethanol.

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

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

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

11.12 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.***

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

11.14 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***.

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

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

11.17 Transfer the clear supernatant to a new plate/tube(s) and proceed with library QC, target enrichment, or sequencing, as appropriate. Store purified, amplified libraries at 4°C for 1 – 2 weeks, or at -20°C.

Appendix: Library Construction Guidelines for the Roche® NimbleGen™ SeqCap™ EZ Target Capture System

This appendix provides guidelines for the preparation of libraries for targeted sequencing (capture) using the Roche NimbleGen SeqCap EZ system, and specific protocols for pre- and post-capture ligation-mediated (LM)-PCR. Please refer to the most recent version of the **Roche NimbleGen SeqCap EZ Library SR User's Guide** for detailed instructions relating to hybridization of the amplified sample library to the SeqCap EZ library (capture), and washing and recovery of the captured DNA sample.

A1. Library Construction

Prepare libraries from fragmented, human genomic DNA according to the protocol (pp. 10 – 16) of this document. Specific recommendations are as follows:

A1.1 For optimal results with 2 x 100 bp paired-end sequencing, input DNA should be fragmented to an average length in the range of 180 – 220 bp. For 2 x 250 bp paired-end sequencing, a slightly longer fragment length may be preferred. The size distribution of the fragmented DNA should be confirmed electrophoretically prior to library construction, using an LabChip® GX, GXII, or GX Touch (PerkinElmer®), Bioanalyzer or TapeStation (Agilent® Technologies), Fragment Analyzer™ (Advanced Analytical®) or similar instrument.

A1.2 The recommended input into fragmentation for the standard SeqCap EZ protocol is 100 ng, but higher or lower inputs can be accommodated. For information regarding exceptions to the standard protocol, please contact Technical Support at **kapabiosystems.com/support**.

A1.3 SeqCap Index Adapters from Roche NimbleGen are recommended for adapter ligation.

- The standard protocol (100 ng input) recommends 3 µL of SeqCap Adapter (of the appropriate index; reconstituted as described in the **SeqCap EZ Library SR User's Guide**) per 50 µL ligation reaction.
- To accommodate this, the adapter ligation master mix (Table 7A, p. 11) must be modified to contain 32 µL PCR-grade water per 50 µL reaction (instead of 30 µL). The compositions of the modified adapter ligation master mix and adapter ligation reaction are outlined in the following tables.

Adapter ligation master mix	Volume
PCR-grade water	32 µL
Ligation Buffer (5X)	10 µL
DNA Ligase	5 µL
Total volume:	**47 µL**

Adapter ligation reaction	Volume
Beads with A-tailed DNA	—
SeqCap Index Adapter	3 µL
Adapter ligation master mix	47 µL
Total volume:	**50 µL**

A1.4 If you are using adapters from a different source, or an input other than 100 ng, adhere to the guidelines provided in **Important Parameters: Adapter Design and Concentration** (p. 5). When using Illumina TruSeq® adapters from an Illumina TruSeq Library Preparation Kit v2:

- For 1 µg input, dilute the adapter 1:5 in 10 mM Tris-HCl (pH 8.0 – 8.5) and use 5 µL per 50 µL ligation reaction.
- For 100 ng input, dilute the adapter 1:50 in 10 mM Tris-HCl (pH 8.0 – 8.5) and use 5 µL per 50 µL ligation reaction.

A1.5 After adapter ligation, a double-sided size selection is recommended. This is achieved by performing **1st Post-ligation Cleanup** (step 7), eluting the DNA in 100 µL elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5), and proceeding to **Double-sided Size Selection** (step 9). The double-sided size selection is designed to eliminate adapter-ligated library fragments >450 bp and <250 bp.

Note: size selection typically results in a significant loss of adapter-ligated material. If there is no need to eliminate large fragments (>450 bp) from the adapter-ligated library, two consecutive 1X bead-based cleanups are recommended to remove unused adapter and adapter-dimer. This strategy results in a simpler workflow with less loss of material prior to pre-capture amplification, and should be considered in cases where the quantity and/or quality of input DNA is limiting.

A1.6 Irrespective of whether the no size selection (two consecutive 1X bead-based cleanups) or double-sided size selection strategy is followed, the final, dried beads with cleaned/size-selected, adapter-ligated library must be thoroughly resuspended in 25 µL of elution buffer. Proceed directly to **Pre-capture LM-PCR** (step A2).

A2. Pre-capture LM-PCR

Note: SeqCap™ Adapter Kits A and B contain library amplification primers for pre- and post-capture LM-PCR. Library Amplification Primer Mix (10X) included in the KAPA HTP Library Preparation Kit is compatible with SeqCap Index Adapters and may be used instead of the SeqCap primers in both the pre- and post-capture LM-PCR for optimal amplification efficiency.

A2.1 Assemble each pre-capture LM-PCR in a PCR tube or wells of a PCR plate as follows:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Pre-LM-PCR Oligos 1 & 2*	5 µL
Purified (size-selected) adapter-ligated library DNA**	20 µL
Total volume:	**50 µL**

*Prepared as described in the **SeqCap EZ Library SR User's Guide**.
**Or PCR-grade water for negative control.

A2.2 Perform the pre-capture LM-PCR with the following thermocycling parameters:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	9
Annealing*	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

*8 cycles are recommended for 100 ng input DNA. For lower inputs or FFPE DNA, additional cycles may be needed to produce sufficient material for capture. Please refer to Tables 3 and 4 in **Important Parameters: Library Amplification** for guidelines.

A2.3 Store the plate/tube(s) at 4°C for ≤72 hrs or proceed directly to **Cleanup and QC of the Pre-capture LM-PCR Amplified Sample Library** (step A3).

A3. Cleanup and QC of the Pre-capture LM-PCR Amplified Sample Library

A3.1 Perform the pre-capture LM-PCR cleanup (1X) as described in **Library Amplification Cleanup** (step 11, p. 16). ***Pre-capture libraries must be eluted in PCR-grade water, not elution buffer.***

A3.2 Quantify the amplified library with the KAPA Library Quantification Kit or a NanoDrop™ spectrophotometer, and analyze 1 µL of the sample using an Agilent® 2100 Bioanalyzer DNA 1000 Kit. For optimal results, the library fragment length distribution should be 150 – 500 bp.

For single captures (one DNA sample library per capture), the yield of each amplified library should be >1 µg. For multiplexed capture (>1 DNA sample libary per capture), a total of 1 µg of the pool of DNA sample libraries is required. Depending on QC and/or archiving requirements, <1 µg of each individual library may therefore be needed, and the number of pre-capture amplification cycles may be adjusted accordingly.

A4. Hybridization of the Amplified Sample Library to the SeqCap EZ Library

Set up hybridization reactions according to instructions outlined in the **SeqCap EZ Library SR User's Guide**.

A5. Washing and Recovery of the Captured DNA Sample

Follow the instructions outlined in the **SeqCap EZ Library SR User's Guide** for binding of the captured DNA sample to streptavidin beads and washing of the bead-bound DNA.

A6. Post-capture LM-PCR

Two 50 µL post-capture LM-PCRs—each containing 20 µL of washed and resuspended streptavidin (capture) beads—are performed for each library.

The composition of each post-capture LM-PCR is given in the table below. If convenient, double the amount of each component may be premixed (for a total of 60 µL of post-capture LM-PCR mix per library) and split equally between two PCR tubes or wells of a PCR plate.

A6.1 Assemble **two** post-capture LM-PCRs for each captured library in PCR tubes or wells of a PCR plate. Each reaction consists of:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Pre-LM-PCR Oligos 1 & 2*	5 µL
Total volume:	**30 µL**

*Prepared as described in the **SeqCap EZ Library SR User's Guide**. May be replaced with Library Amplification Primer Mix (10X).

A6.2 Vortex the streptavidin bead-bound captured Multiplex DNA Sample Library Pool to ensure that the solution is homogeneous before proceeding to the next step.

A6.3 Transfer 20 µL of streptavidin bead-bound capture DNA Sample Library (or PCR-grade water for negative controls) to each of the two wells/tubes containing post-capture LM-PCR mix. Mix thoroughly by pipetting up and down. Store the remaining bead-bound captured DNA Sample Library at -15°C to -25°C.

A6.4 Perform the post-capture LM-PCR with the following thermocycling parameters outlined below:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	14*
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

*The number of post-capture amplification cycles may be reduced if standard parameters produce library yields that far exceed what is needed for QC, sequencing and archiving.

A6.5 Store the plate/tube(s) for ≤72 hrs at 4°C, or proceed directly to **Cleanup and QC of the Post-capture LM-PCR Amplified Sample Library** (step A7).

A7. Cleanup and QC of the Post-capture LM-PCR Amplified Sample Library

A7.1 Perform the post-capture LM-PCR cleanup (1X) as described in **Library Amplification Cleanup** (step 11, p. 16). The standard protocol recommends pooling the two post-capture LM-PCRs into a 1.5 mL tube, for a single cleanup. It also indicates that post-capture libraries should be eluted in PCR-grade water, but elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) should be considered for longer-term stability.

A7.2 Quantify the final, amplified library with the KAPA Library Quantification Kit or a NanoDrop™ spectrophotometer, and analyze 1 µL of the sample using an Agilent® 2100 Bioanalyzer DNA 1000 Kit. The yield of final, amplified library should be >500 ng, and the library fragment length distribution should be 150 – 500 bp.

A8. Determine the Sample Enrichment using qPCR

A8.1 Follow the instructions outlined in the **SeqCap™ EZ SR User Guide** for estimating the relative fold enrichment achieved for the amplified, captured DNA sample.

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